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Signaling Pathways Regulating Cartilage Growth Plate Formation and Activity

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Abstract

The growth plate is a highly specialized and dynamic cartilage structure that serves many essential functions in skeleton patterning, growth and endochondral ossification in developing vertebrates. Major signaling pathways initiated by classical morphogens and by other systemic and tissue-specific factors are intimately involved in key aspects of growth plate development. As a corollary of these essential functions, disturbances in these pathways due to mutations or environmental factors lead to severe skeleton disorders. Here, we review these pathways and the most recent progress made in understanding their roles in chondrocyte differentiation in growth plate development and activity. Furthermore, we discuss newly uncovered pathways involved in growth plate formation, including mTOR, the circadian clock, and the COP9 signalosome.

Keywords

Chondrocytes; Osteoblasts; Transcriptional Regulator; Transdifferentiation; COP9 Signalosome

1. Introduction

The vertebrate skeleton has evolved as a dynamic system that serves numerous functions, such as protecting internal organs, creating attachment sites for muscles to produce locomotion, providing a reservoir for minerals, and serving as a hematopoietic niche. Many signaling pathways control the patterning, growth and maturation of skeletal structures from early development to adulthood, as demonstrated by the various skeletal diseases that occur in humans due to their dysregulation. The vertebrate bony skeleton is formed through two

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processes: most craniofacial bones form through intramembranous ossification, whereby mesenchymal condensations directly differentiate into bone-forming osteoblasts; whereas all other bones are created through endochondral ossification, whereby cartilage templates form first, and are replaced by bone later [1] [2] [3]. These cartilage templates are produced by chondrocytes that undergo successive, well-coordinated steps of differentiation (Figure 1A). Early chondrocytes actively proliferate and produce a highly specific cartilage extracellular matrix, and later become hypertrophic. Along with chondrocyte maturation, the cells present in the perichondrium flanking hypertrophic chondrocytes differentiate into osteoblasts to form a bone collar (interim bone cortex), wherein vascular invasion occurs. Following terminal maturation of hypertrophic chondrocytes, the mineralized cartilage matrix is invaded by blood vessels and associated pericytes, osteoclasts, and osteoblast progenitors. It is ultimately replaced by vascularized trabecular bone as well as bone marrow, forming the primary ossification center. A similar process occurs in epiphyses during the early postnatal stage, forming secondary ossification centers. The area between the primary and secondary ossification centers is the epiphyseal growth plate, which persists in humans until puberty to sustain longitudinal bone growth (Figure 1B).

The epiphyseal growth plate is comprised of discrete zones representing chondrocytes undergoing stepwise maturation in a staggered manner, from resting, proliferative, and prehypertrophic stages, to hypertrophic and terminal stages (Figure 1B). In the reserve zone, chondrocytes are relatively inactive mitotically and express such markers as *Col2a1* (encoding type II collagen). Underneath this zone, chondrocytes rapidly proliferate and highly express *Col2a1* and *Acan* (aggrecan). Next, chondrocytes exit the cell cycle and become prehypertrophic, expressing *Col10a1* (type X collagen) and *Ihh* (Indian Hedgehog) [3]. Prehypertrophic chondrocytes increase in size to become hypertrophic cells that stop expressing *Col2a1* but continue expressing *Col10a1*. Hypertrophic chondrocytes eventually stop expressing *Col10a1* and reach terminal stages, undergoing apoptosis, autophagic cell death, or transdifferentiation.

Not surprisingly, signaling cascades need to be finely tuned to ensure proper skeletal development. The complex regulatory system allows various developmental cues from the environment to transduce into proper spatial- and temporal-specific cellular responses. These signaling cascades converge on master chondrocyte transcription factors, including Sox9, Runx2, and Osterix. Here, we review critical signaling pathways involved in growth plate development and representative skeletal diseases associated with signaling dysregulation. For each signaling pathway, there is a considerable degree of combinatorial mixing and matching at the level of the ligands, receptors, antagonists, and downstream mediators. This produces a large diversity in transcriptional outputs and exerts precise context-dependent control. As of 2015, 436 genetic skeletal disorders have been recognized; as such, this topic has been intensively studied and reviewed [4, 5]. Thus, after a brief overview, we here focus only on the most recent progress regarding growth plate formation for each signaling pathway.

2. Transforming Growth Factor β (TGF- β)/Bone Morphogenetic Protein (BMP) Signaling

The TGF- β superfamily is a highly conserved group of more than 40 members, including TGF- β s, Activins, and BMPs, and many of them play an important role in skeletogenesis. The large number of TGF- β superfamily ligand-receptor combinations makes elucidating the role of each one very challenging. This difficult task is further compounded by functional redundancy among members and by the contextual-dependent nature of TGF- β signaling output [6]. Briefly, the binding of TGF- β superfamily ligands to single-pass transmembrane serine/threonine receptors results in the formation of a heteromeric receptor ligand complex, where the ligand-bound type II receptor phosphorylates the type I receptor. The type I receptor then recruits and phosphorylates regulatory Smad transcription factors (R-Smads), which form a complex with the coregulatory Smad (Co-Smad) Smad4. The complex accumulates in the nucleus and regulates gene transcription. TGF- β signaling primarily targets the R-Smads Smad2 and 3, whereas BMP signaling mostly mediates its response via Smad1, 5, and 8. Another group of Smads, the inhibitory Smads (I-Smads), act as negative regulators of R-Smads to inhibit TGF- β /BMP signaling.

Due to space limitations, we refer readers to a most recent review highlighting the well-established role of BMP signaling in skeletal development, particularly its role in bone and joint formation [7]. BMPs and their receptors are expressed in proliferating chondrocytes, hypertrophic chondrocytes, and perichondrium cells. BMP signaling is vital to endochondral bone formation through its roles in regulating chondrocyte proliferation and differentiation. Most importantly, BMP and TGF- β signaling are required for the expression and maintenance of SOX9, a master transcriptional regulator of chondrogenesis [8] [9] [10] and are thereby important inducers of chondrogenesis.

Mice with a global knockout of *Bmp2* or *Bmp4* die before E10.5, prior to the onset of skeletal development [11] [12]. In mice with *Bmp2* deleted specifically in chondrocytes (using a *Col2a1-CreER* transgene), there was severe chondrodysplasia caused by impaired differentiation and maturation of chondrocytes. In contrast, *Bmp4* deficiency (achieved using the same system) caused only a mild cartilage phenotype [13]. Thus, *Bmp2* is a major ligand in chondrocytes. Interestingly, deletion of *Bmp2* and *Bmp4* in osteochondroprogenitors (*Prx1-Cre*) revealed that the genes are only required for mesenchymal condensations of the posterior digits in the forelimbs, possibly because other BMP genes are required for other condensations [14]. Accordingly, the overexpression of Noggin, a BMP inhibitor, blocked the formation of all prechondrogenic condensations [15, 16]. *Noggin* knockout mice have defects in all skeletal elements, resulting in shorter limbs, fused vertebrae, and fewer ribs [17]. On the other hand, when BMP2/4 was overexpressed in the developing chick limb, there was an increased number of chondrocytes and an increased production of cartilage extracellular matrix, and joints failed to form [18]. Recently, Dullard was discovered as a critical player in chondrogenesis. Although this phosphatase was originally identified as an inhibitor of BMPRII/2 *in vitro* [19], deletion of the gene *Dullard* using a *Prx1-Cre* driver resulted in a significant reduction in the proliferative and

hypertrophic layers in growth plates of newborn mice, and these defects were associated with upregulated TGF- β signaling, but not BMP signaling [20]

