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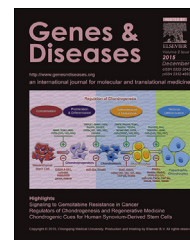


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REVIEW ARTICLE

Multifaceted signaling regulators of chondrogenesis: Implications in cartilage regeneration and tissue engineering



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Abstract Defects of articular cartilage present a unique clinical challenge due to its poor self-healing capacity and avascular nature. Current surgical treatment options do not ensure consistent regeneration of hyaline cartilage in favor of fibrous tissue. Here, we review the current understanding of the most important biological regulators of chondrogenesis and their interactions, to provide insight into potential applications for cartilage tissue engineering. These include various signaling pathways, including fibroblast growth factors (FGFs), transforming growth factor β (TGF- β)/bone morphogenic proteins (BMPs), Wnt/ β -catenin, Hedgehog, Notch, hypoxia, and angiogenic signaling pathways. Transcriptional and epigenetic

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Sox9;
TGF β

regulation of chondrogenesis will also be discussed. Advances in our understanding of these signaling pathways have led to promising advances in cartilage regeneration and tissue engineering.

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Introduction

Articular cartilage injury and repair is an issue of growing importance around the world. Kurtz et al predict that the number of total knee replacements will jump from 700,000 to 3.48 million annually by the year 2030.¹ Although common, articular cartilage defects continue to hold a unique clinical challenge due to its poor self-healing capacity, which is largely due to its avascular nature. Current surgical treatment options do not reliably produce hyaline cartilage tissue in favor of fibrous tissue, and therefore remain controversial. Deposition of fibrocartilage instead of hyaline cartilage can lead to poor durability and rapid decline in activity levels after 1 year. Therefore, there is a critical need for more effective cartilage regenerative therapies. The difficulty in obtaining a sufficiently large number of chondrocytes for implantation could be overcome by utilizing gene therapy to promote chondrogenesis from bone marrow-derived mesenchymal stem cells (MSCs). However, research regarding exogenous control over this multi-step process has yielded only limited clinically applicable results. For this reason, the ability to differentiate MSCs into chondrocytic cells, and subsequently implant these chondrocytes into cartilaginous defects is of significant therapeutic value.

MSCs are multipotent progenitor cells that can differentiate into several lineages, including bone, cartilage, fat, and muscle (Fig. 1). The proliferation and differentiation of mesenchymal cells to chondrocytes, or chondrogenesis, is a complex process that is regulated by a host of factors. These factors include intracellular proteins, receptor ligands, and transcription factors; and a disruption in signaling can result in defective chondrocyte production. Here, we review the current understanding of the most important biological regulators of chondrogenesis and how these regulators interact to provide insight into their potential for cartilage tissue engineering.

Fibroblast growth factor (FGF) signaling pathway

FGFs are a family of heparin-binding growth factors that are involved in the proliferation and differentiation of a variety of tissues. In general, FGFs are known to be positive regulators of chondrogenesis and they appear to be involved throughout the process. The most well studied and generally accepted function of the FGF family with regards to chondrogenesis is its role in the proper patterning of developing cartilage. The developing limb is divided into two components: the apical ectodermal ridge (AER) and the

limb bud mesenchyme. The FGFs that are produced in the AER, zone of polarizing activity (ZPA), and dorsal ectoderm are necessary for early patterning events in the developing embryo.² The AER, which serves as the primary source of FGF2, FGF4, FGF8, and FGF9 in the developing embryo, is maintained by Wnt3 and FGF10 signaling.^{3,4} The effect of FGF10 on the apical ectodermal ridge is the result of a positive feedback loop with FGF8. FGF2, 4, and 8 serve as important regulators of proximal-distal growth, and FGF2 has the additional function of priming MSCs for chondrocytic differentiation.^{5,6} The latter effect is mediated by an increase in basal expression of prochondrogenic transcription factor Sox9 and inactivation of IGF-I and TGF- β signaling.^{5,7}

Sequential expression of three FGFs has been shown to impact the progression of chondrocyte differentiation and maturation: FGF2, FGF9, and FGF18.^{8–10} As previously stated, FGF2 administration is known to be sufficient to enhance chondrocyte proliferation speed and prepare chondroprogenitor cells for terminal differentiation; however, the mechanism by which FGF2 exerts these effects has been largely unknown until recently.⁸ FGF2's ability to enhance chondrocyte proliferation speed is due to its ability to enhance TGF β 1 expression.¹¹ Likewise, FGF2-induced chondrocyte differentiation is a consequence of FGF2-mediated downregulation of TGF- β 2 expression.⁷ Given FGF2's interactions with TGF- β 1 and TGF- β 2, research sought to elucidate the impact of combining FGF2 with TGF- β 3. It has now been determined that FGF2 is able to induce chondrocyte differentiation and enhance proliferation in the presence of TGF- β 3, however these effects are accompanied by inhibition of chondrocyte hypertrophy.¹² In addition to its interactions with the TGF- β family of proteins, combination of FGF2 with Wnt3a or a combination of platelet-derived growth factor (PDGF), insulin, and TGF- β 1 has been shown to be sufficient to support long-term production of fully functional cartilage.^{13,14}

While FGF2 is known to have a major impact on priming chondroprogenitors for the chondrocyte differentiation program, FGF9 and FGF18 are regulators for early chondrogenic differentiation, augmentation of ECM production, and terminal chondrocyte hypertrophy.⁹ FGF9 can be both prochondrogenic and proosteogenic depending on its expression level and the cell population in which it is expressed.¹⁵ FGF9 has not only been found to promote chondrocyte hypertrophy at early stages but also to drive the vascularization of the growth plate prior to osteogenesis.¹⁶ The multifunctionality of FGF9 is believed to be the result of its ability to upregulate expression of other prochondrogenic proteins, such as Sox9, Indian hedgehog (Ihh), and Col2a1. FGF9 also upregulates proosteogenic

