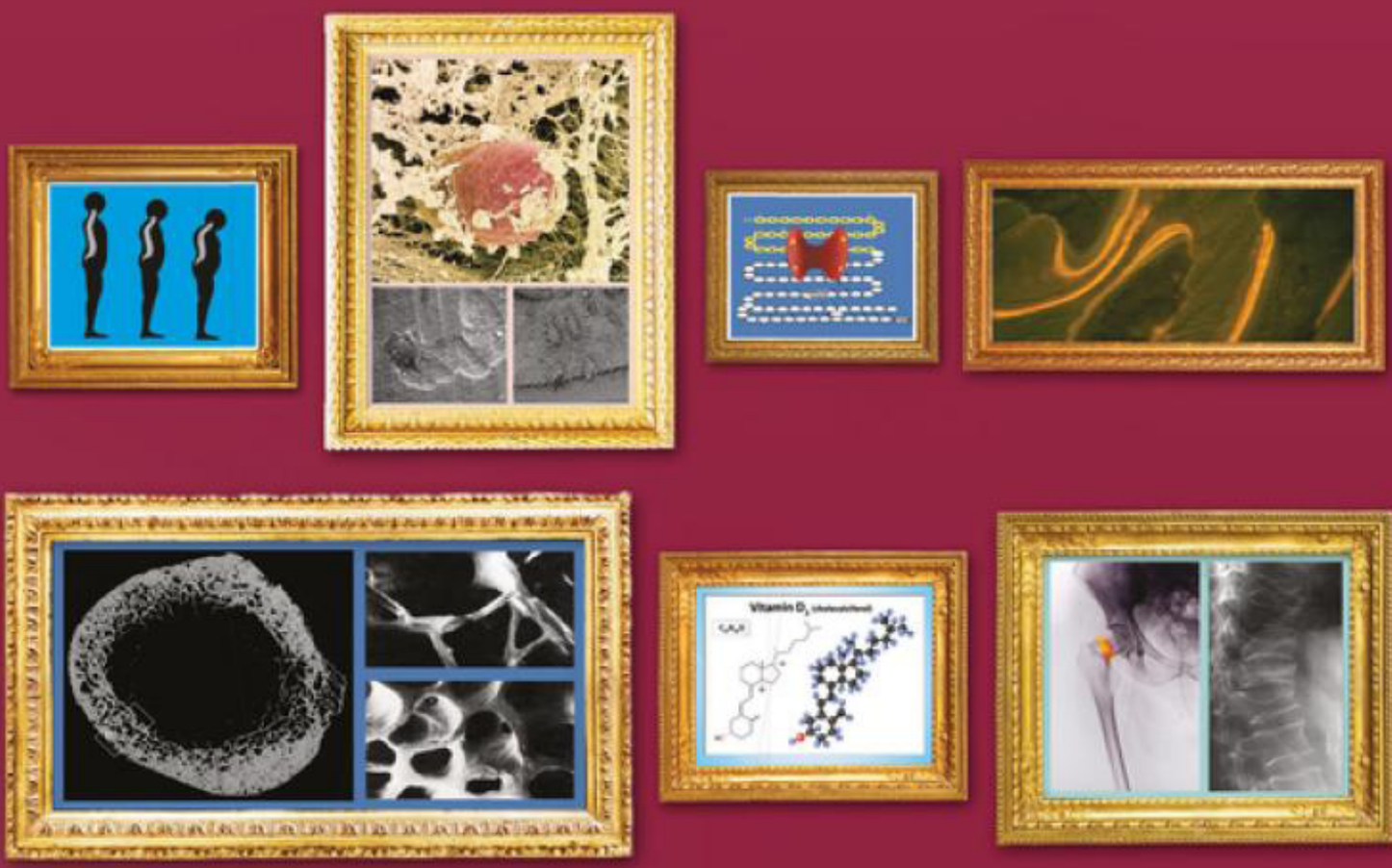
PRIMER ON THE METABOLIC BONE DISEASES AND DISORDERS OF MINERAL METABOLISM



NINTH EDITION

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Ninth Edition

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Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism

Ninth Edition

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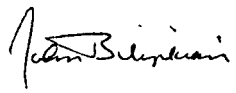
Preface to the Ninth Edition of the *Primer*

Over 30 years ago, pioneers of the ASBMR, in its early years of existence, had a remarkable vision, namely to create a *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism,* to provide '*a comprehensive, yet concise description of the clinical manifestations, pathophysiology, diagnostic approaches, and therapeutics of diseases that come under the rubric of bone and mineral disorders*.' At that time, they pointed out that there was no repository of such information in specialty textbooks. The pioneers also had the remarkable vision to appoint Murray Favus as the inaugural Editor-in-Chief of the *Primer*. The first edition was published in 1990. With enviable longevity, Murray served as Editor-in-Chief of the *Primer* for the next 18 years, when Cliff Rosen assumed the role in 2008. Under Cliff's leadership, the *Primer* continued to reign as the undisputed source of key information in our field, not only for those being introduced to our specialty but also to those of us who periodically need to be refreshed. I am pleased and honored to have been selected to serve as the Editor-in-Chief of this ninth edition of the *Primer*.

The goals of the ninth edition are to continue to provide the most accurate, up-to-date evidence-based information on basic and clinical bone science to beginners and experts, in segments that are concise and eminently readable. Attesting to the vibrancy of our field, we have seen much change since the last edition was published in 2013. The revised chapters and the new ones reflect these changes. We have broadened the authorship to include our younger generation as well as greater international representation. Fully half of the 290 authors are new to the ninth edition. Many of them are our younger stars. One-third of the authorship is from outside the USA. These two points, namely younger and international representation, represent substantial increments over the eighth edition. The highlights of our 30-year history, as represented regularly in all nine editions of the *Primer*, are illustrated on the cover. They display great advances, framed and ready to be shown in a museum!

I am grateful to Juliet Compston and Roger Bouillon for returning as Senior Associate Editors of the ninth edition and to Tom Clemens for joining us at the Senior Associate Editor level. I am grateful also to the returning Section Editors who served in this capacity in the previous edition (Doug Bauer, Suzanne Jan de Beur, Theresa Guise, Karen Lyons, Laurie McCauley, Paul Miller, Socrates Papapoulos, Ego Seeman, Raj Thakker, and Mone Zaidi) and to the Section Editors who are new to this edition (Peter Ebeling, Klaus Engelke, David Goltzman, Harald Jüppner, Mike McClung, David Roodman, Cliff Rosen, and Mike Whyte). Kudos to Ann Elderkin, Executive Director of ASBMR, who took on this added administrative responsibility, when it became necessary, and to Katie Duffy, Publications Director of ASBMR, who worked tirelessly with me, literally from the moment she joined ASBMR. I am also grateful to the Publications Committee under Bob Jilka and its current Chair, Michael Mannstadt.

Many outstanding texts in our field have been published since 1990, when there were virtually none. The *Primer*, however, still stands tall as a unique resource for the broadest, most comprehensive, and easily readable text of them all. I hope all of you gain the knowledge, wisdom, and insights that are contained in these pages. As a result, your work, whether it is basic or clinical research, or patient care, or any combination will be enhanced every time you take the *Primer* down from your real or electronic bookshelf.



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July 2018

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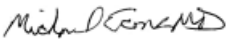
President's Preface

The ninth edition of the *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism* was developed during the 40th anniversary year of American Society for Bone and Mineral Research, a huge milestone for this seminal text that has introduced students and fellows to the field of bone, mineral, and musculoskeletal research since the first edition in 1990. We are grateful for the leadership of Editor-in-Chief John Bilezikian as well as Senior Associate Editors Tom Clemens, Juliet Compston, and Roger Bouillon and their outstanding team of 18 luminary Section Editors.

In this new edition, 11 sections capture the very cutting edge of research covering mineral homeostasis, osteoporosis, and other metabolic bone diseases, skeletal measurement technologies, genetics, and much, much more. The 135 chapters – 15 of them new for this edition – feature over 275 figures and almost 300 contributing authors from wide-ranging international research centers. Although the breadth of the *Primer* coverage is wide, John Bilezikian, the Associate Editors, and the Section Editors endeavored to condense essential materials into chapters with more compact reference lists, for easier reading and teaching.

The *Primer* represents the highest standards of collated scientific content and has evolved to include digital and print formats as well as a companion site at www.wiley.com/go/asbmrprimer, where researchers, instructors, clinicians, and students can download valuable teaching slides of tables and figures from the chapters. We hope that you will enjoy and value the extraordinary effort to capture the most current state of the field in the pages that follow.



