Development of the Endochondral Skeleton

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SUMMARY

Much of the mammalian skeleton is composed of bones that originate from cartilage templates through endochondral ossification. Elucidating the mechanisms that control endochondral bone development is critical for understanding human skeletal diseases, injury response, and aging. Mouse genetic studies in the past 15 years have provided unprecedented insights about molecules regulating chondrocyte formation, chondrocyte maturation, and osteoblast differentiation, all key processes of endochondral bone development. These include the roles of the secreted proteins IHH, PTHrP, BMPs, WNTs, and FGFs, their receptors, and transcription factors such as SOX9, RUNX2, and OSX, in regulating chondrocyte and osteoblast biology. This review aims to integrate the known functions of extracellular signals and transcription factors that regulate development of the endochondral skeleton.

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1 INTRODUCTION

The osseous tissues in mammals are formed via two distinct processes during embryogenesis. Intramembranous bone formation produces many of the craniofacial bones directly from mesenchymal condensations. In contrast, endochondral ossification, the principal process responsible for forming much of the mammalian skeleton, generates bone via a cartilage intermediate. The transition from cartilage to bone is tightly coupled with chondrocyte, osteoblast, and vascular differentiation. This article focuses on the current understanding of the molecular basis for several key processes of endochondral ossification, including formation of the mesenchymal condensation, chondrocyte differentiation and maturation, and osteoblast development.

2 MESENCHYMAL CONDENSATION

Endochondral bone development begins with the condensation of mesenchymal cells of either neural crest in the craniofacial region (e.g., middle ear bones and temporal bones) or mesoderm elsewhere in the body (Fig. 1A). Formation of a mesenchymal condensation is a prerequisite for subsequent chondrogenic differentiation (Thorogood and Hinchliffe 1975). In the limb bud the condensation forms mainly through active congregation of cells without changes in cell proliferation. These condensations can be visualized by the dense packing of cells, the high affinity to the lectin peanut agglutinin, and the transient up-regulation of versican, tenascin, syndecan, N-CAM, and N-cadherin (Hall and Miyake 2000).

The molecular drivers for mesenchymal condensation are not well understood. Based on expression analyses as well as studies with neutralizing antibodies, N-CAM and N-cadherin, which mediate Ca^{2+} -dependent and -independent cell–cell adhesion, respectively, are implicated in mediating the cell–cell adhesion during condensation (DeLise et al. 2000). However, N-CAM-deficient mice developed to adulthood with no known defect in chondrogenesis (Cremer et al. 1994), and N-cadherin-deficient embryos died prematurely (at embryonic day 10 [E10]), precluding analyses of mesenchymal condensation (Radice et al. 1997). *Hoxa13* and *Hoxd13* have been shown genetically to control mesenchymal condensation in the autopod (Box 1) of the mouse (Fromental-Ramain et al. 1996; Stadler et al. 2001). *Hoxa13*- and *Hoxd13*-deficient embryos display defects in cell–cell adhesion mediated through Eph–ephrin signaling molecules that are involved in self-sorting of undifferentiated mesenchyme into skeletal precursors (Wada et al. 1998; Lu et al. 2008). Specifically, in the *Hoxa13*^{−/−} autopod, the mesenchyme expressed a reduced

level of EphA7 that normally demarcates the digit primordia, whereas ephrin A3, a high-affinity ligand for the EphA7 receptor normally marking the periphery of presumptive digits, was expressed diffusively in the autopod mesenchyme in the mutant embryo (Stadler et al. 2001). Importantly, the expression of other cell surface and proadhesion molecules including E-cadherin, N-CAM, EphA2, EphA4, ephrin A1, and ephrin A2 was not altered in the *Hoxa13*^{−/−} embryos, indicating a specific relationship between *Hoxa13* and EphA7 in the distal limb mesenchyme. Future research is needed to determine whether other Hox genes similarly control the condensations for the other limb elements and the axial skeleton.

Bone morphogenetic proteins (BMPs), members of the transforming growth factor- β (TGF- β) superfamily, are essential for the formation of chondrogenic mesenchymal condensations. BMPs transduce signals by binding to complexes of type I and II serine/threonine kinase receptors. Ligand binding induces phosphorylation of the receptors, which in turn phosphorylates and activates receptor SMADs (R-SMADs) 1, 5, and 8. The activated R-SMADs then complex with SMAD4 to enter the nucleus, eventually regulating gene expression (Feng and Derynck 2005; Massague et al. 2005). Additionally, BMPs can activate p38 and function through SMAD-independent mechanisms (Pogue and Lyons 2006). Studies with micromass cultures of limb bud mesenchyme have indicated that BMP signaling is required for the formation of condensations, although the relative contribution of SMAD-dependent versus -independent mechanisms is not clear (Pizette and Niswander 2000). Indeed, simultaneous deletion of *Bmp2* and *Bmp4* in prechondrogenic limb mesenchyme (with *Prx1-Cre*)³ led to the loss of zeugopod elements and to defective joint articulations (Bandyopadhyay et al. 2006). Conversely, genetic deletion of Noggin, a secreted antagonist of BMPs, resulted in enlarged cartilage elements and the failure to form joints (Brunet et al. 1998). Similarly, in humans, mutations in NOGGIN cause two autosomal dominant disorders: proximal symphalangism and multiple synostosis syndrome, both characterized by multiple joint fusions (Gong et al. 1999). The fact that in the mouse study not all cartilage elements were equally affected by the loss of *Bmp2* and *Bmp4* suggests that different skeletal elements may require different levels or specificity of BMP signaling.

The role of BMP signaling in chondrogenic mesenchymal condensation has been further dissected. Live imaging of limb mesenchymal cells undergoing successive phases of chondrogenesis in vitro revealed that BMP signaling was

³*Prx1-Cre* targets early limb bud mesenchyme in the mouse at E9.5, and a subset of craniofacial mesenchyme (Logan et al. 2002).

Figure 1. Endochondral bone development. (A) Mesenchymal condensation. (B) Chondrocyte differentiation. Cells centrally located within the mesenchymal condensation differentiate to chondrocytes (blue), whereas the peripheral cells form the perichondrium (gold). (C) Chondrocyte maturation. After the initial proliferation, chondrocytes at the center of the cartilage primordium undergo progressive maturation through prehypertrophy (brown), hypertrophy (green), and terminal hypertrophy (orange). (D) Cartilage vascularization and bone collar formation. Following terminal hypertrophy of chondrocytes, blood vessels (red lines) from the surrounding tissue invade the center of the hypertrophic zone, concurrent with formation of the bone collar (black) from the surrounding perichondrium. Vascular invasion leads to resorption of cartilage matrix, formation of the marrow (red), and deposition of bone (black) within the marrow cavity.

required for the coalescence of smaller aggregates into a tight cluster with a distinct outer boundary, a prerequisite step for chondrogenic differentiation (Barna and Niswander 2007). This event occurred independently of SOX9 (a critical factor for chondrocyte differentiation; see below), because *Sox9*-null cells can coalesce, even though they subsequently segregate from the condensations and adopt a distinctive “fibroblastoid” morphology. This finding appears to be at odds with that of conditional inactivation of SOX9 in limb mesenchyme (with *Prx1-Cre*), which led to the absence of mesenchymal condensations (Akiyama et al. 2002). However, it is likely that SOX9 is dispensable for the initial formation, but necessary for maintaining the condensation. Alternatively, the lack of mesenchymal condensations could be secondary to the failure in chondrocyte differentiation, which is known to require SOX9 (see below). Nonetheless, the molecular basis for this early

SOX9-independent requirement of BMP signaling is not yet understood.