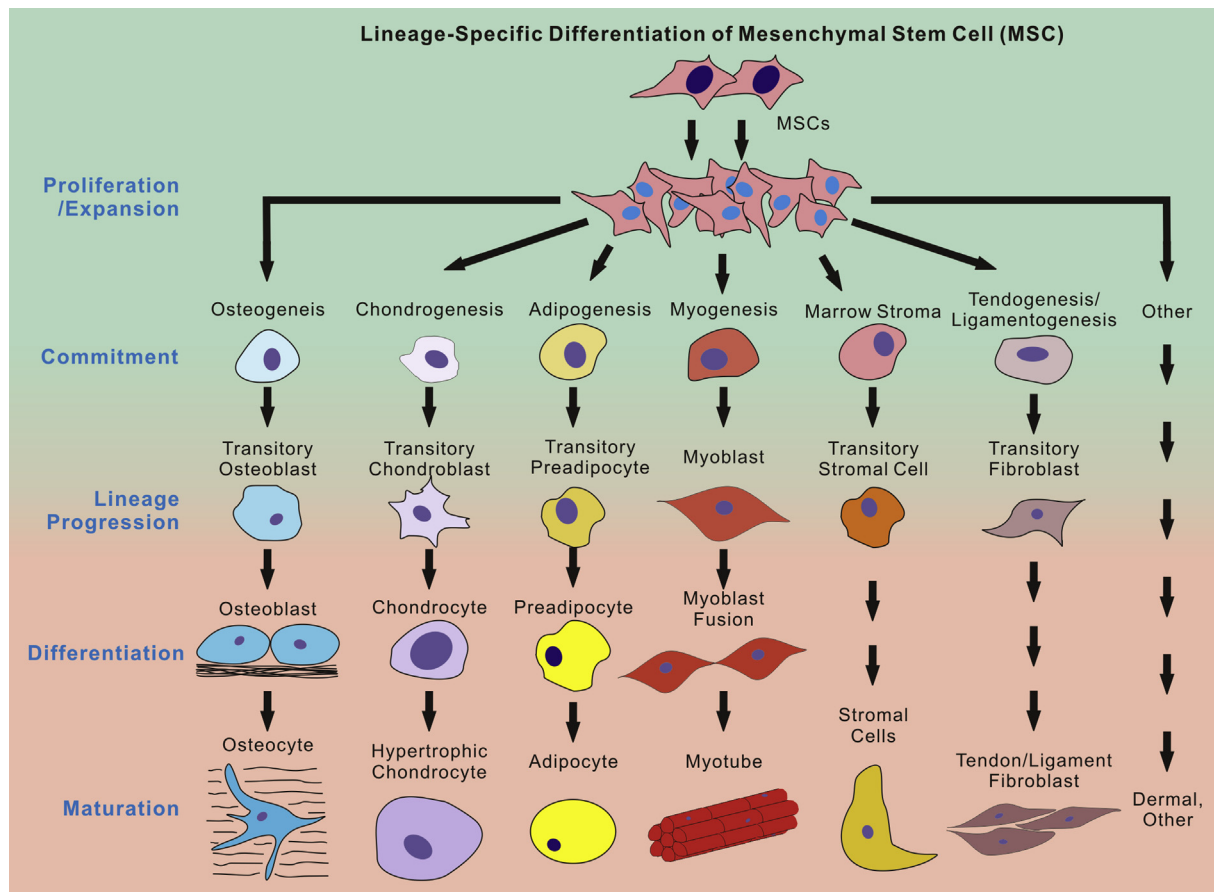


Figure 1 Currently hypothesized lineage-specific differentiation of mesenchymal stem cells (MSCs). Upon receiving appropriate differentiation cues, MSCs are multipotent stem cells and may undergo a cascade of differentiation stages, including progenitor proliferation and expansion, lineage commitment and progression, differentiation and maturation, and subsequently differentiate into multiple types of tissues, such as bone, cartilage, fat, muscle, tendon, etc. It remains a challenge to direct MSCs to differentiate into a specific type of cells/tissues. Furthermore, it is not well understood how early different lineages diverge from MSCs. Nonetheless, MSCs represent one of the most promising populations of progenitors for regenerative medicine and tissue engineering.

proteins, such as Col10a1.¹⁷ Research regarding FGF18 has yielded conflicting results in the *in vitro* setting.^{18,19} However, FGF18 has been shown to stimulate repair of damaged cartilage in the setting of osteoarthritis in rats so it is generally accepted that FGF18 is prochondrogenic.²⁰

Transforming growth factor β (TGF- β)/bone morphogenic protein (BMP) signaling pathway

Members of the transforming growth factor β (TGF- β) superfamily, particularly TGF- β s and BMPs, are essential for multiple stages of embryonic chondrogenesis.^{21,22} This pathway is often utilized to induce chondrocyte differentiation in MSCs and expanded chondrocyte populations.^{21,23–27} TGF- β 1 and TGF- β 2 have long been known to serve as major regulators in chondrogenesis and osteogenesis.^{28–36} Members of the BMP family are required for condensation of chondroprogenitor cells and chondrocyte differentiation both *in vivo* and *in vitro*.^{37–39} TGF- β also mediates the end of proliferation and the subsequent initiation of chondrocyte differentiation.²² This shift from

proliferation to differentiation has been extensively studied both *in vivo* during embryonic cartilage development and *in vitro* using MSCs.⁴⁰ Recent investigation in tissue engineering has focused on developing suitable molecular scaffolds to deliver TGF- β to damaged articular cartilage *in vivo* in order to induce chondrogenic repair of the damaged cartilage.⁴¹ Proper development of joints—another process involving chondrogenesis—requires the activity of several BMPs, including BMP-2, BMP-4, and growth and differentiation factor-5 (GDF5), another member of the BMP family.^{38,42–45} However, exogenous addition of GDF5 to synovial joints during development results in fused joints and chondrocyte overgrowth, suggesting that BMP signaling effects are dose-dependent.^{38,43,44}

TGF- β /BMP signaling primarily activates Smad-dependent signal transduction pathways in the target cell.²² There are two types of receptors for TGF- β s, types I and II; which, upon activation, phosphorylate type-specific receptor-Smads (R-Smads). Following BMP signaling, activated Smad1, Smad5, and Smad8 associate with Smad4 and translocate to the nucleus in order to regulate the

expression of genes.^{37,46,47} In contrast, Smad6 and Smad7 are inhibitory (I-Smads). Smad6 selectively inhibits Smad1/5/8 signaling, whereas Smad7 inhibits all R-Smad signaling pathways. Thus, Smad7 can inhibit chondrocyte differentiation at several different stages.^{22,48} TGF- β can also signal via the mitogen activated protein kinase (MAPK) proteins p38, ERK, and JNK in MSCs; which can contribute to the progression from condensation to differentiation.^{49,50,51}

Though TGF- β is generally associated with differentiation, BMP signaling appears to play a key role in condensation. The coalescence of small aggregates of chondroprogenitor cells into a single distinct cluster *in vitro*, a prerequisite of differentiation, and the condensation of limb bud mesenchyme in culture both require BMP-mediated signaling.^{37,52,53} Prior to the differentiation of chondroprogenitor cells, TGF- β counteracts FGF-mediated cell proliferation.²² TGF- β 2 and TGF- β 3 are highly expressed in the AER of the developing chick embryo limb bud, which corresponds to a reduction in cell proliferation.⁵⁴ Simultaneous downregulation of Gremlin (Grem), an inhibitor of BMP, was also observed, suggesting that TGF- β signaling might indirectly inhibit proliferation by inhibiting Grem; thus, the aforementioned downregulation enables BMPs to counteract the proliferative effects of FGF.⁵⁵ BMP-2 has also been shown to have anti-proliferative effects in the mouse limb bud AER, opposing the effects of FGF-4.^{2,56,57} In addition to inhibiting Grem, TGF- β signaling has been found to activate the cyclin D kinase (CDK) inhibitors p16, p21, and p53, which arrest MSCs in the G1 phase of the cell cycle and induce cellular senescence.²² FGF2 promotes proliferation by inhibiting the expression of TGF- β 2, preventing this senescence.⁵⁸ TGF- β R1 and Smad3 expression increased steadily over time in a 3-month long MSC chondrocyte culture, corresponding directly to an observed decrease in proliferation and increase in differentiation.⁵⁹

In addition to inhibiting proliferation, TGF- β has been shown to mediate the differentiation of chondroprogenitor cells into chondrocytes.^{28,32,60} TGF- β has long been used as the prototype for growth factor-induced chondrogenesis in MSCs *in vitro*.⁶¹ TGF- β -mediated Smad2/3 and Smad1/5/8 signaling is essential for the initiation of chondrogenic differentiation.²² TGF- β also activates MAPK proteins p38, ERK, and JNK in MSCs, which lead to downregulation of N-cadherin expression by blocking Wnt-mediated β -catenin nuclear translocation.⁴⁹ Reducing N-cadherin expression is a necessary step to proceed from condensation to differentiation.^{62–64} Inhibiting TGF- β signaling during chondrogenesis by blocking TGF- β R2 expression alters differentiation and digital joint formation without affecting prior stages of chondrogenesis.^{65–67}

BMPs also take part in the process of chondrocyte differentiation. Simultaneous removal of either BMPRI1A and BMPRI1B or SMAD1 and SMAD5 from chondrogenic cells blocks the formation of the endochondral skeleton in mice, suggesting that those cells are unable to form cartilage.^{37,39,68} Application of BMP-2, 4, 6, 7, 9, 13, or 15 can increase *in vitro* articular chondrocyte synthesis of type II collagen.^{22,38,69,70} Although BMPs take part in the seemingly mutually exclusive condensation and differentiation stages, it is likely that the effects of BMP signaling are time-dependent and concentration-dependent.³⁸

Axin, a protein best known for its role in the β -catenin degradation complex, also interacts with the TGF- β signaling pathways during chondrogenesis.²² During chondrocyte differentiation/maturation, Axin acts as an adaptor between TGF- β R and SMAD3. This adaptor interaction allows for greater phosphorylation and activation of SMAD3 by TGF- β R, which enhances the effect of TGF- β signaling. Axin further enhances this signal by a similar facilitation of TGF- β R phosphorylation of the inhibitor SMAD7, resulting in SMAD7 degradation.^{71,72} Disrupting Axin2 signaling has also been shown to accelerate chondrocyte maturation, further supporting Axin's role in chondrocyte differentiation.⁷³ Given that Axin also mediates the downregulation of Wnt/ β -catenin signaling, this activity is further suggestive of the importance of the switch from Wnt-mediated signaling during condensation to TGF- β signaling during differentiation.

