Strategies to minimize hypertrophy in cartilage engineering and regeneration

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Abstract Due to a blood supply shortage, articular cartilage has a limited capacity for self-healing once damaged. Articular chondrocytes, cartilage progenitor cells, embryonic stem cells, and mesenchymal stem cells are candidate cells for cartilage regeneration. Significant current attention is paid to improving chondrogenic differentiation capacity; unfortunately, the potential chondrogenic hypertrophy of differentiated cells is largely overlooked. Consequently, the engineered tissue is actually a transient cartilage rather than a permanent one. The development of hypertrophic cartilage ends with the onset of endochondral bone formation which has inferior mechanical properties. In this review, current strategies for inhibition of chondrogenic hypertrophy are comprehensively summarized; the impact of cell source options is discussed; and potential mechanisms underlying these strategies are also categorized. This paper aims to provide guidelines for the prevention of hypertrophy in the regeneration of cartilage tissue. This knowledge may also facilitate the retardation of osteophytes in the treatment of osteoarthritis.

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Introduction

Articular cartilage is an avascular and aneural translucent tissue that functions by resisting compression, preventing adjacent bone contact, and maintaining a low-friction surface for joint articulation. Anatomically, normal articular cartilage is composed of four main zones: the superficial zone, the middle zone, the deep zone, and the zone with calcified cartilage; a tide-mark separates articular cartilage from subchondral bone. Articular cartilage is composed of chondrocytes and extracellular matrix (ECM). Cartilaginous ECM contains collagen type II (COLII), non-collagenous proteins, and proteoglycans, such as aggrecan (AGC), that have sulfated glycosaminoglycans (GAGs) that absorb water. Chondrocytes are responsible for the generation and maintenance of the ECM and are the sole, differentiated cellular resident of articular cartilage.

Articular cartilage has a relatively high incidence of damage and deterioration from common trauma such as sports injury and diseases such as osteoarthritis. Due to the avascular nature of articular cartilage, the progenitor cells in blood and bone marrow are inaccessible to the injured cartilage and the chondrocytes cannot move to the injured cartilage and generate ECM to repair the damaged cartilage; consequently, articular cartilage is weak in self-repairing and generally ends up with a fibrous repair tissue (fibrocartilage) which lacks the biomechanical characteristics necessary to withstand compressive stress during articulation. This fibrocartilage generally deteriorates over time, resulting in a return of the original symptoms and occasional progression to osteoarthritis.

Taking the knee joint as an example, current methods for treatment of articular cartilage lesions consist of (1) Palliative Strategies, such as physiotherapy, weight loss, and systemic pain relief medications; (2) Non-reparative, Non-restorative Strategies, such as debridement, chondral shaving, and knee joint lavage; (3) Reparative Strategies, such as arthroscopic abrasion arthroplasty, microfracture, and subchondral drilling; (4) Restorative Strategies, such as high tibial osteotomy, unicompartimental knee arthroplasty, and total knee arthroplasty; and (5) Transplantation Strategies, such as osteochondral transplantation (osteochondral grafting), mosaicplasty, and autologous chondrocyte transplantation (ACT). Although these methods can relieve the patient to a certain extent and improve knee joint function, the effect is controversial.

Of the above methods, ACT shows promise in clinical follow-up. This approach consists of harvesting chondrocytes from the non-weight bearing healthy area of articular cartilage followed by cell culturing for approximately 6 weeks and then transplantation of the cultured cells during open surgery. Despite the advantages of reducing the risk of immunological rejection and transmissible disease, ACT has many disadvantages including donor-site morbidity, the requirement for two surgeries, possible leakage of chondrocytes from the recipient site, uneven distribution of the cells in the defect, chondrocyte dedifferentiation in monolayer culture, long recovery time after operation, and finally, periosteal hypertrophy. The loss of chondrocyte phenotype prior to utilization in implantation is of grave concern for cartilage engineering and regeneration.

Unlike chondrocytes, mesenchymal stem cells (MSCs) are becoming a promising cell source for cartilage regeneration due to in vitro expansion without running the risk of losing their phenotype; however, MSCs tend to simultaneously acquire hypertrophic properties during chondrogenic induction, indicating the possibility of further differentiation toward endochondral bone formation. It is becoming crucial to systematically assess current strategies for minimizing hypertrophy of chondrogenically differentiated cells to provide a high-quality cartilage tissue for clinical defect repair. A previous review covered molecular and biophysical mechanisms regulating hypertrophic differentiation in chondrocytes and MSCs; this review will focus on strategies for preventing chondrogenic hypertrophy, including some new findings, such as the impact of different MSC sources and culture substrates. Potential mechanisms underlying the above strategies will also be delineated.

Definition and characterization of chondrogenic hypertrophy

Chondrogenic hypertrophy is marked by a more than 10-fold increase in cell volume and ECM structural remodeling. Cell volume expansion affects cell function. The explosive increase in the volume of hypertrophic chondrocytes involves changes in intracellular and extracellular osmolarity, ECM degradation around the cell, and an increase in the amount of organelles per cell. Osmotic swelling has been shown stereologically to be responsible for most of the cell volume increase. Swelling can be the result of either an increase in cytoplasmic concentration or a decrease in extracellular osmolarity followed by aquaporin-mediated movement of water to re-establish iso-osmotic conditions. Of all the ECM molecules, AGC is the prime contributor to the osmotic pressure generated in cartilage, both due to its abundance and its high negative fixed charge. It is not completely understood if expression of terminal markers results in increased cell volume or vice versa.

Chondrocyte hypertrophic differentiation is the gradual development process from chondrogenic differentiation to cartilage mineralization, which is characterized by a series of markers; each of these markers has its own function in the process of cartilage mineralization. For example, the transcription factors, runt-related transcription factor 2 (RUNX2) and myocyte enhancer factor-2C (MEF2C), drive the expression of terminal differentiation markers, including matrix metalloproteinase 13 (MMP13), collagen type X (COLX), Indian hedgehog (IHH), alkaline phosphatase (ALP), and vascular endothelial growth factor (VEGF), which all functionally contribute to endochondral ossification. Secreted MMP13 degrades COLII and AGC, key ECM components of functional cartilage; COLX serves as a framework for subsequent calcification through matrix vesicles (MV); ALP hydrolyses pyrophosphate (PPi) to inorganic phosphate (Pi) which, in the presence of calcium, forms hydroxyapatite; and IHH induces the proliferation of non-hypertrophic chondrocytes.