Fibroblast growth factor (FGF) signaling functions in the limb mesenchyme before the formation of condensations. FGFs are a large family of proteins (22 members in humans or mice) that carry out diverse biological functions in vertebrates (Itoh and Ornitz 2008). Most FGFs function by binding to cell surface tyrosine kinase FGF receptors (FGFR1–FGFR4 in humans and mice), leading to phosphorylation and activation of multiple signaling modules, including mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), signal transducer and activator of transcription 1 (STAT1), and protein kinase C (PKC) (Eswarakumar et al. 2005; Turner and Grose 2010). Mutations in FGFRs are responsible for a large range of congenital skeletal disorders in humans (Ornitz and Marie 2002; Su et al. 2008). FGFRs 1 and 2 are expressed throughout

BOX 1. AUTOPOD, ZEUGOPOD, AND STYLOPOD OF THE LIMB

The vertebrate limb proximal–distal axis is subdivided into three morphogenetic domains, from distal to proximal, the autopod, the zeugopod, and the stylopod. Autopod, the most distal skeletal element, gives rise to the carpal bones (wrist), metacarpals, and phalangeal bones (digits) in the forelimb and the tarsal bones (ankle), metatarsals, and phalangeal bones (toes) in the hindlimb. Zeugopod, the middle skeletal element, gives rise to the radius and the ulna in the forelimb and the tibia and the fibula in the hindlimb. Stylopod, the most proximal element, gives rise to the humerus in the forelimb and the femur in the hindlimb.

limb bud mesenchyme before formation of condensations. Although conditional inactivation of *Fgfr2* in mesenchymal precursor cells (with *Dermo1-Cre*)⁴ does not cause a major effect on cartilage (Yu et al. 2003), inactivation of both FGFRs 1 and 2 in the limb bud mesenchyme (with *Prx1-Cre*) results in smaller skeletal elements. Analysis of precondensation limb buds shows that FGF signaling provides an essential cell survival signal for the mesenchyme (Yu and Ornitz 2008).

3 CHONDROCYTE DIFFERENTIATION

Following mesenchymal condensation, cells in the core of the condensations differentiate into chondrocytes that secrete a cartilage matrix rich in types II, IX, and XI collagen and specific proteoglycans such as aggrecan, whereas the expression of type I collagen is suppressed. Cells at the periphery of the condensation form the perichondrium, which continues to express type I collagen and demarcates the developing skeletal element from the surrounding mesenchyme (Fig. 1B) (Caplan and Pechak 1987).

3.1 Sox Proteins

SOX9, a transcription factor of the SRY-related high mobility group box family of proteins, is the earliest known nuclear factor that is required for chondrogenesis. The importance of SOX9 in chondrogenesis is evident from the findings that heterozygous mutations within and around the *Sox9* gene cause campomelic dysplasia, a severe form of human chondrodysplasia (Foster et al. 1994; Wagner et al. 1994). Similarly, haploinsufficiency of SOX9 results in chondrodysplasia in the mouse, and complete loss of SOX9 in prechondrogenic limb mesenchyme abolishes chondrogenesis altogether (Bi et al. 1999, 2001; Akiyama et al. 2002; Kist et al. 2002). More recently, live imaging techniques applied to micromass cultures of limb mesenchyme indicated that SOX9 is dispensable for the initial

formation of mesenchymal condensations but necessary for the subsequent steps toward chondrocyte differentiation (Barna and Niswander 2007). Although SOX9 is known to regulate expression of a number of genes that encode chondrocyte-specific matrix proteins such as collagen II and aggrecan (de Crombrughe et al. 2000), it is not clear whether these genes mediate the role of SOX9 during the early steps of chondrogenesis.

In addition to its early role, SOX9 regulates subsequent chondrocyte differentiation in collaboration with SOX5 and SOX6 (Smits et al. 2001; Akiyama et al. 2002). At the molecular level, the three Sox proteins cooperatively bind and activate the genes for many cartilage-specific extracellular matrix components (Lefebvre et al. 1998; Han and Lefebvre 2008). Thus, SOX9, SOX5, and SOX6 constitute a trio of transcription factors essential for chondrocyte differentiation.

3.2 Extracellular Signals Regulating Chondrocyte Differentiation

In addition to its role in mesenchymal condensation, BMP signaling also stimulates chondrocyte differentiation after the condensation stage. Simultaneous deletion of the BMP type I receptors, BMPRI1A and BMPRI1B, in chondrogenic cells (with *Col2a1-Cre*)⁵ resulted in a severe form of general chondrodysplasia in which a majority of the skeletal elements formed through endochondral ossification were absent (Yoon et al. 2005). Moreover, simultaneous removal of SMAD1 and SMAD5 by the same approach similarly resulted in severe chondrodysplasia (Retting et al. 2009). Thus, BMP signaling through R-SMADs is indispensable for the formation of cartilage following the activation of the *Col2a1* gene. Interestingly, deletion of SMAD4, the binding partner for SMAD1, 5, or 8, with a similar *Col2a1-Cre* line did not severely affect cartilage formation (Zhang et al. 2005). The discrepancy between the R-SMAD- and SMAD4-deficient mice raises the possibility that BMP–

⁴*Dermo1(Twist2)-Cre* is expressed in limb and other mesenchyme beginning at E10.5 (Yu et al. 2003).

⁵*Col2a1-Cre* targets the differentiating chondrogenic cells (Ovchinnikov et al. 2000).

SMAD1/5/8 signaling may control cartilage development in a SMAD4-independent manner. Indeed, BMPs were shown to induce R-SMAD nuclear localization in SMAD4-null colon cancer cells (Liu et al. 1997), but whether this occurs during chondrocyte differentiation remains to be investigated.

In contrast to BMP, some WNT proteins inhibit chondrocyte differentiation. The mammalian WNT family includes 19 members, and WNT proteins control morphogenesis and tissue patterning in a wide variety of organs (Wodarz and Nusse 1998). Depending on the cell context, WNTs interact with several types of membrane receptors, including those of the FRIZZLED (FZ) family and the low-density lipoprotein receptor-related proteins LRP5 and LRP6, to activate a variety of intracellular signaling cascades. In the β -catenin-dependent pathway, binding of WNTs stabilizes β -catenin, which enters the nucleus to interact with the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors and activates the transcription of downstream target genes. β -Catenin-independent pathways include those mediated by PKC or the Rho family of small GTPases, the latter of which can also participate in WNT/ β -catenin signaling (Wu et al. 2008). In chick embryonic limbs and limb bud micromass cultures, ectopic expression of WNT1 or WNT7A inhibited chondrocyte differentiation but did not affect formation of mesenchymal condensations (Rudnicki and Brown 1997; Stott et al. 1999). The antichondrogenic role of WNT proteins may be mediated through β -catenin, as overexpression of a stabilized form of β -catenin in mouse embryonic limb mesenchyme resulted in a near complete loss of all limb cartilage elements (Hill et al. 2005). Continued exposure to WNT/ β -catenin signaling appears to redirect limb mesenchymal cells to the soft connective tissue, but this process can be prevented by FGF signaling (ten Berge et al. 2008). WNT/ β -catenin signaling also has further inhibitory action at stages after *Col2a1* expression has been activated. Conditional overexpression of a stabilized β -catenin by *Col2a1*-Cre can lead to severe achondrodysplasia (Akiyama et al. 2004).