After chondrocytes have differentiated, continued TGF- β -mediated SMAD1/5/8 signaling leads to cartilage hypertrophy.^{22,74} This finding is primarily relevant to efforts to use tissue engineering methods to medically repair damaged articular cartilage (e.g., in osteoarthritis), because TGF- β -induced chondrogenesis in MSCs produces hypertrophic hyaline cartilage.⁷⁵

Wnt/ β -catenin signaling pathway

It is well established that Wnt signaling is involved in chondrogenesis, but the exact nature of its involvement remains to be fully elucidated. Several studies have demonstrated that Wnt signaling is capable of both enhancing and inhibiting chondrogenic differentiation of adult and embryonic progenitor cells.^{75–78} These antagonistic effects may be the product of a variety of factors including the phase of differentiation of target cells, level of Wnt activity, particular type of Wnt signal, and even crosstalk with other signaling pathways, such as the TGF- β /BMP signaling pathway.^{77,79,80} The canonical Wnt/ β -catenin pathway is the most studied with regards to chondrogenesis and will be focused on herein.

Although Wnt signaling's role in the early phase of chondrogenesis has not been well characterized, functional analysis of a few specific Wnt proteins has revealed Wnt to be an important player in chondrogenic cell migration and condensation.^{81,82} It has been demonstrated, for example, that Wnt5b plays a regulatory role in chondroprogenitor cell migration through activation of the planar cell polarity pathway (to be discussed further), and decreases adhesion by inhibiting expression of a destabilizing cadherin receptor as well as exerting regulatory effects on a variety of cadherins.⁹⁴ Wnt5a has also been associated with cellular migration and focal adhesion dynamics in embryonic fibroblasts, but in contrast to Wnt5b, it has been shown to promote cellular adhesion.^{81,83,84}

Progenitor cell migration and cell–cell adhesion regulation are crucial steps in early chondrogenesis and are, in part, mediated by Wnt signaling.⁵² During development of pharyngeal arches, cranial neural crest cells (CNCCs) undergo convergence and extension to form a cartilaginous framework for future ossification.^{85–88} Wnt activation of the non-canonical planar cell polarity pathway is a

fundamental regulatory process in these preliminary CNCC migration and condensation patterns.^{89–91} It has been well documented that the absence of Wnt signaling results in craniofacial deformities in vertebrates, confirming the importance of Wnt signaling in chondrogenic cell migration and condensation.^{92–95} Wnt-mediated CNCC migration and condensation have been studied in zebrafish, in which it has been demonstrated that Wnt9a knockout results in severe craniofacial malformations.^{96,97} These findings do not provide extensive insight into the mechanisms of Wnt's mediating effects on cell migration and condensation, but they do demonstrate the importance of Wnt signaling in these processes.

Several Wnt proteins, including Wnt1, Wnt3a, Wnt4, Wnt5b, and Wnt8 have been shown to signal via the canonical pathway *in vitro*.^{76,77,98–100} However, their effects on chondrogenesis are inconsistent. Wnt1, Wnt4, and Wnt8 have reportedly been shown to inhibit differentiation of chondrocytes whereas Wnt3a and Wnt5b stimulate it. Similarly, Wnt3a and Wnt5b have been shown to inhibit chondrocyte hypertrophy, an important process in the latter stages of chondrogenesis, while Wnt4 and Wnt8 stimulate it.⁸⁹ Other Wnt proteins have similar contradictory effects: Wnt7a inhibits chondrocyte differentiation, Wnt9a inhibits both chondrocyte differentiation and hypertrophy, Wnt5a stimulates chondrocyte differentiation but delays hypertrophy, and Wnt11 seems to have no effect on chondrogenesis.^{101–103} This complicated network of seemingly contradicting effects, which can be found in Table 1, may be the result of Wnt activating two or more of the aforementioned intracellular cascades with effects that modulate each other. For example, Wnt5b has been shown to both stimulate and repress chondrocyte differentiation via the canonical β -catenin and non-canonical JNK pathways, respectively.^{78,81} Similarly, Yan et al showed that Wnt3a signals via the canonical signal cascade as well as the JNK pathway.¹⁰⁴ Additionally, as already mentioned, crosstalk between other signaling pathways such as TGF- β and BMP may be partially responsible for the opposing effects of different Wnt proteins.

Furthermore, the expression patterns of Wnt proteins differ based on the proliferating cell type. Hartmann et al demonstrated that Wnt4, Wnt5a, Wnt5b, and various components of the Wnt cascade show remarkably different expression patterns in perichondrial cells versus chondrocytes (Fig. 2). Artificial induction of inappropriate Wnt

signals in either cell type resulted in severe chick wing malformation.⁷⁶ Other research has found that Wnt5a and Wnt11 are expressed preferentially in the perichondrium, Wnt5b in the prehypertrophic chondrocytes, and Wnt7a and Wnt8 in the mature chondrocytes.^{76,98,102,105,106} The highly localized and sensitive expression pattern of Wnt genes suggests that Wnt signaling is a tightly regulated and highly specific process critical for chondrogenesis.

The positive mediating effect of Wnt and Wnt agonists on chondrogenesis has been observed in pericytes, adult marrow stromal cells, chondroblast precursors, and other multipotent mesenchymal cell types.^{76,77,80} This has been documented to occur through the canonical pathway involving downstream stabilization of cytoplasmic β -catenin and resultant lymphoid enhancing factor (LEF)/T-cell factor (TCF)-induced gene transcription, which, among other functions, promotes chondrogenesis.^{107–109}

Hedgehog signaling pathways

Sonic hedgehog (Shh) signaling

Shh is a morphogenic protein that plays an important role in the developing limb and midline structures of the brain.¹¹⁰ While it is primarily known for its morphogenic properties in the developing limb cartilage, Shh remains an important regulator of chondrogenesis in adults and therefore warrants attention regarding its potential therapeutic impact. Shh signaling, along with the BMP pathway, is critically important in early chondrogenesis due to its role in determining cell fate. For example, the relative timing of Shh and BMP signaling controls whether presomitic mesoderm will adopt either a chondrogenic or lateral plate mesoderm fate. Shh, like other hedgehog proteins, signals through effector molecules known as Gli factors. These Gli factors can be silenced through a direct interaction with GATA factors, whose transcription is BMP-dependent.¹¹¹ The strict regulation of this feedback loop explains why the timing of Shh and BMP signaling is crucial during the early stages of chondrogenesis.

In addition to its role in early chondrogenesis, Shh helps drive chondrogenesis through robust synergism with other prochondrogenic factors.^{112–114} Shh and BMPs work together to establish a positive regulatory loop with Sox9 and Nkx3.2, a transcription factor that increases Sox9 expression.^{115–118} Along with establishing this regulatory loop, transient Shh expression increases target cellular response to BMP signaling.^{27,119} In conjunction with Shh's synergistic ability, it has been shown to significantly enhance chondrogenesis in the presence of two members of the FGF family, FGF2 and FGF8.^{120,121} Altogether, Shh is an important prochondrogenic factor that can have a substantial impact on cartilage tissue engineering because of its synergistic activity with many other chondrogenesis-mediating factors.