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July 2018

About the Companion Website

This book is accompanied by a companion website:

www.wiley.com/go/asbmrprimer

The website includes:

- Videos from the ninth edition
- Editors' biographies
- Slide sets of all figures and tables from the book for downloading
- Useful website links

Section I
Molecular and Cellular Determinants of Bone Structure and Function

Section Editor: Karen Lyons

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1

Early Skeletal Morphogenesis in Embryonic Development

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INTRODUCTION

Formation of the skeletal system is one of the hallmarks that distinguish vertebrates from invertebrates. In higher vertebrates (ie, 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 to 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 are derived 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 forms the limb mesenchyme, from which limb skeletons are derived (Fig. 1.1). Ossification is one of the most critical processes in skeletal 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, differentiation, and polarity 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 morphogens including Wnts, Hedgehogs (Hhs), bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), and Notch/Delta. Recently, the Turing model [1] of pattern formation that determines skeletal formation spatially and temporally has drawn increasing attention. In his seminar paper [1], Turing proposed an ingenious hypothesis that the patterns we observe during embryonic development arise in response to a spatial prepattern in morphogens. Cells would then respond to this prepattern by differentiating in a threshold-dependent way. Thus, Turing hypothesized that the patterns we see in nature, such as skeletal structures, are controlled by a self-organizing network of interacting morphogens. The Turing model has been successfully tested in limb skeletal patterning with combined computational modeling and experimental approaches [2–5].

EARLY SKELETAL PATTERNING

In the craniofacial region, neural crest cells are major sources of cells establishing the craniofacial skeleton [6]. It is the temporal and spatial-dependent reciprocal signaling between and among the neural crest cells and the epithelial cells (surface ectoderm, neural ectoderm,

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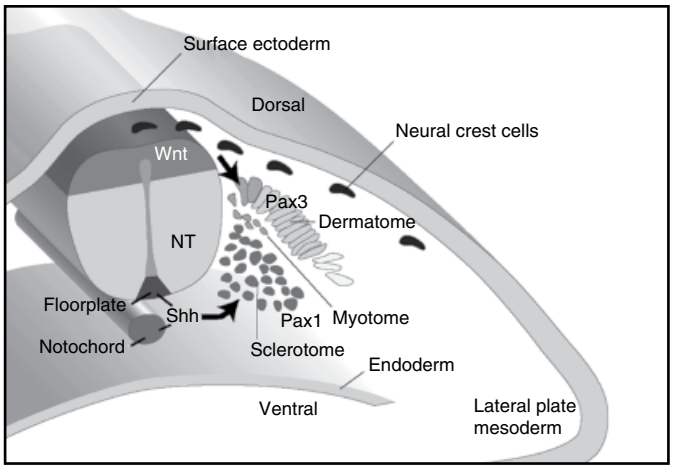

Fig. 1.1. Cell lineage contribution of chondrocytes and osteoblasts. Neural crest cells are born at the junction of dorsal neural tube and surface ectoderm. In the craniofacial region, neural crest cells from the branchial arches differentiate into chondrocytes and osteoblasts. In the trunk, axial skeletal cells are derived from the ventral somite compartment, sclerotome. Shh secreted from the notochord and floor plate of the neural tube induces the formation of sclerotome which expresses Pax1. Wnts produced in the dorsal neural tube inhibits sclerotome formation and induces dermomyotome that expresses Pax3. Cells from the lateral plate mesoderm will form the limb mesenchyme, from which limb skeletons are derived. Source: [16,17]. Reproduced with permission of Elsevier.

or endodermal cells) that ultimately establish the pattern of craniofacial skeleton formed by neural crest cells [7].

The most striking feature of axial skeleton patterning is the periodic organization of the vertebral columns along the anterior–posterior (A–P) axis. This pattern is established when somites, which are segmented mesodermal structures on either side of the neural tube and the underlying notochord, bud off at a defined pace from the anterior tip of the embryo's presomitic mesoderm (PSM) [8]. Somites give rise to axial skeleton, striated muscle, and dorsal dermis [9]. The repetitive and left–right symmetrical patterning of axial skeleton is controlled by a molecular oscillator or the segmentation clock that act in the PSM (Fig. 1.2*A*). The segmentation clock is operated by a traveling wave of gene expression (or cyclic gene expression) along the embryonic A–P axis, which is generated by an interacting molecular network of the Notch, Wnt/β-catenin, and FGF signaling pathways (Fig. 1.2*B*). Understanding molecular control of vertebrate segmentation has provided a conceptual framework to explain human diseases of the spine, such as congenital scoliosis [10].

The Notch signaling pathway mediates short-range communication between contacting cells [11]. 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 (Lfng), and the Notch ligand Delta. The Wnt/β-catenin and FGF signaling pathways mediate long-range signaling across several cell diameters. Upon activation, β-catenin is stabilized and translocates to the nucleus where it binds Lef/Tcf factors and activates expression of downstream genes. Axin2, Dkk1, Dac1, and Nkd1 are Wnt-activated negative regulators that are rhythmically expressed in the PSM. The FGF signaling pathway is also activated periodically in the posterior PSM, indicated by the dynamic phosphorylation of ERK in the mouse PSM. FGF-negative feedback inhibitors, such as Sprouty homolog 2 and 4 (Spry2 and Spry4) and Dual specificity phosphatase 4 and 6 (Dusp4 and 6), are cyclically expressed. There are extensive cross-talks among these major oscillating signaling pathways. However, current studies suggest that none of the three signaling pathways individually acts as a global pacemaker. If there is no unidentified master pacemaker, it likely that each of the three pathways has the capacity to generate its own oscillations, while interactions among them allow efficient coupling and entrain them to each other.

The retinoic acid (RA) signaling controls somitogenesis by regulating the competence of PSM cells to undergo segmentation via antagonizing FGF signaling (Fig. 1.2*A*) [12]. RA signaling has an additional role in maintaining left–right bilateral symmetry of somites by buffering asymmetric signals that establish the left–right axis of the body, particularly Fgf8 [13].

The functional significance of the segmentation clock in human skeletal development is highlighted by congenital axial skeletal diseases. Abnormal vertebral segmentation (AVS) in humans is a relatively common malformation. For instance, mutations in 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 of which exhibit vertebral column defects. However, the identified mutations explain only a minor fraction of congenital scoliosis cases. More work needs to be performed to elucidate the pathological mechanism underlying congenital and idiopathic scoliosis in human.

The formed somite is also patterned along the dorsal–ventral axis by cell signaling from the surface ectoderm, neural tube, and the notochord (Fig. 1.1). Ventralizing signals such as Sonic hedgehog (Shh) from the notochord and ventral neural tube is required to induce sclerotome formation on the ventral side [14,15], 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. 1.1) [16,17]. The sclerotome gives rise to the axial skeleton and the ribs. In the mouse mutant that lacks Shh function, the vertebral column and posterior ribs fail to form. The paired domain transcription factor Pax1 is expressed in the sclerotome and Shh is required to regulate its expression [18,19]. However, axial skeletal phenotypes in Pax1 mutant mice [20] were far less severe than those in the Shh mutants.