Similar to WNT/ β -catenin signaling, Notch signaling also suppresses chondrogenesis. NOTCH signaling mediates communication between neighboring cells to control cell fate decisions in all metazoans (Artavanis-Tsakonas et al. 1999; Chiba 2006). The mammalian genome encodes four NOTCH receptors (NOTCH1 to -4) and at least five ligands (Jagged1 and -2 and Delta-like 1, 3, and 4). In the canonical NOTCH pathway, binding of the ligands to the NOTCH receptors present on the neighboring cell surface triggers two successive intramembranous proteolytic cleavages of the receptors, with the second event mediated by the γ -secretase complex and resulting in the release of the

Notch intracellular domain (NICD) (Schroeter et al. 1998; Kopan and Goate 2000; Kopan and Ilagan 2009). On its release from the plasma membrane, NICD translocates to the nucleus, where it interacts with a transcription factor of the CSL family (RBPJ κ /CBF-1 in mammals) to activate transcription of target genes (Honjo 1996). Expression studies revealed that multiple NOTCH ligands and receptors are expressed in the prechondrogenic mesenchyme. Abolition of NOTCH signaling in the embryonic limb mesenchyme by inactivating either the catalytic subunits of γ -secretase or the transcriptional effector RBPJ κ accelerated chondrocyte differentiation (Dong et al. 2010). Conversely, enforced expression of the constitutively active NICD in limb mesenchyme completely abolished chondrogenesis, whereas simultaneous removal of RBPJ κ fully rescued the chondrogenic defect caused by NICD overexpression (Dong et al. 2010). Similarly, overexpression of NICD in chondrogenic precursors (with *Col2a1*-Cre) resulted in generalized chondrodysplasia (Mead and Yutzev 2009). Thus, NOTCH signaling may inhibit both mesenchymal condensation and subsequent chondrocyte differentiation.

Retinoid signaling via the retinoic acid receptors (RARs) also acts to inhibit early chondrocyte differentiation. In transgenic mice expressing a constitutively active RAR α in limb mesenchyme from the *Hoxb-6* promoter, mesenchymal cells participated in condensations but failed to differentiate into chondrocytes (Cash et al. 1997; Weston et al. 2000). Conversely, the addition of an RAR α -selective antagonist to cultures of these cells was sufficient to stimulate chondrocyte differentiation. However, a more recent study with mice harboring deletions of different RARs established a predominant role for RAR γ in mediating the inhibitory role of retinoic acid in chondrogenesis (Shimono et al. 2011). Mechanistically, RAR γ agonists appear to suppress the response to BMP signaling in mesenchymal cells and to alter their differentiation potential.

4 GROWTH PLATE DEVELOPMENT

Following formation of the cartilage primordia, initially all chondrocytes undergo rapid proliferation that drives the linear growth of the skeletal elements. At a certain stage specific for each element (e.g., $\sim$ E14.5 for mouse tibia), chondrocytes in the center undergo progressive maturation. These cells eventually exit the cell cycle and undergo hypertrophy associated with the secretion of type X collagen (Poole 1991). The terminal hypertrophic chondrocytes express additional molecular markers such as matrix metalloproteinase 13 (MMP13) and are generally believed to undergo apoptosis (Fig. 1C). These changes are accompanied by vascular invasion of the hypertrophic cartilage and by differentiation of the inner perichondrium cells into

osteoblasts, which secrete bone matrix to form the bone collar (Fig. 1D) (Caplan and Pechak 1987). Mouse knock-out studies have shown that MMP13 plays a critical role in cleaving the extracellular matrix proteins within the hypertrophic cartilage and thus facilitating vascular invasion (Inada et al. 2004; Stickens et al. 2004). Vascular invasion of the hypertrophic cartilage leads to further degradation of the mineralized matrix by MMP9 produced by a resorptive cell type (the so-called chondroclast) (Vu et al. 1998), whose formation may be stimulated by RANKL (receptor activator of NF- κ B ligand) produced by hypertrophic chondrocytes (Xiong et al. 2011). Vascular invasion also brings with it osteoprogenitors that differentiate

into osteoblasts, which establish the primary ossification center to generate the trabecular bone (Maes et al. 2010). The orderly maturation of chondrocytes in the embryonic growth plate produces zones of proliferation, hypertrophy, and bone formation, linearly progressing from the articular ends (epiphysis) to the midshaft (diaphysis) of the skeletal element. The proliferative zone can be further divided into regions of round versus columnar chondrocytes based on their distinct morphology (Fig. 2). Continued proliferation of the less mature chondrocytes at the epiphysis, followed by their hypertrophy and their eventual replacement by trabecular bone near the diaphysis, results in a distal displacement of the growth plate and longitudinal

Figure 2. Extracellular signals regulating growth plate development. Depicted is a longitudinal section through one of two growth plates of a mouse long bone during late embryogenesis (E15.5–E19). The growth plate at this stage is without a secondary ossification center and is organized into distinct domains as indicated. (1) IHH and PTHrP coordinate chondrocyte proliferation and maturation through a negative-feedback mechanism. IHH produced by pre- and early hypertrophic chondrocytes stimulates chondrocyte proliferation and PTHrP transcription through derepression of GLI3. PTHrP in turn suppresses chondrocyte maturation associated with IHH expression. Direct IHH signaling also regulates the formation of columnar chondrocytes from round chondrocytes (not depicted here). (2) FGF9/18 from the perichondrium suppresses chondrocyte proliferation and maturation. FGFR3 expressed in chondrocytes is a likely receptor for FGF9/18 to suppress proliferation in the growth plate late in embryonic development and during postnatal bone growth. FGF9/18 may use other yet-to-be-established mechanisms to suppress chondrocyte maturation in the early embryo. (3) BMPs expressed by both chondrocytes and perichondrial cells promote proliferation and maturation. (4) NOTCH signaling in chondrocytes promotes proliferation and maturation. (5) WNT5A expressed by prehypertrophic chondrocytes stimulates hypertrophy.

growth of each skeletal element (Long 2012b). The orderly progression of the growth plate is critical for proper development of the endochondral skeleton (Kronenberg 2003) and is subject to regulation by key extracellular signals and nuclear factors that are discussed below.

4.1 Extracellular Signals Regulating Growth Plate Development

4.1.1 PTHrP

PTHrP (parathyroid hormone-related peptide) is a paracrine factor expressed at highest levels by cells of the periarticular perichondrium and at a lower level by proliferating chondrocytes near the articular surface (Fig. 2) (Lee et al. 1995). The receptor for PTHrP (PTHR1) is expressed at low levels by proliferating chondrocytes but at high levels by the maturing chondrocytes before they become hypertrophic (Vortkamp et al. 1996; St-Jacques et al. 1999). Targeting inactivation of the gene encoding either PTHrP or its receptor in the mouse resulted in neonatal lethal short-limb dwarfism caused by premature hypertrophy of chondrocytes (Karaplis et al. 1994; Lanske et al. 1996), thus identifying a key role for PTH signaling in suppressing the onset of chondrocyte hypertrophy in the growth plate. Conversely, misexpression of *PTHrP* in all chondrocytes markedly delayed chondrocyte hypertrophy, resulting in a completely cartilaginous endochondral skeleton at birth (Weir et al. 1996). Similarly in humans, inactivating mutations in *PTHR1* cause Blomstrand chondrodysplasia, characterized by advanced skeletal maturation with shortened long bones and increased bone density (Jobert et al. 1998; Karaplis et al. 1998; Karperien et al. 1999), whereas gain-of-function mutations in *PTHR1* cause Jansen metaphyseal chondrodysplasia (Schipani et al. 1995, 1996), which was recapitulated in mice overexpressing such a mutant receptor (Schipani et al. 1997). Mechanistically, PTHrP appears to delay chondrocyte hypertrophy, mainly by activating cAMP-dependent signaling that both increases the activity of SOX9 (Huang et al. 2001; Guo et al. 2002), and regulates the HDAC4–MEF2C complex (see below).