Indian hedgehog (Ihh) signaling

Ihh, another member of the mammalian hedgehog family of proteins, is encoded by a mechanosensitive gene and serves as a regulator for both osteogenesis and chondrogenesis.¹²²

Table 1 Effect of Wnt signaling on chondrocyte differentiation and hypertrophy.

Wnt	Differentiation	Hypertrophy	References
Wnt1	Inhibits	—	318
Wnt3a	Stimulates	Inhibits	77,319
Wnt4	Inhibits	Stimulates	76,320
Wnt5a	Stimulates	Inhibits	76,321
Wnt5b	Stimulates	Inhibit	76,81
Wnt8	Inhibits	Stimulates	3,19,322
Wnt7a	Inhibits	—	77
Wnt9a	Inhibits	Stimulates	96,97,124
Wnt11	No effect	No effect	103

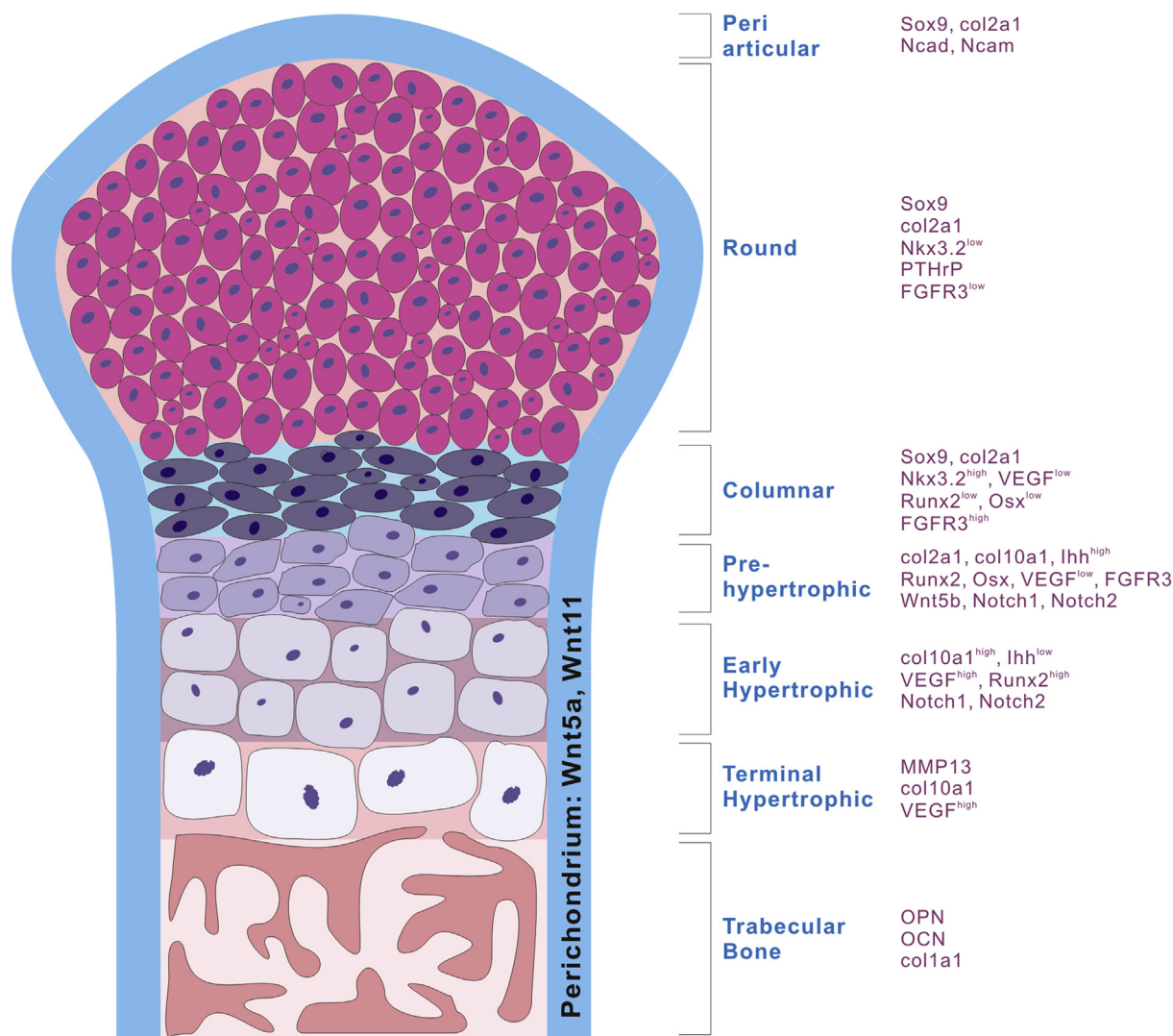


Figure 2 Marker/regulator gene expression in different zones of the growth plate and the perichondrium. The important and representative marker genes are listed.

Ihh is synthesized in prehypertrophic and hypertrophic chondrocytes and couples chondrogenesis to osteogenesis through signaling mechanisms involving chondrocytes and preosteoblasts (Fig. 2).¹²³ Expression of Ihh in prehypertrophic and hypertrophic chondrocytes is dependent on expression of Wnt9a, whose effector β -catenin/Lef1 complex interacts directly with an Ihh promoter.¹²⁴ Due to its ability to drive both osteogenesis and chondrogenesis, precise homeostatic regulation of Ihh expression is crucial for promoting chondrogenesis over osteogenesis.

Although Ihh is not considered one of the master regulators of chondrogenesis, studies show that in primary MSCs it is an equally potent inducer of chondrogenesis in comparison to TGF- β 1 and BMP-2. Solo Ihh expression is sufficient to drive chondrogenesis in an acute injury model, but its major prochondrogenic effects are carried out through interactions with other factors.¹²⁵ For example, endogenously produced Ihh is thought to mediate TGF- β 1-induced chondrogenesis in human bone marrow stromal cells (hBMSCs).^{126,127} Similarly, synergistic interactions between Ihh and other central regulators of chondrogenesis have

been characterized and include Runx proteins and FGfs. Runx2 and Runx3 are essential for chondrocyte maturation and regulation of limb growth, and these effects are carried out through Runx-induced expression of Ihh.^{128,129} Similarly, Ihh works in conjunction with FGF8 during digit primordia elongation. Like its mammalian hedgehog family member Shh, Ihh promotes chondrocyte differentiation via expansion of Sox9 expression. Additionally, Ihh is known to upregulate the expression of FGF8 in MSCs. It is believed that these effects stem from FGF8-induced upregulation of Ihh in MSCs, creating a positive feedback loop that is antagonized by BMP signaling.^{130,131}

Along with interactions with major signaling pathways, Ihh expression is regulated by a variety of peptides. The most well-studied peptide interaction is the Ihh/PTHrP negative feedback loop. In the growth plate, Ihh produced by the prehypertrophic and hypertrophic chondrocytes increases expression of PTHrP, which antagonizes Ihh-mediated differentiation via a protein kinase A-mediated mechanism. This feedback loop helps to coordinate chondrocyte proliferation and differentiation, and this system is

negatively regulated by retinoic acid.^{132–135} Sirtuins (Sirts), a class of proteins with deacetylase activity, have been found to enhance *Ihh*-mediated chondrocyte differentiation. *Sirt6* knockdowns reveal markedly decreased expression of *Ihh*, and consequently depressed *Ihh*-mediated chondrocyte differentiation.¹³⁶ In contrast, the oncogene *delta-EF1* serves as a negative regulator of *Ihh* expression, implying that knockdown of *delta-EF1* would actually enhance *Ihh*-mediated chondrocyte differentiation at the growth plate.¹³⁷