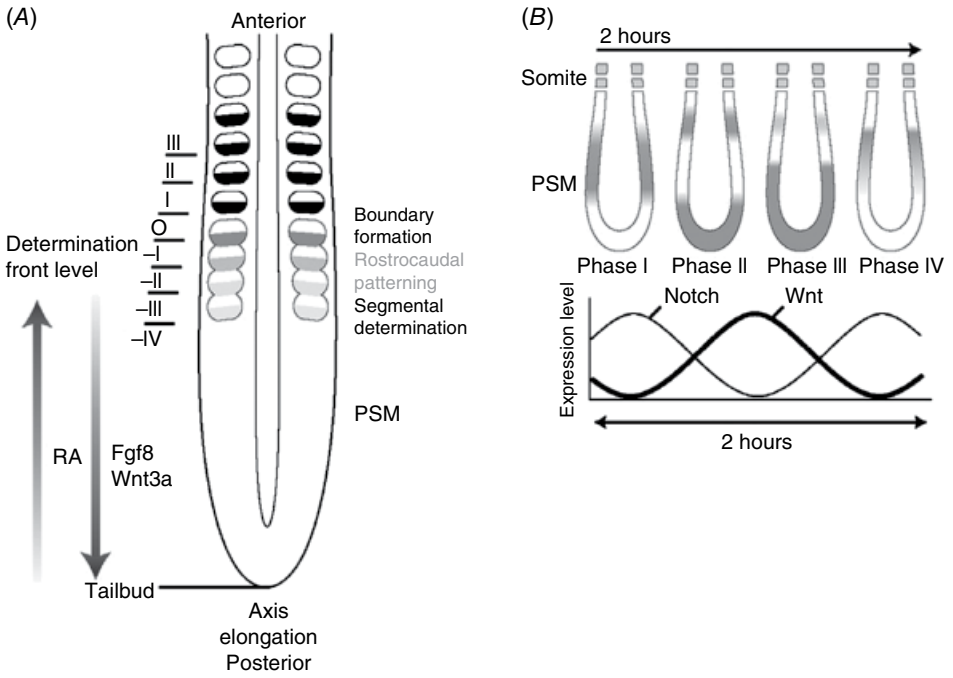

Fig. 1.2. Periodic and left–right symmetrical somite formation is controlled by signaling gradients and oscillations. (*A*) Somites form from the presomitic mesoderm (PSM) on either side of the neural tube in an anterior to posterior (A–P) wave. Each segment of the somite is also patterned along the A–P axis. Retinoic acid signaling controls the synchronization of somite formation on the left and right side of the neural tube. The most recent visible somite is marked by "0," whereas the region in the anterior PSM that is already determined to form somites is marked by a determination front that is determined by Fgf8 and Wnt3a gradients. This FGF signaling gradient is antagonized by an opposing gradient of retinoic acid. (*B*) Periodic somite formation (one pair of somite/2 hours) is controlled by a segmentation clock, the molecular nature of which is oscillated expression of signaling components in the Notch and Wnt pathway. Notch signaling oscillates out of phase with Wnt signaling.

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) axes (Fig. 1.3) [21,22]. 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, as do 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 three-dimensional limb patterning events are regulated by three signaling centers in the early limb primodium, known as 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. 1.3). Canonical Wnt signaling activated by Wnt3 induces AER formation, whereas BMP signaling leads to AER regression. FGF family members Fgf4, Fgf8, Fgf9, and Fgf17 are expressed specifically in the AER and Fgf8 alone is sufficient to mediate the function of AER. 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. It is interesting that exposure to the combined activities of distal signals (Wnt3a and Fgf8) and the proximal signal (RA) in the early limb bud or in culture maintains the potential to form both proximal and distal structures. As the limb bud grows, the proximal cells fall out of range of distal signals (Wnt3a and Fgf8) that act, in part, to keep the cells undifferentiated. Cells closer to the flank therefore differentiate and form proximal structures under the influence of proximal signals such as RA. The potential of distal mesenchymal cells becomes restricted over time to distal fates as they grow beyond the range of proximally produced RA [23,24]. Patterning of the limb bud progenitor cells into distinct segments along the P–D axis may also result in region-specific unique cellular properties such as cell sorting and aggregation behaviors that may direct their contribution toward specific skeletal elements such as the humerus or digits [25].

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. 3.3*B*). 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 [26]. Shh is expressed in the ZPA and is

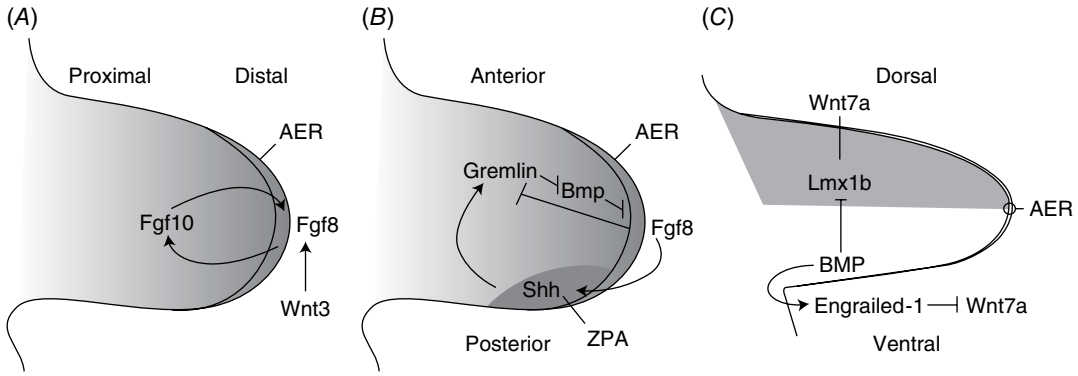

Fig. 1.3. Limb patterning and growth along the proximal–distal (P–D), anterior–posterior (A–P), and dorsal–ventral (D–V) axes are controlled by signaling interactions and feedback loops. (*A*) A signaling feedback loop between Fgf10 in the limb mesoderm and Fgf8 in the AER is required to direct P–D limb outgrowth. Wnt3 is required for AER formation. (*B*) Shh in the ZPA controls A–P limb patterning. A–P and P–D limb patterning and growth are also coordinated through a feedback loop between Shh and FGFs expressed in the AER. FGF signaling from the AER is required for Shh expression. Shh also maintains AER integrity by regulating Gremlin expression. Gremlin is a secreted antagonist of BMP signaling which promotes AER degeneration. The inhibitory feedback loop between Gremlin in the limb mesenchyme and FGFs in the AER is critical in terminating limb bud outgrowth. (*C*) D–V patterning of the limb is determined by Wnt7a and BMP signaling through regulating the expression of Lmx1b in the limb mesenchyme.

both necessary and sufficient to mediate ZPA activity in patterning digit identity along the A–P axis [27]. 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, Alx4, and basic helix-loop-helix (bHLH) transcription factors dHand and Twist1. The Gli3 repressor form (Gli3R) and Alx4 establish the anterior limb territory by restricting dHand expression to the posterior limb, which in turn activates Shh expression [28,29]. The activity of dHand in the posterior limb is also antagonized by Twist1 via a dHand-Twist1 heterodimer. Recently, the zinc finger factors Sall4 and Gli3 have been found to cooperate for proper development of the A–P skeletal elements and also function upstream of Shh-dependent posterior skeletal element development [30].

Mutations in the human *TWIST1* gene cause Saethre–Chotzen syndrome (SCS, OMIM #101400), one of the most commonly inherited craniosynostosis conditions. The hallmarks of this syndrome are premature fusion of the calvarial bones and limb abnormalities. Mutations in the *GLI3* gene also cause limb malformations including Greig cephalopolysyndactyly syndrome (GCPS, OMIM #175700) and Pallister–Hall syndrome (PHS, OMIM #146510).

The third signaling center is the non-AER limb ectoderm that covers the limb bud. It sets up the D–V polarity of not only the ectoderm but also the underlying mesoderm (Fig. 1.3*C*) (review by [21,31]). Wnt and BMP signaling are required to control D–V limb polarity. *Wnt7a* is expressed specifically in the dorsal limb ectoderm and it activates the expression of *Lmx1b*, which encodes a dorsal-specific LIM homeobox transcription factor that determines the dorsal identity. *Wnt7a* expression in the ventral ectoderm is suppressed by En-1, which encodes a transcription factor that is expressed specifically in the ventral ectoderm. The BMP signaling pathway is also ventralizing in the early limb (Fig. 1.3*C*). It appears that the effects of BMP signaling are mediated by Msx1 and Msx2, two transcription factors that are also transcriptionally regulated by BMP signaling. The function of BMP signaling in the early limb ectoderm is upstream of En-1 in controlling D–V limb polarity [32]. However, when BMPRIA is specifically inactivated only in the mouse limb bud mesoderm, the distal limb is dorsalized without altering the expression of Wnt7a and En-1 in the limb ectoderm [33]. Thus, BMPs also have En-1-independent ventralization activity by directly signaling to the limb mesenchyme to inhibit *Lmx1b* expression.

Limb development is a coordinated three-dimensional event. Indeed, the three signaling centers interact with each other through interactions of the mediating signaling molecules. First, there is a positive feedback loop between Shh and FGFs expressed in the AER, which connects A–P limb patterning with P–D limb outgrowth (Fig. 1.3*B*) [21,22]. This positive feedback loop is antagonized by an FGF/Grem1 inhibitory loop that attenuates strong FGF signaling and terminates limb outgrowth signals in order to maintain a proper limb size [34]. Second, the dorsalizing signal Wnt7a is required for maintaining the expression of Shh that patterns the A–P axis [35,36]. Third, Wnt/β-catenin signaling has been found to be both distalizing and dorsalizing [37–39].