4.1.2 IHH

The expression of PTHrP in the periarticular region is strictly dependent on Indian hedgehog (IHH), a member of the Hedgehog (HH) family that in mammals also includes Sonic hedgehog (SHH) and Desert hedgehog (DHH). The HH family of proteins plays fundamental roles in animal development and is conserved from flies to humans (Ingham and McMahon 2001; McMahon et al. 2003; Huangfu and Anderson 2006). The HH signal is trans-

duced through Smoothened (SMO), a seven-pass transmembrane domain protein, and ultimately controls the processing and subcellular localization of the Gli transcription factors (GLI1 to -3) that regulate expression of downstream target genes. In the developing cartilage, IHH is primarily expressed by chondrocytes immediately before hypertrophy (prehypertrophic chondrocytes) and also by early hypertrophic chondrocytes. IHH signals to both the proliferative chondrocytes and the overlying perichondrial cells (Vortkamp et al. 1996; St-Jacques et al. 1999). *Ihh*^{-/-} embryos exhibited a severe reduction in chondrocyte proliferation and premature hypertrophy of chondrocytes (St-Jacques et al. 1999; Long et al. 2001). Subsequent genetic manipulation of *Smo* in chondrocytes revealed that direct IHH input was required for chondrocyte proliferation (Long et al. 2001), but not for the regulation of chondrocyte hypertrophy. Instead, hypertrophy depended primarily on PTHrP, which was induced by IHH (St-Jacques et al. 1999; Karp et al. 2000; Long et al. 2001). Direct IHH signaling also regulates the formation of columnar chondrocytes from round chondrocytes within the proliferating zone (Kobayashi et al. 2005; Hilton et al. 2007). The control of PTHrP by IHH appears to be through direct HH signaling in the target cells, as localized removal of *Smo* (with Col2a1-CreERTM)⁶ led to a corresponding loss of PTHrP expression within the periarticular region (Hilton et al. 2007). Finally, the role of IHH in chondrocyte proliferation and PTHrP expression is mainly mediated through the derepression of GLI3 repressor function, as simultaneous removal of both GLI3 and IHH restored normal proliferation, PTHrP expression, and hypertrophy (Hilton et al. 2005; Kozziel et al. 2005). Overall, these studies support a model in which IHH and PTHrP jointly regulate chondrocyte proliferation and maturation.

4.1.3 FGF

In addition to the IHH/PTHrP regulatory axis, FGF signaling also controls growth plate development. Conditional inactivation of *Fgfr1* in chondrocytes (Col2a1-Cre) delayed maturation of hypertrophic chondrocytes (Jacob et al. 2006), whereas inactivation of *Fgfr2* in skeletal mesenchymal precursor cells (*Dermo1-Cre*) resulted in skeletal dwarfism and decreased bone density, without causing major effects on cartilage (Yu et al. 2003). *Fgfr3*^{-/-} mice have increased levels of chondrocyte proliferation (Deng et al. 1996; Eswarakumar and Schlessinger 2007) and an expanded hypertrophic zone that is most evident postnatally (Colvin et al. 1996; Deng et al. 1996). In contrast,

⁶Col2a1-CreERTM targets mainly chondrocytes upon administration of tamoxifen at proper times during embryogenesis (Hilton et al. 2007).

overexpression of activated FGFR3 (achondroplasia or thanatophoric dysplasia mutation) in the growth plate of transgenic mice reduced chondrocyte proliferation and resulted in decreased numbers of cells in the prehypertrophic and hypertrophic zones (Naski et al. 1998; Chen et al. 2001; Iwata et al. 2001). Interestingly, in *Fgfr3*^{-/-} mice, IHH expression and signaling in chondrocytes was increased relative to wild-type mice, whereas in the mice overexpressing activated FGFR3, IHH expression and signaling was inhibited (Naski et al. 1998; Chen et al. 1999; Li et al. 1999). In contrast to phenotypes observed in mice, IHH and PTHR1 expression were not altered in human achondroplasia or thanatophoric dysplasia growth plate tissue (Cormier et al. 2002). Thus, FGFR3 signaling may inhibit chondrocyte proliferation and hypertrophy in part through indirect modulation of the IHH/PTHrP axis, but additional mechanisms may also mediate the pathological consequences of FGFR3 activating mutations.

The mechanisms by which FGFR3 signaling regulates chondrocyte proliferation are not well defined. Early studies showed that activation of FGFR3 in growth plate chondrocytes leads to phosphorylation of STAT1 and inhibition of chondrocyte proliferation (Li et al. 1999). However, more recent studies have shown that activation of FGFR3 down-regulates AKT activity to cause decreased chondrocyte proliferation (Priore et al. 2006). Additionally, activation of MEK1 in chondrocytes leads to a chondrodysplasia phenotype accompanied by decreased chondrocyte differentiation (Murakami et al. 2004). Furthermore, MEK1 activation inhibited the skeletal overgrowth seen in mice lacking *Fgfr3*. Thus, FGFR3 may regulate chondrocyte proliferation through STAT1 and AKT, but differentiation through the MAPK pathway.

The physiologic FGF ligand(s) for FGFR3 in the growth plate remain to be identified. However, FGF9 and FGF18 are good candidates because both are expressed in the adjacent perichondrium (Liu et al. 2002; Ohbayashi et al. 2002; Hung et al. 2007), and FGF18 is also expressed at low levels by growth plate chondrocytes (Lazarus et al. 2007). In addition, both ligands are known to activate FGFR3 in vitro (Zhang et al. 2006). More importantly, studies of *Fgf9*^{-/-} and *Fgf18*^{-/-} mice and mice with an activating mutation of FGFR3 show that FGF9, FGF18, and FGFR3 can promote chondrocyte proliferation at early stages of embryonic development (E14.5–15.5) (Iwata et al. 2000; Liu et al. 2002; Hung et al. 2007). In the late embryonic growth plate, loss-of-function studies show that FGF18 and FGFR3 function to inhibit proliferation and accelerate maturation (Colvin et al. 1996; Deng et al. 1996; Liu et al. 2002). The observation that *Fgf9*^{-/-} and *Fgf18*^{-/-} mice begin to manifest accelerated chondrocyte maturation during embryogenesis when the *Fgfr3*^{-/-} embryos are relatively normal suggests that FGF9

and FGF18 may use receptor(s) other than FGFR3 to regulate growth plate development at early embryonic stages.

4.1.4 BMP

BMP signaling regulates multiple aspects of growth plate development (Pogue and Lyons 2006). Although mouse embryos completely lacking both *Bmpr1a* and *Bmpr1b* in chondrogenic cells (with Col2a1-Cre) (*Bmpr1a*CKO/*Bmpr1b*^{-/-}) failed to form many cartilage elements, those carrying one remaining *Bmpr1b* allele (*Bmpr1a*CKO/*Bmpr1b*^{+/-}) developed all elements but exhibited marked defects in chondrocyte proliferation, survival, and hypertrophy (Yoon et al. 2006). Similar defects were observed in the limbs of mice lacking *Smad1* and *Smad5* in chondrogenic cells (with Col2a1-Cre) (Retting et al. 2009). In embryos lacking either *Bmpr1a/b* or *Smad1/5*, IHH expression and signaling was reduced and may have contributed to the overall phenotype (Yoon et al. 2006; Retting et al. 2009). On the contrary, FGF signaling was increased in these mutant growth plates. Further supporting the antagonism between BMP and FGF signaling, the expression of *Bmp4* was suppressed in both growth plate chondrocytes and in the surrounding perichondrium of mice overexpressing activated FGFR3 (Naski et al. 1998). Thus, proper balancing of BMP and FGF signaling may be important for normal development of the embryonic growth plate.