Notch signaling pathway

The Notch signaling pathway is a cell signaling pathway involving one of four transmembrane notch receptors: NOTCH1–4. The Notch signaling pathway is highly conserved and it is known to play roles in many developmental and cell type specification processes.¹³⁸ Previous studies have demonstrated differential expression of markers for Notch signaling during cartilage development, underscoring its importance in chondrogenesis and suggesting a role in cartilage tissue engineering.^{139,140}

The function of the Notch intracellular domain (NICD) is central to Notch-mediated regulation of chondrogenesis. The NICD, one of the end products of the Notch signaling pathway, translocates into the nucleus where it complexes with CSL-type DNA binding proteins CBF1(RBP-J), Su(H), and Lag-1. During this interaction, NICD converts the aforementioned DNA binding proteins from transcriptional repressors to transcriptional activators.¹⁴¹ These transcriptional activators go on to induce the transcription of members of the HES gene family. The HES gene family encodes for basic helix-loop-helix (bHLH) nuclear proteins that suppress transcription.¹⁴² Members of this family, HES-1 and HEY-1 act on the Sox9 binding site in the *col2a1* enhancer and consequently prevent Sox9-mediated transcriptional activation of *col2a1*.^{143,144} Thus, it is clear that Notch signaling has the ability to inhibit chondrocytic differentiation through the upregulation of HES-1 and HEY-1 activity.

Although a mechanism detailing one way Notch signaling can regulate chondrogenesis has been elucidated, there are no data which suggest a mechanism behind the differential expression of Notch receptors. NOTCH1 is the most well-studied receptor with regards to chondrogenesis and is highly expressed in mesenchymal condensations, prehypertrophic and hypertrophic zones in embryos, and the surface layer of joint cartilage in mice (Fig. 2). Likewise, NOTCH2, a modulator of NOTCH1 and NOTCH3 activity, is broadly distributed throughout proliferating, prehypertrophic, and hypertrophic chondrocytes in articular cartilage and the epiphyseal growth plate.^{139,140,145,146} As a result of these findings, it is commonly accepted that Notch receptors can serve as markers for chondroprogenitor cells.¹⁴⁷ This differential expression suggests a dual role for Notch signaling during the early and late stages of chondrogenesis.

Recent findings have determined that the impact of Notch signaling on chondrogenesis is conditional. This is evident by the finding that both overexpression of NICD and loss of NICD activity result in skeletal malformations due to defective endochondral ossification.¹⁴⁸ These results

suggest that Notch signaling is necessary for chondrogenesis to occur completely, although overactivity of the pathway creates inhibitory effects. We have previously discussed that increased Notch signaling can inhibit Sox9-induced chondrogenesis, but the fact that Notch receptors are highly expressed in chondroprogenitor cells implies that Notch signaling can maintain proliferating chondrocytes in their progenitor stage. Karlsson et al have confirmed this hypothesis by showing that there is high expression of HES-5 in undifferentiated chondrocytes and that downregulation of HES-5 is required for expression of genes with a hyaline phenotype.^{149,150} In summary, current research suggests that transient Notch signaling is required to prime MSCs for chondrogenesis, but Notch signaling must be switched off for chondrogenesis to proceed to completion. As a result, finding ways to regulate Notch signaling activity may prove to have a significant impact on cartilage tissue engineering.

Hypoxia and angiogenic signaling pathways

Hypoxia signaling

Hypoxia-inducible factors (HIFs) are transcription factors that respond to decreases in oxygen, or hypoxia, in the cellular environment. It is well known that cartilage is a largely avascular tissue, and as a result, chondrocytes almost always reside in a state of mild hypoxia.^{151,152} Severe hypoxia (1% O₂ saturation) slows cell differentiation, including chondrogenesis, through decreases in gene expression secondary to cell damage.¹⁵³ The link between mild hypoxia (5% O₂ saturation) and the presence of chondrocytes is of tremendous importance for cartilage tissue engineering, and HIFs play a crucial role in establishing this link. Because chondrocytes primarily reside in a hypoxic environment, it is conceivable that HIFs are involved throughout the entirety of chondrogenesis. In early chondrogenesis, HIFs help maintain the chondrocyte phenotype and ensure survival of proliferating chondroprogenitor cells.^{154–156} HIFs also enhance chondrocyte secretion of ECM and alter gene expression in chondroprogenitor cells. Specifically, HIFs enhance expression of *col2a1*, *Sox9*, aggrecan, CD44, CD105, and PDGF receptor α ; while inhibiting the expression of *col1a1*, *col1a2*, and *col3a1*.^{157–162} There are 6 members of the HIF family, but HIF-1 α and HIF-2 α are the primary HIFs that have been shown to promote chondrogenesis.

There is a competing body of evidence regarding HIF-1 α 's role in chondrogenesis, but many researchers have shown that HIF-1 α is critical to the survival of the hypoxic chondrocyte.^{163–166} HIF-1 α accomplishes this function primarily through two mechanisms, the first of which involves interaction of HIF-1 α with the cAMP response element-binding protein binding protein/p300. This complex serves as a coactivator for HIF-1 α and enhances the transcription of HIF-1 α 's target genes.¹⁶⁷ The second mechanism by which HIF-1 α promotes hypoxic chondrocyte survival is through upregulating expression of heat shock proteins Hsp90 and Hsp70. HIF-1 α -mediated increases in Hsp90 and Hsp70 expression is also believed to be the mechanism of action of protection against chondral inflammation.^{168,169} These important signaling pathways initiated by HIF-1 α

are stabilized *in vivo* by Runx2, which blocks the interaction between von Hippel-Lindau protein and HIF-1 α .¹⁷⁰

While it has been established that HIF-1 α serves as the primary HIF expressed to maintain the chondrocyte phenotype, HIF-2 α has emerged as the primary HIF involved in chondrocyte differentiation. HIF-2 α induces chondrocyte differentiation by enhancing the transcription of many prochondrogenic genes, including the Sox trio, aggrecan, versican, Col2a1, Col9a1, Col10a1, and Col11a1.¹⁷¹ HIF-2 α -mediated chondrogenesis is reinforced via increased activity of the PI3K-Akt signaling pathway.¹⁷²

Vascular endothelial growth factor (VEGF) signaling pathway

VEGF, which is secreted by hypertrophic chondrocytes, is a signaling protein that stimulates vasculogenesis and angiogenesis, thus making a proosteogenic factor.^{173,174} As previously stated, angiogenesis reduces the severity of hypoxic states, which is needed to maintain the chondrocyte phenotype. Vascularization of cartilage tissue results in loss of phenotype, and subsequently, differentiation of chondrocytes to bone. Therefore, regulation of VEGF expression is important in maintenance of cartilage tissue.

Much of the research regarding VEGF's impact on chondrogenesis comes in the form of knockout studies. Here, we have attempted to summarize the most pertinent results. Antibodies against VEGF inhibit migration of chondroprogenitor cells *in vitro*. These results are also seen when chondroprogenitor cells are introduced to media containing antibodies against VEGF receptor 2, and are consistent with the findings that VEGF plays a key role in chondrocyte metabolism and accelerates the proliferation of chondroprogenitor cells *in vitro*.^{175–178} In contrast, Flt-1, a soluble VEGF antagonist, has been shown to improve BMP-4- and TGF- β 3-induced chondrogenesis.^{178,179} Similar results were seen with administration of sFlk-1 or Bevacizumab.¹⁸⁰

In addition to helping determine cell fate by disrupting hypoxia, VEGF is involved in different phases of cartilage tissue development. VEGF is important for the formation of blood vessels needed for limb development. In this capacity, VEGF serves as the effector molecule of Sphingosine-1-Phosphate receptor, a G-protein coupled receptor central to endothelial cell morphogenesis and vascular maturation.¹⁸¹ Exogenous administration of VEGF to the developing limb markedly increases blood vessel number and density, but it inhibits digit development due to repression of Sox9 expression.¹⁸² Alternatively, complete inactivation of VEGF-A in areas of col2a1 expression results in embryonic lethality.¹⁸³ These results imply that a closely regulated level of VEGF expression is necessary for proper limb development, a process that includes extensive chondrogenesis.