Identification of these interacting signaling networks in early limb patterning has provided a fertile ground to test the self-organizing Turing models [1] that simulate the pattern of digit formation in the limb. By combining experiments and modeling, a self-organizing Turing network implemented by BMP, Sox9, and Wnt has been found to drive digit specification. When modulated by morphogen gradients, the network is able to recapitulate

the expression patterns of Sox9 in the wild type and in perturbation experiments [2]. Interestingly, the Turing model is also found to explain the dose effects of distal *Hox* genes in modulating the digit period or wavelength [3]. Progressive reduction in *Hoxa13* and *Hoxd11-Hoxd13* genes from the Gli3-null background results in progressively more severe polydactyly, displaying thinner and densely packed digits.

Recently, the generality and contribution of this Turing network implemented by BMP, Sox9, and Wnt to the morphological diversity of fins and limbs has been further explored [5]. Is has been suggested that the skeletal patterning of the catshark *Scyliorhinus canicula* pectoral fin is likely driven by a deeply conserved BMP–Sox9–Wnt Turing network. Therefore, the union of theory and experimentation is a powerful approach to not only identify and validate the minimal components of a network regulating digit pattern, but also to ask a new set of questions that will undoubtedly be answered as a result of the continued merging of disciplines.

EMBRYONIC CARTILAGE AND BONE FORMATION

The early patterning events determine where and when the mesenchymal cells condense, though the mechanism remains to be elucidated. Subsequently, osteochondral 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 [40,41], are both expressed in osteochondral progenitor cells, but Sox9 expression precedes that of Runx2 in the mesenchymal condensation in the limb [42]. Early Sox9-expressing cells give rise to both chondrocytes and osteoblasts regardless of ossification mechanisms [43]. Loss of Sox9 function in the limb leads to loss of mesenchymal condensation and Runx2 expression [42]. Coexpression of Sox9 and Runx2 is terminated upon chondrocyte and osteoblast differentiation when Sox9 and Runx2 expression is quickly segregated into chondrocytes and osteoblasts respectively. The mechanism controlling lineage-specific Sox9 and Runx2 expression is fundamental to the regulation of chondrocyte and osteoblast differentiation and the determination of ossification mechanisms. 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 Wnt/β-catenin signaling is detected in the developing calvarium and perichondrium where osteoblasts differentiate through either intramembranous or endochondral ossification. Indeed, enhanced Wnt/β-catenin signaling enhanced bone formation and Runx2 expression, but inhibited chondrocyte differentiation and Sox9 expression [44–46]. Conversely, removal of β-catenin in osteochondral progenitor cells resulted in ectopic chondrocyte differentiation at the expense of osteolasts during both intramembranous and endochondral ossification [46–48]. Therefore, during intramembranous ossification, Wnt/β-catenin signaling levels in the condensation are higher, which promotes osteoblast differentiation while inhibiting chondrocyte differentiation. During endochondral ossification, however, Wnt/β-catenin signaling in the condensation is initially lower, such that only chondrocytes can differentiate. Later, when Wnt/β-catenin signaling is upregulated in the periphery of the cartilage, osteoblasts will differentiate. It is likely that by manipulating Wnt signaling, mesenchymal progenitor cells, and perhaps even mesenchymal stem cells, can be directed to form only chondrocytes, which are needed to repair cartilage damage in osteoarthritis, or only form osteoblasts, which will lead to new therapeutic strategies to treat osteoporosis. These studies have provided new insights into 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 [49]. Ihh is expressed in newly differentiated chondrocytes and Ihh signaling does not seem to affect chondrocyte differentiation from mesenchymal progenitors. However, when Hh signaling is inactivated in the perichondrium cells, they ectopically form chondrocytes that express Sox9 at the expense of Runx2. This is similar to what has been observed in the Osterix (Osx) mutant embryos, except that in the $Osx^{-/-}$ embryos, ectopic chondrocytes express both Sox9 and Runx2 [50], suggesting that Runx2 is not sufficient to inhibit Sox9 expression and chondrocyte 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 in the developing calvarium or Hh signaling is activated in a ligand-independent manner in the developing calvarium. Indeed, it has been recently found that in the rare human genetic disease progressive osseous heteroplasia (POH), which is caused by null mutations in GNAS that encodes Gαs, Hedgehog signaling is upregulated. Such activation of Hh signaling is independent of Hh ligands and is both necessary and sufficient to induce ectopic osteoblast cell differentiation in soft tissues [51]. Importantly, GNAS gain-of-function mutations upregulate Wnt/β-catenin signaling in osteoblast progenitor cells, resulting in their defective differentiation and fibrous dysplasia [52]. Therefore, studies of human genetic diseases identify Gαs as a key regulator of proper osteoblast differentiation through its maintenance of a balance between the Wnt/β-catenin and Hedgehog pathways.

Both Wnt/β-catenin and Ihh signaling pathways are required for endochondral bone formation. To understand which one acts first, a genetic epistatic test was carried out [53]. These studies found that β-catenin is required downstream of not just Ihh, but also Osx in promoting osteoblast maturation. By contrast, Ihh signaling is not required after Osx expression for osteoblast differentiation [54]. 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.

BMPs are the transforming growth factor (TGF) superfamily members that were identified as secreted proteins able to promote ectopic cartilage and bone formation [55]. 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 for the past two decades. During this time, our understanding of BMP action in chondrogenesis and osteogenesis has benefited greatly from molecular studies of BMP signal transduction [56]. Reducing BMP signaling by removing BMP receptors leads to impaired chondrocyte and osteoblast differentiation and maturation [57].

FGF ligands and FGF receptors (FGFR) are both expressed in the developing skeletal system. 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 as complete genetic inactivation of FGF signaling in mesenchymal condensation has not been achieved. Nevertheless, it is clear that FGF signaling acts in mesenchymal condensation to control osteoblast differentiation during intramembranous bone formation. Mutations in FGFR 1, 2 and 3 cause craniosynostosis (premature fusion of the cranial sutures). The craniosynostosis syndromes involving FGFR 1, 2, 3 mutations include Apert syndrome (AS, OMIM #101200), Beare-Stevenson cutis gyrata (OMIM #123790), Crouzon syndrome (CS, OMIM #123500), Pfeiffer syndrome (PS, OMIM #101600), Jackson-Weiss syndrome (JWS, OMIM #123150), Muenke syndrome (MS, OMIM #602849), crouzonodermoskeletal syndrome (OMIM #134934) and osteoglophonic dysplasia (OGD, OMIM #166250), a disease characterized by craniosynostosis, a prominent supraorbital ridge, and a depressed nasal bridge, as well as rhizomelic dwarfism and nonossifying bone lesions. All these mutations are autosomal dominant and many of them are activating mutations of FGF receptors. FGF signaling can promote or inhibit osteoblast proliferation and differentiation depending on the cell context. It does so either directly or through interaction with the Wnt and BMP signaling pathways.

Apart from having the right types of cells and proper size, cartilage and bone also have distinct morphologies which are required for their function. For example, the limb and long bones preferentially elongate along the P–D axis. It is well understood that Wnts can act as morphogens by forming gradients that specify distinct cell types in distinct spatial orders by inducing the expression of different target genes at threshold concentrations. In this regard, morphogen gradients provide quantitative information to generate a distinct pattern by coordinating cell proliferation and differentiation. Because the limbs are elongated organs instead of a three-dimensionally symmetrical ball, directional information has to be provided during limb and long bone elongation.

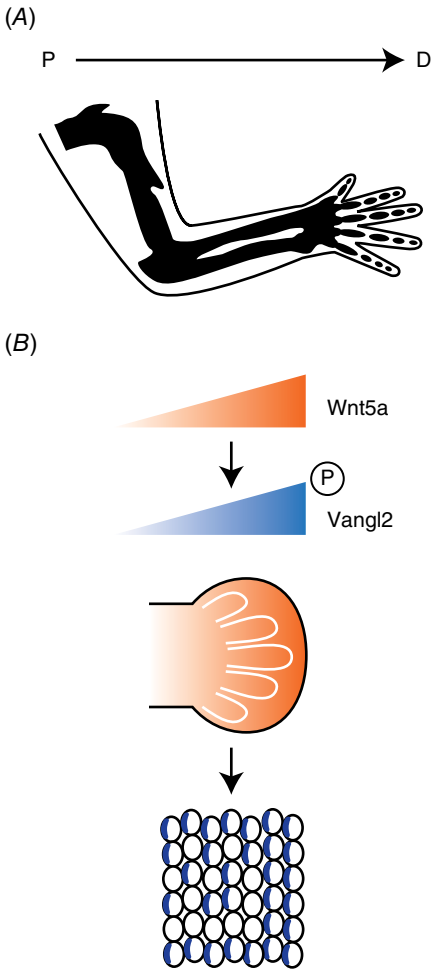

Fig. 1.4. Wnt5a gradient controls directional morphogenesis by regulating Vangl2 phosphorylation and asymmetrical localization. (*A*) Schematics of skeletons in a human limb that preferentially elongates along the proximal–distal (P–D) axis. (*B*) A model of a Wnt5a gradient controlling P–D limb elongation by providing a global directional cue. Wnt5a is expressed in a gradient (orange) in the developing limb bud and this Wnt5a gradient is translated into an activity gradient of Vangl2 by inducing different levels of Vangl2 phosphorylation (blue). In the distal limb bud of an E12.5 mouse embryo showing the forming digit cartilage, the Vangl2 activity gradient then induces asymmetrical Vangl2 localization (blue) and downstream polarized events.