Calcification of cartilage ECM originates at MV. ECM mineralization to endochondral bone formation consists of
three steps (Fig. 1): (1) Hydroxyapatite crystals are formed inside the MV; (2) Hydroxyapatite crystals penetrate MV into the ECM; and (3) Endochondral ossification. The final stages of endochondral ossification, including degradation of the calcified matrix, VEGF-mediated vascular invasion of the calcified zone, and deposition of osteoid on the calcified trabeculae by osteoblasts, are all under the control of MMPs. MMP is indispensable for the development of MV and it can calcify the growth plate; finally, calcification is substituted by endochondral bone. MMP13 binding to the MV membrane and cooperating with MMP9 could promote the release of VEGF in apoptotic chondrocytes, further accelerating the formation of vascularity in the growth plate.24

Impact by cell sources chosen

Adult MSCs can differentiate into osteoblasts, adipocytes, muscle, and chondrocytes and are a promising cell source for tissue regeneration.25 Recent evidence indicates that great variability in differentiation capacity exists among tissue-specific stem cells,26 which might provide a theoretic foundation in regenerating a high-quality hyaline cartilage with minimum potential for hypertrophy.27

Articular chondrocytes

Articular chondrocytes are the earliest used cell sources for ACT.28 Due to limited availability, harvested chondrocytes need to be expanded in monolayer to obtain sufficient cells before implantation. However, monolayer expansion leads to a rapid chondrocyte dedifferentiation and causes loss of phenotype29 despite the limited restoration of chondrogenic properties of articular chondrocytes using growth factors and/or three dimensional (3D) culture.30–32 Dedifferentiated chondrocytes mostly become fibrous cartilage rather than hyaline cartilage33 with inferior biomechanical properties,34,35 thus limiting the application of articular chondrocytes in cartilage repair.

Articular cartilage progenitor cells

Articular cartilage progenitor cells (ACPCs) usually exist in one third of the superficial zone of articular cartilage.36–39 Recent findings show that ACPCs also exist in two thirds of the deep zone; the cell number in the deep zone is inferior to that in the superficial zone, but the chondrogenic and osteogenic differentiation capabilities are superior.40 McCarthy and colleagues found that both ACPCs and bone marrow stromal cells (BMSCs) could be induced for chondrogenic differentiation while COLX, RUNX2, and matrilin-1 existed only in the differentiated cells from BMSCs.41 ACPCs may therefore be considered superior to BMSCs in producing cartilage capable of functional repair despite a report showing ACPCs with inferior capability of producing cartilaginous matrix compared to articular chondrocytes.42

ACPCs isolated from the healthy area of articular cartilage could cause donor-site morbidity.43 When articular cartilage is injured severely, ACPCs begin to proliferate and migrate to the damaged site and act as alarmins promoting the development of inflammation44 while articular chondrocytes remain in a stationary state.45 The research on ACPCs is still in the early stage and needs further investigation to elucidate its role in cartilage regeneration.

Embryonic stem cells

Embryonic stem cells (ESCs) have the ability of infinite proliferation and self-renewal as well as differentiating into all kinds of human cells.46 The use of human ESCs to replace damaged cells and tissues is promising for cartilage tissue engineering in the future. However, in vitro chondrogenic
induction of mouse ESCs also shows a tendency to hypertrophically differentiate. Complex ethical and legal questions as a result of the research needed to develop these cell-replacement therapies restrict the use of ESCs in research and clinical settings.

Mesenchymal stem cells

Mesenchymal stem cells are promising candidate cells for cartilage tissue engineering. High expression of cartilage hypertrophic markers such as COLX, MMP13, and phosphorylated ALP by BMSCs undergoing chondrogenic induction were observed in in vitro pellet culture. Like BMSCs, chondrogenesis of adipose-derived stem cells (ADSCs) was also associated with hypertrophy according to premature COLX expression and up-regulation of ALP activity as well as in vivo calcification of spheroids after ectopic transplantation in SCID mice.

Recent evidence indicates that tissue-specific stem cells exhibit lineage-specific differentiation potential (Fig. 2). Compared to MSCs from adipose, bone marrow, and muscle, synovium-derived stem cells (SDSCs) display superior chondrogenic capacity with a limited potential toward hypertrophy. The lineage-specific differentiation ability of MSCs is also influenced by matrix microenvironment deposited by tissue-specific stem cells. For example, decellularized matrix deposited by SDSCs enhanced expanded SDSCs’ chondrogenic potential rather than osteogenic capacity, while decellularized matrix deposited by BMSCs promoted expanded BMSCs’ endochondral bone formation.

Besides various site-dependent MSCs, cell composition also plays a critical role in MSC-based cartilage engineering and regeneration. BMSCs are multi-lineage potential non-hematopoietic cells, accounting for only a small percentage of nucleated cells in bone marrow. Hematopoietic cells could yield short-term selected (STS) cells; passaging enriches more primitive, uniformly Sca-1 expressing, long-term selected (LTS) cells. In STS cells, chondrogenesis progressed rapidly to terminal differentiation while LTS cells differentiated at a slower rate with no hypertrophy. These data indicate the impact of stromal cell composition on the chondrogenic differentiation profile, which is an

Figure 2  Adult stem cells can be derived from various tissues in the body. These viable and undifferentiated stem cell populations can be expanded in vitro and induced to undergo lineage-specific differentiation for chondrogenesis (C), osteogenesis (O), myogenesis (M), or adipogenesis (A). Although the cells may appear similar in morphology upon harvest, they are anything but identical. From the data presented in the paper entitled “Impact of tissue-specific stem cells on lineage-specific differentiation: a focus on the musculoskeletal system”, the efficacy of adult stem cells in lineage-specific differentiation is greatly affected by the type of resident tissue from which they are harvested. In the heatmap, the differentiation capacity is visualized by color ranging from low differentiation (blue) to high differentiation (red).
Strategies for inhibition of chondrogenic hypertrophy

Specific intervention

Current evidence indicates that chondrogenic hypertrophy can be prevented with interventions (Table 1) at a protein level, such as parathyroid hormone-related peptide (PTHrP), transforming growth factor beta1 (TGF-β1), MMP13 inhibitor, and extracellular signal-regulated protein kinases 1 and 2 (Erk1/2) inhibitor, and at a gene level, such as NK3 homeobox 2 (Nkx3.2), histone deacetylase 4 (HDAC4), and Chondromodulin 1 (ChM1).

Protein-level intervention

PTHRP. During the development of cartilage, PTHR P, belonging to the family of parathyroid hormones (PTHs), maintained chondrocytes in a proliferative state and inhibited their terminal differentiation into hypertrophic chondrocytes. The addition of PTHR P in a dose-dependent fashion increased DNA and GAG contents in the pellets of both human BMSCs and ADSCs with downregulation of COL10A1 and RUNX2 and up-regulation of SRY (sex determining region Y)-box 9 (SOX9) and COL2A1, suggesting PTHR P could promote chondrogenesis and suppress hypertrophy during in vitro chondrogenesis. Mueller and coworkers found that PTHR P(1–40) treatment reduced ALP expression in human BMSC pellets cultured under standard chondrogenic conditions in a dose-dependent manner; however, when cultured under hypertrophy-enhancing conditions, PTHR P(1–40) could not diminish the induced enhancement of hypertrophy in the MSC pellets. Kafienah and colleagues also found that the inclusion of PTHR P at a dose of 1 μM or 10 μM in chondrogenic induction medium resulted in significant suppression of COL10A1 and ALP activity in cartilage constructs engineered from BMSCs of patients with osteoarthritis. Of four PTHR P isoforms (1–34, 1–86, 7–34, and 107–139), Lee and Im found that PTHR P(1–34) most significantly enhanced chondrogenesis and suppressed hypertrophy in human BMSCs, supporting its use for cartilage tissue engineering.