Transcriptional regulation of chondrogenesis

SRY-box 9 (Sox9)

Sox9 is a prochondrogenic transcription factor that is arguably the single most important regulator of

chondrogenesis because high levels of Sox9 are required for full expression of the chondrocyte phenotype.¹⁸⁴ Sox9 expression is induced by compressive force: a variety of prochondrogenic factors, including RelA, Pref-1, Arib5b; and two other Sox transcription factors, Sox5 and Sox6.^{185–190} As a transcription factor, Sox9's mechanism of action is directly related to the genes that it regulates. Akiyama et al have elucidated that Sox9 interacts with two binding sites for HMG-domain proteins and activates elements in the promoters of col2a1, col9a1, col11a2, Bbf2h7-sec23a, and aggrecan.^{191–193} Furthermore, Sox9 induces its own transcription in addition to the transcription of Sox5 and Sox6, forming the Sox Trio. The individual components of the Sox Trio act cooperatively to increase the expression of the previously discussed gene targets of Sox9.^{191,194–196}

Sox9 primarily regulates chondrocyte proliferation and differentiation through direct control of the expression of many chondrocyte-specific genes. In addition to this observation, many studies have elucidated Sox9's role in earlier phases of chondrogenesis, such as condensation. Numerous studies show that Sox9 expression levels are markedly elevated in condensing mesenchymal progenitors, both *in vitro* and *in vivo*, while Sox9 knockout mice are unable to condensate mesenchymal cells.^{197–199} As previously stated, FGF2 induces chondrocyte proliferation by upregulating basal expression levels of Sox9.^{200,201} Sox9 then potentiates BMP2's prochondrogenic properties and inhibits BMP2-induced osteogenic differentiation and endochondral ossification.²⁰² While this relationship has been well studied, it is only one of many Sox9 interactions that help regulate chondrogenesis.

Sox9's ability to promote chondrocyte differentiation and maturation is dependent upon the expression of coactivators of Sox9-mediated transcription and the silencing of factors that stimulate osteogenesis. Many coactivators of Sox9 expression have been identified in MSCs, including Paraspeckle regulatory protein 54-kDa nuclear RNA-binding protein (p54nrb), Smad proteins 2 and 3, peroxisome proliferator-activated receptor γ co-activator 1 α (PGC-1 α), Tip60, Lc-Maf, CypA, Scleraxis/E47, and p300.^{203–209} Having so many coactivators most likely plays a role in fine-tuning local expression levels of Sox9, which may prove advantageous within the field of cartilage tissue engineering. Sox9's major antagonist is Runx2, the master regulator of osteogenesis. Expression of Sox9 inhibits early and late osteogenic differentiation by depressing the expression of Runx2, as well as osterix, a transcription factor for osteogenic differentiation, col1a1, and col10a1.^{197,202,210} In addition to regulating the expression of proosteogenic transcription factors and genes, Sox9 also targets Wnt/ β -catenin promoters for degradation by the ubiquitination/proteasome system.^{198,211} Finally, Sox9 activity can be blunted by its two primary negative regulators, Runx2 and RhoA protein.^{211,212}

Runx-related transcription factors (Runx)

Runx proteins, including Runx1, Runx2, and Runx3, are a family of transcription factors that play significant roles in organogenesis and embryonic patterning. While all 3

members of the Runx protein family are expressed during chondrogenesis, Runx1 is believed to have a minimal effect on chondrogenesis and will not be discussed in this review.²¹³

Runx2 and Runx3 are essential for chondrocyte maturation, but Runx2 serves a secondary role as a potent inducer of osteogenesis. The regulation of osteogenesis and chondrocyte proliferation by Runx2 is accomplished through an interaction with PI3K-Akt signaling.²¹⁴ Regulatory control of Runx2 is handled primarily by Sox9, as stated previously, helping to explain Sox9's apparent dominance over Runx2 in MSCs destined for a chondrogenic lineage.²¹⁵ This interaction is further complicated by interactions with CypA, a positive regulator of both Runx2 and Sox9 expression in chondroprogenitor cells. Predictably, CypA knockdowns have depressed chondrogenesis and endochondral ossification.²⁰⁸ Nkx3.2 serves as another method for regulatory control of Runx2. Nkx3.2 represses Runx2 activity through direct interaction with the Runx2 promoter, and this repression is required for the progression of BMP-induced chondrogenesis.^{216,217}

The regulation of Runx2 expression is of extreme importance with regards to chondrogenesis, given the variety of effects Runx2 expression can have on MSCs. Col10a1, a type of collagen expressed in hypertrophic chondrocytes destined for endochondral ossification, is a direct transcriptional target of Runx2.²¹⁸ Runx2's ability to drive osteogenesis is most likely related to its ability to cooperate with HIFs to stimulate angiogenesis. As already discussed, angiogenesis decreases the severity of local hypoxia which inhibits chondrogenesis while promoting osteogenesis.²¹⁹ In addition to driving osteogenesis, Runx2 has also been shown to induce adipogenic differentiation *in vitro*.²²⁰ Finally, Hinoi et al have shown that Runx2 enhances expression of FGF18, a negative regulator of chondrocyte maturation, in the perichondrium.²²¹

Trico-rhino phalangeal syndrome type 1(Trps1)

Trps1 is a GATA-like transcription factor that serves as a novel regulator of chondrocyte differentiation and proliferation through regulation of a variety of genes. As a GATA-like transcription factor, Trps1 acts as a downstream effector of the Gdf5 signaling pathway to promote both differentiation and apoptosis.²²² Trps1's versatility as a transcription factor is linked to its ability to effect expression levels of key regulators, like Sox9, as well as less researched gene targets, such as Stat3.^{223,224} In addition to its function as a transcription factor, Trps1 also interacts with many microRNAs (miRs) and other signaling pathways. Specifically, Trps1 is responsible for maintaining low levels of miR-221, which allows MSCs to commit toward the chondrocyte lineage.²²⁵ The interaction between Trps1 and miR-221 may be a part of a feedback loop, as it has been shown that Trps1 expression is regulated by seven Runx2-targeting miRs (miRs-23a, -30c, -34c, -133a, -135a, -205, and -217).²²⁶ Furthermore, Trps1 interacts with Ihh/Gli3 signaling, as evidenced by studies in which Trps1 knockout mice overexpress PTHrP and inhibit Ihh-mediated chondrogenesis via the Ihh/PTHrP negative feedback loop.^{227,228}

MIA/CD-RAP in chondrogenic differentiation

The MIA/CD-RAP gene is a gene locus that encodes for the melanoma inhibitory activity protein (MIA) and the cartilage-derived retinoic acid-sensitive protein (CD-RAP). Normal expression of this gene locus is limited to cartilage, but pathological expression does occur in melanoma and chondrosarcoma.²²⁹ Given the cartilage-selective expression of the MIA/CD-RAP gene, it makes sense that these proteins are noteworthy regulators of chondrogenesis.