Combined deletion of *Bmpr1a* (BMP Receptor Type IA) and *Bmpr1b* (BMP Receptor Type IB) in mouse chondrocytes resulted in a generalized chondrodysplasia with no growth plates [21]. These type I BMPR were shown to promote growth plate chondrocyte proliferation and late-stage differentiation by promoting *Ihh* expression and inhibiting *Fgfr1* expression [22]. On the other hand, over-expression of a constitutively active form of *Bmpr1a* in mouse chondrocytes led to premature chondrocyte hypertrophy [23]. Another Type I receptor, Activin receptor IA/Activin-Like Kinase 2 (ACVR1/ALK2), binds to multiple BMP, TGF- β and Activin ligands. The activating mutations in *ACVR1/ALK2* cause Fibrodysplasia Ossificans Progressiva (FOP), a rare and devastating genetic disorder characterized by endochondral ossification occurring at extra-skeletal sites, such as muscle and ligaments [24] [25]. Human mesenchymal stem cells overexpressing the ACVR1 activating mutation R206H, which occurs in 98% of FOP patients, were shown to cause ectopic bone formation in nude mice [26], and mice lacking *Acvr1* in chondrocytes had significantly reduced BMP signaling, as well as craniofacial defects, axial skeleton defects, and thoracic kyphosis [27] [28].

The chondrocyte-specific deletion of *Smad1/5/8* caused severe chondrodysplasia in mice [29]. These mice had small cartilage anlagen and reduced *Sox9*, *Runx2*, *Col2a1* and *Col10a1* expression. Growth plate chondrocytes displayed reduced proliferation and increased apoptosis, and lack of columnar organization and hypertrophy. While *Smad4* expression was not altered in these mice, the expression of BMP receptor genes was lower, as was the expression of *Tak1* and *Mekk3*, which encode kinases activating p38 MAPK through BMP signaling. Lastly, the *Ihh*/PTHrP feedback loop and functional antagonism between BMPs and FGFs were also disrupted [29]. There was a very similar lethal chondrodysplasia phenotype in the *Smad1/5* double mutant mice, while *Smad8*-deficient mice had mostly normal growth plates and chondrogenesis [29]. Thus, these results point to a more important role of *Smad1/5* over *Smad8* in chondrocytes. Furthermore, the phenotype of *Smad1/5* mutant mice was very similar to that of *Bmpr1a/Bmpr1b* receptor mutant mice [21], indicating that BMP signaling is mostly mediated through *Smad1/5* in the growth plate. Interestingly, the chondrocyte-specific deletion of *Smad4* using the same *Col2a1* transgene resulted in a mild growth plate phenotype [7]. Thus, *Smad1/5* likely may have many *Smad4*-independent actions in chondrocytes.

The inhibitory *Smad6* and *Smad7* add another layer of regulation to TGF- β /BMP signaling. *In vitro*, *Smad7* can inhibit both TGF- β and BMP signaling, whereas *Smad6* inhibits primarily BMP signaling [6]. *Smad6/7* acts at multiple levels, including ubiquitin-mediated degradation of R-Smads and TGF- β receptors, competitive inhibition of Co-*Smad4* binding, and binding of TGF- β receptors to prevent *Smad* phosphorylation. Both *Smad6* and *Smad7* play important roles in cartilage [30] [31]. *Smad7*-deficient mice are dwarfs, with delayed vascular invasion of growth plates and short hypertrophic zones. They show increased expression of *Col2a1* and *Col10a1*, with a concomitant decrease in the expression of *Mmp13*, a terminal chondrocyte marker [31]. They have increased levels of phospho-*Smad1/5/8*, indicating enhanced BMP signaling in growth plates. *Smad6*-deficient mice

displayed similar impairment of skeletal development [30]. They also showed an increase in BMP type 1 receptor expression, consistent with a study showing that Smad6 is responsible for the degradation of this receptor [30]. Therefore, Smad6/7's control of chondrocyte proliferation and hypertrophy is at least in part through inhibiting BMP signaling.

In addition to the Smad-mediated canonical BMP pathway, BMPs also signal through noncanonical pathways in which BMP receptors initiate signaling via MAPKs, ultimately resulting in p38 MAPK family activation by downstream mediators including TGF- β activated kinase 1 (TAK1) [32]. TAK1 can be activated downstream of TGF- β and BMP4 *in vitro*. Both chondrocyte-specific and limb mesenchyme-specific deletion of *Tak1* resulted in severe chondrodysplasia, with impaired formation of secondary ossification centers, absence of joints, and reduced BMP signaling [32] [33] [34]. Kindlins are a group of conserved cytoplasmic proteins involved in extracellular matrix adhesion, migration, and signaling. Knockout of *Kindlin-2*, a newly identified regulator of TGF- β signaling, in mice led to reduced Smad2 phosphorylation and neonatal lethal chondrodysplasia, disorganized chondrocyte columns, decreased chondrocyte proliferation, and increased apoptosis [35]. Lastly, Kartogenin, a small biomolecule, was able to stimulate limb skeletal growth and facilitate joint formation through the modulation of TGF- β and *Ihh* pathways [36]. All these latest results highlight that intricate TGF- β and BMP regulatory networks are directly involved in chondrogenesis and growth plate formation.

In humans, many skeletal dysplasias result from disrupted BMP signaling. In addition to FOP mentioned earlier, acromesomelic dysplasia is a group of rare osteochondrodysplasias characterized by short limbs and rudimentary fingers and toes. It results from mutations in *BMPT1B* or in the gene for the Growth and Differentiation Factor 5 (*GDF5*), a TGF- β superfamily member [4] [37]. Heterozygous mutations in the BMP antagonist *NOGGIN* results in multiple synostosis syndrome and proximal symphalangism, diseases characterized by failure to form joints in the hands and feet [38]. Moreover, many mutations in genes that alter BMP signaling through disrupting either ligand or receptor function can cause brachydactyly [5].

3. Fibroblast Growth Factor (FGF) Signaling

The FGF family is composed of at least 18 secreted ligands, which can bind to 4 different receptors (FGFR1–4). Many of the FGF ligands, including FGF4, FGF8, and FGF10, are responsible for limb bud formation and patterning [39]. Additionally, FGF9 and FGF18 are important regulators of endochondral bone formation [39]. FGF9 and FGF18 have both redundant and distinct functions. FGF18 has a biphasic effect, promoting chondrocyte proliferation and hypertrophy in early gestation (E14.5) and inhibiting them in late gestation (E16.5) [40] [41]. *Fgf18*^{-/-} and *Fgfr3*^{-/-} mice have similar growth plate phenotypes of expanded proliferating and hypertrophic zones, whereas *Fgf9*^{-/-} mice have a normal proliferative zone. FGF9 and FGF18 promote and maintain *Ihh* and *Runx2* expression [40]. Both are also involved in growth plate vascularization.

Alternative splicing creates FGFR isoforms with different specificities for FGF ligands. Upon ligand binding, FGFRs homodimerize and trans-autophosphorylate, leading to the

phosphorylation of the docked adaptor protein FGFR substrate 2 α (FRS2 α). This allows the binding of other adaptor proteins and activation of different signaling cascades, involving MAPKs, PI3K, PLC γ , and JAK-STAT. FGFR3 signaling results in the activation of STAT1, ERK1/2, and p38 MAPK to regulate growth plate formation and endochondral ossification. FGFR3 can also indirectly control growth plate chondrocytes through modulating BMP, WNT, IHH, PTHrP and other developmental signaling pathways. The deletion of *Fgfr3* in mice results in increased chondrocyte proliferation and an expansion of the hypertrophic zone, whereas its overexpression results in achondrodysplasia, a severe form of dwarfism due to very short growth plates [39]. In mice overexpressing *Fgfr3* in limb bud mesenchyme, differentiation of chondrocytes from prehypertrophy to hypertrophy was blocked. Persistent *Sox9* expression also occurred, possibly due to down-regulation of β -catenin [42].