TGF-β. Shintani and coworkers reported that TGF-β1 enhanced the bone morphogenetic protein 2 (BMP-2)-induced chondrogenesis of bovine synovial explants, improved the hyaline-like properties of the neo-cartilage, and arrested the downstream differentiation of cells at an early stage of hypertrophy. Mello and Tuan found that TGF-β1 stimulated limb mesenchymal cell proliferation and suppressed hypertrophy from chick embryos, while the thyroid hormone triiodothyronine stimulated hypertrophy and apoptosis. Intriguingly, Cals and coworkers found that human BMSC pellets cultured with TGF-β1 had significantly less mineralization than pellets cultured with TGF-β3. Consistent with this finding, Pei and colleagues found that, despite a comparable chondrogenic capacity during in vitro induction, the supplementation of TGF-β3 up-regulated COL10A1 and ALP in porcine SDSC pellets compared to TGF-β1. However, Mueller and coworkers did not observe significant TGF-β subtype-dependent differences in suppressing hypertrophy in chondrogenic induction of human BMSCs. Interestingly, TGF-β-depletion during expansion improved the re-differentiation capacity of chondrocytes and inhibited hypertrophy while TGF-β1 administration during expansion of human articular chondrocytes in a serum-free medium redirected cell phenotype toward hypertrophy.

BMP-4/7/13 and Dorsumorphin. Belonging to the superfamily of TGF-β, BMP enhanced the TGF-β-induced chondrogenesis of MSCs. BMP-4 promoted MSC chondrogenic differentiation while suppressing hypertrophy. Caron and coworkers found that BMP-2 acted as a specific inducer of chondrocyte hypertrophy while BMP-7 appeared to increase or maintain chondrogenic potential and prevent chondrocyte hypertrophy; bagpipe homeobox homolog 1 (Bapx1)/Nkx3.2 was involved in the BMP-7 mediated suppression of chondrocyte hypertrophy in ATDC5 cells (clonal mouse embryonal carcinoma cells). BMP-13 inhibited osteogenic differentiation of human BMSCs in vitro. Those studies indicated that BMP-4/7/13 can

Protein-level intervention

| Hypertrophy inhibitors | Target molecules |
|------------------------|-----------------|
| **Protein level**       |                 |
| PTHrP                  | Nkx3.2/PKC/cAMP/CaMKII |
| TGF-β                  | Smad 2/3 or Smad1/5/8 |
| BMP-4/7/13             | Bapx1/Nkx3.2 |
| GG86/2                 | MMP13 |
| Dorsumorphin           | BMPIR |
| PD98059                | Erk1/2 |
| NC23766                | Rac1 |
| FK506                  | Calcinurin |
| Nkx3.2                 | RUNX2 |
| SOX9                   | β-catenin/PTHrP/RUNX2 |
| Smad6                  | Smad1/5/8 |
| HDAC4                  | Runx2/MEF2C |
| ChM1                   | p21 |
| sFlt-1                 | VEGF |
| C-1-1                  | RUNX2/COLX/ALP |

**Abbreviation:** ALP: alkaline phosphatase; Bapx1: bagpipe homeobox homolog 1; BMP: bone morphogenetic protein; BMPIR: bone morphogenetic protein receptor I; cAMP: cyclic adenosine monophosphate; CaMKII: Ca²⁺/calmodulin-dependent protein kinase II; ChM1: Chondromodulin 1; COLX: type X collagen; Erk1/2: extracellular regulated protein kinases 1/2; HDAC4: histone deacetylase-4; MEF2C: myocyte-specific enhancer factor 2C; MMP13: matrix metalloproteinase 13; Nkx3.2: NK3 homeobox 2; PKC: protein kinase C; PTHrP: parathyroid hormone-related peptide; Rac1: Ras-related C3 botulinum toxin substrate 1; RUNX2: runt-related transcription factor 2; sFlt-1: soluble Flt-1; Smad: mothers against decapentaplegic homolog (Drosophila); SOX9: sex determining region Y-type high mobility group box 9; TGF-β: transforming growth factor beta 1; VEGF: vascular endothelial growth factor.
increase or maintain chondrogenic potential and prevent chondrocyte hypertrophy. Terminal differentiation and mineralization of chondrocytes can be regulated by the Smad1/5/8 pathway.\textsuperscript{74-76} Yu and colleagues found that Dorsomorphin inhibited the BMP type I receptors activin receptor-like kinase 2 (ALK-2), ALK-3, and ALK-6 and thus blocked BMP-mediated Smad1/5/8 phosphorylation.\textsuperscript{77}

**MMP13 inhibitor.** As one of the hypertrophic markers, proteolysis involving MMP13 was required for chondrocyte differentiation that occurs as part of growth plate development and was associated with matrix mineralization.\textsuperscript{78} Bertram and coworkers found that broad spectrum pan-MMP inhibitors suppressed proteoglycan deposition, COLII and COLX staining, ALP activity, and reduced SOX9 and COL2A1 expression in a dose-dependent fashion; a selective MMP13 inhibitor GG86/2 allowed chondrogenesis and showed only weak effects on ALP activity, indicating that, in future therapeutic applications of diseased joints, the tested MMP13-specific inhibitor GG86/2 promises suppression of COLII degradation without imposing a risk of impairment of MSC-driven regeneration processes.\textsuperscript{81}

**Erk1/2 inhibitor.** Erk1/2 signaling, one of the mitogen-activated protein kinase (MAPK) pathways, increased chondrogenic hypertrophy.\textsuperscript{79} Kim and Im found that the addition of PD98059, an Erk1/2 inhibitor, in chondrogenic medium suppressed hypertrophy and promoted chondrogenesis of human MSCs in a pellet culture system.\textsuperscript{80} Furthermore, the in vitro culture results showed that the PD98059-impregnated poly(lactic-co-glycolic acid) (PLGA) scaffold is more effective in suppressing hypertrophy than the TGF-\textbeta-2-immobilized scaffold while both scaffolds enhance chondrogenesis from human BMSCs. After 10 weeks of in vivo implantation in rabbits, osteochondral defects were successfully repaired in both PD98059-impregnated and TGF-\textbeta-2-immobilized scaffolds seeded with rabbit BMSCs when evaluated grossly and microscopically. However, COLX was not observed from regenerated cartilage in PD98059-impregnated scaffold, whereas it was detected around chondrocytes in the TGF-\textbeta-2-impregnated scaffolds. These results suggested that the use of the PD98059-impregnated scaffold led to articular cartilage regeneration of better quality and prevented hypertrophy when implanted in osteochondral defects.\textsuperscript{85}

**Rac1 inhibitor.** Rac1 belongs to the Rho family of small GTPases and can promote chondrocyte hypertrophy within the growth plate.\textsuperscript{81} Rac1 inhibitor was also reported to decrease the expression of COL10A1 in human ADSCs.\textsuperscript{82} Activated Rac1 promoted expression of MMP13, a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5), and COLX in chondrocytes, and accelerated osteoarthritis progression while inhibition of Rac1 activity by NSC23766 delayed osteoarthritis development.\textsuperscript{83} Furthermore, TGF-\textbeta-3 promoted ADSC chondrogenic differentiation and NSC23766 prevented differentiated cells from hypertrophy in vitro. The combination of ADSCs, TGF-\textbeta-3, and NSC23766 promoted the quality of osteochondral defect repair in rats with much less chondrocyte hypertrophy and significantly higher International Cartilage Repair Society macroscopic and microscopic scores.\textsuperscript{84}