Regulation of chondrogenesis by MIA/CD-RAP occurs through two primary mechanisms. First, transcription factor YBX1 has been shown to mediate MIA/CD-RAP-dependent activation of p54^{nrb} transcription.²³⁰ Second, consistent data support the concept of MIA/CD-RAP-mediated inhibition of ERK signaling. ERK is known to block chondrogenic differentiation, and its inhibition helps drive chondrogenesis.²³¹ Through these two mechanisms, it has become clear that MIA/CD-RAP proteins are required for chondrocyte differentiation. This is confirmed by the finding that MIA knockout mice show enhanced chondrocyte proliferation but delayed and defective differentiation, which can be rescued by decreasing AP1 and CRE activity.^{230,232} Given the close proximity of the MIA and CD-RAP genes, alterations in transcription often change the expression of both proteins. Therefore, interactions involving the promoter region of MIA/CD-RAP have a significant impact on the regulation of chondrogenesis. Known regulators of MIA/CD-RAP expression include delta-EF1, USF1/USF2, Sox9, retinoic acid, AP-2, and HMGA. Sox9 serves as a positive regulator, while the others serve as negative regulators.^{233–236}

Epigenetic regulations of chondrogenesis

Histone deacetylase superfamily

Histone deacetylases (HDACs) are a class of enzymes that remove acetyl groups from histone proteins, allowing higher affinity DNA binding and consequent inhibition of transcription. Therefore, it is no surprise that HDACs primarily function as repressors of the transcription of pro-chondrogenic genes. For example, HDAC1 and HDAC2 repress the expression of cartilage-specific genes in human chondrocytes.²³⁷ Furthermore, LRF-mediated repression of COMP gene expression and chondrocyte differentiation requires HDAC1 activity.²³⁸ HDACs are also known negative regulators of osteogenesis. Specifically, inhibition of HDAC activity potentiates BMP9-induced osteogenic signaling in mouse MSCs and attenuates chondrocyte proliferation and differentiation.^{239–242} Similarly, HDAC4 overexpression promotes chondrogenesis while inhibiting chondrocyte hypertrophy via modulating TGF- β 1 activity.²⁴³ Negative regulation of osteogenesis by HDACs may help explain these findings.

microRNAs (miRs)

MicroRNAs are small, non-coding RNA fragments whose function involves mRNA silencing and post-transcriptional

gene regulation. Since their discovery in the early 1990's, it has been well known that microRNAs are important for developmental timing in both vertebrates and invertebrates.^{244,245} More recent findings have confirmed this notion, showing that universal disruption of Dicer, a RNA endonuclease required for microRNA production, results in embryonic lethality and depletion of pluripotent stem cells.^{246,247} These findings, among others, have sparked significant research interest in the role of microRNAs in chondrogenesis and their potential therapeutic effects.

MicroRNAs have well characterized effects on both pre- and post-natal chondrogenesis. Pre-natally, microRNA expression profiles are extremely important for the development of cartilaginous tissues, such as the pharyngeal arches. For example, Weinholds et al have shown that 10 microRNAs (miRs-23a, -27a, -27b, -140, -140*, -145, -146, -199a, -199a*, and -214) were specifically expressed in the developing pharyngeal arches of zebrafish embryos.²⁴⁸ In addition, post-natal expression profiles of microRNAs and their effects on chondrogenesis are currently large areas of research. Studies of many cell types have been conducted, and the results are much too expansive to be detailed in this review. With this in mind, we have organized what we believe to be the most compelling results into [Table 2](#).

A large number of microRNAs have been implicated in regulating chondrogenesis, but three major target genes have been implicated in mediating their effects: C/EBPb, master regulator Sox9, and Adam9. C/EBPb is believed to protect articular cartilage from degenerative diseases such as osteoarthritis, and it has been shown to be negatively regulated by several miRNAs during chondrogenic differentiation of hADSCs.²⁴⁹ Sox9 and Adam9 expressivity are similarly regulated by a variety of microRNAs. Adam9 is a negative regulator of chondrogenesis, and its upregulation could lead to apoptosis of chondrogenic progenitors and inhibition of MSC migration.²⁵⁰

Implications in cartilage regeneration and tissue engineering

Cartilage tissue engineering is an area of significant research amongst engineers and physicians. This research is of significant therapeutic importances given the high prevalence of osteochondrodysplasias, including but not limited to achondroplasia, cleidocranial dysostosis, and ovine chondrodysplasia. Over the past decade, cartilage tissue engineering has become dependent upon the selection of an appropriate cell line, subsequent chondrogenic differentiation of the selected cell line, and the fabrication of a biocompatible and mechanically suitable scaffold.²⁵¹ Given these challenges, current progress has been unable to show significant improvement in tissue restoration over proven surgical techniques. Here, we review the most recent advances in cartilage tissue engineering regarding overcoming these challenges.

An appropriate cell line for cartilage tissue engineering must have the ability to differentiate into mature, fully functional cartilage and maintain a chondrocyte phenotype long-term. A clear starting point was chondrocytes extracted from the patient, which have the ability to maintain a chondrocyte phenotype and remodel. Unfortunately, these

Table 2 List of known microRNA expression in human and mouse MSCs undergoing chondrogenesis.

microRNA	Expression	References
miR-26b	Upregulated	323
miR-28	Upregulated	323
miR-130b	Upregulated	323
miR-152	Upregulated	323
miR-193b	Upregulated	323
miR-15b	Upregulated	324
miR-16	Upregulated	324
miR-23b	Upregulated	3,25,325
miR-27b	Upregulated	324
miR-140	Upregulated	324
miR-148	Upregulated	324
miR-197	Upregulated	324
miR-222	Upregulated	324
miR-328	Upregulated	324
miR-505	Upregulated	324
miR-127	Upregulated	326
miR-140	Upregulated	326
miR-125b*	Upregulated	326
miR-99a	Upregulated	326
miR-140*	Upregulated	326
miR-181a-1	Upregulated	326
let-7f	Upregulated	326
miR-30a	Upregulated	326
miR-24	Upregulated	327
miR-101	Upregulated	327
miR-124a	Upregulated	327
miR-199a	Upregulated	3,28,328
miR-199b	Upregulated	327
miR-130b	Upregulated	323
miR-193b	Upregulated	323
miR-455	Upregulated	329
miR-337	Upregulated	330
miR-574-3p	Upregulated	331
miR-212	Downregulated	326
miR-132	Downregulated	326
miR-143	Downregulated	326
miR-125b-3p	Downregulated	326
miR-18	Downregulated	326
miR-96	Downregulated	326
miR-145	Downregulated	332
miR-490-5p	Downregulated	333
miR-124	Downregulated	328
miR-199*	Downregulated	334,335

cells are limited in number, and their expansion leads to dedifferentiation with resultant phenotypic loss.²⁵¹ Other cell types explored as candidates for cartilage tissue engineering include human MSCs (hMSCs), adipose-derived stem cells, and embryonic stem cells. Adipose-derived stem cells and embryonic stem cells have the ability to undergo chondrogenic differentiation in response to certain signals, but they are limited by specific growth requirements and ethical controversy, respectively.^{252,253} hMSCs are the most promising candidate, and use of combinations of growth factors in hMSCs is the current gold standard for scaffold-based cartilage tissue engineering.^{251,254,255}

The many biological regulators of chondrogenesis, many of which are detailed in this review, provide various ways to induce chondrogenic differentiation in progenitor cells (Fig. 3). Current research suggests that among the many growth factors implicated to have a regulatory effect on chondrogenesis, TGF- β proteins are the most potent inducers of chondrogenesis in hMSCs. In contrast, other growth factors, including insulin-like growth factors (IGFs), FGFs, and PDGFs, appear to mediate chondrocytic physiology to a greater degree than they promote chondrogenesis in hMSCs.^{19,61,254–271} In addition to members of the TGF- β family of proteins, VEGF, hypoxia, and Sox9 have been shown to induce chondrocytic differentiation in a variety of 3D scaffolds.^{272–276} The challenge in using signaling molecules to optimize cartilage regeneration is that their regulatory effects depend on a large number of factors that must be tightly controlled: the dosing and timing of administration, the cell type, and the medium in which they act. This phenomenon could help explain the variability in the results regarding the effects of different growth factors in cartilage regeneration. Researchers circumvent this inconsistency by infecting target cells with adenoviruses expressing a gene of interest and through the use of gene therapy.^{277–281}

