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REVIEW

Regulatory mechanisms for the development of growth plate cartilage

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Abstract In vertebrates, most of the skeleton is formed through endochondral ossification. Endochondral bone formation is a complex process involving the mesenchymal condensation of undifferentiated cells, the proliferation of chondrocytes and their differentiation into hypertrophic chondrocytes, and mineralization. This process is tightly regulated by various factors including transcription factors, soluble mediators, extracellular matrices, and cell–cell and cell–matrix interactions. Defects of these factors often lead to skeletal dysplasias and short stature. Moreover, there is growing evidence that epigenetic and microRNA-mediated mechanisms also play critical roles in chondrogenesis. This review provides an overview of our current understanding of the regulators for the development of growth plate cartilage and their molecular mechanisms of action. A knowledge of the regulatory mechanisms underlying the proliferation and differentiation of chondrocytes will provide insights into future therapeutic options for skeletal disorders.

Keywords Chondrocyte · Transcription factors · Growth factors · Extracellular matrix · Differentiation

Introduction

Most of the skeleton, including the long bones of the limbs and the vertebral columns, is formed through endochondral ossification, involving a cartilaginous intermediate [1–3]. Endochondral bone formation starts with the condensation of mesenchymal cells, which differentiate into chondrocytes characterized by the production of specific extracellular matrix (ECM) proteins such as type II collagen (Col II) and aggrecan. The chondrocytes proliferate unidirectionally to form orderly parallel columns which accumulate a cartilaginous matrix [2]. These cells then exit the cell cycle, differentiate further to become hypertrophic, and produce type X collagen (Col X) [4]. In growth plates, maturing chondrocytes are organized into zones, including a resting zone, a proliferating zone, a prehypertrophic zone, and a hypertrophic zone [5]. Once the hypertrophic chondrocytes have terminally differentiated, the cartilaginous matrix is mineralized and the cells undergo apoptosis. These mature chondrocytes express vascular endothelial growth factor (VEGF) to induce blood vessel invasion and matrix metalloproteinases (MMPs) to aid in the degradation of the cartilaginous matrix by chondroclasts, and the primary ossification center is developed [6, 7] (Fig. 1).

The process of chondrocyte proliferation and differentiation is regulated by various transcription factors, growth factors, ECMs, and cell–matrix interactions [2, 8–10]. In addition, recent studies have revealed the importance of epigenetic and microRNA-mediated control in cartilage development. Defects in the factors involved in the development of growth plate cartilage are often associated with skeletal dysplasias and short stature [11]. In this review article, I will mainly address the mechanisms regulating the development of growth plate cartilage.

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Transcriptional control of chondrogenesis

Sox9 is a member of the Sox family of transcription factors characterized by a high-mobility-group-box DNA binding

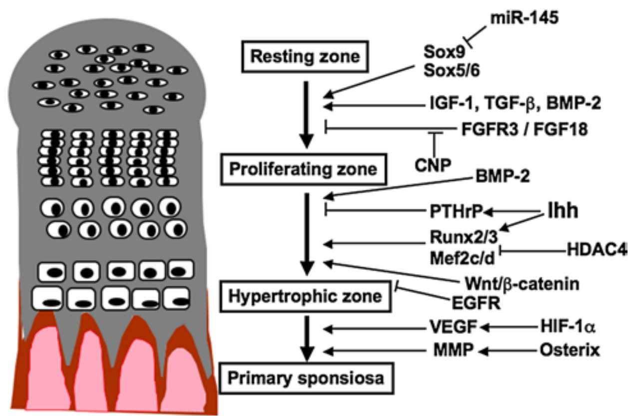

Fig. 1 Schematic representation of molecules involved in the development of growth plate cartilage

motif related to that of the sex determining factor SRY, and plays a central role in chondrogenesis. In chondrocyte lineage cells, the expression of Sox9 starts at the mesenchymal osteochondroprogenitor stage and remains high during differentiation. Mutations in the human *SOX9* gene result in camptomelic dysplasia characterized by severe skeletal malformation, indicating the critical role of SOX9 in skeletogenesis [12, 13]. Accumulating evidence in mice also has revealed that Sox9 is indispensable for chondrocyte differentiation [3, 14]. Sox9 transcriptionally controls the expression of cartilage-specific genes such as *Col2a1* encoding Col II [15, 16]. Two other Sox family members, Sox5 (L-Sox5) and Sox6, cooperate with Sox9 to activate the chondrocyte-specific enhancers in the genes for ECM components [15, 17, 18]. Other transcription factors, such as members of the activating transcription factor (ATF)/cyclic AMP response element binding protein (CREB) family and the AP1 family member c-Fos, are required to maintain the proliferative capacity of early chondrocytes [19–21]. RhoA is a Rho GTPase, which functions as a regulator of cytoskeletal dynamics. RhoA signaling through its main effector ROCK inhibits chondrogenesis by suppressing the expression of Sox9 [22, 23].