Mutations in the FGF receptors lead to human skeletal dysplasias with variable severity. Activating mutations in *FGFR3* cause achondroplasia, the most common form of dwarfism among live births. Some of these mutations, such as G380R, located in the FGFR3 transmembrane domain, stimulate FGF signaling in a ligand-dependent fashion. Others, like R248C in the extracellular domain or K650E in the intracellular domain, act in a ligand-independent manner and result in a more severe form of dwarfism, thanatophoric dysplasia [39]. The growth plates of humans with *FGFR3* mutations show disrupted chondrocyte columns and reduced numbers of hypertrophic chondrocytes [39]. A recent study showed that the efficacy of five small molecule FGFR inhibitors, while effective in chondrocytes *in vitro*, did not improve skeletal growth in mice. Troublingly, they had no selectivity for FGFR3, but had significant off-target activity for IGFR1 [43]. These data show that new innovative approaches to treat achondrodysplasia are still required. Encouragingly, several candidate therapies are in progress [44]. These include competitive inhibitors such as a decoy receptor and a synthetic peptide that binds to FGFR3. Additionally, drugs such as statins decrease the activity of mutant FGFR3, whereas meclozine blocks MAPK and ERK signaling downstream of FGFR3. Both drugs increased the mouse *Fgfr3* mutant limb length. The most promising therapy, an analog drug of C-type natriuretic peptide (CNP), is in phase II clinical trials, and was shown to increase growth velocity in children with achondrodysplasia through antagonism of MAPK signaling [44]. The tyrosine kinase inhibitor NVP-BGJ398, which is more selective for FGFR3 compared to the other FGF receptors, was shown to inhibit the hyperactivity of FGFR3 in a mouse model of achondrodysplasia and in human cartilage cells, as evidenced by restoration of chondrocyte proliferation and improvement of chondrocyte differentiation [45].

In addition to *FGFR3*, many human diseases are associated with loss and gain of function mutations in *FGFR1* and *FGFR2*. Both activating and inactivating missense mutations in *FGFR1* results in several different craniosynostosis syndromes, and missense mutations, deletions, and insertions in *FGFR2* cause craniosynostosis syndromes, as well as defects in the appendicular skeleton and other organs [39]. FGFR1 and FGFR2 play many essential and mostly redundant roles during development, including growth plate formation. *Fgfr2*-deficient embryos fail to form limb buds [39]. On the other hand, overexpression of *Fgfr1* in chondrocytes causes joint fusion. Deletion of both *Fgfr1* and *Fgfr2* in mice using an *Osx-Cre* driver caused a decreased length of the growth plate with a reduced number of proliferating chondrocytes [46]. Interestingly, the inactivation of *Fgfr1/2* resulted in suppression of *Ihh*

and *Pthrp*, and the growth plate phenotype was rescued by administration of PTH(1–34), suggesting an *in vivo* link between FGF signaling and IHH signaling in relaying information between growth plate chondrocytes [46].

MAPK/ERK Kinase 1 (MEK1) is activated by FGF signaling, and phosphorylates as well as activates ERK1/2 MAPKs (Extracellular signal-Regulated Kinase/Mitogen-Activated Protein Kinase). Constitutive activation of MEK1 in chondrocytes resulted in achondroplasia through inhibiting hypertrophic differentiation [47]. *Erk1/2* inactivation in osteochondroprogenitor cells (*Prx1-Cre*) caused ectopic chondrocyte differentiation in the perichondrium, whereas expression of constitutively active *MEK1* inhibited chondrocyte differentiation [48]. Furthermore, chondrocyte-specific deletion of *Erk1/2* (*Col2a1-Cre*) caused severe chondrodysplasia [48], whereas *Erk1/2* inactivation in hypertrophic chondrocytes (*Osx-Cre*) resulted in expansion of the hypertrophic zone and impairment of chondrocyte terminal differentiation [49]. MEK1 and ERK1/2 can also be activated by RAF kinases [50]. The deletion of *a-Raf* and *b-Raf* specifically in chondrocytes did not affect endochondral bone development [51]. However, chondrocyte-specific ablation of *c-Raf* caused expansion of the hypertrophic zone, decreased apoptosis of chondrocytes, and impaired vascular invasion of the growth plate concomitantly with reduced *Vegf* expression and decreased phospho-Erk1/2 levels *in vivo* [52]. These results indicate that c-Raf, which is the predominant isoform expressed by hypertrophic chondrocytes, plays a more important role in growth plate maturation than a- and b-Raf [52].

FGF and BMP signaling act antagonistically on chondrocyte proliferation and hypertrophic differentiation [1]. In a mouse model of achondrodysplasia, BMP2 treatment partially rescued the growth plate defect by increasing the size of the hypertrophic zone and increasing chondrocyte proliferation in limb explants [53]. BMP-mediated activation of a *Msx2* promoter linked to a luciferase reporter was antagonized by FGF2 via the ERK/MAPK pathway. BMP induction of *Ihh* was also inhibited by ERK [29]. On the other hand, BMP signaling inhibits the activation of STAT and ERK1/2 MAPKs, which are key effectors of FGF signaling [22]. Interestingly, BMP and FGF signaling can also act synergistically to induce early chondrogenesis. Indeed, it was shown that FGF is necessary and sufficient to induce *Sox9* expression and specification of a prechondrogenic identity in neural crest-derived mesenchymal cells. However, FGF was unable to maintain *Sox9* expression and could not initiate the full *Col2a1*-expressing chondrogenic program. In contrast, BMP4 was able to maintain *Sox9* expression, but only in combination with FGFs [54]. These findings highlight the complex nature of growth plate regulation, as many signaling pathways intersect in a delicate fashion.

4. WNT/ β -Catenin Signaling

There are at least 19 WNT ligands and most signal through the canonical β -catenin pathway. When WNT ligands are absent, β -catenin is bound in a destruction complex consisting of glycogen synthase kinase 3 β (GSK3 β), axin, and adenomatosis polyposis coli (APC). GSK3 β phosphorylates β -catenin and thereby triggers its ubiquitination and degradation. Wnt ligands bind to seven-pass transmembrane receptors Frizzled (Fzd) along with the co-receptors lipoprotein-related protein 5 (LRP-5) and LRP-6. Upon this interaction, GSK3 β -

mediated phosphorylation of β -catenin is inhibited, resulting in β -catenin stabilization. β -catenin then translocates to the nucleus and acts as a co-activator of TCF/LEF transcription factors. Members of the Dkkopf (DKK) family, secreted Frizzled-related proteins (sFRP), and Wnt inhibitory factor 1 (WIF-1), act as antagonists of Wnt signaling [55]. Mouse genetic studies have shown that Wnt signaling is intimately involved in the development of many tissues and organs, and both gain- and loss-of-function mutations in Wnt signaling cause various human diseases, including bone diseases [56] [57, 58].