**FK506.** FK506 (Tacrolimus) exerts its immunosuppressive effect via a common mechanism, calcineurin inhibition, after binding to intracellular proteins FK506-binding protein (FKBP). FK506 was found to induce chondrogenic differentiation of ATDC5 cells in a concentration-dependent manner (0.1–1000 ng/ml).\textsuperscript{85} van der Windt and colleagues found that inhibition of calcineurin activity by FK506 increased the expression of chondrogenic markers via endogenous TGF-\textbeta-1 production in human articular chondrocytes.\textsuperscript{86} Furthermore, FK506 at physiologic tonicity (380 mOsm) exerted a superior effect compared to the physiologic toxicity or FK-506 alone, increasing anabolic markers while suppressing hypertrophic and catabolic markers.\textsuperscript{87}

**Gene-level intervention**

**Nkx3.2.** Nkx3.2 is a transcription factor inhibitor promoting chondrogenesis\textsuperscript{88} which acts as a negative regulator of chondrocyte maturation in vivo.\textsuperscript{89} Lengner and colleagues found that transfection of Nkx3.2 into pluripotent C3H10T1/2 cells showed dose-responsive repression of the RUNX2 promoter. Bypassing RUNX2 repression by adenoviral-mediated introduction of RUNX2 into C3H10T1/2 cells prevented the induction of chondrogenesis, but could not reverse the chondrogenic phenotype once it was initiated, as evidenced by SOX9 and COL2A1 expression and ECM deposition. These results suggest that RUNX2 is a direct transcriptional target of Nkx3.2 and that repression of RUNX2 at the onset of chondrogenesis is a prerequisite for the activation of a chondrocyte-specific program of gene expression.\textsuperscript{90} RUNX2 is a critical link in BMP-2-mediated initiation of mesenchymal chondrogenesis that results in activation of SOX9 at least in part through the Nkx3.2-dependent repression of RUNX2.\textsuperscript{91}

**SOX9 and/or SOX5/SOX6.** Sox proteins are necessary in the process of cartilage formation. SOX9 mutation can cause serious cartilage dysplasia, in terms of campomelic dysplasia.\textsuperscript{92} SOX5 and SOX6 knockout also can cause serious cartilage dysplasia, leading to death at birth in a mouse model.\textsuperscript{93} Overexpression of SOX9 promoted the ability for chondrogenesis in mouse BMSCs.\textsuperscript{92} The SOX5, SOX6, and SOX9 combination (the SOX trio) successfully induced chondrocyte differentiation of mouse ESCs.\textsuperscript{94} Moreover, Ikeda and coauthors found that, among various combinations examined, only the SOX trio successfully induced chondrocyte differentiation in all cell types tested, including nonchondrogenic types, and the induction occurred regardless of the culture system used (monolayer, spheroid, and 3D). Contrary to the conventional chondrogenic techniques, the SOX trio suppressed hypertrophic and osteogenic differentiation at the same time.\textsuperscript{95} Venkatesan and coworkers found that recombinant adeno-associated virus (rAAV)-FLAG-hSOX9 delivery in human BMSCs strongly enhanced chondrogenic differentiation and reduced the levels of markers of hypertrophy and terminal and osteogenic/adipogenic differentiation.
SMAD6. Smad6 and Smad ubiquitin regulatory factor 1 (Smurf1) blocked the signal transduction of the BMP-Smad1/5/8 pathway.96,97 Smad6 transgenic mice showed postnatal dwarfism with osteopenia and inhibition of Smad1/5/8 phosphorylation in chondrocytes.90 In cultured human articular chondrocytes, stimulation with interleukin 1β (IL-1β) showed up-regulation of SMAD7, whereas SMAD6, aggrecan (ACAN), and COL2A1 were down-regulated,98 suggesting a potentially important role of IL-1β signaling in chondrocytes via indirect influencing of the BMP/TGF-β signaling cascade. Histological analysis of appendicular bones revealed delayed onset of hypertrophic differentiation and mineralization at midgestation in SMAD6−/− mice.99 By late gestation, however, an expanded hypertrophic zone associated with an increased pool of proliferating cells undergoing hypertrophy was evident in SMAD6 mutant growth plates. Loss of SMAD6 in mice led to defects in both axial and appendicular skeletal development.99 Overall, these results show that Smad6 is required to limit BMP signaling during endochondral bone formation.

**HDAC4.** HDACs modulate cell growth and differentiation by governing chromatin structure and repressing the activity of specific transcription factors.100,101 HDAC4-null mice display premature ossification of developing bones due to ectopic and early onset chondrocyte hypertrophy, mimicking the phenotype that results from constitutive RUNX2 expression in chondrocytes. Conversely, overexpression of HDAC4 in proliferating chondrocytes in vivo inhibited chondrocyte hypertrophy and differentiation, mimicking a RUNX2 loss-of-function phenotype.102 These results establish HDAC4 as a central regulator of chondrocyte hypertrophy and skeletogenesis and suggest general roles for class II HDACs in the control of cellular hypertrophy. Similarly, Pei and coauthors found that, in the presence of TGF-β1, adenovirus-mediated HDAC4 transduction in porcine SDSCs sped up and maintained a high level of chondrogenesis while down-regulating COL10A1 expression.103 Shimizu and coworkers found that HDAC4 interacted with RUNX2, bound the MMP13 promoter, and suppressed MMP13 gene transcription in the rat osteoblastic cell line, UMR 106-01. PTH induces the rapid cyclic adenosine monophosphate (cAMP)-dependent protein kinase-dependent release of HDAC4 from the MMP13 promoter and subsequent transcription of MMP13. Knock-out of HDAC4 either by small interfering RNA (siRNA) in vitro or by gene deletion in vivo led to an increase in MMP13 expression; overexpression of HDAC4 decreased the PTH induction of MMP13, indicating that HDAC4 represses MMP13 gene transcription in bone.103

**Chondromodulin 1/soluble Flt-1.** ChM1 is significant in articular cartilage and could induce the chondrocyte phenotype and inhibit the invasion of vessel structures.104 Klinger and colleagues found that transplantation of porcine osteochondral progenitor cells infected with AAV-ChM1 to cartilage lesions in the knee joints of miniature pigs that were treated by the microfracture technique stabilized the chondrocyte phenotype by supporting chondrogenesis but inhibiting chondrocyte hypertrophy and endochondral ossification.105 The underlying mechanism is unclear; some studies indicated that ChM1 promotes expression of the cell cycle inhibitor p21WAF1/Cip1,106 preventing expression of ALP and COL10A1.58,59 VEGF increased the BMP-4-induced endochondral bone formation of muscle-derived stem cells (MDSCs) in vitro.107 The soluble Flt-1 (sFlt1) gene, a VEGF antagonist, delayed the development of mouse osteoarthritis.108 Kubo and coworkers found that sFlt-1 gene therapy improved BMP-4- and TGF-β3-induced in vitro chondrogenesis of MDSCs and promoted the persistence of articular cartilage repair by preventing vascularization and bone invasion into the repaired articular cartilage.109 Matsumoto and colleagues also reported that, after intraarticular injection of stem cells for cartilage repair in an immunodeficient rat osteoarthritis model, a combination of sFlt-1- and BMP-4-transduced MDSCs demonstrated better repair without osteophyte formation macroscopically and histologically when compared with a combination of VEGF- and BMP-4-transduced MDSCs or with BMP-4-transduced MDSCs alone.110 However, some reports also indicated that VEGF is a necessary survival factor for stem cells and chondrocytes in the process of cartilage development. In VEGF knockout mice, a lot of chondrocytes died in the epiphyseal region during the process of cartilage development.111,112