A decrease in the expression and/or activity of the Sox proteins is required for the hypertrophic maturation of chondrocytes. In addition to the negative regulation by Sox proteins, other transcription factors, such as the Runt domain family members Runx2 and Runx3, function to promote chondrocyte hypertrophy [24]. Mice lacking both Runx2 and Runx3 lack hypertrophic chondrocytes [24]. Runx2 directly binds and activates the genes *Ihh (Indian hedgehog)*, *Col10a1* encoding Col X, and *MMP13* [25–27]. A recent study using a doxycycline-inducible conditional knockout of *Sox9* has revealed that Sox9 suppresses the expression of Runx2 and β-catenin signaling and thereby inhibits the progression from proliferation to prehypertrophy of chondrocytes [28]. Twist-1 is a basic helix-loop-helix-type transcription factor, which represses the expression of Runx2 in the perichondrium. Runx2 enhances the expression of *fibroblast growth factor 18* (*Fgf18*) and exerts an indirect negative effect on chondrocyte maturation [29]. Osterix regulates the calcification and degradation of cartilaginous matrix through MMP13 expression in association with Runx2 [30].

MADS-box transcription factors Mef2c and Mef2d (myocyte enhancer factor 2c and 2d) are also involved in chondrocyte hypertrophy. Genetic deletion of Mef2c in endochondral cartilage impairs hypertrophic maturation, while the forced expression of a superactivating form of Mef2c resulted in precocious chondrocyte hypertrophy [31]. The activity of Runx2/3 and Mef2c/d is inhibited by the histone deacetylase HDAC4 [31–33]. Other transcription factors, such as Msx2, the AP1 family member Fra2, and FoxA family transcription factors, also positively control chondrocyte hypertrophy [34–37].

The developmental growth plate is hypoxic, especially in its interior. The transcription factor hypoxia-inducible factor I (HIF-1) is one of the major regulators of the hypoxic response in mammals. Genetic evidence obtained from mice lacking HIF-1α suggests its role in chondrocyte survival and the regulation of *Vegf* expression [38]. Conditional overexpression of VEGF164 in chondrocytes lacking HIF-1α rescued the phenotype of HIF-1α-deficient growth plate only partially, indicating VEGF-independent functions of HIF-1α in developing growth plate cartilage [39]. It is also reported that HIF-1α regulates collagen hydroxylation and secretion in developing cartilage [40].

Soluble mediators involved in chondrogenesis

Ihh, a member of the hedgehog family of signaling molecules, is expressed in prehypertrophic chondrocytes, and regulates the onset of hypertrophic differentiation through a negative feedback loop with parathyroid hormone-related protein (PTHrP). Ihh increases the expression of PTHrP in perichondrial cells and chondrocytes at the ends of long bones, which inhibits chondrocyte hypertrophy through its cognate receptor expressed in proliferating chondrocytes and keeps the cells in the proliferating stage [41]. Moreover, it is also reported that Ihh stimulates the proliferation and maturation of chondrocytes independently of PTHrP [42, 43]. Activation of Wnt and bone morphogenetic protein (BMP) signaling is suggested to be involved in the PTHrP-independent role of Ihh to regulate chondrocyte hypertrophy [44].

Fibroblast growth factors (FGFs) also play important roles in skeletogenesis by activating signaling through FGF receptors (FGFRs) [45]. Gain-of-function mutations

