Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism

Seventh Edition

An Official Publication of the American Society for Bone and Mineral Research
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Section XI. Appendix

(Section Editor: Roger Bouillon)

Please visit the Seventh Edition Primer website at www.asbmrprimer.org for the expanded and updated Appendix.
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Preface to the Seventh Edition

The mission of the ASBMR Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism is to present core knowledge in this field in a comprehensive yet concise fashion. In the two years since publication of the Sixth Edition, substantial new information has advanced our understanding of bone biology, mineral homeostasis, hormonal regulation, and the associated disease states.

The Seventh Edition reflects these rapid and exciting advances in the basic sciences and translational research. Each of the major sections now includes an overview which provides the reader with an opportunity to understand the scope and implications of the chapters contained within it. Under the guidance of Jane Lian and Juliet Compston, we added several new chapters, and expanded the text by nearly one third. We increased our international representation on the editorial board and among authors. In the Seventh Edition, we added a new section on animal models that includes everything from mouse genetics to phenotyping and structural correlates. We expanded the section related to malignancy and bone under the guidance of Dr. Theresa Guise and included sections on the management of bone pain from cancer and the treatment of metastatic bone disease. Genetics is now covered more extensively, for mouse and humans, and methods for measuring bone mass have been expanded to keep pace with recent technology. Dr. Laurie McCauley spearheaded an expansion of the oral and maxillofacial section to include new concepts, and expand on a discussion of osteonecrosis of the jaw. The appendix is now much larger and includes not only reference data, but Web-based links to multiple sites for ease of use. This appendix will appear on the Primer website (www.asbmrprimer.org) and is free to access. In addition, Dr. Bouillon did an outstanding job updating approved drugs for metabolic bone diseases for both national and international members. In total, some chapters have been consolidated, others expanded, but all continue the role of the Primer as an essential resource for house staff, graduate students, fellows, and junior faculty. We believe the 7th edition will prove even more valuable for the many scientists, trainees, and physician practitioners who are intrigued and challenged by the complexities of the bone, its regulatory hormones, and the related diseases.

The Seventh Edition of the Primer is the first without Dr. Murray Favus as Editor in Chief. We continue to owe him a debt of gratitude for his previous successes in modeling the Primer as the most complete and up to date source of information in the field. This edition also represented a complete change in the editorial board, with two new associate editors, Jane Lian and Juliet Compton, as well as new assistant editors: Drs. Vicki Rosen, Socrates Papapoulos, Pierre Delmas, Raj Thakker, Paul Miller, Laurie McCauley, Richard Keene, Ego Seeman, Marie Demay, Roger Bouillon, Theresa Guise, and Suzanne Jan de Beur. As such, this edition is a remarkable accomplishment and a testimony to the hard work, collegiality, and fellowship of this editorial board. It is even more remarkable considering the increase in breadth and depth that was undertaken in this publication. Our initial goal was to expand the number of chapters by nearly one third, and enhance the appendix, both as a reference component and as a gateway for Web-based references.

The Seventh Edition of the Primer is the product of a tripartite collaboration involving an experienced editorial board, skilled authors and an enthusiastic and supportive ASBMR editorial staff. The ASBMR staff, seasoned by the initial experience of self-publication with the Fifth Edition, has improved the publication process in many ways that benefited the authors and editors. The JBMR staff under the leadership of Matt Kilby must also be credited with expanding the distribution of the Primer and delivering it into the hands of so many more students, trainees, scientists, and physician practitioners than we ever envisioned. Matt’s tireless efforts on the part of the Primer are greatly appreciated.

The evolution of the Primer is an ongoing process, and just as we complete the Seventh Edition we look forward to continuing this work in the Eighth Edition.

Of course, it is the contributing authors who have made the Primer a valuable source of information in the bone field. The editorial board is deeply indebted to the many authors who over the years have articulated in print the state of their areas of expertise and to the new authors who have given so much of their valuable time to this endeavor. This impressive group has successfully taken on the challenge of organizing the ever-expanding scientific information into a coherent presentation.

Clifford J. Rosen, M.D.
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The untimely death of Dr. Pierre Delmas, one of the associate editors of the Primer, is a tragic loss to the Society, to the editors of this book, and to all who work in the field of metabolic bone diseases. His commitment to research, teaching, and promoting public health will be valued for years to come. The Seventh Edition of the Primer is dedicated to his legacy.
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Preface for Primer

The American Society for Bone and Mineral Research is proud to present the Seventh Edition of the Primer on the Metabolic Bone Disease and Disorders of Mineral Metabolism. This book marks the first edition published under its new Editor-in-Chief, Dr. Clifford Rosen, and many notable changes have been made to the text and format as a result of this transition.

A major objective of Dr. Rosen and the new editorial team was to increase international coverage with the Seventh Edition and the accomplishment of this goal has exceeded all expectations. The editorial board and the list of contributing authors closely match the international depth of the ASBMR.