WNT signaling is well known to regulate chondrocyte differentiation and cartilage development [3]. It is well established that β -catenin and SOX9 directly antagonize each other's stability in differentiating chondrocytes [59] [60] [61] [62] [63]. The inactivation of β -catenin specifically in chondrocytes of mouse embryos led to severe dwarfism with decreased chondrocyte proliferation, delayed hypertrophy, and impaired endochondral bone formation accompanied by increased *Sox9* expression [59]. The defect in chondrocyte proliferation and maturation was similar in another mouse model with deletion of *β -catenin* using a *Dermo1-Cre* driver [64]. Additionally, the deletion of *β -catenin* in hypertrophic chondrocytes (*Col10a1-Cre*) impaired chondrocyte maturation, as assessed by reduced expression of *Vegf*, *Mmp13*, *Runx2*, and *Osx* [65]. Further demonstrating the sensitivity of chondrocytes to β -catenin levels, β -catenin stabilization in chondrocytes of mouse embryos led to severe chondrodysplasia with decreased *Sox9* expression [59]. Although *Lrp5* and *Lrp6* serve largely redundant functions, deletions of *Lrp5* and *Lrp6* using a *Dermo1-Cre* driver caused a severe shortening of the endochondral skeletal elements as a result of reduced hypertrophic chondrocytes and concomitant with a decreased expression of *Col10a1* [66]. Additionally, the *Col2a1-Cre*-targeted deletion of *Lrp5/6* resulted in lethality either *in utero* or shortly after birth, and analysis of E18.5 embryos showed abnormal growth plate development [67]. Inhibition of β -catenin in chondrocytes by expressing ICAT (Inhibitor of β -catenin and TCF), a Wnt inhibitor, resulted in delayed skeletal growth postnatally with decreased proliferative and hypertrophic zones [68]. Wnt signaling has also been shown to promote chondrocyte hypertrophy by inhibiting PTHrP activity [69].

Multiple Wnt ligands signal through non-canonical pathways, including the planar cell polarity (PCP) and the Wnt/ Ca^{2+} pathways [57]. Non-canonical Wnt signaling has been implicated in promoting chondrocyte differentiation due to its ability to inhibit canonical Wnt signaling. The PCP pathway plays a unique role in inducing chondrocyte column formation in the growth plate [70]. Recently, time-lapse movies of live tissue revealed that chondrocyte columnar arrangement in the growth plate involves cadherin- and β -catenin-dependent expansion of surface adhesion [71].

5. Hedgehog Signaling and Parathyroid related Peptide (PTHrP) Signaling

Hedgehog (Hh) signaling is involved in many developmental processes. Mutations in hedgehog genes cause various congenital limb deformities [5]. Vertebrates produce three Hh ligands: Sonic (Shh), Indian (Ihh), and Desert Hedgehog (Dhh). If Hh ligands are absent, the G protein-coupled receptor Smoothened (Smo) is inhibited catalytically by Patched (Ptc). Upon Hh ligand binding to Ptc, the inhibition of Smo is released and Gli transcription factors are activated [72] [73]. The two Patched homologues, Patched1 and Patched2, were

recently found to play overlapping and complementary roles in limb development [74]. *Shh*-deficient mouse embryos die at birth with holoprosencephaly and oligodactyly, a disorder caused in humans by *SHH* mutations, whereas *Shh* overexpression causes polydactyly [75] [76]. *Ihh* is produced by prehypertrophic chondrocytes and plays a crucial role in chondrocyte differentiation. *Ihh*-deficient mouse embryos display severe skeletal growth retardation. They show reduced chondrocyte proliferation, and premature and abnormal hypertrophy [77] [78] [79]. *Scube2* encodes a secreted and cell membrane-bound protein that mediates long-range *Ihh* signaling, probably through increasing *Ihh* solubility by binding to its cholesterol moiety. Genetic ablation of *Scube2* led to defective chondrocyte differentiation and proliferation, manifested by short growth plates [80]. Thus, post-translational modifications play an important role in modulating *Ihh* activity.

Ihh plays a crucial role in chondrocyte hypertrophy. In a negative feedback loop, *Ihh* promotes the expression of the gene for the parathyroid hormone-related peptide (PTHrP), which maintains growth plate chondrocytes in a proliferative state. Overall, the concerted actions of *Ihh* and PTHrP regulate chondrocyte proliferation and maturation. *Pthrp* is expressed in resting chondrocytes and maintains chondrocyte proliferation, whereas chondrocytes farther away from the resting zone in the growth plate do not receive PTHrP signals, and undergo hypertrophy. Indeed, *Pthrp*-deficient mice had accelerated hypertrophic differentiation and premature mineralization [81].

PTHrP signals through parathyroid hormone type I receptor (PTH1R), a G-protein coupled receptor, to control skeletogenesis and *Pth1r* knockout mice also showed accelerated hypertrophy [82]. Disruption of PTH1R signaling is associated with a number of osteochondrodysplasias, such as brachydactyly type E, caused by a mutation in *PTHrP*, and metaphyseal dysplasia and Eiken dysplasia, which are rare skeletal disorders caused by mutations in *PTH1R* [82].

6. Notch Signaling

Notch signaling is another evolutionarily conserved pathway involved in cell fate determination, differentiation, proliferation, and apoptosis [5]. It occurs when the Notch receptor is activated by binding to Delta or Jagged, ligands anchored on adjacent cells as type 1 transmembrane proteins [83]. The Notch receptor then undergoes cleavage to generate a Notch Extracellular Truncation (NEXT) fragment. This fragment is further cleaved by γ -secretase proteinase to produce the Notch Intracellular Domain (NICD). NICD translocates to the cell nucleus to interact with the transcription factor RBPJ and the transcriptional coactivator Mastermind-like 1 (MAML1). The ternary complex promotes the expression of basic helix-loop-helix (bHLH) transcription factors of HES and HEY families, which then transcriptionally repress downstream target genes [83].

Notch signaling plays important roles in bone and cartilage formation. Mouse genetic deletions and human genetic mutations in Notch signaling are associated with a variety of defects in patterning, axial skeleton development, and skeletal diseases [83, 84]. Notch signaling is important for chondrocyte hypertrophy in part via regulating *Sox9* expression [83] [85]. Mouse models with loss and gain of function in Notch signaling have revealed that

Notch acts upstream of *Sox9* and *Runx2* in the regulation of osteochondro-progenitor cell proliferation and lineage specification. In *Prx1-Cre* targeted osteochondral progenitor cells, the loss of Notch enhanced chondrocytic lineage commitment but impaired fracture healing due to depletion of the bone marrow stromal cell pool, whereas gain of Notch function suppressed chondrocyte differentiation and proliferation [86] [87]. Additionally, the overexpression of NICD specifically in chondrocytes (*Col2a1Cre* and *Col2a1CreER*) caused skeletal malformations with decreased proliferation and impaired chondrocyte hypertrophy, whereas the loss of Notch function in these cells increased the hypertrophic zone size [88] [89, 90]. Notch negatively regulates chondrocyte differentiation in the axial skeleton by suppressing *Sox9* expression [89]. A RBPjk-dependent pathway mediates the chondrocyte maturation, whereas a RBPjk-independent pathway is incriminated in the suppression of chondrocyte proliferation, survival and columnar organization, as well as impaired responsiveness to Ihh signaling [90]. Again, the role of Notch signaling in growth plates is likely to be even more complex as Notch signaling was recently shown to regulate chondrocyte hypertrophy in part in a *Sox9*-independent manner [85]. Furthermore, *Prx1-Cre* deletion of *Hes1* and *Hes5*, the key targets of RBPjk-dependent Notch signaling, confirms that deletion of Notch effectors in osteochondral progenitors accelerates chondrocyte differentiation [91]. While *Hes5* alone controls *Sox9* transcription, both *Hes1* and *Hes5* are required to suppress chondrogenesis and promote the onset of hypertrophy. Additionally, the overexpression of *Hes1* in osteochondral progenitor cells delays chondrocyte hypertrophy and reduces proliferation. On the other hand, the HEY family of transcription factors play no role in chondrogenesis or matrix catabolism. However, this contrasts with an earlier study of combined deletion of *Hes1* and *Hes5* using a *Col2a1-Cre* driver showed that they are dispensable for endochondral ossification in chondrocytes [92]. This again highlights the stage-specific role that Notch signaling plays in growth plate homeostasis. Another new study has shown that Notch may inhibit chondrogenic differentiation of limb bud mesenchyme by upregulating the expression of *Twist1*, which encodes a bHLH transcription factor involved in skeletal formation [93]. These data show that a key action of Notch signaling in chondrogenesis and growth plate formation is to control the expression of such master cell fate regulators as *Sox9* and *Twist1*, and understanding of the downstream Notch targets provides insight into normal development and the role of Notch in skeletal disorders.