4.1.5 WNT

Members of the WNT family also regulate the proper progression of growth plate chondrocytes. WNT5A and WNT5B are expressed by chondrocytes during the transition from proliferative to hypertrophic zone in the mouse. *Wnt5a*^{-/-} mice exhibited a marked delay in chondrocyte hypertrophy (Yang et al. 2003). Interestingly, overexpression of either WNT5A or WNT5B in chondrocytes also delayed hypertrophy in both mouse and chicken embryos (Hartmann and Tabin 2000; Yang et al. 2003). Thus, a proper level of WNT5A/B signaling is necessary for the normal transition of chondrocytes to hypertrophy.

4.1.6 NOTCH

NOTCH signaling modulates development of the growth plate chondrocytes. Deletion of both NOTCH1 and NOTCH2 or of RBPJ κ in the limb mesenchyme (with Prx1-Cre) before chondrogenesis did not grossly affect the formation of cartilage anlagen but reduced chondrocyte proliferation and delayed both the onset and the terminal progression of chondrocyte hypertrophy in the mouse embryo (Hilton et al. 2008; Dong et al. 2010; Kohn et al. 2012). Conversely, forced expression of the intracellular domain of

Notch1 (NICD) in differentiated chondrocytes (with tamoxifen induction of Col2-CreERT2)⁷ accelerated both the onset and terminal progression of hypertrophy in an RBPJ κ -dependent manner (Kohn et al. 2012). Thus, proper Notch signaling within the growth plate is critical for normal proliferation and maturation of chondrocytes. Future studies are necessary to elucidate how NOTCH intersects with the other extracellular signals to ensure proper development of the growth plate (Fig. 2).

4.2 Nuclear Factors Regulating Growth Plate Development

In addition to the extracellular signals described above, a number of nuclear factors have also been shown to regulate chondrocyte hypertrophy. RUNX2, a runt-domain transcription factor, plays an important role in promoting chondrocyte hypertrophy. RUNX2 is initially expressed within the chondrogenic mesenchyme, subsequent to and dependent on SOX9 expression (Akiyama et al. 2002, 2005). Following the formation of cartilage anlagen, RUNX2 becomes more restricted to the perichondrial cells and osteoblasts, but it is reexpressed in the prehypertrophic and early hypertrophic chondrocytes. Several lines of evidence have supported RUNX2 as an important positive regulator of the hypertrophic program. First, *Runx2*^{-/-} mice lack hypertrophic chondrocytes in multiple skeletal elements (Inada et al. 1999; Kim et al. 1999). Second, forced expression of RUNX2 in all chondrocytes leads to premature chondrocyte hypertrophy, and also rescues the defects in hypertrophy observed in *Runx2*^{-/-} mice (Takeda et al. 2001; Ueta et al. 2001; Tu et al. 2012b), whereas expression of a dominant-negative RUNX2 mutant inhibits chondrocyte hypertrophy (Ueta et al. 2001). The role of RUNX2 in chondrocyte hypertrophy appears to be shared with RUNX3, as simultaneous deletion of both completely blocked hypertrophy in all endochondral skeletal elements (Yoshida et al. 2004). Finally, RUNX2 may also function in the perichondrium to regulate development of the growth plate through indirect mechanisms, as RUNX2 regulates the perichondrial expression of FGF18, which in turn signals to chondrocytes (Hinoi et al. 2006).

The direct effect of RUNX2 on chondrocyte hypertrophy appears to be regulated by HDAC4, a member of the class II histone deacetylases (Vega et al. 2004). Like RUNX2, HDAC4 is expressed in the prehypertrophic and early hypertrophic chondrocytes. Genetic deletion of HDAC4, like overexpression of RUNX2, greatly accelerated chondrocyte hypertrophy. Conversely, overexpression of HDAC4

in all chondrocytes inhibited hypertrophy, mimicking the effect of RUNX2 deletion. Biochemically, HDAC4 physically interacts with RUNX2 and reduces RUNX2 binding to DNA. Thus, HDAC4 prevents premature hypertrophy of chondrocytes in part by directly suppressing RUNX2 activity.

HDAC4 also suppresses chondrocyte hypertrophy by inhibiting MEF2C activity. MEF2C, a member of the myocyte enhancer factor-2 (MEF2) family, is expressed by prehypertrophic and early hypertrophic chondrocytes (Arnold et al. 2007). Conditional deletion or expression of a dominant-negative mutant of MEF2C in chondrocytes impairs hypertrophy, whereas an activated form induces precocious chondrocyte hypertrophy. MEF2C stimulates hypertrophy partly by increasing RUNX2 expression. Moreover, HDAC4 and MEF2C antagonize each other genetically, and HDAC4 functions as a corepressor of MEF2C to suppress chondrocyte hypertrophy.

The activity of HDAC4 in turn is regulated by other factors. PTHrP induces dephosphorylation of HDAC4 and promotes its nuclear translocation and repression of MEF2 transcriptional activity (Kozhemyakina et al. 2009). Conversely, salt-inducible kinase 3 (SIK3) is required for the exclusion of HDAC4 from the nuclei so that hypertrophy could occur, as *Sik3*^{-/-} mice exhibited a marked delay in chondrocyte hypertrophy (Sasagawa et al. 2012). Thus, the HDAC4-MEF2C complex appears to be a central node of regulation for chondrocyte hypertrophy.

Additional regulators of chondrocyte hypertrophy have been identified. SOX9 has been proposed to both prevent cells from prematurely entering the prehypertrophic stage and to promote the subsequent hypertrophy (Dy et al. 2012). The transcription factors FoxA2 and FoxA3 are necessary for chondrocyte hypertrophy (Ionescu et al. 2012). SHOX2, a homeodomain-containing transcription factor, is necessary for RUNX2/RUNX3 expression and hypertrophy within the stylopod (see Box 1) (Cobb et al. 2006; Yu et al. 2007). Finally, Zfp521, a zinc finger-containing transcriptional coregulator, suppresses chondrocyte hypertrophy and partly mediates the function of PTHrP in this process (Correa et al. 2010; Seriwatanachai et al. 2011). Overall, chondrocyte hypertrophy is tightly regulated by a multitude of extracellular signals and nuclear factors. How the extracellular signals integrate with the nuclear factors represents an important area of future research.

5 OSTEOBLASTOGENESIS

Coupled with chondrocyte hypertrophy, osteoblast differentiation first occurs within the perichondrium and continues within the marrow cavity following vascularization of the hypertrophic cartilage. The differentiation of

⁷Col2-CreERT2 targets chondrocytes upon injection of tamoxifen (Chen et al. 2007).

osteoblasts from their mesenchymal progenitors is regulated by specific extracellular signals and transcription factors as discussed below.