While current research suggests that administration of biologic agents can delay progression of articular cartilage degeneration in an experimental setting, few are available clinically, necessitating consideration for surgical treatment. Although total joint arthroplasty may be the ultimate end point in many cases, early surgical treatment options include microfracture, osteochondral auto- or allograft transfer, and autologous chondrocyte implantation (ACI). The determination of which method provides the ideal solution depends on the size and location of the lesion, patient age and activity level, and concomitant pathology. In ACI, a full-thickness sample from a low-weight-bearing region of the joint is collected by punch biopsy and then implanted into the debrided cartilage defect and covered by a membrane. When successful, this technique can

produce a greater amount of hyaline-like cartilage at the repair site. Limitations of the technique include the need for two separate surgical procedures, a prolonged recovery time, and increased procedural complexity.²⁸² In addition, the procedure has a comparatively high reoperation rate and cost is prohibitive for many patients. Other early surgical interventions, including matrix-induced autologous chondrocyte implantation (MACI), have had promising histological results, but superiority over ACI remains unproven.^{283,284}

The function of a tissue engineering scaffold is to provide a temporary structure on which cells seeded within the biodegradable matrix synthesize new, natural tissue. Scaffold-based techniques are preferred over scaffold-free approaches because they increase control over filling defects, eliminate the complications associated with a surgical donor site, shorten recovery time, and are associated with a reduced risk of dedifferentiation.²⁸⁵ *Ex vivo* studies have shown that successful cartilage regeneration is dependent on both the chondrocyte proliferation rate and on the differentiation capacity of stem cells within a 3D scaffold. Taken together with evidence that cartilage production varies with scaffold architecture, the importance of choosing the appropriate scaffold for cartilage tissue engineering is clear.^{286–288} The two most well-studied types of scaffolds with regards to cartilage tissue engineering are poly(α -hydroxy esters) and hydrogels. Poly(α -hydroxy esters) are the most commonly used synthetic polymers for cartilage tissue engineering, while poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) are the most investigated. Both *in vitro* and *in vivo* studies have demonstrated that progenitor cells cultured on PLA or PLGA scaffolds retain chondrocyte phenotype and produce cartilage-specific ECM.^{289–297} In contrast, hydrogels are cross-linked polymer networks that have the ability to absorb large amounts of water, making them attractive scaffolds for engineering tissues with high water content, such as cartilage.²⁵¹ Despite theoretical strengths in the design, use of hydrogel scaffolds has failed to improve

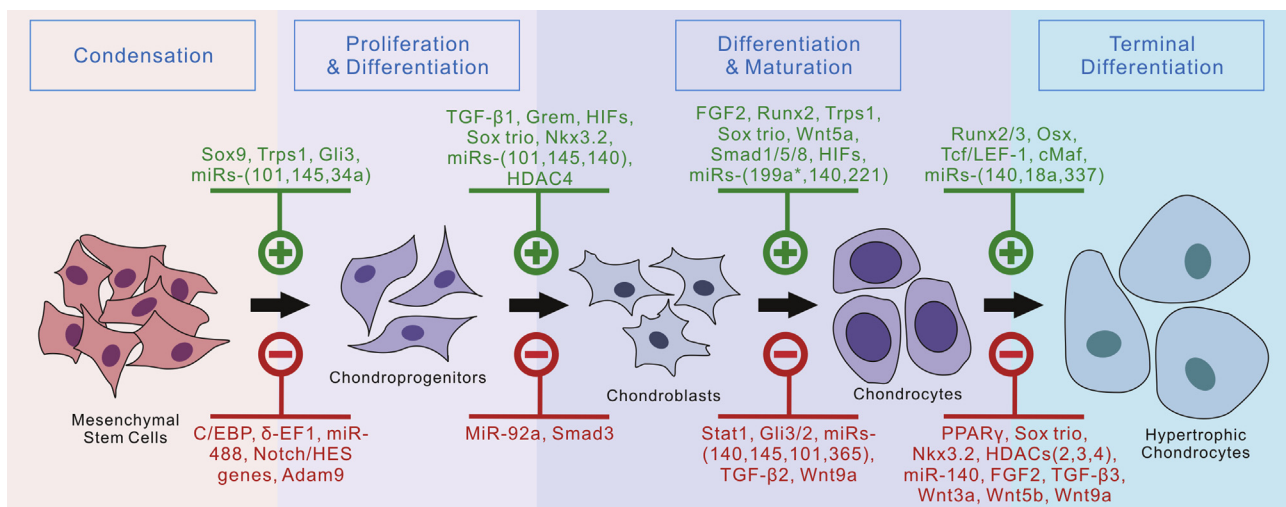


Figure 3 Currently identified regulators of chondrogenic differentiation from MSCs. Currently reported signaling molecules, transcription factors, microRNAs and other regulators are listed. Positive regulators are shown on the top panel in green, while the inhibitory regulators are shown in red at the bottom panel.

clinical outcomes when compared to the less expensive poly(α -hydroxy esters).

Recently, there has been a growing interest in the understanding of the mechanobiology of MSCs, given that mechanical factors are known to influence the structure and function of musculoskeletal tissues at all stages of life.^{298,299} A significant proportion of the current research in cartilage tissue engineering is directed at discerning how mechanical stimulation induces chondrogenesis in chondroprogenitor cells, and how scientists can use these signals to drive cartilage tissue production. However, the molecular mechanisms of mechanical signal transduction in chondrocytes and chondroprogenitor cells are still unclear. Current theories implicate ion channels, primary cilia, the nucleus, and the cytoskeleton as potential transducers of mechanical signals in these cells.^{300–303} Regardless of the molecular mechanism, it has been well established that a variety of forms of mechanical stimulation in MSCs can produce a prochondrogenic state that is favorable for cartilage regeneration and repair. For example, Choi et al showed that low-intensity ultrasound enhanced chondrogenesis in MSCs cultured in fibrin-hyaluronic acid.³⁰⁴ Likewise, Kupcsik et al showed that mechanical stimulation of multipotent stem cells enhanced collagen type X and lubricin expression.³⁰⁵

Another method employed by researchers to overcome the previously described challenges of cartilage tissue engineering is the use of bioreactor systems to enhance the growth of cartilaginous constructs. Bioreactor systems have emerged as valuable enhancers of cartilage tissue engineering because they accelerate chondrocyte growth by supplying nutrients or mechanical stimulation.²⁵¹ Bioreactor systems come in two forms: loading reactors and hydrodynamic reactors. Loading reactors, hydrodynamic reactors, and combinations of the two have all been shown to expedite the rate and quality of cartilaginous tissue growth.^{263,297,306–312} Promising results in cartilage tissue engineering have been achieved through the utilization of self-assembling MSCs in a bioreactor model. Self-assembling MSCs were directed to form condensed mesenchymal bodies (CMBs) and were fused together with the aid of press molding.^{313–317} This novel approach has enabled a breakthrough in the ability to engineer human cartilage from MSCs with the same physiological Young modulus and friction coefficient.³¹³

Concluding remarks and future directions

Regulation of chondrogenesis and cartilage tissue engineering remain areas of significant research given the increasing prevalence of osteodegenerative disease and orthopedic trauma. Cartilage tissue engineering has been complicated by three main challenges: production of a chondrogenic progenitor cell line that can proliferate and differentiate into mature chondrocytes, finding biological factors that can facilitate the proliferation and differentiation of chondrogenic seed cells, and finding biomaterial scaffolds that provide a 3D architecture resembling the cartilaginous matrix. While many biological regulators have been identified, implantation of chondrocytes grown *in vitro* has failed to yield significant clinical results. The

application of bioreactor systems, which allows scientists and physicians to deliver nutrients to a largely avascular tissue, has yielded the most promising results and appears to be the future of cartilage tissue engineering.

Conflict of interest

The authors declare no conflict of interest.

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