7. Retinoic Acid Signaling

Retinoic acid (RA), the main metabolite of vitamin A, elicits a key pathway involved in growth plate regulation. Signaling occurs when RA binds to RA Receptors ($RAR\alpha/\beta/\gamma$), which will then form a heterodimer with Retinoid X Receptors ($RXR\alpha/\beta/\gamma$), and bind to RA Response Elements in DNA to directly regulate gene transcription. Importantly, the RAR and RXR heterodimers can also act as transcriptional repressors in the absence of ligand through the recruitment of co-repressors and histone deacetylase complexes to promote cartilage growth and chondrocyte differentiation [94]. The amount of RA available to a cell is dependent on the balance between the RA synthesizing enzymes retinol dehydrogenases (RDH) and retinaldehyde dehydrogenases (RALDH), and cytochrome P450 26 (CYP26), which degrades RA [94]. The genetic deletion of any single member of the *RAR* family (*RAR α* , *RAR β* , *RAR γ*) in mice results in a minor phenotype, while deletions of multiple

RAR receptors (e.g. *RARα/γ* or *RARβ/γ*) results in perinatal lethality with severe skeletal abnormalities [95] [96]. While most *RAR* and *RXR* genes are functionally redundant, *RXRα*-deficient mice die *in utero* [94].

RA signaling regulates anteroposterior and proximodistal patterning in early limb development [94]. RA signaling in the proximal limb promotes the expression of transcription factors *Meis1/2* in the limb bud, whereas *Fgf8* signaling in the distal limb represses the expression of *Meis1/2* to promote proper proximodistal patterning [94]. Furthermore, in mouse and zebrafish models deficient for *Raldh2*, the enzyme catalyzing the conversion of retinaldehyde to RA, there is a severely reduced expression of *T-box 5* (*Tbx5*), a transcription factor expressed in the lateral plate mesoderm and important for forelimb initiation [94]. Indeed, the deletion of *Raldh2* in mice causes the absence of forelimb bud development.

Elucidating the role of RA signaling in the growth plate has been challenging since activating as well as arresting RA signaling can result in similar phenotypes. Multiple animal models, including quail, zebrafish, chick and murine models, show that RA is important in forelimb development and nearly dispensable in the hindlimb [94]. Furthermore, exogenous treatment with RA has a teratogenic effect on chondrocyte differentiation in murine limb development. Indeed, increasing RA signaling through global and *Prx1-Cre*-mediated deletion of *Cyp26b1* in mice resulted in truncated limbs and abnormal digit formation. Furthermore, the addition of a *RARα* antagonist in culture stimulates chondrogenesis, whereas a *RARγ* agonist can inhibit BMP signaling response in chondrogenesis. Thus, RA signaling generates an important signaling cascade for limb bud development, and interacts with many pathways involved in control of growth plate homeostasis, including FGF, WNT, BMP, and SHH [94]. For example, BMP signaling inhibits the expression of *Aldh1a2*, an enzyme that synthesizes RA [97]. Furthermore, inhibition of RA signaling in primary cultures of mouse limb mesenchyme repressed *Sox9* expression, and p38 MAPK and PKA signaling is activated when RA signaling is inhibited [98]. Interestingly, global deletion of RA Receptor γ (*RARγ*) in *Cyp26b1*-deficient mice only partially rescues limb morphology, indicating that there are two distinct effects of RA signaling in the limb, a teratogenic effect and a patterning effect [99]. Additionally, deletion of *RARα/γ* or *RARβ/γ* in chondrocytes (*Col2a1-Cre*) caused severe growth retardation by 3 weeks of age, with shorter growth plates, reduced proliferating chondrocytes and decreased *Aggrecan* expression, whereas *RARα/β*-deficient mice are essentially normal [96]. In contrast, RA treatment of mouse epiphyseal chondrocytes in culture stimulates Wnt signaling and enhances the inhibitory role of Wnt signaling on matrix accumulation [100]. Therefore, the RA signaling pathway is also likely to be context-dependent, and further study is warranted to better understand the mechanism with which RA metabolism controls growth plate development.

8. mTOR Signaling

The mammalian Target of Rapamycin (mTOR) pathway is a ubiquitous, central regulator of cell metabolism, survival and proliferation and is thereby critical for organismal growth and homeostasis. This pathway integrates various types of extracellular and intracellular signals.

It can be activated by various growth factors, such as epidermal growth factor and insulin-like growth factor, which activate Akt/PI3K signaling, as well as nutrients such as amino acids, glucose, and oxygen levels. It can be inhibited by AMP-activated protein Kinase (AMPK) in response to the absence of nutrients in the cell [101].

The importance of mTOR in the cartilage growth plate has only recently started to emerge. mTOR is a highly conserved serine/threonine kinase that takes part in two distinct complexes: mTORC1 and mTORC2. These complexes share several common protein subunits in addition to mTOR, and differ by other components, namely RAPTOR for mTORC1 and RICTOR for mTORC2 [101]. The deletion of *mTOR* specifically in limb bud mesenchyme (*Prx1-Cre*) resulted in impaired embryonic skeletal growth [102]. Mutant mice died at birth due to exencephaly, and displayed smaller appendicular bones, although the authors did not describe any growth plate abnormalities in these mutants. The disruption of mTORC1 specifically by deleting *Raptor* (*Prx1-Cre*) also resulted in neonatal lethality [102]. Cartilage elements showed reduced production of cartilage extracellular matrix due to deficient protein synthesis. There was no effect on growth plate chondrocyte proliferation or survival, but chondrocyte hypertrophy was delayed and cells showed limited enlargement [102]. The disruption of mTORC2 specifically by deleting *Rictor* (mTORC2) in limb bud mesenchyme caused a similar phenotype, with shorter skeletal elements and delayed chondrocyte hypertrophy. However, these mutants had no defect in cartilage matrix production and although the hypertrophic zone was shortened, there was no change in hypertrophic chondrocyte cell size [103].

Interestingly, a recent study demonstrated that mTORC1 promotes early stage chondrocyte proliferation and prevents later stage terminal differentiation [104]. Indeed, mice treated with rapamycin showed thinner growth plates and a reduced number of proliferating and prehypertrophic chondrocytes. Furthermore, the deletion of TSC1, an upstream negative regulator of mTORC1, specifically in chondrocytes (*Col2a1-Cre*) delays postnatal endochondral bone formation and causes chondrodysplasia and dwarfism. The mutants had enhanced chondrocyte proliferation but impaired chondrocyte hypertrophy and terminal differentiation. This phenotype was rescued by rapamycin treatment, indicating there is an uncoupling of the proliferation and differentiation program in the growth plate as a result of mTOR signaling disruption. mTORC1 activity is also crucial for PTHrP transcription by phosphorylating and promoting the nuclear accumulation of Gli2, a Hedgehog signaling mediator, in a smoothened-independent manner. [104]. Finally, the deletion in mice of *Lkb1*, a serine/threonine kinase inhibitor of mTOR signaling, in chondrocytes (*Col2a1-Cre*) resulted in marked growth retardation in postnatal mice, concurrent with a disorganized growth plate that had increased proliferating chondrocytes and ectopic hypertrophic chondrocytes [105]. Thus, mTOR signaling is an important coordinator of chondrocyte proliferation and hypertrophy. mTORC1 and mTORC2 play important and distinct roles in the growth plate and the disruption of mTOR signaling might affect growth plate homeostasis through disrupting other signaling pathways. However, much more work is still needed to further elucidate how mTOR signaling regulates the growth plate.