Along with these changes, Dr. Roger Bouillon was tasked with creating an international appendix, which can be seen on the Primer Website (www.asbmrprimer.org). Among the tables is a formulary of drugs used in the treatment of bone disorders and their availability throughout the world. The appendix will be located online only to allow extra room for broad coverage of topics in the book, as well as to make this information freely accessible online.

The Seventh Edition of our Primer upholds the tradition of excellence set by its predecessors while carving its own niche into our science and our lives. We commend the efforts of everyone involved.

Barbara E. Kream, Ph.D.
President
American Society for Bone and Mineral Research
SECTION I

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(Section Editor: Vicki Rosen)

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Chapter 1. Skeletal Morphogenesis and Embryonic Development

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INTRODUCTION

Formation of the skeletal system is one of the hallmarks that distinguish vertebrate animals from invertebrate ones. In higher vertebrates (i.e., birds and mammals), the skeletal system contains mainly cartilage and bone that are mesoderm-derived tissues and formed by chondrocytes and osteoblasts, respectively, during embryogenesis. A common mesenchymal progenitor cell also referred as the osteochondral progenitor gives rise to both chondrocytes and osteoblasts. Skeletal development starts from mesenchymal condensation, during which mesenchymal progenitor cells aggregate at future skeletal locations. Because mesenchymal cells in different parts of the embryo come from different cell lineages, the locations of initial skeletal formation determine which of the three mesenchymal cell lineages contribute to the future skeleton. Neural crest cells from the branchial arches contribute to the craniofacial bone, the sclerotome compartment of the somites gives rise to most axial skeletons, and lateral plate mesoderm form the limb mesenchyme, from which limb skeletons are derived (Fig. 1). Ossification is one of the most critical processes in bone development, and this process is controlled by two major mechanisms: intramembranous and endochondral ossification. Osteochondral progenitors differentiate into osteoblasts to form the membranous bone during intramembranous ossification, whereas during endochondral ossification, osteochondral progenitors differentiate into chondrocytes instead to form a cartilage template of the future bone. The location of each skeletal element also determines its ossification mechanism and unique anatomic properties such as the shape and size. Importantly, the positional identity of each skeletal element is acquired early in embryonic development even before mesenchymal condensation through a process called pattern formation.

Cell-cell communication that coordinates cell proliferation and differentiation plays a critical role in pattern formation. Patterning of the early skeletal system is controlled by several major signaling pathways that also regulate other pattern formation processes. These signaling pathways are mediated by Wnts, Hedgehogs (Hhhs), Bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and Notch/Delta. Later in skeletal development in a different cell context, these signaling pathways also control cell fate determination, proliferation, and maturation in the skeleton.

EARLY SKELETAL PATTERNING

In the craniofacial region, neural crest cells are major sources of cells establishing the craniofacial skeleton. It is the temporal- and spatial-dependent reciprocal signaling between and among the neural crest cells and the epithelial cells (surface ectoderm, neural ectoderm or endodermal cells) that ultimately establish the pattern of craniofacial skeleton formed by neural crest cells. Patterning of the axial skeleton can be traced back to the formation of somites, which are segmented mesodermal structure on either side of the neural tube and the underlying notochord. Somites give rise to axial skeleton, striated muscle, and dorsal dermis. The repetitive and left-right symmetrical patterning of axial skeleton is controlled by a molecular oscillator or the segmentation clock and gradients of signaling molecules that act in the presomitic mesoderm (PSM) (Fig. 2A). The segmentation clock is operated by cyclic expression of genes, most of which are components of the Notch and canonical Wnt signaling pathways (Fig. 2B).

The Notch signaling pathway mediates short-range communication between contacting cells. The majority of cyclic genes are downstream targets of the Notch signaling pathway and code for Hairy/Enhancer of split (Hes) family members, Lunatic fringe (Lfgn), and the Notch ligand Delta. The canonical Wnt signaling pathway mediates long-range signaling across several cell diameters. On activation of canonical Wnt signaling, β-catenin is stabilized and translocates to the nucleus where it binds Lef/Tcf factors and activates expression of downstream genes including Axin2 and Nkd1. Axin2 and Nkd1 expression oscillates out of phase with Notch signaling components in the mouse PSM (Fig. 2B). The Notch and Wnt pathways interact to certain extent within the mechanism of the segmentation clock.

The FGF and retinoic acid (RA) signaling also control somatogenesis by regulating the competence of PSM cells to undergo segmentation. They form two opposing and functionally antagonistic gradients within the PSM (Fig. 2A). RA signaling has an additional role in maintaining left-right bilateral symmetry of somites by interacting and coordinating with the signaling pathways establishing left-right asymmetry of the body axis and the segmentation clock of the somites.

The functional significance of segmentation clock in human skeletal development is highlighted by congenital axial skeletal diseases. Abnormal vertebral segmentation (AYS) in humans is a relatively common malformation. For instance, mutations in the NOTCH signaling components cause at least two human disorders, spondylocostal dysostosis (SCD, 277300, 608681, and 609813) and Alagille syndrome (AGS, OMIM 118450 and 610205); both exhibit vertebral column defects.