Although the molecular mechanism underlying such directional morphogenesis was poorly understood in the past, there is evidence that alignment of the columnary chondrocytes of the growth plate might be regulated by planar cell polarity (PCP) during directional elongation of the formed cartilage [58]. PCP is an evolutionarily conserved pathway that is required in many directional morphogenetic processes including left–right asymmetry, neural tube closure, body axis elongation and brain wiring [59]. Recently, a major breakthrough has been made by demonstrating that newly differentiated chondrocytes in the developing long bones in the limb are polarized along the P–D axis. For the first time it was found with a definitive molecular marker, Vangl2 protein, a core regulatory component in the PCP pathway. Vangl2 protein is asymmetrically localized on the proximal side of the Sox9 positive chondrocytes, not in Sox9 negative interdigital mesenchymal cells [60]. Importantly, Vangl2 protein asymmetrical localization requires a Wnt5a signaling gradient. In the $Wnt5a^{-/-}$ mutant limb, the cartilage forms a ball-like structure and Vangl2 is symmetrically distributed on the cell membrane (Fig. 1.4). PCP mutations in the *WNT5a* and *ROR2* genes have been found in skeletal malformations such as the Robinow syndrome and brachydactyly type B1, which both exhibit short-limb dwarfisms [61–65]. In addition, mutations in PCP signaling components such as VANGL1 has been found in adolescent idiopathic scoliosis (AIS).

CONCLUSION

Skeletal formation is a process that has been perfected by nature in embryos during vertebrate evolution. Understanding the underlying molecular mechanisms of cartilage and bone formation in embryonic development will advance our knowledge of vertebrate embryonic morphogenesis in general. This knowledge will allow us to develop the strategy to promote skeletal tissue repair by endogenous cells or rejuvenate old skeletal tissues without having to use cells cultured in vitro. In addition, to use autologous cells and tissues or iPS (induced pluripotent stem) cells to repair bone and cartilage damaged during injury and disease, we require a more complete knowledge of skeletal development so that cartilage or bone can be fabricated using the body's own cells. Understanding skeletal development is indispensable for understanding pathological mechanisms of skeletal diseases, finding therapeutic targets, promoting consistent cartilage or bone repair in vivo, and eventually growing functional cartilage or bone in vitro.

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2
Endochondral Ossification

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INTRODUCTION

A defining feature of vertebrates is the presence of a mineralized skeleton. Aside from giving animals their characteristic shape, the skeleton provides diverse functions including protecting internal organs, supporting body mass and movement, production of blood cells, calcium storage, and endocrine signaling. The skeleton is comprised primarily of two tissues, cartilage and bone, which are formed embryonically by chondrocytes and osteoblasts respectively. During skeletal development, these specialized cells are derived from a common mesenchymal progenitor of either neural crest origin in the craniofacial region or mesodermal origin for bones formed elsewhere in the body. Bones develop via two distinct mechanisms: intramembranous or endochondral ossification. Intramembranous ossification is responsible for forming specific parts of the skull and the clavicle, whereby mesenchymal progenitors differentiate directly into osteoblasts responsible for secreting bone matrix. In contrast, endochondral ossification, the process responsible for generating most of the skeleton, requires a cartilage intermediate before forming bone. Here we will discuss the major cellular events of endochondral ossification: chondrogenesis, chondrocyte hypertrophy, and osteoblast differentiation, as well as important molecular mediators governing each of these processes.

CHONDROGENESIS AND CHONDROCYTE HYPERTROPHY DURING ENDOCHONDRAL OSSIFICATION

Bones within the limbs serve as the model for endochondral ossification. Development of the limb skeleton initiates during embryogenesis via the migration of multipotent mesenchymal progenitors from the lateral plate mesoderm into the developing limb field. These progenitors rapidly proliferate, expanding the limb bud, followed by the formation of condensations ultimately giving rise to cartilage anlagen (Fig. 2.1*A*,*B*). During the condensation phase, mesenchymal progenitors express multiple cell adhesion related molecules such as: *N-cadherin* (*Ncad*), *N-cam* (*Ncam1*), and *tenascin C* (*Tnc*) aiding in mesenchymal cell compaction. Cells within condensations undergo differentiation to generate mature chondrocytes (cartilage cells), a process known as chondrogenesis (Fig. 2.1*A*,*B*). Newly formed chondrocytes take on a characteristic round shape, continue to proliferate, and begin producing an extracellular matrix rich in type II, type IX, and type XI collagens (COL2A1, COL9A1, COL11A1) and the proteoglycan, aggrecan (ACAN). As cartilage rudiments continue to grow, chondrocytes nearest the epiphyseal ends maintain their round appearance and reduce their proliferative index, whereas chondrocytes near the center of rudiments

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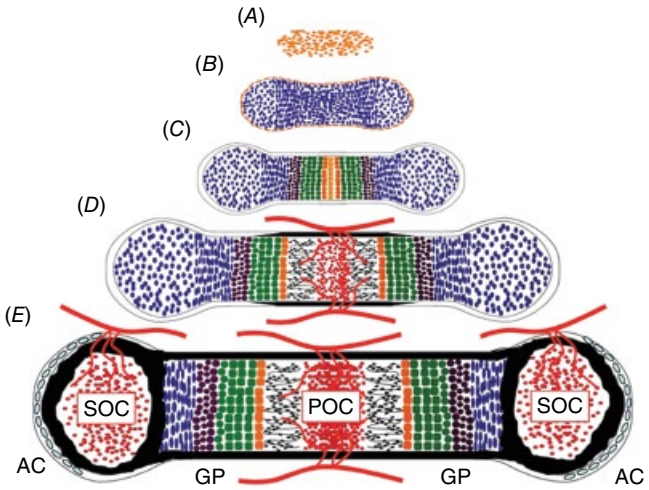

Fig. 2.1. Stages of endochondral ossification. (*A*) Mesenchymal condensation (orange cells = mesenchymal progenitors). (*B*) Chondrogenesis (blue cells = chondrocytes; orange cells = perichondrial progenitors. (*C*) Chondrocyte hypertrophy (blue cells = epiphyseal round chondrocytes and flat columnar chondrocytes; purple cells = prehypertrophic chondrocytes; green cells = hypertrophic chondrocytes; orange cells = late stage hypertrophic chondrocytes. (*D*) Formation of the primary ossification center (POC) (all cells colored as described above; blood vessels and marrow cells in red; osteoblasts and bone matrix in black). (*E*) Formation of secondary ossification center (SOC) separates the articular chondrocytes (AC) from growth plate (GP) chondrocytes (light blue cells = articular chondrocytes; all other cells/tissues as previously described).

enhance their rate of proliferation, adopt a flattened appearance, and align into columns, driving longitudinal growth of the cartilage elements (Fig. 2.1*C*). A combination of chondrogenesis and chondrocyte proliferation establishes the early cartilaginous skeleton, which serves as the template for endochondral bone development.