9. Insulin-like Growth Factor (IGF) Signaling

Insulin-like growth factor signaling has key roles in endochondral ossification. It consists of two ligands (IGF1 and IGF2), two receptors (IGFR1 and IGFR2), and six IGF-binding proteins (IGFBP1 to 6). IGF1 is the main signaling ligand. IGF1 binds to IGFR1 with 20 times more affinity than IGF2. However, IGF1 barely binds IGFR2. Furthermore, IGFR1 is a receptor with tyrosine kinase activity that is highly homologous to the insulin receptor, whereas IGFR2 is a single pass transmembrane receptor with no kinase activity.

The binding of IGFs or insulin to IGFR1 initiates PI3K/AKT and RAS/RAF/MEK/ERK signaling to control growth and survival through activation of the mTOR pathway, inactivation of caspase-mediated cell death, and induction of cell cycle progression. IGFBPs act extracellularly as endocrine factors, although they can also be found intracellularly, to regulate IGF half-life or to modulate its signaling [106]. The global deletion of *Igf1* resulted in shortening of the skeleton and dwarfism, and *Igfr1* deletion caused a more severe growth reduction and neonatal lethality [107]. *Igf1* deletion is associated with reduced proliferation, decreased survival, and impaired maturation of growth plate chondrocytes [108]. Moreover, *Igfr1* deficiency specifically in chondrocytes (*Col2a1-Cre*) resulted in neonatal lethality, and the growth plates of these mutants showed disorganized chondrocyte columns and delayed ossification and vascularization [109]. Mutant chondrocytes exhibited decreased proliferation and increased apoptosis, as well as increased *Pthrp* expression. Mice with the deletion of *Igfr1* in prehypertrophic chondrocytes and osteoblasts (*Osx-Cre*) were viable, but resulted in impaired postnatal growth [108, 110]. The morphology of growth plates was abnormal, and chondrocytes showed decreased proliferation and delayed maturation. Interestingly, Wnt-induced secreted protein 3 (WISP3), a member of the CCN family of secreted growth factors, inhibits IGF1 signaling by binding to IGF1 and preventing its secretion. This results in impaired chondrocyte hypertrophy, with reduced *Col10a1* expression [111]. *WISP3* mutations in humans results in the autosomal recessive skeletal disease progressive pseudorheumatoid dysplasia (PPD), a spondylo-epiphyseal dysplasia characterized by cartilage loss and joint failure by early adulthood [112]. However, its deletion or overexpression in mice does not result in an apparent phenotype [112]. Therefore, more study is needed to elucidate its role in the growth plate.

While IGF1 signaling mostly predominates IGF2 signaling, IGF2 does play a role in growth plate chondrocyte differentiation. For example, treatment of mouse E18.5 limb explant cultures with IGF2 and BMP9 led to increased endochondral ossification with an expanded hypertrophic zone [113]. IGF2 synergistically acted with BMP9 in activating the Smad pathway, and this effect was antagonized by IGFBP3 and IGFBP4. Additionally, in fibroblasts isolated from patients with 3-M Syndrome, a human disorder characterized by pre and postnatal growth impairment, there was reduced expression of *IGF2* [114].

In humans, homozygous deletions of *IGF1* result in severe growth retardation *in utero* and postnatally, accompanied with severe osteopenia [107]. Various other *IGF1* mutations have also been reported, including a partial deletion of exons 4 and 5, a homozygous missense mutation, and a nucleotide substitution. These *IGF1* mutations caused various degrees of retarded skeletal maturation, microcephaly, deaf-mutism, and mental retardation [107].

Furthermore, humans with heterozygous mutations in *IGFR1* display a similar phenotype to *IGF1* mutations, underlying the importance of IGF1-IGFR1 signaling axis in human skeletal development [107].

10. The Circadian Clock

Another important emerging area of growth plate research is the regulation of endochondral ossification by the circadian clock and its effect on developmental signaling pathways. The circadian clock is an endogenous time-keeping system present in nearly all organisms. It is involved in the regulation of gene expression, physiology, and behavior with an approximately 24-hour periodicity. It is composed of a positive and negative regulatory feedback loop that is entrained by external zeitgebers (temporal cues), such as the sun, with the positive arm consisting of the transcription factors BMAL1 and CLOCK, and the negative arm consisting of the transcription factors PERIODS (PER) and CRYPTOCHROMES [115]. Increasing evidence indicates that circadian clocks also regulate skeletal homeostasis [116] [117]. Interestingly, some chondrocyte-specific genes, such as *Col2a1* and *Col10a1*, are expressed in a diurnal pattern along with the core clock genes in chondrogenic ATDC5 cells as well as in articular cartilage and growth plates [118] [119]. Mouse *Per1* and *Per2* expression is increased by PTH through PKA-CREB signaling [120]. Indeed, PTH can reset the circadian clock in femurs in a time- and dose-dependent manner [121] [122]. Thus, there might exist a chondrocyte-specific peripheral clock that is critical to growth plate development. In *Bmal1*-deficient mice, the femora and tibiae were significantly shorter, with impaired *Ihh*-mediated chondrocyte differentiation [123] [124]. More importantly, the reduced bone length and decreased *Ihh* expression persisted when *Bmal1* was deleted specifically in chondrocytes, confirming that the effect is likely to be cell-autonomous [124]. Moreover, the chondrocyte-specific ablation of *Bmal1* (*Col2a1-Cre*) also resulted in severe cartilage degeneration by 3 months of age due to dysregulated TGF- β signaling [125]. Given that the circadian clock signaling pathway is implicated in many physiological processes, it will be interesting to understand the detailed mechanism of circadian control of the musculoskeletal system, especially in regards to its interaction with other signaling pathways.

11. Novel Transcriptional Co-Factors involved in Growth Plate Signaling

The effects of diverse signaling pathways reviewed above all ultimately impinge on a multi-layered transcriptional network in which key transcription factors, such as Sox9, Runx2 and Osterix, have been extensively studied and their role in growth plates has been well established. On the other hand, some transcriptional cofactors, such as β -catenin, CBF β , JAB1, and YAP1, have been shown in recent years to also play a key role in growth plate formation. Such transcriptional cofactors, although they do not have identifiable DNA-binding domains themselves, can interact with key transcription factors such as Sox9, Runx2, and key signaling downstream effectors, such as Smads, to regulate chondrocyte differentiation and skeletal development in a spatio-temporal manner. In this regard, β -catenin serves as a prime example as a crucial transcriptional co-activator that plays essential roles at multiple steps of growth plate formation [57]. Overall, various transcription co-factors often interpret various developmental signaling pathways in an exquisite fashion,

serving as a crucial bridge between upstream signaling cues and downstream events in the nucleus [1]. We refer the readers to the review by Liu and colleagues in the same journal issue for a review on master transcription factors involved in chondrocyte specification and differentiation. We here below review very recent studies on two novel, and crucial transcriptional co-factors, the COP9 signalosome and YAP1

11.1. The COP9 signalosome

In recent years, chondrocyte differentiation and growth plate formation have been shown to be regulated by a novel transcriptional co-factor Jab1 (c-Jun activation domain binding protein-1). Jab1, also known as Cops5 and Csn5, is the fifth member of the COP9 Signalosome complex (CSN), an evolutionarily conserved proteolysis regulator consisting of 8 subunits and playing an essential role in the regulation of development in mammals, plants and flies [126]. The CSN has the deneddylase activity that regulates the stability of the SCF E3 ubiquitin ligase complex, and is involved in the regulation of protein stability, the control of protein subcellular localization, cell cycle control, DNA repair and many developmental processes [127]. Jab1 interacts with many master transcription factors and key signaling pathways, and is also overexpressed in various cancers [127] [128]. Jab1 directly interacts with the TGF- β /BMP pathway. Jab1 can bind Smad4 *in vitro* and induce Smad4 degradation, and this ultimately attenuates TGF- β signaling [129]. However, Jab1 was also shown to interact with and induce the degradation of Smad7, resulting in increased TGF- β signaling [130]. Interestingly, Jab1 can interact with Smad5 to inhibit BMP signaling in human chondrocyte cultures [131]. Thus, the role of Jab1 in TGF- β /BMP signaling is very likely to be highly context-dependent.