The formed somite is also patterned along the dorsal-ventral axis by cell signaling from the surface ectoderm, neural tube and the notochord (Fig. 1). Ventrailizing signals such as Sonic hedgehog (Shh) from the notochord and ventral neural tube is required to induce sclerotome formation on the ventral side, whereas Wnt signaling from the surface ectoderm and dorsal neural tube is required for the formation of dermomyotome on the dorsal side of the somite (Fig. 1). The sclerotome gives rise to the axial skeleton and the ribs. In the mouse mutant that lacks Shh function, vertebra number and posterior ribs failed to form. The paired domain transcription factor Pax1 is expressed in the sclerotome, and Shh is required to regulate its expression. However, axial skeletal phenotypes in Pax1 mutant mice were far less severe than those in the Shh mutants.

Key words: bone, cartilage, embryonic development, chondrocyte, osteoblast, cell signaling, skeletal development, pattern formation, PTH-related peptide, Ihh, Wnt, fibroblast growth factor, bone morphogenetic protein, retinoic acid, Notch, Sox9, Runx2, ossification mechanism, intramembranous ossification, endochondral ossification, chondrocyte proliferation, chondrocyte hypertrophy, somatogenesis, segmentation clock
Limb skeletons are patterned along the proximal-distal (P-D, shoulder to digit tip), anterior-posterior (A-P, thumb to little finger), and dorsal-ventral (D-V, back of the hand to palm) axis (Fig. 3). Along the P-D axis, the limb skeletons form three major segments: humerus or femur at the proximal end, radius and ulna or tibia and fibula in the middle, and carpal/tarsal, metacarpal/metatarsal and digits in the distal end. Along the A-P axis, the radius and ulna have distinct morphological features; so does each of the five digits. Patterning along the D-V limb axis also results in characteristic skeletal shapes and structures. For instance, the sesamoid processes are located ventrally whereas the knee patella forms on the dorsal side of the knee. The 3D limb patterning events are regulated by three signaling centers in the early limb primordium called the limb bud before mesenchymal condensation.

The apical ectoderm ridge (AER), a thickened epithelial structure formed at the distal tip of the limb bud, is the signaling center that directs P-D limb outgrowth (Fig. 3). Canonical Wnt signaling activated by Wnt3 indices AER formation. Fgf family members that are expressed in the AER, mainly Fgf8 and Fgf4, are necessary and sufficient to mediate the function of AER (29-34). Fgf10 expressed in the presumptive limb mesoderm is required for limb initiation and it also controls limb outgrowth by maintaining Fgf8 expression in the AER (29-31).

The second signaling center is the zone of polarizing activity (ZPA), which is a group of mesenchymal cells located at the posterior distal limb margin and immediately adjacent to the AER (Fig. 3B). When ZPA tissue is grafted to the anterior limb bud under the AER, it leads to digit duplication in mirror image of the endogenous ones. Shh is expressed in the ZPA and is both necessary and sufficient to mediate ZPA activity in patterning digit identity along the A-P axis. However, the A-P axis of the limb is established before Shh signaling. This pre-Shh A-P limb patterning is controlled by combined activities of Gli3, A1x4, and basic helix-loop-helix (bHLH) transcription factors dHand and Twist1. The Gli3 repressor form (Gli3R) and A1x4 establish the anterior limb territory by re-
The early patterning events determine where and when the mesenchymal cells condense. After that, osteochondrogenic progenitors in the condensation form either chondrocytes or osteoblasts. Sox9 and Runx2, master transcription factors that are required for the determination of chondrocyte and osteoblast cell fates, respectively, are both expressed in osteochondral progenitor cells, but Sox9 expression precedes that of Runx2 in the mesenchymal condensation in the limb. Early Sox9-expressing cells give rise to both chondrocytes and osteoblasts regardless of ossification mechanisms. In addition, loss of Sox9 function in the limb leads to loss of mesenchymal condensation and Runx2 expression. Coexpression of Sox9 and Runx2 is terminated on chondrocyte and osteoblast differentiation. Sox9 and Runx2 expression are quickly segregated into chondrocytes and osteoblasts, respectively. Understanding the mechanism controlling this expression segregation of Sox9 and Runx2 in specific cell lineages is fundamental to elucidate the regulation of not only chondrocyte and osteoblast differentiation but also the determination of ossification mechanism. It is clear that cell–cell signaling, particularly those mediated by Wnts and Indian hedgehog (Ihh), are required for cell fate determination of chondrocytes and osteoblasts by controlling the expression of Sox9 and Runx2.