5.1 Extracellular Signals Regulating Osteoblast Differentiation

5.1.1 IHH Signaling

IHH critically regulates osteoblast differentiation during endochondral skeletal development. *Ihh*^{-/-} mice completely lack osteoblasts within the endochondral skeleton, although intramembranous osteoblasts still form (St-Jacques et al. 1999). Moreover, IHH signaling is directly required for perichondrial cells to initiate osteoblast differentiation, as cells genetically deficient in SMO (an obligatory transducer of the HH signal) exhibit a cell-autonomous defect in osteoblast differentiation (Long et al. 2004). In the absence of IHH, the mesenchymal progenitors in the perichondrium fail to express RUNX2, which is indispensable for osteoblast differentiation. Interestingly, although the activation of RUNX2 expression by IHH in perichondrial cells can be achieved through removal of the GLI3 repressor, induction of Osterix (OSX or SP7), an essential transcription factor acting downstream from RUNX2, requires both the suppression of GLI3 and activation of GLI2 (Hilton et al. 2005; Joeng and Long 2009). In addition to osteoblast differentiation in the perichondrium, HH signaling is also necessary for trabecular bone formation, as indicated by lack of trabecular bone in mouse embryos lacking SMO in osteoprogenitor cells (Long et al. 2004). Thus, IHH, secreted by the prehypertrophic and early hypertrophic chondrocytes, controls the onset of osteoblast differentiation in the endochondral skeleton.

5.1.2 WNT Proteins

The WNT proteins have important roles in regulating osteoblast differentiation. Most notably, mice lacking β -catenin in mesenchymal progenitors fail to develop mature osteoblasts (Day et al. 2005; Hill et al. 2005; Hu et al. 2005; Rodda and McMahon 2006). Specifically, β -catenin is required for the progression from the RUNX2-positive to the OSX-positive stage, and from OSX-positive cells to mature osteoblasts (Hu et al. 2005; Rodda and McMahon 2006). Similarly, deletion of both LRP5 and LRP6 in mesenchymal progenitors results in loss of osteoblasts (Joeng et al. 2011). Interestingly, deletion of β -catenin in either mesenchymal progenitors or OSX-positive cells caused ectopic cartilage formation in place of bone, possibly owing to a fate switch for the bipotential progenitors (Day et al. 2005; Hill et al. 2005; Hilton et al. 2005; Rodda and McMahon 2006). Finally, β -catenin signaling appears to function downstream

from IHH, as β -catenin deletion does not impair IHH signaling in the perichondrium (containing osteoblast progenitors), whereas IHH removal abolishes β -catenin signaling in that compartment (Hu et al. 2005; Mak et al. 2006). Thus, IHH and WNT signaling sequentially regulate osteoblast differentiation in the endochondral skeleton during embryogenesis.

WNT signaling has been further implicated in regulating bone accrual during postnatal life. In humans, loss-of-function mutations in the gene encoding the WNT coreceptor LRP5 cause osteoporosis–pseudoglioma syndrome (Gong et al. 2001), a form of juvenile-onset osteoporosis. Conversely, a different set of mutations in *Lrp5* that render the coreceptor refractory to extracellular WNT inhibitors DKK1 and Sclerostin (encoded by *Sost*) cause high-bone-mass syndrome (Boyden et al. 2002; Little et al. 2002; Ai et al. 2005; Ellies et al. 2006; Semenov and He 2006). Further supporting the role of LRP5 signaling in bone formation, loss-of-function or loss-of-expression mutations in *Sost*, which are expected to enhance LRP5 signaling, result in the bone-thickening diseases known as sclerosteosis or van Buchem disease, respectively (Balemans et al. 2001, 2002; Brunkow et al. 2001; Staehling-Hampton et al. 2002). Furthermore, mice deficient in *Lrp5* (Kato et al. 2002) or *Wnt10b* (Bennett et al. 2005) exhibit lower bone mass postnatally, whereas *Lrp6* haploinsufficiency further decreases bone mass in *Lrp5*-null mice (Holmen et al. 2004). Conversely, mice expressing LRP5 with mutations that cause high bone mass in humans also exhibit a high-bone-mass phenotype (Yadav et al. 2008; Cui et al. 2011). Analyses of the LRP5 mutant mice revealed that LRP5 predominantly affects osteoblast number and function in postnatal animals, with no obvious effect on osteoclasts (Kato et al. 2002; Yadav et al. 2008; Cui et al. 2011). Thus, genetic evidence has shown the importance of LRP5 signaling in postnatal bone formation.

The mechanism through which LRP5 controls postnatal bone accrual is not well understood. Because deletion of β -catenin in mature osteoblasts or osteocytes did not produce a similar bone phenotype to *Lrp5*-deficient mice (i.e., increase in osteoblast number and activity with no obvious effect on osteoclasts) (Glass et al. 2005; Holmen et al. 2005; Kramer et al. 2010), LRP5 could function independently of β -catenin, at an earlier stage within the osteoblast lineage, or extrinsic to skeletal tissue. The potential stage specificity of LRP5 function was addressed by two different groups but with conflicting results. Whereas one group reported that deletion of *Lrp5*, either in mature osteoblasts (*Col1-Cre*)⁸

⁸Col1-Cre driven by the 2.3-kb promoter of mouse Col α 1(I) targets mature osteoblasts (Dacquin et al. 2002).

(Yadav et al. 2008) or in mesenchymal progenitors (*Dermol-Cre*, and therefore all cells of the osteoblast lineage) (Yadav et al. 2010), did not produce any bone phenotype, a second group showed that deletion of *Lrp5* predominantly in osteocytes (differentiated from osteoblasts; *Dmp1-Cre*)⁹ resulted in less bone (Cui et al. 2011). The different findings support competing models. Whereas the first group argues that LRP5 functions in the duodenum to suppress circulating levels of serotonin, which in turn directly inhibits osteoblast proliferation (Yadav et al. 2008), the second group believes that LRP5 functions directly in the osteoblast lineage (Cui et al. 2011). Thus, the precise mechanism for LRP5 regulation of osteoblasts remains to be further elucidated.

β -Catenin-independent WNT signaling also has been implicated in regulating osteoblast differentiation. WNT-induced G-protein-coupled phosphatidylinositol signaling and PKC δ activation have been shown to promote osteoblast differentiation by stimulating the progression from RUNX2- to OSX-positive cells through an unknown mechanism (Tu et al. 2007). In addition, WNT5A is believed to promote osteoblast differentiation by inducing suppressive histone methylation at the promoters of peroxisome proliferator-activated receptor- γ (PPAR γ) target genes, thereby inhibiting the adipogenic program (Takada et al. 2007). On the other hand, WNT10B inhibits adipogenesis by suppressing the expression of the adipogenic transcription factors PPAR γ and C/EBP α (CCAAT/enhancer-binding protein- α) (Ross et al. 2000). Exactly how inhibition of adipogenesis by either WNT5A or WNT10B leads to osteoblast differentiation is not clear. Finally, deletion of *Frizzled9* decreased bone mass in part through down-regulation of ISG15, a ubiquitinlike protein modifier, without impairing β -catenin signaling (Albers et al. 2011). Thus, WNT signaling regulates osteoblast differentiation through multiple mechanisms.

5.1.3 NOTCH Signaling

Mouse genetic studies suggest that NOTCH signaling suppresses osteoblast differentiation. Removal of either presenilin 1 (PS1), PS2, NOTCH1, NOTCH2, or RBPJ κ in the embryonic limb mesenchyme markedly enhanced osteoblast formation, resulting in high bone mass in adolescent mice (Hilton et al. 2008; Tu et al. 2012a). The high-bone-mass phenotype in NOTCH-deficient mice coincided with decreased numbers of bone marrow mesenchymal progenitors, supporting a model in which NOTCH signaling normally suppresses osteoblast differentiation from

progenitor cells. The suppression seems to occur at a stage before OSX activation, as deletion of RBPJ κ in OSX-positive cells did not cause a similar phenotype (Tu et al. 2012a). Mechanistically, NOTCH signaling inhibits osteoblast differentiation partly by inducing HEY1 and HEYL, which suppress osteoblast differentiation, both through decreasing RUNX2 transcriptional activity (Hilton et al. 2008), and by reducing the expression of NFATC1, a positive regulator of osteoblast differentiation (Tu et al. 2012a).