in human FGFR3 result in chondrodysplasias and dwarfism [46–49]. As to FGF ligands, *Fgf2* was first identified to be expressed in chondrocytes [50], and is also expressed in periosteal cells and osteoblasts [51, 52]. However, *Fgf2*-knockout mice demonstrate no defects in chondrogenesis [52, 53]. *Fgf9* is also expressed in immature chondrocytes in mesenchymal condensation. In the perichondrium, the expression of *Fgf7*, *Fgf8*, *Fgf9*, *Fgf17*, and *Fgf18* has been reported [54–58]. Evidence obtained from mouse models indicates profound role for Fgf18 in chondrogenesis [56, 57]. Fgf9 was also proven to regulate early hypertrophic chondrocyte differentiation and skeletal vascularization by the defects in chondrogenesis in *Fgf9*-knockout mice [59]. Among the FGFRs, *Fgfr3* is expressed in chondrocytes undergoing mesenchymal condensation and proliferating chondrocytes, whereas *Fgfr1* is expressed in prehypertrophic and hypertrophic chondrocytes [60–62]. Genetic and functional studies demonstrated that the signaling through FGFR3 negatively regulates the chondrocyte proliferation and differentiation [63–66]. The effects of FGFR3 in chondrogenesis are partly exerted by direct signaling in chondrocytes, and in part indirectly through the regulation of Ihh/PTHrP/BMP signaling [67]. In achondroplasia, constitutive activation of FGFR3 results in the activation of downstream pathways including STAT1 and ERK signaling [45]. The growth plates of mice lacking Fgf18 have a similar histology to those of *Fgfr3*-knockout mice, suggesting that FGF18 is a physiological ligand for FGFR3 in chondrocytes [56, 57].

C-type natriuretic peptide (CNP) controls cell behavior through the activation of two transmembrane receptors, NPR1 and NPR2 [68–70]. Since these receptors synthesize cyclic GMP in response to ligand binding, NPR1 and NPR2 are also called guanylyl cyclase A and B (GC-A and GC-B), respectively. CNP exerts its signal mainly through NPR2/GC-B. The importance of CNP signaling in chondrogenesis was shown by the severe dwarfism of CNP-knockout mice and the finding that CNP stimulated the longitudinal growth of cartilage in organ cultures [71, 72]. NPR2-null mice display a similar phenotype to CNP-knockout mice [73]. CNP promotes endochondral bone growth through several mechanisms, including the stimulation of chondrocyte proliferation, an acceleration of chondrocyte hypertrophy, and an increase of ECM production. In humans, loss-of-function mutations in the *NPR2* gene cause acromesolic dysplasia, type Maroteaux, characterized by severe dwarfism [74]. We have recently identified a novel gain-of-function type mutation of the *NPR2* gene in a family with overgrowth [75]. Such evidence indicates the critical role of CNP/NPR2 signaling in chondrogenesis both in humans and in mice.

The skeletal phenotype in CNP-deficient mice resembles that in cases of achondroplasia. Overexpression of CNP in cartilage rescued the skeletal phenotypes in a mouse model of achondroplasia, suggesting an intimate link between the FGF and CNP signaling [73]. CNP signaling inhibited the activation of the ERK pathway induced by FGF signaling, while FGF signaling blocked CNP-induced cGMP production in a MAPK-dependent manner [73]. In addition to the ERK pathway, recent studies demonstrated the possible involvement of the p38MAPK and PI3K/Akt pathways in the regulation of chondrocyte development by CNP [76]. CNP analogues are promising as new drugs for the dwarfism associated with skeletal dysplasias [77].

Studies have established the involvement of signaling mediated by epidermal growth factor receptor (EGFR) in chondrogenesis. Delayed primary endochondral ossification associated with defective osteoclast recruitment was reported in mice lacking EGFR [78]. Ubiquitous overexpression of betacellulin, a ligand for EGFR, resulted in defects in growth plates characterized by a smaller zone of hypertrophic chondrocytes in mice [79]. In addition, cartilage-specific inactivation of EGFR in mice as well as the administration of an EGFR-specific small-molecule inhibitor, gefitinib, into rats caused hypertrophic cartilage enlargement [80].

Other growth factors such as Wnts, BMPs, transforming growth factor-beta (TGF-β), insulin-like growth factors (IGFs), thyroid hormone, and connective tissue growth factor (CTGF) also play roles in chondrogenesis. There are several excellent review articles on their actions [81–83].

Regulation of chondrogenesis by the ECM

In the early stages of chondrogenesis, cell–cell interaction via adhesion molecules such as N-cadherin and N-CAM plays a role in cellular condensation and the subsequent chondrogenesis [84, 85]. As chondrocytes mature, they produce abundant matrix proteins, and the cell–matrix interactions come to have important roles. Integrins bind various extracellular components such as ECMs and other cell surface proteins [86]. The binding of ligands to integrins leads to the formation of focal adhesion complexes, and transduces the signaling from the ECM to intracellular effectors such as cytoskeleton [87, 88]. Integrins exist as dimers of an α subunit and a β subunit, and chondrocytes express several integrin subunits including fibronectin receptors (α5β1, αnβ3, αnβ5), a laminin receptor (α6β1) and collagen receptors (α1β1, α2β1, α10β1) [89–92]. The importance of β1 integrin-mediated signaling in chondrogenesis was demonstrated by the chondrodysplasia-like phenotype of chondrocyte-specific β1 integrin-knockout mice [93]. Growth plates of these mice exhibited unorganized proliferative columns and an abnormal cell shape due to the loss of adhesion to Col II. The chondrocytes isolated

from these mice displayed reduced proliferation caused by a defect in G1/S transition and cytokinesis.