Active canonical Wnt signaling is detected in the developing calvarium and perichondrium where osteoblasts differentiate through either intramembranous or endochondral ossification or endochondral ossification. Indeed, enhanced canonical Wnt signaling enhanced bone formation and Runx2 expression, but inhibited chondrocyte differentiation and Sox9 expression. Conversely, removal of β-catenin in osteochondral progenitor cells resulted in ectopic chondrocyte differentiation at the expense of osteoblasts during both intramembranous and endochondral ossification. Therefore, the mesenchymal progenitor cells in the condensation are at least bipotential in their final cell fate determination. During intramembranous ossification, Wnt signaling in the condensation is higher, which promotes osteoblast differentiation while inhibiting chondrocyte differentiation. During endochondral ossification, however, Wnt signaling in the condensation is kept low such that only chondrocytes can differentiate. Later, when Wnt signaling is upregulated in the periphery of the cartilage, osteoblasts will differentiate. Thus, by manipulating Wnt signaling, mesenchymal progenitor cells, and perhaps even mesenchymal stem cells, can be directed to form only chondrocytes, which is needed in repairing cartilage damage in osteoarthritis, or only form osteoblasts, which will lead to new therapeutic strategies to treat osteoporosis. These studies also provide new insights to tissue engineering that aims to fabricate cartilage or bone in vitro using mesenchymal progenitor cells or stem cells.

Ihh signaling is required for osteoblast differentiation by activating Runx2 expression only during endochondral bone formation. Ihh is expressed in newly differentiated chondrocytes, and Ihh signaling does not seem to affect chondrocyte differentiation from mesenchymal progenitors. However, when Ihh signaling is inactivated in the perichondrium cells, they ectopically form chondrocytes and express Sox9 at the expense of Runx2. This is similar to what has been observed in the Osterix (Ox) mutant embryos, except that, in the Ox/– embryos, ectopic chondrocytes express both Sox9 and Runx2 because Ox acts downstream of Runx2 in osteoblast differentiation. It is still not clear what controls Ihh-independent Runx2 expression during intramembranous ossification. One likely scenario is that the function of Ihh is compensated by Shh during intramembranous ossification in the developing calvarium.

Both the canonical Wnt and Ihh signaling pathways are required for endochondral bone formation. It is important to understand their genetic epistasis. All vertebrate Hhs including Shh and Ihh signal through the same pathway. Two multipass transmembrane proteins Patched1 (Pch1) and Smoothened (Smo) receive Hh signaling on the cell membrane. Ihh signal-
TGF superfamily. BMPs were identified as secreted proteins signaling, whereas loss of 

The sequential actions of Hh and Wnt signaling in osteoblast repair and tissue engineering.

In this double mutant, Osx is concurrently inactivated at the same time in the same cells in the developing bone. In this double mutant, B-catenin is required downstream of not just Ihh, but also Osx in promoting osteoblast maturation. In contrast, Ihh signaling is not required after Osx expression for osteoblast differentiation.

The sequential actions of Hh and Wnt signaling in osteoblast differentiation and maturation suggest that Hh and Wnt signaling need to be manipulated at distinct stages during fracture repair and tissue engineering.

The BMP family of secreted growth factors belong to the TGF superfamily. BMPs were identified as secreted proteins that have the ability to promote ectopic cartilage and bone formation. Unlike Ihh and Wnt signaling, BMP signaling promotes the differentiation of both osteoblast and chondrocyte differentiation from mesenchymal progenitors. The mechanisms underlying these unique activities of BMPs have been under intense investigation in the last two decades. During the course, understanding BMP action in chondrogenesis and osteogenesis has benefited greatly from molecular studies of BMP signal transduction. Reducing BMP signaling by removing BMP receptors leads to impaired chondrocyte and osteoblast differentiation and maturation.

FGF ligands and FGF receptors (FGFRs) are both expressed in the developing skeletal system, and the significant role of FGF signaling in skeletal development was first identified by the discovery that achondroplasia (ACH; OMIM 100800), the most common form of skeletal dwarfism in humans, was caused by a missense mutation in FGFR3. Later, hypochondroplasia (HCH; OMIM 146000), a milder form of dwarfism, and thanatophoric dysplasia (TD; OMIM 187600 and 187601), a more severe form of dwarfism, were also found to result from mutations in FGFR3. FGFR3 signaling acts to regulate the proliferation and hypertrophy of the differentiated chondrocytes. However, the function of FGF signaling in mesenchymal condensation and chondrocyte differentiation from progenitors remains to be elucidated because complete genetic inactivation of FGF signaling in mesenchymal condensation has not been achieved. Nevertheless, it is clear that FGF signaling acts in the mesenchymal condensation to control osteoblast differentiation during intramembranous bone formation. Mutations in FGFR1, 2, and 3 cause craniosynostosis (premature fusion of the cranial sutures). The craniosynostosis syndromes involving FGFR1, 2, and 3 mutations include Apert syndrome (AS; OMIM 101200), Crouzon syndrome (CS; OMIM 123500), Pfeiffer syndrome (PS; OMIM 101600), Jackson-Weiss syndrome (JWS; OMIM 123790), Crouzon syndrome (CS; OMIM 123500), Pfeiffer syndrome (PS; OMIM 101600), Jackson-Weiss syndrome (JWS; OMIM 123150), Muenke syndrome (MS; OMIM 133000), and osteoglophonic dysplasia (OMIM 166250), a disease characterized by craniosynostosis, a prominent supraorbital ridge, and a depressed nasal bridge, as well as the rhizomelic dwarfism and nonossifying bone lesions. All these mutations are autosomal dominant, and many of them are activating mutations of FGFRs. FGF signaling can promote or inhibit osteoblast proliferation and differentiation depending on the cell context. It does so either directly or through interacting with the Wnt and BMP signaling pathways.