Calcification and ossification of the endochondral skeleton begins with chondrocyte hypertrophy. During this process, columnar chondrocytes located at the center of growing cartilage rudiments, also known as prehypertrophic and hypertrophic chondrocytes, undergo further differentiation after exiting the cell cycle. Hypertrophic differentiation consists of genetic programs responsible for dramatically increasing chondrocyte cell size, switching the production of type II collagen to type X collagen (COL10A1), and inducing factors responsible for calcification and vascularization of the cartilage matrix such as ALP and VEGF respectively (Fig. 2.1*C*). Hypertrophic chondrocytes express transcriptional regulators and a myriad of growth factors that not only coordinate the hypertrophic chondrocyte differentiation process, but also induce osteoblast differentiation of surrounding perichondrial cells and promote vascularization of the calcified cartilage by surrounding blood vessels, establishing a marrow cavity and primary ossification center (POC) (Fig. 1.1*D*). Late stage hypertrophic chondrocytes secrete the catabolic enzyme matrix metalloprotease 13 (MMP13), which helps to degrade the cartilage matrix. Previously coined as terminal hypertrophic chondrocytes, these cells were thought to undergo exclusively a form of programmed cell death; however, lineage tracing studies recently showed that many hypertrophic chondrocytes undergo transdifferentiation into the osteoblast lineage. The combination of calcified cartilage degradation and hypertrophic chondrocyte transdifferentiation provides both a scaffold and a cell source for the generation of bone within the POC. Concominant with the ossification process directly associated with cartilage, osteoblasts derived from perichondrial cells and perivascular mesenchymal progenitors also utilize the degrading cartilage as a scaffold for further bone formation. The continuous processes of chondrocyte proliferation, hypertrophy, calcification, vascularization, cartilage matrix degradation, transdifferentiation, and bone formation drive embryonic and postnatal endochondral bone growth.

During early postnatal endochondral ossification, round chondrocytes maintained near the epiphyseal ends of bones undergo a maturation process similar to chondrocytes during embryonic skeletogenesis. Epiphyseal chondrocytes hypertrophy, generate a calcified matrix, degrade the matrix, undergo apoptosis and/or transdifferentiation, and eventually are replaced by invading vasculature and osteoblasts to create the secondary ossification center (SOC) (Fig. 2.1*D*,*E*). This SOC serves an important support role within weight-bearing articulating joints and separates the only two areas of remaining cartilage within the adult endochondral skeleton: the articular cartilage (AC) and growth plate (GP) cartilage (Fig. 2.1*E*). As cartilage growth and turnover decreases in the postnatal or adult skeleton, the contribution of cartilage to bone formation dramatically decreases, ultimately terminating the process of endochondral ossification.

MOLECULAR MEDIATORS OF CARTILAGE DEVELOPMENT

Specific transcriptional regulators are critical in establishing the cartilage phase of the endochondral skeleton. Several Sry-box (SOX) factors are required for chondrogenesis and early cartilage development. The master regulator of cartilage development, SOX9, is expressed in mesenchymal progenitors, osteochondral progenitors, and immature chondrocytes. SOX9 controls cell morphology at the mesenchyme to chondrocyte transition [1], while also directly regulating the expression of *Col2a1*, *Col9a1*, *Col11a1*, *Acan*, and other cartilage-related genes [2]. Much of the transcriptional regulation imposed by SOX9 occurs via interactions with other SOX factors, specifically SOX5 and SOX6, which together form the SOX trio. Mouse genetic studies in which *Sox9* is either removed from the germline or specifically within the limb mesenchyme highlight the requirement for SOX9 in forming organized condensations capable of undergoing

chondrogenesis [2, 3]. Interestingly, Sox5$^{-/-}$ Sox6$^{-/-}$ double mutant mice develop normal mesenchymal cell condensations, but subsequently show impaired chondrogenesis, columnar chondrocyte disruption, and failure to maintain the chondrocyte phenotype, even though *Sox9* expression is maintained [4]. Similarly, oseteochondral progenitor cell deletion of *Sox9* results in normal condensations, but mutants subsequently develop a severe chondrodysplasia phenotype. These mouse genetic studies underscore the critical and sequential roles for SOX9, SOX5, and SOX6 during cartilage development.

Numerous developmental signaling pathways are critical during chondrogenesis and early phases of endochondral ossification. Factors such as the BMPs play key roles in the compaction of mesenchymal cells and shaping of condensations [1]. Both the BMP and related TGFβ pathways induce *Sox9* expression to promote chondrogenesis and cartilage development. Conditional mutant mouse models with *Bmpr1a* floxed alleles deleted in osteochondral progenitors of a *Bmpr1b*$^{-/-}$ background display a severe generalized chondrodysplasia in which formation of mesenchymal condensations and chondrogenic rudiments fail because of a lack of *Sox9*, *Sox5*, and *Sox6* expression [5]. SMAD proteins are intracellular mediators of BMP signaling. Genetic removal of *Smad1 and Smad5* floxed alleles in osteochondral progenitors results in reduced condensation size, more compacted cells with less cartilage matrix, decreased chondrocyte proliferation, and an increased incidence of immature chondrocyte cell death, a phenotype slightly less severe than that of BMP receptor mutant mice. These data suggest that whereas BMPs mostly exert their prochondrogenic functions via SMAD activation, alternative signaling mechanisms important for BMP-mediated regulation of endochondral ossification likely exist (Fig. 2.2) [6]. The TGFβ pathway signals through the TGFβ-related SMADS, SMAD2, and SMAD3. TGFβ1, TGFβ2, and TGFβ3 are each sufficient to induce chondrogenesis and cartilage matrix synthesis (Fig. 2.2); however, individual mutant mice have not defined a requisite role

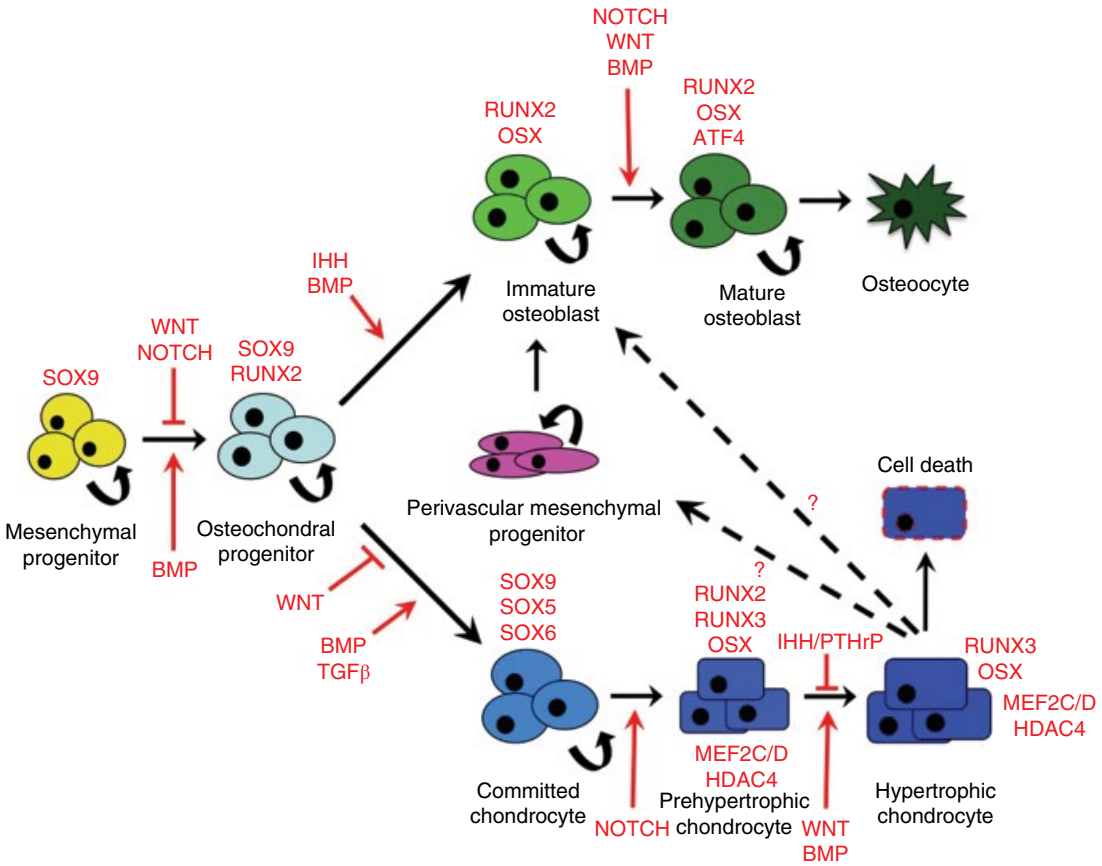

Fig. 2.2. Cell differentiation and signaling regulators of endochondral ossification. Mesenchymal progenitor cells (yellow) differentiate into osteochondral progenitors (light blue) before committing to either the osteoblast (shades of green; osteoblastogenesis) or chondrocyte lineages (shades of blue; chondrogenesis). Osteoblast differentiation proceeds from immature osteoblasts to mature osteoblasts before becoming an osteocyte. Chondrocyte differentiation proceeds from committed chondrocytes to prehypertrophic and hypertrophic chondrocytes before undergoing cell death or transdifferentiation into the osteoblast lineage (pink cells = perivascular mesenchyme progenitors). The NOTCH, BMP, TGFβ, IHH, and WNT pathways play important roles in regulating chondrogenesis, chondrocyte hypertrophy, and osteoblast differentiation during endochondral ossificaiton. Question marks indicate unknown molecular mechanisms.