Consistent with the negative role of NOTCH signaling in osteoblast differentiation, NOTCH1 haploinsufficiency in humans causes ectopic ossification in the aortic valves (Garg et al. 2005; Mohamed et al. 2006), whereas gain-of-function mutations in NOTCH2 are responsible for Haddju–Cheney syndrome, a disorder of severe and progressive bone loss (Isidor et al. 2011; Simpson et al. 2011). Similarly, transgenic mice globally overexpressing HEY1 show progressive osteopenia (Salie et al. 2010). Interestingly, whereas NOTCH activation suppressed osteoblast differentiation from early precursors (Zanotti et al. 2008), at a later stage it caused pathological overproduction of immature osteoblasts, similar to what occurs in certain human osteosarcomas (Engin et al. 2008, 2009; Tao et al. 2010). These studies highlight the stage-specific functions of constitutive NOTCH activation in the osteoblast lineage.

5.1.4 BMP Signaling

Genetic studies have uncovered important roles for BMP2 and BMP4 in promoting osteoblast differentiation. Knockout experiments showed that a critical threshold level of BMP2 and BMP4 signaling is required for the formation of mature osteoblasts by controlling the transition from RUNX2- to OSX-positive cells (Bandyopadhyay et al. 2006). Interestingly, mice lacking only BMP2 in the limb mesenchyme form bone during embryogenesis but display a clear defect in postnatal bone mineral density, resulting in frequent fractures that fail to heal (Tsuji et al. 2006). Thus, BMP2 and BMP4 appear to redundantly control osteoblast differentiation during embryogenesis, with BMP2 having a unique role in postnatal bone formation.

BMP3 may counteract the activities of BMP2 and BMP4 to maintain a proper bone mass in vivo, as *Bmp3*-null mice had more trabecular bone than wild-type mice (Daluisi et al. 2001). Recent studies have shown that BMP3 engages the BMP type II receptor *Acvr2b* to inhibit signaling by BMP2 or BMP4 (Kokabu et al. 2012). In the postnatal skeleton, BMP3 is mainly produced by osteoblasts and osteocytes and it in turn reduces differentiation from the earlier-stage osteoblast lineage cells (Kokabu et al.

⁹*Dmp1-Cre* mainly targets osteocytes (Lu et al. 2007).

2012). Thus, BMP3 may function in a negative-feedback mechanism to ensure the production of a proper number of osteoblasts.

The importance of BMP signaling in bone has also been supported by studies of the BMP receptor, BMPRII. Deletion of BMPRII in preosteoblasts and osteoblasts, either in utero or postnatally, unexpectedly increased bone mass as a result of reduced bone resorption, even though bone formation was also decreased (Kamiya et al. 2008a,b, 2010). However, the precise role of BMPRII signaling during osteoblast differentiation has not yet been elucidated, nor is it clear whether BMPRII signals predominantly through the SMAD-dependent pathway. Nonetheless, BMPRII appears to function through SMAD4 to promote the function of mature osteoblasts, as deletion of either molecule in mature osteoblasts decreased osteoblast function (Mishina et al. 2004; Tan et al. 2007).

5.1.5 FGF Signaling

FGF signaling has been implicated in osteoblast differentiation. A number of *Fgfs* are expressed in the perichondrium, where osteoblast differentiation occurs during endochondral bone development. These include *Fgf1*, -2, -6, -7, -8, -9, -17, and -18 (Colvin et al. 1999; Xu et al. 1999; Liu et al. 2002; Hung et al. 2007; Lazarus et al. 2007), although they are not all expressed uniformly in all skeletal elements. Importantly, *Fgf18*^{-/-} embryos exhibited defects in the formation of mature osteoblasts despite normal RUNX2 expression (Ohbayashi et al. 2002; Liu et al. 2007).

Studies of FGF receptors have revealed their diverse roles in the regulation of osteoblasts. In particular, FGFR1 signaling at an early developmental stage promotes osteoblast differentiation without affecting RUNX2 expression, but FGFR1 signaling in mature osteoblasts inhibits their mineralization activity (Jacob et al. 2006). In contrast, FGFR2 promotes the proliferation of preosteoblasts and the anabolic function of mature osteoblasts (Yu et al. 2003), partly through up-regulation of RUNX2 expression (Eswarakumar et al. 2002, 2004). Finally, mice lacking FGFR3 showed an increase in osteoblast number but a decrease in osteoid mineralization (Valverde-Franco et al. 2004). Thus, FGF signaling through different receptors has diverse roles in regulating preosteoblast proliferation, osteoblast differentiation, as well as the activity of mature osteoblasts, but the precise stages at which FGFs regulate proliferation and differentiation, potential redundancy between FGFRs, and the intracellular signaling cascades responsible for each function remain to be elucidated. In summary, all major developmental signaling pathways regulate specific aspects of osteoblast differentiation and/or function (Fig. 3).

5.2 Transcription Factors Controlling Osteoblast Differentiation

5.2.1 RUNX2 Is Essential for Osteoblast Differentiation

RUNX2 is indispensable for osteoblast differentiation during both endochondral and intramembranous ossification. Homozygous deletion of RUNX2 or its nuclear targeting signal in mice resulted in a complete lack of osteoblasts (Komori et al. 1997; Otto et al. 1997), whereas haploinsufficiency of RUNX2 in either mice or humans causes cleidocranial dysplasia (Lee et al. 1997; Mundlos et al. 1997; Choi et al. 2001). Interestingly, in the absence of RUNX2, the perichondrium (normally containing osteogenic progenitors) becomes hypoplastic (Komori et al. 1997; Otto et al. 1997), indicating that RUNX2 may be necessary for the production and/or maintenance of the progenitors. In addition to osteoblast differentiation, RUNX2 is necessary for the proper function of mature osteoblasts (Ducy et al. 1999).

5.2.2 OSX Is Required Downstream from RUNX2

OSX, a transcription factor containing three C2H2-type zinc fingers, is necessary for osteoblast differentiation. Deletion of OSX results in a complete absence of osteoblasts in the mouse embryo, even though expression of RUNX2 is relatively normal (Nakashima et al. 2002). These results, together with the observation that *Osx* expression is abolished in *Runx2*^{-/-} mice (Nakashima et al. 2002), indicate that OSX functions downstream from RUNX2 as another indispensable regulator of osteoblast differentiation. However, there are important differences between the phenotypes of *Runx2*^{-/-} and *Osx*^{-/-} mice. Whereas RUNX2 deletion leads to a hypoplastic perichondrium, loss of OSX causes ectopic cartilage formation beneath a thickened perichondrium at the midshaft of long bones (where a bone collar normally forms), possibly because of a fate switch of progenitors from osteoblasts to chondrocytes (Nakashima et al. 2002). Beyond its role in embryonic osteoblast differentiation, OSX is also critical for postnatal osteoblast and osteocyte formation and function (Zhou et al. 2010).