CHONDROCYTE PROLIFERATION AND DIFFERENTIATION IN THE DEVELOPING CARTILAGE

During endochondral bone formation, chondrocytes differentiated from the mesenchymal condensation form the cartilage, which provides a growth template for the future bone. Differentiated chondrocytes inside the cartilage undergo tightly controlled progressive proliferation and hypertrophy, which is required for endochondral bone formation. In the developing cartilage of the long bone, chondrocytes with different properties of proliferation and differentiation are located in distinct zones along the longitudinal axis, and such organization is required for long bone elongation (Fig. 4A). Proliferating chondrocytes express ColII, whereas hypertrophic chondrocytes express ColX. The chondrocytes that already exit cell cycle, but have not yet become hypertrophic, are prehypertrophic chondrocytes. These cells and early hypertrophic chondrocytes express Ihh, which is a master regulator of endochondral bone development by coupling chondrocyte hy-
pertrophy with osteoblast differentiation as Ihh is produced by prehypertrophic chondrocytes and signals to the adjacent perichondrium to induce osteoblast differentiation.  

Ihh"" mice have striking skeletal development defects. Apart from lack of endochondral bone formation, all cartilage elements are small because of a marked decrease in chondrocyte proliferation.  

Ihh also controls the pace of chondrocyte hypertrophy by activating the expression of PTH-related peptide (PTHrP) in articular cartilage and pericartilaginous cells. PTHrP acts on the same G protein–coupled receptor used by PTH. This PTH/PTHrP receptor (PPR) is expressed at much higher levels by prehypertrophic and early hypertrophic chondrocytes. PTHrP signaling is required to inhibit precocious chondrocyte hypertrophy primarily by keeping proliferating chondrocytes in the proliferating pool.  

Ihh signaling is also required to mediate the activity of Ihh in regulating chondrocyte hypertrophy but not proliferation.  

Therefore, Ihh and PTHrP form a negative feedback loop to control the decision of chondrocytes to leave the proliferating pool and become hypertrophy (Fig. 8B). In this model, PTHrP, secreted from cells at the ends of cartilage, acts on prehypertrophic chondrocytes to keep them proliferating. When chondrocytes are further away from the end of cartilage as a result of cartilage elongation, they are no longer sufficiently stimulated by PTHrP. These cells exit cell cycle and become Ihh-producing prehypertrophic chondrocytes. Ihh stimulates PTHrP expression at the ends of cartilage to slow down cell cycle exit of proliferating chondrocytes. This model is supported by experiments using chimeric mouse embryos.  

Clones of PPR"" chondrocytes differentiate into hypertrophic chondrocytes and produce Ihh within the wildtype proliferating chondrocyte domain. This ectopic Ihh expression leads to ectopic osteoblast differentiation in the perichondrium, an upregulation of PTHrP expression, and a consequent lengthening of the columns of wildtype proliferating chondrocytes. These studies show that the lengths of proliferating columns, hence the growth potential of cartilages, are critically determined by the Ihh–PTHrP negative feedback loop. Indeed, it has been found that mutations in IHH in humans cause brachydactyly type A1 (OMIM 112500), which exhibit shortened digits, philangies and short body stature.  

This feedback loop may also act to assure that cells at the hypertrophic front exit cell cycle and undergo hypertrophy at the same time.  

Several Wnt ligands including Wnt5a, Wnt5b, Wnt4, and Wnt9a are expressed in the cartilage and perichondrium of mouse embryos. Wnt4 and Wnt9a signal through the canonical Wnt pathway, whereas Wnt5a and Wnt5b signal through the beta-catenin–independent (noncanonical) pathways to regulate chondrocyte proliferation and hypertrophy. Although the mechanisms vary, in the absence of either canonical or noncanonical Wnt signaling, chondrocyte proliferation is altered, and chondrocyte hypertrophy is delayed.  

Furthermore, both the canonical and noncanonical Wnt pathways act in parallel with Ihh signaling to regulate chondrocyte proliferation and differentiation.  

MAPK pathways. FGFR3 signaling also interacts with the Ihh/PTHrP/BMP signaling pathways. The proliferation-inhibitory role of Fgf signaling in chondrocytes is not unique to FGFR3. When expressed in growth plate chondrocytes in vivo, both FGFR1 and FGFR3 kinase domains seem to have similar activities.  