Inactivation of the α10 integrin gene also resulted in growth plate dysfunction, which was associated with an abnormal cell shape and increased apoptosis of chondrocytes [94]. On the other hand, knockout of the gene for α1 integrin resulted in osteoarthritis but no abnormalities in the growth plates, despite this gene's predominant expression in hypertrophic chondrocytes [95].

Activation of integrin-mediated signaling triggers the formation of a complex consisting of multiple proteins, which regulate various cellular processes. Integrin-linked kinase is one of the components, and the knockout of its gene caused a chondrodysplasia-like phenotype resembling that of chondrocyte-specific β1 integrin-knockout mice [96]. In these mice, reduced proliferation of chondrocytes was the main cause for the skeletal phenotype, and the expression of chondrocyte-specific genes such as *Col2a1* was comparable with that in wild-type mice.

CD44 is a cell surface glycoprotein that functions as a receptor for collagens and hyaluronan. It was reported that the blocking of CD44-hyaluronan binding on chondrocytes resulted in degradation of the cartilage matrix, suggesting a role for CD44 in cartilage homeostasis [97]. Annexin V acts as a receptor for collagen, specifically for a fragment of Col II in articular chondrocytes [98]. Antibodies against annexin V inhibited the binding of chondrocytes to Col II [99]. It is also suggested that annexin V is involved in regulating the apoptosis of growth plate chondrocytes [100].

Cartilage contains abundant proteoglycans. The sulfate transporter SLC26A2 is responsible for sulfate uptake by chondrocytes, and mutations in its gene lead to undersulfation of cartilaginous proteoglycans, resulting in a chondrodysplasia called diastrophic dysplasia. In *dtd* mice with a knock-in *Slc26a2* mutation, the resulting undersulfation of glycosaminoglycans such as chondroitin destroys the articular surface and correlates with the rate of chondroitin synthesis across epiphyseal cartilage [101]. Chondroitin sulfate *N*-acetylgalactosaminyltransferase 1 (CSGalNAcT-1) is an enzyme that participates in the initiation of the biosynthesis of chondroitin sulfate. Mice lacking the gene encoding CSGalNAcT-1 exhibit shorter, disorganized chondrocyte columns in the growth plates with a rapid catabolism of aggrecan [102].

In addition to providing signals to cells by binding to integrins and other ECM receptors, ECM proteins regulate chondrogenesis through the binding, storage, and release of soluble factors. TGF-β is produced by chondrocytes as a high molecular weight macromolecule in association with latent TGF-β binding protein (LTBP), which functions in the storage of TGF-β in the ECM [103, 104]. Proteoglycans such as decorin, biglycan, and fibromodulin also regulate TGF-β activity by sequestering TGF-β in the ECM [105].

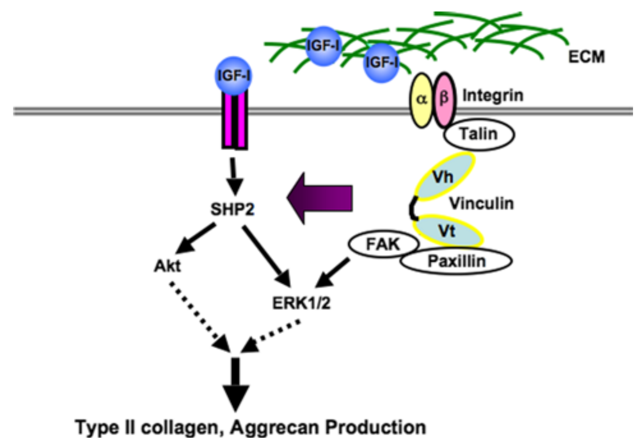

Fig. 2 Vinculin regulates the production of type II collagen and aggrecan by orchestrating the signal of extracellular matrix and that of IGF-1

Most FGFs bind to heparan sulfate proteoglycans. They bind to cognate receptors in the context of heparan sulfate proteoglycans and evoke signaling into the cells. Genetic evidence obtained with mice lacking sulfate-modifying factor 1 (Sumf1) has suggested that the desulfation of proteoglycans regulates chondrocyte proliferation and differentiation by limiting FGF signaling [106].