5.2.3 ATF4 Regulates Osteoblast Differentiation

ATF4, a member of the bZIP family of transcription factors, is believed to play important roles at later stages of osteoblast differentiation. Misregulation of ATF4 activity has been linked with the skeletal abnormalities seen in human patients with Coffin–Lowry syndrome and neurofibromatosis type I (Yang et al. 2004; Eleftheriou et al. 2006). Mechanistically, ATF4 directly regulates the expression of osteocalcin and RANKL in osteoblasts (Yang et al. 2004;

Figure 3. Extracellular signals regulating osteoblast differentiation. Model is based on studies of the mouse limb skeleton. Osteoblasts differentiate from mesenchymal progenitors (MP) through distinct developmental stages marked by expression of key transcription factors including SOX9, RUNX2, and OSX. Mature osteoblasts (OB) can further differentiate to osteocytes (OCY) or bone lining cells (not depicted) or undergo apoptosis (not depicted). (A) Indian hedgehog (IHH) signaling is required for osteoblast differentiation during endochondral bone development. IHH binding to the receptor Patched homolog 1 (PTCH1) activates signaling through Smoothed (SMO), thereby inhibiting the generation of the proteolytically cleaved GLI3 repressor (GLI3R) and promoting the generation of the full-length GLI2 activator (GLI2A). Whereas derepression of GLI3R is sufficient to generate RUNX2⁺ cells, both derepression of GLI3R and activation of GLI2A are necessary for progression to the RUNX2⁺OSX⁺ stage. (B) NOTCH signaling inhibits osteoblast differentiation. Following binding to their ligands, Jagged (JAG) or Delta-like (DLL), Notch receptors are proteolytically cleaved by the γ -secretase complex, leading to release of the Notch intracellular domain (NICD) from the plasma membrane. NICD interacts with RBPJ κ and together they activate downstream target genes, including HES (Hairy and Enhancer of Split) and HEY (HES-related with YRPW motif) family transcription factors, ultimately leading to inhibition of osteoblast differentiation, seemingly at a stage before OSX activation. (C) WNT signaling promotes osteoblast differentiation. During β -catenin-dependent WNT signaling, β -catenin is stabilized following binding of WNT to its receptors Frizzled (FZD) and lipoprotein receptor-related protein 5 (LRP5) or LRP6, leading to the transcription of β -catenin target genes and ultimately stimulating progression from the RUNX2⁺ stage to the RUNX2⁺OSX⁺ stage, and from RUNX2⁺OSX⁺ cells to mature osteoblasts. WNT can also signal independently of LRP5/6 and β -catenin through protein kinase C δ (PKC δ), promoting progression to the RUNX2⁺OSX⁺ stage through an unknown mechanism. (D) Bone morphogenetic protein (BMP) signaling stimulates osteoblast differentiation and function. Binding of BMP2 or BMP4 to their receptors results in phosphorylation of SMAD1, SMAD5, or SMAD8. These can then form a complex with their partner, SMAD4, and enter the nucleus to regulate gene expression, ultimately promoting the transition to RUNX2⁺OSX⁺ cells and enhancing the function of mature osteoblasts; however, a direct role for SMAD signaling in osteoblast differentiation remains to be shown. (E) Fibroblast growth factor (FGF) signaling has diverse roles in osteoblast lineage cells. FGFs function by binding to cell surface Tyr kinase FGF receptors (FGFR1–FGFR4 in humans and mice), leading to the activation of multiple signaling modules. FGF signaling regulates preosteoblast proliferation and osteoblast differentiation, as well as the function of mature osteoblasts. However, the precise stages at which FGFs regulate proliferation and differentiation, and the intracellular signaling cascades responsible for each function, remain to be elucidated. BMPR, BMP receptor; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; STAT1, signal transducer and activator of transcription 1.

Eleftheriou et al. 2005) and also promotes amino acid import into osteoblasts to support robust protein synthesis (Yang et al. 2004). Interestingly, members of the forkhead box O (FOXO) family of transcription factors may interact with ATF4 to promote amino acid import in osteoblasts (Ambrogini et al. 2010; Rached et al. 2010). Conversely, the leucine-zipper-containing nuclear protein FIAT seems to antagonize ATF4 activity (Yu et al. 2005). More recently, ATF4 was shown to act in chondrocytes to promote osteo-

blast differentiation in the mouse embryo, possibly through stimulation of IHH (Wang et al. 2012). Thus, ATF4 may regulate osteoblast differentiation during embryogenesis in part through an indirect mechanism.

5.2.4 Other Transcription Factors

Numerous nuclear factors have been shown to synergize with RUNX2 to promote osteoblast differentiation (Kar-

senty 2009; Long 2012a). These include MAF, TAZ, SATB2, RB, GLI2, DLX5, MSX2, and BAPX1, which function in different ways, including stimulating *Runx2* expression, enhancing RUNX2 activity, or acting as coactivators (Tribioli and Lufkin 1999; Satokata et al. 2000; Thomas et al. 2001; Robledo et al. 2002; Cui et al. 2003; Hong et al. 2005; Lee et al. 2005; Dobrev et al. 2006; Shimoyama et al. 2007; Calo et al. 2010; Nishikawa et al. 2010). Conversely, some other nuclear factors (TWIST1, HAND2, ZFP521, STAT1, Schnurri 3, GLI3, HOXA2, and the HES/HEY proteins) have been found to suppress RUNX2 levels or activity (Kanzler et al. 1998; Kim et al. 2003; Bialek et al. 2004; Garg et al. 2005; Jones et al. 2006; Hilton et al. 2008; Ohba et al. 2008; Funato et al. 2009; Hesse et al. 2010). These factors also use a range of mechanisms, including blocking RUNX2 DNA binding, nuclear translocation, and protein expression.

Several transcription factors participate in the regulation of osteoblast differentiation by modulating OSX (Long 2012a). These include the tumor suppressor p53, which decreases OSX levels through an unknown mechanism (Wang et al. 2006), and NFATC1, a calcium-sensitive transcription factor that stimulates osteoblast differentiation by enhancing OSX transcriptional activity (Koga et al. 2005; Winslow et al. 2006).

Members of the AP1 transcription factor family have been shown to regulate osteoblast differentiation or function, but their relationship with RUNX2, OSX, or ATF4 is not yet clear. In particular, loss of the AP1 protein FRA1 in the mouse embryo led to a low-bone-mass phenotype owing to reduced production of bone matrix proteins (Eferl et al. 2004), whereas transgenic overexpression of FRA1 resulted in high bone mass resulting from increased osteoblast differentiation (Jochum et al. 2000). Similarly, transgenic overexpression of Δ FosB, a naturally occurring isoform of the AP1 protein FOSB, increased bone mass through cell-autonomous stimulation of osteoblast differentiation and function (Sabatakis et al. 2000; Kveiborg et al. 2004). Conversely, genetic deletion of the AP1 factor JUNB reduced osteoblast differentiation in a cell-autonomous manner (Kenner et al. 2004).

6 CLOSING REMARKS

Genetic studies in the mouse in the past 15 years have greatly enhanced our understanding about the molecular mechanisms governing endochondral development. In particular, the roles of several major developmental pathways and key transcription factors in chondrocyte differentiation, hypertrophy, and osteoblast differentiation have been elucidated. For clarity, we have presented the regulation of chondrocytes and osteoblasts separately. In reality,

development of the two cell lineages is tightly coupled during normal development. For instance, IHH is expressed by chondrocytes but regulates both chondrocyte maturation and osteoblast differentiation. A major challenge for future research is to understand how the various extracellular signals and nuclear factors integrate to execute specific developmental, homeostatic, and reparative functions. In addition, vascularization of the hypertrophic cartilage is of critical importance for proper development of the endochondral skeleton. Although a number of secreted factors including vascular endothelial growth factor A (VEGFA), IHH, and FGFs have been implicated (Gerber et al. 1999; Zelzer et al. 2002; Hung et al. 2007; Liu et al. 2007; Joeng and Long 2009), exactly how cartilage vascularization is regulated in a spatially and temporally specific manner remains an important topic for future research.

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