Because Fgf18 mice show an increase in chondrocyte proliferation that closely resembles the cartilage phenotypes of Fgfr3"" mice, FGF18 is likely a physiological ligand for FGFR3 during skeletal development. However, the phenotype of the Fgf18"" mouse is more severe than that of the Fgfr3"" mice, suggesting that Fgf18 may also signal through Fgfr1 in hypertrophic chondrocytes and Fgfr2 and -1 in the perichondrium. Mice conditionally lacking Fgfr2 develop skeletal dwarfism with decreased BMD. Osteoblasts also express Fgfr3, and mice lacking Fgfr3 have decreased BMD and develop osteopenia. Thus, in osteoblasts, FGF signaling promotes bone growth by promoting osteoblast proliferation. Interestingly, mice lacking Fgfr2 also show osteopenia, although much later in development than in Fgfr2"" mice, suggesting that FGF2 may be a homeostatic factor that replaces the developmental growth factor, FGF18, in adult bones. It is still not clear which FGFR (1, 2, or 3) is actually responding to FGF18 in osteoblasts.  

Like the other major signaling pathways mentioned above, BMP signaling also acts during later stages of cartilage development. Both in vitro limb explant experiments and in vivo genetic studies showed that BMP signaling promotes chondrocyte proliferation and Ihh expression. Addition of BMPs to bone explants increases proliferation of chondrocytes, whereas Noggin blocks chondrocyte proliferation. In addition, conditional removal of both BmpR1A and BmpR1B in differentiated chondrocytes leads to reduced chondrocyte proliferation and Ihh expression. BMP signaling also regulates chondrocyte hypertrophy because removal of BmpR1A in chondrocytes leads to an expanded hypertrophic zone caused by accelerated chondrocyte hypertrophy and delayed terminal maturation of hypertrophic chondrocytes.  

Given the function of Ihh in promoting chondrocyte proliferation and controlling the pace of chondrocyte hypertrophy, BMP signaling regulates chondrocyte proliferation and hypertrophy at least in part through Ihh expression.  

BMP signaling also interacts with FGF signaling through mutual antagonism. In limb explant cultures, BMP and FGF signaling pathways have opposing functions in the growth plate. Furthermore, comparison of cartilage phenotypes of BMP and FGF signaling mutants indicate that these two signaling pathways antagonize each other in regulating chondrocyte proliferation and hypertrophy. The molecular mechanisms of BMP/FGF antagonism requires further study.  

Chondrocytes in the developing cartilage transduce distinct signals including Ihh, PTHrP, Wnts, FGFs, and BMPs at the same time. A complete understanding of the molecular regulation of cartilage development will not be achieved without deciphering how these signaling pathways interact with each other and coordinately control common downstream effectors inside the cell. Sox9 and Runx2 are two critical transcription factors that can integrate various signaling inputs in controlling chondrocyte proliferation and differentiation. When Sox9 was removed from differentiated chondrocytes, chondrocyte proliferation, expression of matrix genes, and the Ihh–PTHrP signaling components were reduced in the cartilage. These phenotypes are very similar to those mutant mice lacking both Sox5 and Sox6, two other Sox-family members that themselves require Sox9 for expression. Sox5 and Sox6 cooperate with Sox9 in maintaining chondrocyte phenotypes and regulating chondrocyte specific gene expression. Haploinsufficiency
of SOX9 protein in humans causes camptomelic dysplasia (CD; OMIM 114290), and defects of CD patients are recapitulated in Sox9-/- mice that show cartilage hypoplasia and a perinatal lethal osteochondrodysplasia. Chondrocyte hypertrophy is accelerated in the Sox9-/- cartilage but delayed in Sox9-overexpressing cartilage. Sox9 acts in both PTHrP and Wnt signaling pathways to control chondrocyte hypertrophy. PTHrP signaling in chondrocyte activate protein kinase A (PKA), which promotes Sox9 transcription activity by phosphorylating it. In addition, Sox9 inhibits the canonical Wnt signaling activity by promoting degradation of β-catenin, a central mediator of the canonical Wnt pathway. Thus, Sox9 is a master transcription factor that act in many critical stages of chondrocyte proliferation and differentiation as a central node inside pre-chondrocytes and chondrocytes to receive and integrate multiple signaling inputs.

Runx2 is expressed in prehypertrophic and hypertrophic chondrocytes. It is also highly expressed in perichondrial cells and in osteoblasts. The significant role of Runx2 in skeletal development is first shown by the striking phenotypes of Runx2-/- mice. These mutant mice have no osteoblast differentiation at all. Mutations in human RUNX2 cause cleidocranial dysplasia (CCD; OMIM 119600), an autosomal-dominant condition characterized by hypoplasia/aplasia of clavicles, patent fontanelles, supernumerary teeth, short stature, and other changes in skeletal patterning and growth. Runx2 is the earliest known transcription factor that is required for osteoblast differentiation from mesenchymal progenitors. Runx2 also controls chondrocyte proliferation and hypertrophy. Chondrocyte hypertrophy is significantly delayed, and Ihh expression is reduced in Runx2-/- mice, whereas Runx2 overexpression in the cartilage results in accelerated chondrocyte hypertrophy. Furthermore, removing both Runx2 and Runx3 completely blocks chondrocyte hypertrophy and Ihh expression in mice, indicating RunX transcription factors control Ihh expression. Thus, Runx2 can also be viewed as a master controlling transcription factor and a central node through which other signaling pathways are integrated in coordinate chondrocyte proliferation and hypertrophy. In chondrocytes, Runx2 acts in the Ihh–PTHrP pathway to regulate cartilage growth by controlling the expression of Ihh. However, this will not be its only function because Runx2 upregulation leads to accelerated chondrocyte hypertrophy, whereas Ihh upregulation leads to delayed chondrocyte hypertrophy. One of Runx2’s Ihh-independent activities is to act in the perichondrium to inhibit chondrocyte proliferation and hypertrophy indirectly by regulating Fgf18 expression. Interestingly, this role of Runx2 in the perichondrium is antagonistic to its role in chondrocytes. Furthermore, histone deacetylases 4 (HDAC4), which modulates cell growth and differentiation by governing chromatin structure and repressing the activity of specific transcription factors, regulates chondrocyte hypertrophy and endochondral bone formation by interacting with and inhibiting the activity of Runx2.