**ERG/C-1-1.** The ETS-related gene (ERG) belongs to the family of erythroblast transformation-specific (ETS) transcription factors.113 Iwamoto and coworkers found that, during limb development, C-1-1 and ch-ERG had diverse biological properties and distinct expression patterns. Virally driven expression of C-1-1 maintained chondrocytes in a stable and immature phenotype, blocked their maturation into hypertrophic cells, and prevented the replacement of cartilage with bone. In contrast, virally driven expression of ch-ERG significantly stimulated chondrocyte maturation in culture, as indicated by increases in ALP activity and deposition of a mineralized matrix; however, it had modest effects in vivo. Growth of articular chondrocytes in culture was accompanied by decreasing C-1-1 expression after several passages, while expression of hypertrophic markers increased. Expression of C-1-1 in cultured chondrocytes inhibited cell hypertrophy, ALP activity, and cartilage matrix mineralization. In contrast, over-expression of ch-ERG promoted chondrocyte maturation and mineralization.114 Those findings suggest that C-1-1 plays a crucial role in the development process of cartilage formation and is important for steering the cells toward alternative developmental paths in the epiphyseal region; it also makes chondrocytes in the epiphyseal region acquire a permanent articular chondrocyte phenotype.

**Co-culture**

The use of a co-culture system of articular chondrocytes and MSCs could enhance chondrogenesis and suppress
hypertrophy during chondrogenesis of MSCs. Aung and colleagues found that human BMSCs co-cultured with primary osteoarthritic chondrocytes underwent chondrogenic differentiation even in the absence of growth factors; however, the same effect could not be replicated using osteoarthritic chondrocyte-conditioned medium or expanded cells. Additionally, the co-culture environment down-regulated hypertrophic differentiation of human BMSCs. Bian and coworkers found that mixed cell populations (human BMSCs and human articular chondrocytes) encapsulated in hydrogels exhibited significantly higher Young’s moduli, dynamic moduli, GAG levels, and collagen content than did constructs seeded with only BMSCs or chondrocytes. In addition, the deposition of COLX was significantly lower in the co-culture constructs than in the constructs seeded with BMSCs alone.

Articular chondrocyte-derived soluble factors and direct co-culture are potent means of improving chondrogenesis and suppressing the hypertrophic development of MSCs. PTHrP secreted by chondrocytes is an important candidate soluble factor involved in this effect. Two distinct cell types (human BMSCs and rabbit articular chondrocytes) were encapsulated in alginate hydrogels singly or in various ratios and cultured under chondrogenic conditions; Mo and coworkers found that newly synthesized cartilaginous ECM and COL2A1 were up-regulated with greater human BMSC ratios and longer culture periods. However, a specific COL2A1 human gene probe was found only in single human BMSC groups and was absent in all co-culture groups, which indicated that the enhanced cartilaginous phenotype originated from the co-cultured rabbit chondrocytes. Bovine articular chondrocytes co-cultured with BMSCs also resulted in redifferentiation of passaged chondrocytes and the trophic effect of MSCs may significantly increase the chondrogenic potential of articular chondrocytes. Those studies indicated that both MSCs and articular chondrocytes stabilized the chondrocyte phenotype. MSCs drive chondrocytes to synthesize cartilaginous ECM; chondrocytes also inhibit or minimize the hypertrophy of chondrogenic MSCs.

Culture substrates

Culture substrates can be synthetic material, purified single matrix component, or comprehensive decellularized matrix from mature tissue or grown cells. Cell phenotype, adhesion, migration, proliferation, and differentiation can be affected by culture substrates, such as ECM components and structure.

Nitrogen-rich plasma polymer

One of the most important properties of MSCs is adherent growth. The capability for adhesion of stem cells can be enhanced by nitrogen (N)-rich plasma polymer layers (PPE:N). Petit and colleagues found that PPE:N suppressed COL10A1 in fetal bovine growth plate chondrocytes but had no significant effect on COL2A1, ACAN, MMP13, and cyclin B2 (CCNB2) levels, indicating that PPE:N is beneficial for MSC chondrogenesis. Similarly, Mwale and coworkers found that PPE:N almost completely suppressed the expression not only of COL10A1, but also of osteogenic marker genes such as ALP, bone sialoprotein (BSP), and osteocalcin (BGLAP) in human BMSCs. In contrast, neither ACAN nor COL1A2 expression was significantly affected. Rampersad and coworkers further confirmed the potential of two different types of PPE:N surfaces (low-pressure-PPE:N [L-PPE:N] and high-pressure-PPE:N [H-PPE:N]) in suppressing COL10A1 expression, more so on the latter. Interestingly, when human BMSCs were transferred to pellet cultures, the expression level of COL10A1 was further decreased by preincubation on H-PPE:N, suggesting that these kinds of coatings show promise for tissue engineering of cartilage and disc tissues.

Chondroitin sulfate

Chondroitin sulfate (CS), a chemical that is normally found in articular cartilage, has been widely used in cartilage tissue engineering. Varghese and colleagues found that the aggregation of goat BMSCs in poly(ethylene glycol) (PEG)/CS hydrogels resulted in an enhancement of chondrogenic genes and matrix production and a significant down-regulation of COLX expression compared to control PEG hydrogels containing no CS-mieties. Similarly, a 3D alginate micro bead platform was coated with cartilaginous ECM components hyaluronic acid, CS, and COLII to emulate an in vivo chondrogenic microenvironment for the differentiation of BMSCs; Wu and coworkers found that CS- and COLII-coated microbeads enhanced the chondrogenic differentiation of human BMSCs. In addition, COLII-coated microbeads resulted in hypertrophic maturation of the differentiated chondrocytes, similar to conventional pellet culture, while CS-coated microbeads were able to retain the pre-hypertrophy state of the differentiated cells. Those findings demonstrated that CS coatings are beneficial to induce MSCs to differentiate into chondrocytes and prevent hypertrophy.

Decellularized matrix

ECM, an indispensable niche for stem cells in vivo, not only provides support scaffold, but also participates in the regulation of self-renewal, proliferation, and differentiation of stem cells. Decellularization of natural ECM, eliminating the cells and antigen composition, can avoid disease transmission, reduce inflammation and immune response, and maintain the integrity of ECM. The advantages of using ECM in cartilage engineering and regeneration include not only the formation of functional specific tissue dependent on several conditions, such as cytokines inside ECM and unique surface anatomical features, but also the in vitro microenvironment for stem cell rejuvenation during in vitro expansion.