Signals from the ECM itself and those triggered by soluble mediators appear to interplay to regulate chondrogenesis. We have demonstrated that vinculin plays a role in chondrogenesis [107]. Vinculin is a component of multimolecular complexes which function in adhesion and/or signaling between the extracellular microenvironment and the cell, via integrins and cadherins. Impaired functioning of vinculin by knockdown in primary chondrocytes and organ cultures of metatarsal explants resulted in the reduced expression of *Col2a1*, *aggrecan*, *Col10a1*, and *Runx2*. In addition, knockdown of *vinculin* in the metatarsals abrogated IGF-I-induced growth, and inhibited the up-regulation of *Col2a1* and *aggrecan* expression by IGF-I. These results suggest that vinculin regulates the expression of chondrocyte-specific genes via the integration of signaling from the ECM and soluble factors such as IGF- I (Fig. 2). It is also reported that cell adhesion via integrin regulates the activation of growth factor receptors. The orchestration of the signaling of soluble factors and the ECM should be considered a factor in the regeneration of cartilage [107].

Epigenetic and microRNA-mediated regulation of chondrogenesis

There is growing evidence that epigenetic and microRNA-mediated mechanisms play roles in chondrogenesis as well

as in pathogenesis of osteoarthritis [108]. Histone modifications involving acetylation and deacetylation have an impact on the phenotype of chondrocytes. Among the histone deacetylases (HDACs), HDAC4 has been suggested to prevent premature chondrocyte hypertrophy by blocking the activity of Runx2, as described above [32]. HDAC1 and HDAC2 were shown to repress the expression of some cartilage-specific genes including *Col2a1*, and the Snail transcription factor was identified as a mediator of the repression [109]. The up-regulation of HDAC7 expression was suggested to contribute to the cartilage degradation by promoting the expression of MMP13 [110].

SIRT1 is a NAD^{+}-dependent histone deacetylase, and enhances the expression of cartilage-specific ECM genes, such as *Col2a1*, by recruiting co-activators to the enhancer and promoter and facilitating Sox9-mediated transcription [111]. In addition, SIRT1 has been suggested to regulate chondrocyte apoptosis [112].

DNA methylation at CpG dinucleotides is commonly associated with gene repression. An in vitro study demonstrated that the induction of *COL10A1* during the chondrogenesis of mesenchymal stem cells correlated with the demethylation of 2 CpG sites in the *COL10A1* promoter [113]. It was reported that DNA methylation inversely correlated with the expression of cartilage-specific genes including *COL9A1*, but not catabolic genes such as *MMP13*, during fetal femur development in human [114]. Moreover, recent reports have suggested that the methylation of a specific CpG site inhibits the transactivation of *MMP13* by transcription factors HIF-2α and CREB [115, 116].

MicroRNAs (miRNAs) are a class of ~22 nucleotide noncoding RNAs that regulate the expression of other genes at the posttranscriptional level. Knockout of Dicer, an enzyme required for miRNA synthesis, led to severe skeletal growth defects caused by decreased chondrocyte proliferation and accelerated differentiation in mice, indicating the critical roles of miRNAs in chondrogenesis [117]. Specific miRNAs have been identified to have roles in chondrocyte differentiation. miR-199a was shown to be responsive to BMP and to regulate chondrogenesis by directly targeting Smad1 [118]. Mice lacking miR-140 showed a mild skeletal phenotype with a short stature and age-related OA-like changes associated with the elevated expression of ADAMTS-5, suggesting that miR-140 regulates cartilage development and homeostasis [119]. miR-145 was reported to directly target Sox9 and regulate chondrogenic differentiation of mesenchymal stem cells [120]. miR-675, whose production is positively regulated by Sox9, increases the expression of *COL2A1* in human articular chondrocytes [121]. These findings have established the importance of miRNA-mediated regulation in cartilage development.

Conclusion

The development of growth plate cartilage is a complex process, regulated by transcription factors, soluble factors, cell–cell and cell–matrix interactions, and epigenetic factors. These factors interplay to control the proliferation and differentiation of chondrocytes. Failure in the development of growth plate cartilage is often associated with skeletal dysplasias, for which currently there is no effective treatment. Understanding the mechanisms regulating chondrogenesis may lead to new therapeutic drugs for these diseases, such as CNP analogues.

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