REGULATION OF CHONDROCYTE SURVIVAL

Cartilage is an avascular tissue that develops under a hypoxia condition because chondrocytes, particularly the ones in the middle of the cartilage, do not have access to vascular oxygen delivery. As in other hypoxia conditions, a major mediator of hypoxic response in the developing cartilage is the transcription factor hypoxia-inducible factor 1 (Hif-1) and its oxygen-sensitive component Hif-1α. Removal of Hif-1α in cartilage results in chondrocyte cell death in the interior of the growth plate. A downstream target of Hif-1 in regulating chondrocyte hypoxic response is Vegf. The extensive cell death seen in the cartilage of mice lacking Vegf has a striking similarity to that observed in mice in which Hif-1α is removed in the developing cartilage. In addition, Vegf expression in the growth plate is reduced in the absence of Hif-1α. However, Vegf expression is also regulated through Hif-1α–independent mechanisms, possibly as a result of alternative response to hypoxia in the Hif-1α cartilage-specific mutant. In this mutant, upregulation of Vegf expression and ectopic angiogenesis are observed in chondrocytes surrounding areas of extensive cell death.

CONCLUSIONS

Being able to use autologous cells and tissues to repair damaged bone and cartilage during injury and diseases has obvious advantages and will be a big step forward in regenerative medicine. With the possibility of reprogramming somatic cells into embryonic stem cells (ESCs), one may be able to fabricate cartilage or bone using one’s own reprogrammed ESCs. Because bone formation is a process that has been perfected by nature in embryos during vertebrate evolution, understanding the underlying molecular mechanism of cartilage and bone formation in embryonic development will allow us to learn the strategy in achieving directed differentiation of chondrocytes and osteoblasts from ESCs. In addition, such knowledge will significantly promote consistent cartilage or bone repair in vivo or grow functional cartilage or bone in vitro.

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Chapter 2. Signal Transduction Cascades Controlling Osteoblast Differentiation

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INTRODUCTION

Mesenchymal stem cells are pluripotent cells located in bone marrow, muscles, and fat that can differentiate into a variety of tissues, including bone, cartilage, muscle, and fat.123 Divergence toward these lineages is controlled by a multitude of cytokines, which regulate the expression of cell lineage–specific sets of transcription factors. Among the cytokines involved in osteoblast differentiation are the Hedgehogs, bone morphogenetic proteins (BMPs), TGF-β, PTH, and WNTs. The signal transduction cascades initiated by these cytokines and their effect on osteoblast differentiation will be discussed in this chapter. Osteoblasts and chondrocytes are thought to differentiate from a common mesenchymal precursor, the osteo-chondrogenic precursor (Fig. 1). The osteoblastic differentiation process can be divided into several stages, including proliferation, extracellular matrix deposition, matrix maturation, and mineralization.45 To study osteoblast differentiation, the expression level of distinct differentiation markers is used, including alkaline phosphatase (ALP), type I collagen (Col1), bone sialoprotein (BSP), osteopontin (OPN), and osteocalcin (OC). Whereas ALP is used as an early marker, OC is considered to be a late marker for osteoblast differentiation.