Cartilage matrix. A porous scaffold derived from adult porcine articular cartilage has the ability to induce chondrogenic differentiation of human ADSCs without exogenous growth factors, with significant synthesis and accumulation of ECM macromolecules, and with the development of mechanical properties approaching those of native cartilage. In a further study, acellular cartilage matrix (ACM) powders and human SDSCs were mixed into collagen gel for in vitro culture. The data showed that ACM powders had the potential of promoting COL2A1 expression in an environment with no growth factors; a synergistic effect between ACM powders and
chondrogenic growth factors was observed in the formation of engineered cartilage and reduction of hypertrophy in chondrogenesis of SDSCs.  

Stem cell matrix. He and coworkers found that decellularized matrix deposited by stem cells (DSCM) could provide a cell expansion system for the rejuvenation of porcine SDSCs, in terms of "tiny and spindle shaped typical stem cell morphology", "higher cell yields (about 44 times more than those expanded on plastic flasks for two continuing passages)", and "high-quality cells (in subsequent 14-day passages), and "high-quality cells (in subsequent 14-day passages)". 

From the same group, Choi and colleagues demonstrated that basic fibroblast growth factor (FGF2) and DSCM could promote porcine SDSCs' proliferation and chondrogenic potential; however, in the subsequent chondrogenic induction, DSCM expanded SDSCs yielded pellets with significantly lower levels of COL10A1, ALP, and MMP13 compared to FGF2 pretreated SDSCs.

Chondrocyte matrix. A cell-derived ECM scaffold was constructed using cultured porcine chondrocytes via a freeze-drying method; Jin and colleagues demonstrated its ability to promote cartilage formation both in vitro and in vivo. From the same laboratory, Choi and colleagues evaluated the chondrogenic capacity of rabbit BMSCs after growth on ECM scaffold deposited by porcine chondrocytes and found that the ECM scaffold evoked chondrogenic differentiation of BMSCs earlier and produced more cartilaginous tissues than the polyglycolic acid (PGA) scaffold. Next, BMSCs in each scaffold were preconditioned with chondrogenic media in vitro for 1 week and implanted in the backs of nude mice for 6 weeks. The initially formed cartilaginous tissues turned into bone matrix. This phenomenon progressed much more rapidly in the PGA group than in the ECM group. In the ECM group, the chondrogenic phenotypes of BMSCs were also maintained longer than in the PGA group. The loss of chondrogenic phenotypes was accompanied by the calcification of matrix and hypertrophic changes by immunohistochemistry for osteocalcin and COLI and COLX. Blood vessel invasion took place more deeply and intensively in the PGA group. These results suggested that the ECM scaffold not only strongly supports chondrogenic differentiation of rabbit MSCs, but also helps maintain its phenotype in vivo.

Low oxygen

Cartilage cells live in an environment heavily influenced by mechanical forces. Wong and coworkers found that cyclic tension (shear stress) could up-regulate the expression of RUNX2, MMP13, connective tissue growth factor (CTGF), COL10A1, and VEGF and down-regulate the expression of the tissue inhibitor of metalloproteinase-1 (TIMP1) bovine chondrocyte-seeded alginate constructs. Cyclic tension also up-regulated the expression of COL2A1, cartilage oligomeric matrix protein (COMP) and Proteoglycan 4 (PRG4), but did not change the expression of SOX9 and ACAN. Cyclic hydrostatic pressure down-regulated the expression of MMP13 and COL1A1 and up-regulated expression of TIMP1 compared to the unloaded controls. Hydrostatic pressure may slow chondrocyte differentiation and have a chondroprotective, anti-angiogenic influence on cartilage tissue. These findings suggested that cyclic tension activates the RUNX2/MMP13 pathway and increases the expression of terminal differentiation hypertrophic markers. 

Bian and colleagues found that dynamic compressive loading increased the mechanical properties, as well as the glycosaminoglycan (GAG) and collagen contents of human BMSC-seeded hyaluronic acid hydrogel constructs in a seeding density dependent manner and was also shown to significantly reduce the expression of hypertrophic markers and to suppress the degree of calcification in MSC-seeded hyaluronic acid hydrogels.

Low-intensity ultrasound (LUS) was shown to enhance TGF-β-mediated chondrogenic differentiation of human BMSCs in a pellet culture system. Similarly, Cui and coworkers found that LUS pretreatment could significantly increase chondrogenic genes while decreasing hypertrophic genes in rabbit BMSCs seeded on PGA scaffold; four weeks after subcutaneous transplantation into nude mice, the LUS and LUS/TGF groups were significantly better at forming hyaline cartilage-like tissue than the non-LUS groups. The development of osteogenic phenotypes shown by von Kossa staining was highly suppressed until four weeks in the LUS groups, along with compressive strength comparable to the positive control.
Potential mechanisms underlying chondrogenic hypertrophy

Many signaling pathways and biophysical factors have been involved in the process of chondrogenic hypertrophy, so understanding these pathways (Fig. 3) will benefit the control of hypertrophy in cartilage regeneration.

PTHrP/IHH signaling

As one of the key anti-hypertrophy factors, PTHrP functions by activating protein kinase A (PKA) and, to a lesser extent, protein kinase C (PKC). cAMP-dependent PKA phosphorylates SOX9 and activates protein phosphatase II (PP2A) leading to the dephosphorylation of HDAC4 and the inactivation of MEF2C. PKC inhibits the activity of p38 MAPK reducing MEF2C phosphorylation and, ultimately, hypertrophic gene expression. PTHrP also interferes with the calcium pathway by dephosphorylation of calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII) because endogenous CaMKII activity is up-regulated prior to hypertrophy; the loss of CaMKII function substantially blocks the transition from proliferation to hypertrophy.

In addition, PTHrP blocks hypertrophy by stimulating Nkx3.2 and preventing RUNX2 expression. Sonic Hedgehog (SHH) and BMP signals work in sequence to establish a positive regulatory loop between SOX9 and Nkx3.2. Interestingly, IHH and PTHrP signaling play crucial roles in regulating the onset of chondrocyte hypertrophy by forming a negative feedback loop, in which IHH signaling regulates chondrocyte hypertrophy by controlling PTHrP expression. IHH signaling is also found to promote chondrocyte hypertrophy of PTHrP independently.