for any one TGFβ ligand in regulating chondrogenesis. Analyses of *Smad2*$^{-/-}$ and *Tgfβr2* or *Smad3* conditional mutant mice in which floxed alleles were deleted from mesenchymal or osteochondral progenitors have also yet to uncover a requisite role for TGFβ signaling during overt chondrogenesis [7–9]; however, important components of the pathway that may be critical in promoting chondrogenesis have yet to be scruitinized. As an opposition to BMP/TGFβ signaling, the WNT and NOTCH signaling pathways antagonize the formation of mesenchymal condensations and inhibit chondrogenic differentiation. Activation of WNT signaling in mesenchymal progenitors using a stabilized β-catenin floxed allele (β-catenin$^{ex3/ex3}$) inhibits *Sox9*, resulting in impaired condensation formation and suppression of chondrogenesis, ultimately leading to a failure of endochondral ossification (Fig. 2.2) [10]. Conversely, genetic deletion of β-catenin floxed alleles in mesenchymal or osteochondral progenitors resulting in WNT pathway loss of function leads to enhanced *Sox9* expression and accelerated chondrogenesis at the expense of osteoblastogenesis, indicating that WNT/β-catenin signaling regulates a critical fate switch in osteochondral progenitors by inhibiting the chondrogenic fate (Fig. 2.2) [10, 11]. Similar to WNT effects on chondrogenesis, NOTCH signaling suppresses condensation formation and chondrogenic differentiation. Activation of NOTCH signaling via conditional overexpression of the NOTCH intracellular domain (NICD) within mesenchymal progenitors suppresses expression of *Sox9*, *Sox5*, and *Sox6*, disrupts formation of mesenchymal condensations, and completely blocks endochondral ossification, an effect that is overturned via the simultaneous genetic removal of the NOTCH nuclear effector, RBPjk. Analyses of mutant mice in which only *Rbpjk* floxed alleles were deleted in mesenchymal progenitors showed an increase in chondrogenesis and chondrogenic gene expression, indicating the requirement for RBPjk-dependent NOTCH signaling as a regulator of the pace of chondrogenesis and endochondral ossification (Fig. 2.2) [12]. Similar, although less severe, effects on chondrogenesis were also observed in gain- and loss-of-function studies for the NOTCH target genes, *Hes1* and *Hes5*, indicating that NOTCH regulation of chondrogenesis occurs at least partially via a HES-mediated mechanism [13]. Each of these pathways exhibits distinct functions in space and time to regulate mesenchymal condensations and chondrogenesis; however, it is likely all intersect on a common transcriptional program governed by the SOX trio.

Chondrocyte hypertrophy is coordinated by balancing the expression and activities of specific transcriptional regulators including: runt-related transcription factor 2 (RUNX2), runt-related transcription factor 3 (RUNX3), osterix (OSX), myocyte enhancer factor 2c (MEF2C), myocyte enhancer factor 2d (MEF2D), histone deacetylase 4 (HDAC4), and SOX9. RUNX2 is expressed in prehypertrophic and hypertropic chondrocytes and regulates *Col10a1*, *Alpl*, *Vegf*, and *Mmp13* expression, coordinating hypertrophic differentiation with calcification, vascularization, and catabolism of the hypertrophic cartilage matrix (Fig. 2.2). Assessments of various *Runx2*$^{-/-}$ isoform mutants, as well as conditional mutant mice in which *Runx2* floxed alleles were removed from osteochondral progenitors, show a significant delay or absence of hypertrophic differentiation, mineralization, and vascularization of cartilage elements [14, 15]. Interestingly, combined *Runx2*$^{-/-}$; *Runx3*$^{-/-}$ double mutants exhibit a more robust blockade in hypertrophic differentiation leading to a complete failure of endochondral ossification, showing an important and potentially redundant role for RUNX3 in cartilage development [16]. OSX is expressed in prehypertrophic and hypertrophic chondrocytes and is another critical regulator of chondrocyte hypertrophy, calcification, and catabolism of calicified cartilage functioning downstream of RUNX2 and RUNX3 (Fig. 2.2). Germline deletion of *Osx* or conditional removal of *Osx* floxed alleles in mesenchymal or osteochondral progenitors results in severely delayed chondrocyte hypertrophy, failed matrix calcification, and an inability to catabolize the cartilage matrix [17]. The latter effects are probably manifest because of the direct transcriptional regulation of *Mmp13* by OSX. HDAC4 is yet another critical transcriptional regulator expressed in hypertrophic chondrocytes, but not osteoblasts (Fig. 2.2). Germline deletion of *Hdac4* results in accelerated chondrocyte hypertrophy, cartilage calicifiation, and advanced endochondral ossification, whereas transgenic overexpression of *Hdac4* suppresses chondrocyte hypertrophy and endochondral ossification, likely because of the chromatin and transcriptional regulation of *Runx2* imposed by HDAC4 [18]. MEF2C and MEF2D are related transcription factors also expressed in prehypertrophic and hypertrophic chondrocytes (Fig. 2.2). Conditional deletion of *Mef2c* alone or in combination with *Mef2d* from mesenchymal or osteochondral progenitors results in failed chondrocyte hypertrophy, cartilage vascularization, and endochondral ossification reminiscent of *Runx2* mutants [19]. Genetic interactions between HDAC4 and MEF2C were shown by deleting either a single allele of *Mef2c* in a *Hdac4*$^{-/-}$ background or deleting a single *Hdac4* allele in a *Mef2c*$^{+/-}$ background resulting in normalization of their respective mutant phenotypes. Molecular studies further determined that both factors converge on the regulation of *Runx2* in controlling chondrocyte hypertrophy, calcification, vascularization, and overall endochondral ossification [19]. Finally, SOX9 not only regulates chondrogenic gene expression important for inducing and maintaining immature chondrocytes, but also coordinates the onset of hypertrophy (Fig. 2.2). Forced expression of Sox9 in hypertrophic chondrocytes delays chondrocyte maturation and inhibits both calcification and vascularization of the hypertrophic cartilage [20], whereas cartilage-specific loss of *Sox9* showed roles for SOX9 in (i) maintaining early hypertrophic chondrocytes via appropriate transcriptional regulation of *Col10a1* and (ii) acting as a counterbalance to RUNX2 and OSX activities, preventing osteoblast differentiation of chondrocytes and excessive endochondral ossification [21].