RUNX2 AND OSTERIX TRANSCRIPTION FACTORS

An essential event in osteoblast differentiation, and a point of convergence of many signal transduction pathways involved, is activation of the transcription factor Runx2 (also known as Cbfal) (Fig. 2). Runx2 is a master switch for osteoblast differentiation. This is shown by the fact that Runx2-deficient mice completely lack osteoblasts, fail to form hypertrophic chondrocytes, and produce a cartilaginous skeleton that is completely devoid of mineralized matrix.45 In humans, heterozygous insertions, deletions, and nonsense mutations leading to translational stop codons in the DNA-binding domain or in the C-terminal transactivating region of the Runx2 gene underlie the rare skeletal disorder cleidocranial dysplasia (CCD). CCD is characterized by defective development of the cranial bones and the complete or partial absence of the collar bones, emphasizing the importance of Runx2 in bone formation.55 By interacting with many transcriptional activators and repressors and other co-regulatory proteins, Runx2 can either positively or negatively regulate expression of a variety of osteoblast-specific genes including Col1, ALP, OPN, osteonectin (ON), and OC (Fig. 2).6–9 Runx2 also regulates expression of the zinc-finger-containing transcription factor Osterix. The promoter of the Osterix (Sp7) gene (which encodes Osterix) contains a consensus Runx2-binding sequence, which suggests that Osterix is a direct Runx2 target.100 Whereas Runx2 expression is not affected in Osterix-−/− mice, Osterix expression is lost in Runx2−/− mice, emphasizing the importance of Runx2 in bone formation.
deficient mice. Similar to mice deficient in Runx2, Osx-−/− mice lack osteoblasts, showing the requirement of this transcription factor in bone formation. Osterix can interact with nuclear factor for activated T cells 2 (NFAT2), which cooperates with Osterix in controlling transcription of target genes such as OC, OPN, ON, and Coll. Because nuclear localization of NFAT transcription factors is regulated by the Ca²⁺-calcineurin pathway, signaling pathways that modulate intracellular Ca²⁺ levels can potentially control Osterix-mediated osteoblast differentiation through NFAT activation (Fig. 3).

**FIG. 1.** Schematic model of mesenchymal stem cell differentiation toward the osteoblastic lineage and the impact of transcriptional regulators in this process. ATF4, activating transcription factor-4; Dlx, Distal Homeobox; FRA, Fos-related antigen; Osx, Osterix; Runx2, Runt-related transcription factor2.

**FIG. 2.** Schematic model of signaling pathways involved in Runx2-mediated osteoblast differentiation. Coll, collagen type 1; BSP, bone sialoprotein; OPN, osteopontin; ON, osteonectin; OC, osteocalcin; ALP, alkaline phosphatase.
FIG. 3. Schematic model of signaling pathways involved in Osterix/NFAT2-mediated osteoblast differentiation. Col1, collagen type 1; OPN, osteopontin; ON, osteonectin; OC, osteocalcin.

Other transcription factors that are involved in osteoblast differentiation are homeobox proteins such as Msx2, Dlx-3, Dlx-5, Dlx-6, and members of the activator protein 1 (AP-1) family such as Fos, Fra, and ATF4. However, deficiency of these genes does not result in complete loss of osteoblasts like in Runx2-/- mice and Osx-/- mice, pointing at a facilitory role in osteoblastogenesis.

### BMP SIGNALING

BMPs belong to the TGF-β superfamily and were originally identified as the active components in bone extracts capable of inducing bone formation at ectopic sites. BMPs are expressed in skeletal tissue and are required for skeletal development and maintenance of adult bone homeostasis and play an important role in fracture healing. Conditional knockout mice deficient in BMP ligands or Smad proteins, the intracellular mediators of BMP signaling, in bone display skeletal defects. In addition, several naturally occurring mutations in BMPs or their receptors cause inherited disorders, including fibrodysplasia ossificans progressiva (FOP), in which bone is progressively formed at ectopic sites. Thus, the BMP signaling pathway fulfills a key role in skeletal development and bone remodeling.

BMPs bind as dimers to type I and type II serine/threonine receptor kinases, forming an oligomeric complex (Fig. 2). On oligomerization, the constitutively active type II receptors phosphorylate and consequently activate the type I receptors. Subsequently, the activated type I receptors phosphorylate BMP receptor-regulated Smads, Smad1, -5, and -8, at their extreme C termini. The receptor-regulated Smads associate with the Co-Smad, Smad4, and translocate into the nucleus, where they together with other transcription factors bind promoters of target genes and control their expression (Fig. 2).

For example, Runx2 interacts with Smad1 and -5 and cooperates in controlling BMP-induced osteoblast-specific gene expression and osteogenic differentiation. Interestingly, a nonsense mutation found in a CCD patient results in expression of a truncated Runx2 mutant which displayed impaired Smad1 interaction and inhibited BMP-induced ALP activity. Moreover, BMP signaling induces expression of both BMPs and Runx2, thereby generating a positive feedback loop. Id proteins are inhibitors of basic helix-loop-helix proteins that inhibit osteoblast differentiation. Indeed, BMP-induced bone formation in vivo was found to be suppressed in Id1/Id3 heterozygous knockout mice. Furthermore, BMP2 was found to induce expression of Osterix, which besides Runx2 also seems to be mediated by the p38 and c-Jun N-terminal kinase (JNK) MAP kinase (Fig. 3).

### TGF-β SIGNALING

TGF-β is implicated in the control of proliferation, migration, differentiation, and survival of many different cell types. TGF-β is one of the most abundant cytokines in bone matrix and plays a major role in development and maintenance of the skeleton, affecting both cartilage and bone metabolism. Interestingly, TGF-β can have both positive and negative effects on bone formation depending on the context and concentration.

TGF-β signals through a similar mechanism as the related BMPs. However, on binding to its specific receptors, TGF-β induces activation of Smad2 and -3. Smad3 overexpression in mouse osteoblastic MC3T3-E1 cells enhanced the levels of bone matrix proteins, ALP activity, and mineralization. As is the case for Smad1 and -5, Runx2 also interacts with...