Ion channels

Accumulation of cytosolic Ca²⁺ concentration is indispensable to proper chondrogenesis. This cytoplasmic Ca²⁺
accumulation occurs through at least two distinct pathways. Extracellular Ca\(^{2+}\) can directly pass through ion channels in the cell membrane or, alternatively, it can activate G-protein coupled receptors (GPCRs), such as the calcium-sensing receptor, CaR, which is expressed in elevated levels in hypertrophic chondrocytes\(^\text{166}\) that stimulate intracellular Ca\(^{2+}\) release from the endoplasmic reticulum (ER).\(^\text{167}\) Activation of CaR could increase the expression of COLX. The Ca\(^{2+}\) release from the ER is regulated by phospholipase C (PLC) activation, prompting hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3), which triggers the opening of ER ion-channels (IP3R).\(^\text{168}\) A series of changes of signaling pathways are triggered by cytoplasmic calcium via CaM, CaMKII, and calcineurin.\(^\text{169}\)

Phosphorylation of HDAC4 by CaMKII promotes nuclear export and prevents nuclear import of HDAC4 with consequent derepression of chondrocyte hypertrophy.\(^\text{170}\) PTHrP inhibited the activity of p38 MAPK, which led to an increase in Bcl-2 activity which was further shown to inhibit IP3R channel opening.\(^\text{171}\) Administration of PTHrP or the overexpression of its receptor abolished the effect of elevated calcium to increase terminal differentiation markers via dephosphorylation of CaMKII activity.\(^\text{172}\) A synergistic relationship exists between the stimulatory effect of active CaMKII and RUNX2 on IHH expression.\(^\text{173}\) Increasing Ca\(^{2+}\) concentration results in significant up-regulation of Erk1/2 phosphorylation promoting chondrocyte hypertrophy and mineralization.\(^\text{174}\)

**TGF-β/BMP signaling**

Hellingman and colleagues found that blocking Smad2/3 after the onset of chondrogenesis resulted in a halt in COLII production: blocking Smad1/5/8 resulted in decreased expression of MMP13, COLX, and ALP while allowing COLII production. Moreover, blocking Smad1/5/8 prevented mineralization. This finding indicated that, while Smad2/3 is important for continuation of COLII deposition, Smad1/5/8 phosphorylation is associated with terminal differentiation and mineralization.\(^\text{175}\) TGF-β-dependent Smad3 signaling pathways present a key role for the SOX9-dependent transcriptional activation in primary chondrogenesis\(^\text{176}\) and also leads to RUNX2 inhibition through dephosphorylation of HDAC4.\(^\text{177}\) The Smad2/3 and Smad1/5/8 pathways can be blocked by Smad7, while only the Smad1/5/8 pathway can be inhibited by Smad6. Therefore, hypertrophy can be suppressed by up-regulating the expression of Smad6.\(^\text{178}\) Smurf1 can suppress terminal differentiation via blocking the Smad1/5/8 pathway and Smurf2 can decrease chondrogenic differentiation by blocking the Smad2/3 pathway.\(^\text{179}\) Thus, endochondral ossification can be stimulated by overexpression of Smurf2.\(^\text{180}\)

**Wnt signaling**

The Wingless/int (Wnt) signaling, including canonical Wnt (cWnt) and non-canonical Wnt (nCwnt), is another pathway involved in regulation of chondrocyte hypertrophy. cWnt signaling controls the fate of β-catenin via the Frizzled receptor (FzR).\(^\text{181}\) In the absence of cWnt, β-catenin is bound by a degradation complex consisting of glycogen synthase kinase 3 (GSK3), adenomatous polyposis coli (APC), AXIN, and casein kinase 1α (CK1) which phosphorylate β-catenin initiating its ubiquitination and proteosomal degradation.\(^\text{182}\) cWnt activation of the FzR and coreceptor low density lipoprotein receptor-related protein 5 and 6 (LRP5/6) interferes with the degradation complex; β-catenin can translocate to the nucleus where it binds to the lymphoid enhancer factor (LEF) and T cell factor (TCF) proteins. The LEF/TCF/β-catenin complex promotes RUNX2-expression inducing hypertrophy.\(^\text{183}\) SOX9 inhibits this signaling through phosphorylation/degradation of β-catenin.\(^\text{184,185}\) The inhibition of cWnt leads to an increase in COL2A1 and ACAN expression but does not affect COL10A1 expression in MSC pellet culture.\(^\text{186}\) The nCwnts (such as Wnt5a) exhibit dual functions during chondrogenesis of MSCs. At early stages, Wnt5a induces chondrogenesis and hypertrophy through intracellular Ca\(^{2+}\) release via GPCR activation. Later, it acts as an inhibitor of hypertrophy by activating the phosphoinositide 3-kinase (PI3K)/protein kinase B (PIK or Akt)-dependent pathway which, in turn, activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), an inhibitor of RUNX2.

Gremlin 1 (GREM1), frizzled related protein (FRZB), and dickkopf 1 homolog (Xenopus laevis) (DKK1) are enriched in articular cartilage compared with other hyaline cartilage types and these BMP and cWnt antagonists are important regulators of articular cartilage homeostasis by preventing hypertrophic differentiation of chondrocytes.\(^\text{187}\) GREM1, FRZB, and DKK1 mRNA expression levels are down-regulated in osteoarthritis.\(^\text{188,189}\) FRZB and DKK1 are cWnt antagonists and GREM1 is a BMP antagonist. GREM1 is also able to inhibit Wnt signaling via unknown indirect mechanisms and BMP signaling is able to repress Wnt signaling.\(^\text{190}\) The crosstalk between BMP and cWnt signaling might act as a feedback loop that balances the activity of both pathways.\(^\text{191}\)

**MAPK signaling**

MAPKs play a key role in a variety of cellular responses including proliferation, differentiation, and apoptosis of cells.\(^\text{192}\) The Erk1/2 MAPK pathway is activated by growth factors and the JNK/p38 MAPK pathways are activated by cellular stress, cytokines, and hypoxia.\(^\text{193,194}\) Inhibition of p38 MAPK in hypertrophic chondrocytes by either PTH, SB203580, or both together leads to a decrease of COL10A1 mRNA and an increase of the expression of prehypertrophic marker cartilage matrix protein. Therefore, inhibition of p38 converts a hypertrophic cell phenotype to a pre-hypertrophic one, thereby preventing precocious chondrocyte hypertrophy.\(^\text{157,158}\)

Activation of the Erk1/2 pathway promoted chondrocyte hypertrophy.\(^\text{79,195}\) TGF-α, an activator of epidermal growth factor receptor (EGFR) signaling, was up-regulated in articular chondrocytes in experimentally induced and human osteoarthritis; inhibition of MAPK kinase (MEK)/Erk1/2 could prevent TGF-α-induced deterioration of COLII and AGC.\(^\text{196}\) Stimulation of EGFR signaling in articular chondrocytes by TGF-α resulted in the activation of RhoA/
Rho-associated protein kinase (ROCK), MEK/ERK, PI3K, and p38 MAPK pathways and cartilage degradation. Therefore, inhibition of p38 MAPK and Erk1/2 is an effective strategy to minimize hypertrophy in cartilage regeneration.

**HIF signaling**

Hypoxia-inducible factor 1 alpha (HIF-1α), an oxygen-sensitive transcription factor, was reported to promote chondrogenesis of MSCs in part by activating SOX9 via a HIF-1α-dependent mechanism. Strobel and coauthors demonstrated that the chondrogenic and anti-catabolic effect (MMP13) of hypoxia in pellet culture of human articular chondrocytes disappeared with the addition of a HIF-1α inhibitor. The exact role of HIF-2α in chondrogenesis is currently undetermined. Hypoxic conditions enhanced robust chondrogenesis with an up-regulation of HIF-2α but suppressed COL10A1 in BMSC pellet cultures regardless of the oxygen tension during BMSC isolation and propagation. Lafont and coworkers found that hypoxia promoted cartilage matrix synthesis specifically through HIF-2α-mediated SOX9 induction of key cartilage genes. Interestingly, HIF-2α was also reported to enhance promoter activities of COL10A1, MMP13, and VEGFA through specific binding to the respective hypoxia-responsive elements and was essential for endochondral ossification of cultured chondrocytes and embryonic skeletal growth in mice. In addition, HIF-2α caused cartilage destruction by regulating crucial catabolic genes, such as MMPs, ADAMTS4, nitric oxide synthase-2 (NOS2), and prostaglandin-endoperoxide synthase-2 (PTGS2).