Multiple signaling molecules regulate chondrocyte hypertrophy in both direct and indirect manners. Indian hedgehog (IHH) and PTHrP form a negative feedback loop critical for coordinating chondrocyte hypertrophy and endochondral ossification. IHH is secreted from prehypertrophic chondrocytes and directly regulates PTHrP in epiphyseal chondrocytes, which in turn signals to its receptor, PTHrP-R, on prehypertrophic chondrocytes to antagonize the pace of chondrocyte hypertrophy (Fig. 2.2). Germline deletions of *Ihh* and *Ptrhp* both exhibit reduced chondrocyte proliferation and precocious chondrocyte hypertrophy; however, *Pthrp* mutants generate accelerated bone formation whereas *Ihh* mutants fail to form bone because of a critical function of IHH in osteoblast differentiation from perichondrial progenitors (see later) [22–24]. Cartilage specific deletions of *Smoothened* (*Smo*), a critical cell surface protein mediating hedgehog signaling, aided in uncoupling IHH effects on chondrocyte hypertrophy and endochondral ossification from perichodrial bone formation because mutants developed accelerated chondrocyte hypertrophy without defects in perichondrial bone formation [25]. A critical intracellular function of IHH signaling is to antagonize GLI3 repressor function while activating other GLI family members (GLI1 and GLI2). Analyses of $Ihh^{-/-}$; $Gli3^{-/-}$ mutant mice identified the requirement for GLI3 in mediating IHH actions on PTHrP, chondrocyte hypertrophy, and endochondral ossification, but not on vascularization of the cartilage or perichondrial osteoblast differentiation [26, 27]. Finally, PTHrP induces dephosphorylation of HDAC4 in turn decreasing HDAC4 and 14-3-3 protein interactions in the cytoplasm to promote nuclear translocation of HDAC4 and repression of MEF2C transcriptional activation of *Runx2*, thereby establishing an IHH/PTHrP-HDAC4-MEF2C-RUNX2 molecular regulation of chondrocyte hypertrophy and endochondral ossification [28]. Antagonistic to the IHH/PTHrP pathway, both BMP and WNT signaling promote chondrocyte hypertrophy (Fig. 2.2). Conditional removal of *Smad1* and *Smad5* floxed alleles from osteochondral progenitors [6] or *Bmpr1a* floxed alleles from osteochondral progenitors in a $Bmpr1b^{+/-}$ background [29] defined an extracellular to intracellular signaling cascade leading to BMP-mediated chondrocyte proliferation, survival, hypertrophy, and proper endochondral ossification; a cascade culminating in SMAD-mediated regulation of IHH signaling and transcriptional control of *Runx2*. Similarly, conditional deletion of β-catenin floxed alleles in mesenchymal and osteochondral progenitors showed the role WNT/β-catenin plays in promoting chondrocyte hypertrophy, calcification, vascularization, and endochondral ossification via β-catenin induced degradation and antagonism of SOX9 combined with transcriptional activation of *Osx* (Fig. 2.2) [10, 11, 30, 31]. NOTCH signaling also promotes chondrocyte hypertrophy and endochondral ossification (Fig. 2.2), via alternative mechanisms, however. First, both in vivo and in vitro lines of evidence support NOTCH suppression of chondrocyte proliferation and cell cycle exit during early chondrocyte hypertrophy [32]. Second, conditional deletion of *Notch1* and *Notch2* floxed alleles from mesenchymal progenitors or RBPjk floxed alleles from mesenchymal progenitors, osteochondral progenitors, or chondrocytes leads to delayed onset of chondrocyte hypertrophy and cartilage matrix catabolism, whereas overexpression of NICD in chondrocytes promotes these processes [32–34]. Molecular dissection of the pathway indicates that NOTCH indirectly promotes chondrocyte hypertrophy in an RBPjk-dependent manner via HES/HEY-mediated downregulation of *Sox9*, *Col2a1*, *Acan,* and other chondrogenic genes [13], while simultaneously inducing cartilage catabolism and turnover of growth plate cartilage via RBPjk-mediated induction of numerous catabolic genes including *Mmp13* [32, 35]. Each of the aforementioned transcriptional regulators and signaling pathways impart profound effects on chondrocyte hypertrophy and maturation during endochondral ossification. However, the identification of signals positively or negatively regulating hypertrophic chondrocyte transdifferentiation into osteoblast lineage cells (mesenchymal progenitors or committed osteoblasts) remains to be elucidated (Fig. 2.2).

OSTEOBLAST DIFFERENTIATION AND BONE FORMATION

Osteoblasts are responsible for producing and secreting a combination of extracellular proteins that comprise the bone matrix. These include copious amounts of type 1 collagen (COL1A1) and noncollagenous matrix proteins including ALPL, integrin-linked bone sialoprotein (IBSP), and osteocalcin (BGLAP), which serve as markers of distinct stages of osteoblast differentiation in addition to regulating diverse aspects of bone matrix mineralization. The process of osteoblast differentiation begins with condensation of multipotent mesenchymal progenitors, specification of osteochondral progenitors, formation of committed preosteoblasts, differentiation into mature functional osteoblasts, and finally encasement in bone matrix to form osteocytes (Fig. 2.2). Unlike osteoblasts that secrete the bone matrix, osteocytes function as mechanosensory cells that transduce mechanical loads into biochemical signals to regulate osteoblast differentiation and bone formation. Recent studies highlight the diverse cellular sources that can give rise to osteoblasts, including perichondrial cells, perivascular mesenchymal progenitors (pericytes) brought in during vascular invasion, circulating progenitors, hypertrophic chondrocytes [36, 37], and other mesenchymal cells within the bone marrow (see also Chapters 3, 4, and 5). Extensive studies over the past few decades have identified a number of transcription factors and developmental signals that regulate osteoblast differentiation. Differentiating osteoblasts are characterized by the expression of master transcriptional regulators including *Sox9* (expressed in mesenchymal progenitors), *Runx2* (expressed in osteochondral progenitors and mature osteoblasts),

Osterix (Osx, preosteoblasts and mature osteoblasts), and *Atf4* (mature osteoblasts) (Fig. 2.2). RUNX2 is indispensable for osteoblast differentiation and promotes the differentiation of osteochondral progenitors into preosteoblasts as well as the proper function of mature osteoblasts. Homozygous deletion of *Runx2* in mice results in a complete loss of osteoblasts. Like RUNX2, OSX is required for osteoblast differentiation and bone formation but functions downstream of *Runx2*. OSX is required for the transition from the preosteoblast to a functional mature osteoblast. Homozygous deletion of *Osx* in mice results in a thickened perichondrium at the diaphysis because of a failure of osteoblast differentiation. OSX is also crucial for postnatal osteoblast differentiation and function. ATF4 is required for terminal differentiation and regulates bone-forming activities in mature osteoblasts. Homozygous deletion of *Atf4* results in delayed osteoblast differentiation and decreased bone formation. ATF4 is dispensable for *Runx2* and *Osx* expression but coregulates *Ibsp* and *Bglap* expression with RUNX2. ATF4 promotes amino acid uptake to facilitate protein synthesis and bone matrix production by osteoblasts. This appears to be the primary function of ATF in osteoblasts as a high protein diet can correct the bone phenotypes of $Atf4^{-/-}$ mice.

Developmental signals such as NOTCH, IHH, WNT, and BMP are required at different stages and play unique roles in osteoblast differentiation. NOTCH signaling plays an important role in maintaining an osteochondral progenitor pool to provide osteoblasts throughout life. NOTCH signaling maintains the osteochondral progenitor pool by inhibiting RUNX2 transcriptional activity and preventing osteoblast differentiation (Fig. 2.2). Loss of this blockade through genetic inhibition of NOTCH signaling in mesenchymal progenitors results in exuberant differentiation and bone formation early in life with a severe reduction of bone mass later because of exhaustion of the progenitor pool. In committed osteoblasts, forced NOTCH activation inhibits terminal differentiation and stimulates osteoblast activity and bone formation by expanding the number of active osteoblasts resulting in sclerotic bone formation [41]. However, genetic ablation of NOTCH signaling in committed osteoblasts results in no discernable phenotype, underscoring the uncertainty of a physiological role for NOTCH signaling in mature osteoblasts. Conversely, Hedgehog signaling is required to initiate osteoblast differentiation. IHH, expressed in prehypertrophic and hypertrophic chondrocytes, signals to adjacent perichondrial cells to initiate osteoblast differentiation by regulating *Runx2* and *Osx* expression (Fig. 2.2). The WNT pathway similarly promotes osteoblast differentiation but functions downstream of IHH (Fig. 2.2) (also see Chapter 9). The WNT transcriptional effector β-catenin (encoded by *Catnnb1*) is required for osteoblast differentiation. Genetic deletion of *Catnnb1* in mesenchymal progenitors abolishes osteoblast formation and results in ectopic cartilage formation. Both β-catenin dependent and independent WNT signaling are required for progression from the *Runx2* positive progenitor to the *Osx* positive preosteoblast and from the preosteoblast to the mature osteoblast stages [11, 31, 44, 45]. Recent studies implicate cellular metabolism as a target of WNT regulation during differentiation. WNT stimulates glucose uptake, which favors osteoblast differentiation by increasing RUNX2 expression and activity [46, 47]. In mature osteoblasts, WNT stimulates glutamine catabolism which increases ATF4 translation to stimulate osteoblast activity and terminal differentiation [48]. Like WNT signaling, BMPs play multiple roles in regulating osteoblast differentiation [49]. BMP signaling directly regulates both *Runx2* and *Osx* expression and is required to form preosteoblasts (Fig. 2.2) (see also Chapter 8). Later, BMP promotes differentiation by suppressing proliferation in preosteoblasts and stimulating osteoblast activity. BMP signaling ultimately regulates osteoblast activity and bone formation by increasing ATF4 protein expression downstream of the unfolded protein response [50]. Combined, these signals cooperate in an elaborate and elegant web to coordinate endochondral ossification.

CONCLUSION

Here we have provided a general overview of endochondral ossification with a focus on the major cellular events (chondrogenesis, chondrocyte hypertrophy, and osteoblast differentiation), transcription factors (SOX trio, RUNX2, OSX, HDAC4, MEF2C/D, and ATF4), and signaling pathways (BMP/TGFB, WNT, IHH/PTHrP, and NOTCH) governing each stage of the process. In particular, we highlighted critical murine studies using sophisticated genetic approaches to determine the function(s) for many of the transcriptional regulators and signaling molecules important in coordinating proper development of the endochondral skeleton.

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