Interestingly, in both human and mouse growth plates, Jab1 is highly expressed in the resting zone and proliferative zone, but not in the hypertrophic zone, suggesting a critical role for Jab1 in controlling chondrocyte hypertrophy. However, global knockouts of *Jab1* in mice resulted in embryonic lethality by E8.5 prior to skeletogenesis [132] [133]. To better understand the physiological role of Jab1 in growth plate development, using a *Col2a1-Cre* driver, we have recently shown that Jab1 plays a crucial role in chondrocyte differentiation *in vivo*, most likely as a negative regulator of BMP signaling [134]. These mutant mice died at birth with severe chondrodysplasia, and had defects in the development of both the appendicular and axial skeleton, resulting in severe dwarfism [134]. Chondrocytes from these mice had altered BMP signaling, with increased BMP downstream effector Smad1/5 phosphorylation, enhanced response of primary *Jab1* knockout chondrocytes to BMP treatment, and increased expression of BMP ligands and target genes, including Runx2 [134]. Furthermore, we showed that Jab1 is also essential for early limb development in a mouse model using a *Prx1-Cre* driver to delete *Jab1* in osteochondral progenitor cells. The mutant embryos all displayed drastically shorter limbs that progressively worsen with age, with smaller cartilage elements, fewer hypertrophic chondrocytes, and a reduced or absent secondary ossification centers [135]. Interestingly, in micromass culture experiments, BMP response was decreased in the mutants, thus highlighting the differential role of Jab1 at different stages of BMP-mediated growth plate development. Moreover, in *Jab1* knockout mutants, *Sox9* expression and activity was severely compromised, suggesting Jab1 positively regulates Sox9 in early growth plate development. Interestingly, in a recent Sox9

ChIP-seq study in E12.5 limb buds, Jab1 was identified as a putative key downstream target for Sox9, suggesting a possible Jab1-Sox9 positive feedback loop in early limb development [136]. Thus, it is essential to further elucidate the underlying mechanism of the interaction between Jab1 and master transcription factors such as Sox9 and Runx2 in growth plate development.

11.2. YAP1

The transcriptional co-factor Yes-Associated Protein 1 (YAP1) is a key effector of the Hippo signaling, which is primarily involved in the regulation of organ size and tissue regeneration. YAP1 and closely related TAZ regulate downstream target genes by interacting with transcription factors such as TEAD. The evolutionarily conserved Hippo-YAP1/TAZ signaling pathway functions in a unique transduction manner, and we direct readers to a very recent comprehensive overview [137]. Briefly, when the tumor suppressor Hippo pathway is inactive, oncogenic YAP1 and TAZ are unphosphorylated and located in the nucleus. YAP1 and TAZ interact with transcription factors TEADs to promote the expression of target genes involved in survival, proliferation, differentiation, and migration/invasion [137]. When the Hippo pathway is activated by TAO kinases, MTS1/2 kinases are phosphorylated and activated, which subsequently phosphorylate LATS1/2 kinases. LATS1/2 kinases then phosphorylate and inactivate YAP1 and TAZ. As a result, YAP1 and TAZ are retained in the cytoplasm and are inactivated [137]. YAP1 serves as a key transcriptional regulator to integrate multiple signaling cascades, including Wnt, TGF- β /BMP, and hedgehog [138]. While a role for TAZ in Runx2-mediated osteogenesis has been well-established, most recently the role of YAP1 in chondrocyte differentiation has also begun to emerge. YAP1 can inhibit chondrogenic differentiation through downregulating BMP signaling by suppressing Smads [139]. Furthermore, a most recent mouse genetic study showed that *Yap1* promoted chondrocyte proliferation, but inhibited hypertrophy during endochondral ossification and fracture healing [138]. The study shows that *Yap1* expression is high in early chondrocyte differentiation and progressively decreases. Mechanism-wise, the high-level expression of *Yap1* in early chondrogenesis promotes chondrocyte proliferation, at least in part, through inducing the expression of Sox6, a major downstream target of Sox9 during chondrogenesis. On the other hand, lower *Yap1* expression in hypertrophic chondrocytes relieved its inhibition of Runx2-mediated *Col10a1* expression [138]. This provides another example of a transcriptional co-factor that has differential roles at different stages of growth plate development through its interaction with key transcription factors.

12. Chondrocyte Terminal Fates and Transdifferentiation

The precise fates of hypertrophic chondrocytes and the origins of trabecular-forming osteoblasts during endochondral ossification had eluded us for decades. It is conventionally assumed that the hypertrophic chondrocytes, both in the cartilage anlagen prior to the establishment of growth plates and those at the chondro-osseous junction of growth plates, are terminally differentiated and are ultimately eliminated through programmed cell death, by either apoptosis (type I programmed cell death) or autophagy (type II programmed cell death) [140, 141]. Indeed, many studies in various organisms have detected evidences of apoptosis or autophagy in growth plate hypertrophic chondrocytes either under physiological

condition or in response to various stimuli, including altered extracellular matrix and hypoxia in the microenvironment [142] [143] [144] [145] [146] [147, 148] [149]. The findings of these studies vary quite extensively in terms of type and percent of cells involved in programmed cell death. For example, Farnum and Wilsman [144, 150] observed only about 12% of the hypertrophic chondrocytes with condensed morphology, indicative of apoptotic cells, in the lower lacunae adjacent to the cartilage–bone marrow interface in Yucatan swine. However, Gibson [145] reported that more than 50% of hypertrophic chondrocytes displayed characteristics of apoptosis at the chondro-osseous interface in the chick sterna. Similarly, studies with the TUNEL method also yielded divergent results, from as low as 9% of TUNEL-positive cells of hypertrophic chondrocytes isolated from the chick growth plate [151], to as much as 44% of TUNEL-positive cells found in the hypertrophic zone [152].

Unlike cells in other tissues, most of growth plate chondrocytes are well embedded within cartilage matrix and cannot be phagocytosed by macrophages as apoptotic cells in other tissues. While lacking typical apoptotic morphological changes in the terminal hypertrophic chondrocytes, there was the presence of autophagic vacuoles and the expression of autophagy-regulating genes by growth plate chondrocytes, suggesting that these cells may undergo processes more similar to autophagy than apoptosis [140, 144, 153]. Indeed, the autophagy pathway promotes cell survival in the mouse growth plate. The chondrocyte-specific deletion of Autophagy-related (*Atg*) genes, *Atg5* and *Atg7*, resulted in reduced proliferation and enhanced cell death through caspase-dependent mechanisms [154]. Another study showed that autophagy in growth plates regulated the secretion of type II collagen through Fgf18-Fgfr4 signaling via JNK-dependent activation of the VPS34-Beclin1 [155].