**FGF signaling**

FGF receptor 3 (FGFR3) is a tyrosine kinase receptor expressed in proliferating chondrocytes and early hypertrophic chondrocytes in the growth plate; its deficiency in mice resulted in the expansion of both the proliferative zone (PZ) and hypertrophic zone (HZ), indicating FGFR3 suppresses both proliferation and differentiation. The major ligands of FGFR3 in the growth plate include FGF18 and FGF9. Mice lacking FGF18 display expanded PZ and HZ, similar to FGFR3-null mice. Recently, Shung and colleagues found that FGFR3 expression increased SOX9 and decreased β-catenin levels and transcriptional activity in cultured mesenchymal cells. Since both SOX9 overexpression and β-catenin deletion independently block hypertrophic differentiation of chondrocytes, activation of FGF signaling may inhibit the hypertrophic differentiation of chondrocytes.

**Integrin signaling**

The mutual recognition and adhesion between chondrocytes and ECM can translate extracellular stimulation into intracellular cascades via integrin activation. The Rho family of GTPases is a family of small signaling G proteins and a subfamily of the Ras superfamily. Three members of the Rho GTPase family, Cdc42, Rac1, and RhOa, are implicated in the formation of actin cytoskeletal organization in fibroblasts. Activating the Integrin-Rho signal pathway led to the formation of stress fibers and focal adhesions and regulated the signal transduction process between cell membrane receptor and cytoskeleton.

Xu and coworkers found that TGF-β treated SDFcs illustrated the activation of the RhoA/ROCK pathway and concomitantly induced cytoskeletal reorganization and gene expression of SOX9, COL2A1, and ACAN. RhoA overexpression in chondrogenic ATDC5 cells resulted in increased proliferation and a marked delay of hypertrophic differentiation. Overexpression of Rac1/Cdc42 in ATDC5 cells activated the p38 MAPK pathway, which plays a crucial role in hypertrophy, resulting in decreased proliferation and a marked acceleration of hypertrophic differentiation. Integrin α1β1 and integrin α5β1 were demonstrated to induce GTP-bound glutaminase 2 (TG2) mobilization to the cell surface, phosphorylation of p38 MAPK, and expression of MEF2C, indicating that blocking integrin β1, a COLII and COLX binding integrin, is a successful strategy to suppress COLX expression in chondrocytes.

**Epigenetic regulation**

Many differences in gene expression arise during development and are subsequently retained through mitosis. Stable alterations of this kind are said to be ‘epigenetic’ because they are heritable in the short term but do not involve mutations of the DNA itself. Histone (de)acetylation, DNA methylation, and microRNAs (miRs) are epigenetic events strongly involved in chondro-specific differentiation.

**HDAC4**

Up-regulation of HDAC4 increased chondrogenesis as well as suppressing hypertrophy by inhibiting RUNX2 and MEF2C. CaMKIV induced chondrocyte differentiation through regulation of HDAC4 subcellular relocation, from the nucleus to the cytoplasm, which resulted in increased activity of RUNX2 and the transition of chondrocytes from the proliferative to the prehypertrophic stage. Nuclear HDAC4 is not tethered in the nucleus, but instead shuttles between the nucleus and the cytoplasm. Phosphorylation-induced 14-3-3 binding biases the balance of nucleo-cytoplasmic shuttling toward the cytoplasm by inhibiting nuclear import. HDAC4 nuclear localization was enhanced by TGF-β and PTHrP, whereas increased calcium concentration led to its phosphorylation and nuclear export. In addition to HDAC4, HDAC5, and HDAC7 suppressed MEF2C and RUNX2, respectively.

**MicroRNA 140**

MicroRNAs are a class of noncoding regulatory small RNA about 22 nucleotides (~22 nt) in length that probably function as antisense regulators of other RNAs and profoundly affect cell proliferation, differentiation, apoptosis, and individual growth and development. Previous studies found reduced miR-140 expression in human osteoarthritic cartilage, which may contribute to the abnormal gene expression pattern characteristic of osteoarthritis. The expression of miR-140 shadows SOX9 expression, and the deletion of SOX9 diminishes miR-140 expression during embryogenesis, indicating that miR-140 is subject to SOX9 regulation in chondrocytes. Transfection
of human chondrocytes with double-stranded-miR-140 down-regulated IL-1β-induced ADAMTS5 expression. 226,228 Buechli and coworkers demonstrated that miR-140 was highly expressed in normal equine articular cartilage and chondrogenically induced equine cord blood-derived MSCs; miR-140 expression closely paralleled that of the cartilage-specific transcription factor SOX9 while up-regulation of miR-140 repressed chemokine (CXC motif) ligand 12 (CXCL12) and ADAMTS5. 229

DNA methylation
DNA methylation of mammals occurs in the cytosine of the CpG dinucleotide via a reaction catalyzed by proteins called DNA methyltransferases. 230 Ezura and coworkers found that the DNA methylation levels of CpG-rich promoters of genes related to chondrocyte phenotypes were largely kept low during chondrogenesis in human SDSCs. 231 The findings from Zimmermann and colleagues indicated that methylation-based COL10A1 gene silencing is established in cartilage tissue and human articular chondrocytes. Altered methylation levels at 2 CpG sites of COL10A1 in MSCs and their demethylation during chondrogenesis may facilitate induction of COL10A1 as observed during in vitro chondrogenesis of MSCs. 232 These studies show that chondrogenic differentiation can be regulated by the level of DNA methylation. 237 With osteoarthritis, the methylation of MMPs present in healthy cartilage was lost and the de-methylation led to the expression of MMP-3/9/13 and ADAMTS4. 223,224 The application of the nonspecific de-methylating agent 5-aza-deoxyctydine on chondrogenic differentiation yielded mixed results. 235 The role of gene silencing through DNA methylation during chondrogenesis and hypertrophy is still in its infancy and further study is needed.

Conclusion and future perspective

Seed cells, growth factors, and scaffolds are three main elements of tissue engineering; mechanical stimulation also has a great impact on differentiation, so strategies to minimize hypertrophy for cartilage regeneration could be explored from those aspects.

Great variability in the differential capacity certainly exists between tissue-specific stem cells, which may vary within the same cell type. 26,86 For researchers investigating stem cell-based tissue engineering, it is necessary to choose the most appropriate type of MSC naturally suited to the research goals and objectives. SDSCs have a great capacity for proliferation and chondrogenic differentiation, a small potential for hypertrophy, and are tissue specific for cartilage regeneration; to our knowledge, SDSCs are the most appropriate type of MSC naturally suited to the research to hypertrophy. Understanding a series of signaling pathways and biophysical factors which have been involved in the process of chondrocyte hypertrophy will be beneficial to controlling hypertrophy during cartilage regeneration.

Conflicts of interest

All authors have none to declare.

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