Evidently, there are a significant number of hypertrophic chondrocytes that are not accounted for by programmed cell death. As autophagy can lead to cell survival as well as cell death, some autophagy might also lead to reprogrammed progenies. Indeed, the idea that some of these hypertrophic chondrocytes, rather than dying, can transdifferentiate into osteoblasts, was suggested several decades ago, and is now regaining traction [156]. The fact that both osteoblasts and chondrocytes are derived from the same *Sox9*-expressing osteochondro-progenitors hints at their potential common gene regulatory networks. Indeed, the transcription factors involved in chondrocyte and osteoblast differentiation serve complementary functions in the development of cartilage and bone [157]. In addition to their own specific markers such as *Col10a1*, hypertrophic chondrocytes also express many osteoblast markers, including *Alp*, *SPARC*, *BGLAP*, *Opn*, and *Bsp*, as well as *Osx* and *Runx2*, albeit at relatively lower levels compared with their expressions in osteoblasts [158, 159]. In fact, earlier morphological, histological [147], and cell culture experiments [158, 160–162] all observed the presence of potential chondrocyte-derived osteoblasts and likely transdifferentiation events. It was suggested that hypertrophic chondrocytes to osteoblasts transdifferentiation might have occurred through either asymmetrical division or direct transdifferentiation [147] [162] [163] [164]. However, most of such evidence came from *in vitro* studies and was not fully conclusive.

Recently, several groups independently reported the findings of their Cre/reporter lineage tracing studies in mice [165] [166, 167]. In these studies, chondrocytes or hypertrophic

chondrocytes were labelled with reporter genes driven by chondrocyte or hypertrophic chondrocyte-specific promoters either constitutively or in an inducible manner. Collectively, their findings provide strong *in vivo* evidence that a significant number of hypertrophic chondrocytes indeed become osteoblasts, indicating that hypertrophic chondrocytes are a direct major source of osteoblasts in endochondral bone formation [165]. Zhou *et al.* showed that the *Col10a1-Cre* marked reporter⁺ cells started to appear in the primary ossification center coincident with the onset of primary ossification [165]. These cells were gradually increased in numbers and were distributed throughout the primary spongiosa. They were located in the bone marrow, on trabecular surfaces, and embedded within bone matrix (Figure 2). In postnatal mice, these *Col10a1-Cre* marked reporter⁺ cells were also found on endosteum and within cortex. Zhou *et al.* further showed that approximately 60% of the total mature osteoblasts on both trabecular and endosteum surfaces were derived from hypertrophic chondrocytes [165]. In addition to hypertrophic chondrocyte-derived osteoblasts and osteocytes, there were abundant numbers of *Osx*-expressing reporter⁺ bone marrow cells, which did not express *Col1a1* and *Osteocalcin* but displayed skeletal progenitor characteristics, implying that hypertrophic chondrocytes may be the source of some *Osx*-expressing skeletal progenitors [168, 169].

These studies also found that embryonic hypertrophic chondrocyte-derived cells persisted well into adulthood, as long term chasing showed the labeled cells were present as osteoblasts, osteocytes and stromal cells in the metaphysis after birth [167]. It is worth noting that the Cre-induced reporter⁺ cells were not found in the perichondrium and periosteum, in which *Osx* is highly expressed, indicating that the chondrocyte-derived osteoblasts represent a sub-population of *Osx*-expressing osteoblasts [165]. Bone fracture healing occurs mostly through processes similar to developmental endochondral ossification. Recent studies also demonstrated that chondrocytes in the repair callus were a direct source of osteoblasts responsible for bone formation during fracture healing, as hypertrophic chondrocyte-derived osteoblasts were found in the fracture callus [165] [167]. It is rational to speculate that chondrocyte-derived osteoblasts may also be involved in other endochondral ossification processes, such as osteophyte formation in osteoarthritis and heterotopic ossification in FOP patients. It will be important to dissect the underlying mechanism of transdifferentiation, as these results raise many key questions for future studies, such as what determines the final fate of hypertrophic chondrocytes as to undergo apoptosis, autophagy, or transdifferentiation. Hopefully, in the near future, this hypertrophic chondrocyte-to-osteoblast trans-differentiation notion will be more firmly established with more advanced technology, such as better live imaging techniques and more sophisticated cell lineage tracing genetic tools. Nevertheless, the very fact that these chondrocyte-targeted Cre recombinases are capable of marking abundant numbers of osteoblasts, osteocytes and osteo-progenitors should be taken into consideration in our interpretation of bone phenotypes in genetic mouse models when a given gene is specifically deleted in chondrocytes.

10. Concluding remarks

In summary, growth plate formation is a dynamic, well-coordinated, tightly controlled process. Many signaling pathways interact with each other to exert essential roles during the

process. The large number of skeletal diseases that exist in humans, and the limited number and efficacy of currently available treatment options mean that much research remains to be done. Newer methodologies, including Next Generation Sequencing (NGS), the Clustered regularly-interspaced short palindromic repeats CRISPR/Cas9 genome editing system, and induced pluripotent stem cells, will accelerate our uncovering of novel signaling transduction mechanisms involved in the formation and activity of cartilage growth plates [170–172]. Indeed, rapid progress in NGS allows single cell analyses to be performed, and has already provided insightful results. For example, a single cell analysis study showed that the expression patterns of chondrocyte differentiation markers are heterogeneous amongst cells, and do not correlate with functional capacity, challenging the widely-held notion that a marker gene expression profile correlates with a cellular phenotype [173]. Furthermore, a new bioinformatics pipeline, which analyzed single cell RNA-seq data among 217 single cells that represent various stages of growth plate development, allowed spatiotemporal analysis and identification of genes that are involved in regulatory networks controlling growth plate development [174]. In addition to validating previously identified transcription factors involved in growth plate development, this study also identified novel factors, including the Krüppel-like family and Fos family, as having high regulatory potential [174]. Notably, this study shows the potential power of bioinformatics and the ability to practically trace single cells from early to late stages during growth plate development. In the future, the acquisition of detailed, specific knowledge of genes and pathways involved in the control of the growth plate should allow for the development of new therapies and design of new drugs to directly and effectively target culprit signaling pathways or stimulate alternative pathways, and thereby achieve marked efficacy and reduce side effects in musculoskeletal diseases.

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Fig. 1. Successive steps of endochondral ossification in mice

A. Schematic representations of successive steps of cartilage growth plate formation. B. Hematoxylin and eosin staining of mouse limb growth plates at embryonic days E12.5, E15.5, E18.5, and post-natal days P15. Vertical lines indicate the Resting Zones (RZ), Proliferative Zones (PZ), and Hypertrophic Zones (HZ) at each stage. Arrows point to the Primary Ossification Center (POC) and the Secondary Ossification Center (SOC).

Tomato⁺ - Hypertrophic Chondrocyte-Derived Cells

GFP⁺ - Mature Osteoblasts

Tomato⁺/GFP⁺ - Chondrocyte-Derived Mature Osteoblasts, ~60% of total GFP⁺ cells in both trabeculae and endosteum

Fig. 2. Mouse hypertrophic chondrocytes can transdifferentiate into mature osteoblasts

The triple transgenic *Col10a1-Cre;2.3Col1-GFP;ROSA-tdTomato* mice express tomato (red) upon Cre recombination in hypertrophic chondrocytes, express Enhanced Green Fluorescent Protein (EGFP) in *2.3Col1-GFP⁺* mature osteoblasts, and a Tomato Red/EGFP overlay creates yellow, indicating chondrocyte-derived mature osteoblasts. Blue indicates nuclei. Image shows a magnified region of the cortical bone. bm: bone marrow. The image is adapted from reference 165.