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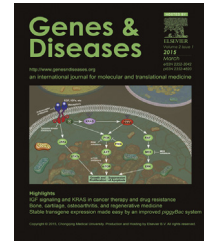


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REVIEW ARTICLE

# 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.<sup>1</sup> 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.<sup>2</sup> 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.<sup>3</sup>

Taking the knee joint as an example, current methods for treatment of articular cartilage lesions<sup>4</sup> 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, unicompartmental 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 pain to a certain extent and improve knee joint function, the effect is controversial.<sup>5</sup>

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.<sup>6</sup> 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.<sup>7,8</sup> 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<sup>9</sup>; 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.<sup>10</sup> Cell volume expansion affects cell function.<sup>11</sup> 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.<sup>12</sup> 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.<sup>13</sup> 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.<sup>14</sup> 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),<sup>9</sup> collagen type X (COLX),<sup>15</sup> Indian hedgehog (IHH),<sup>16</sup> alkaline phosphatase (ALP), and vascular endothelial growth factor (VEGF),<sup>8,17</sup> which all functionally contribute to endochondral ossification. Secreted MMP13 degrades COLII and AGC, key ECM components of functional cartilage<sup>18</sup>; COLX serves as a framework for subsequent calcification through matrix vesicles (MV)<sup>19</sup>; ALP hydrolyses pyrophosphate (PPi) to inorganic phosphate (Pi) which, in the presence of calcium, forms hydroxyapatite<sup>20</sup>; and IHH induces the proliferation of non-hypertrophic chondrocytes.<sup>21</sup>

Calcification of cartilage ECM originates at MV.<sup>22</sup> 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.<sup>23</sup> 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.<sup>24</sup>

## Impact by cell sources chosen

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

## Articular chondrocytes

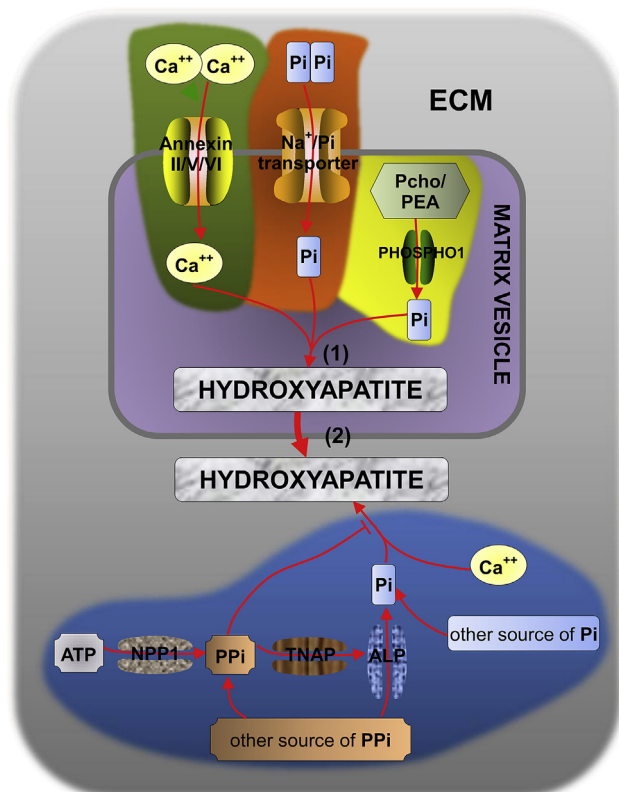
Articular chondrocytes are the earliest used cell sources for ACT.<sup>28</sup> Due to limited availability, harvested chondrocytes need to be expanded in monolayer to obtain sufficient cells before implantation. However, monolayer expansion leads to a rapid chondrocyte dedifferentiation and causes loss of phenotype<sup>29</sup> despite the limited restoration of chondrogenic properties of articular chondrocytes using growth factors and/or three dimensional (3D) culture.<sup>30–32</sup> Dedifferentiated chondrocytes mostly become fibrous cartilage rather than hyaline cartilage<sup>33</sup> with inferior biomechanical properties,<sup>34,35</sup> 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.<sup>36–39</sup> 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.<sup>40</sup> 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.<sup>41</sup> 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.<sup>42</sup> ACPCs isolated from the healthy area of articular cartilage could cause donor-site morbidity.<sup>43</sup> When articular cartilage is injured severely, ACPCs begin to proliferate and migrate to the damaged site and act as alarmins promoting the development of inflammation<sup>44</sup> while articular chondrocytes remain in a stationary state.<sup>45</sup> 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.<sup>46</sup> The use of human ESCs to replace damaged cells and tissues is promising for cartilage tissue engineering in the future. However, *in vitro* chondrogenic



**Figure 1** ECM mineralization process: (1) Hydroxyapatite crystals are formed inside the MV (gray shading) when the concentration of calcium ion (influx through annexinII/IV/VI calcium ion channels) and Pi [produced by the hydrolysis of Pcho and PEA via PHOSPHO1<sup>236,237</sup> and transferred into the MV by type-III Na<sup>+</sup>/Pi cotransporter<sup>238,239</sup> exceeds the solubility values.<sup>20,238</sup> (2) Hydroxyapatite crystals penetrate MV into the ECM (light grey shading). ATP, in the presence of nucleotide NPP1,<sup>240</sup> can generate PPI which would in return inhibit the formation of hydroxyapatite.<sup>241</sup> Pi could be produced through TNAP hydrolyzation of PPI<sup>238</sup> and ALP dephosphorylation of PPI, promoting the formation of hydroxyapatite. PPI and Pi have antagonistic effects on the mineralization process.<sup>20,242</sup> Abbreviation: ALP: alkaline phosphatase; ATP: adenosine triphosphate; ECM: extracellular matrix; MV: matrix vesicles; NPP1: nucleotide pyrophosphatase phosphodiesterase 1; Pcho: phosphatidyl choline; PEA: phosphatidylethanolamine; PHOSPHO1: phosphoethanolamine/phosphocholine phosphatase; Pi: phosphatidylinositol; PPI: extracellular inorganic pyrophosphate; TNAP: tissue non-specific alkaline phosphatase.

induction of mouse ESCs also shows a tendency to hypertrophically differentiate.<sup>47</sup> 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.

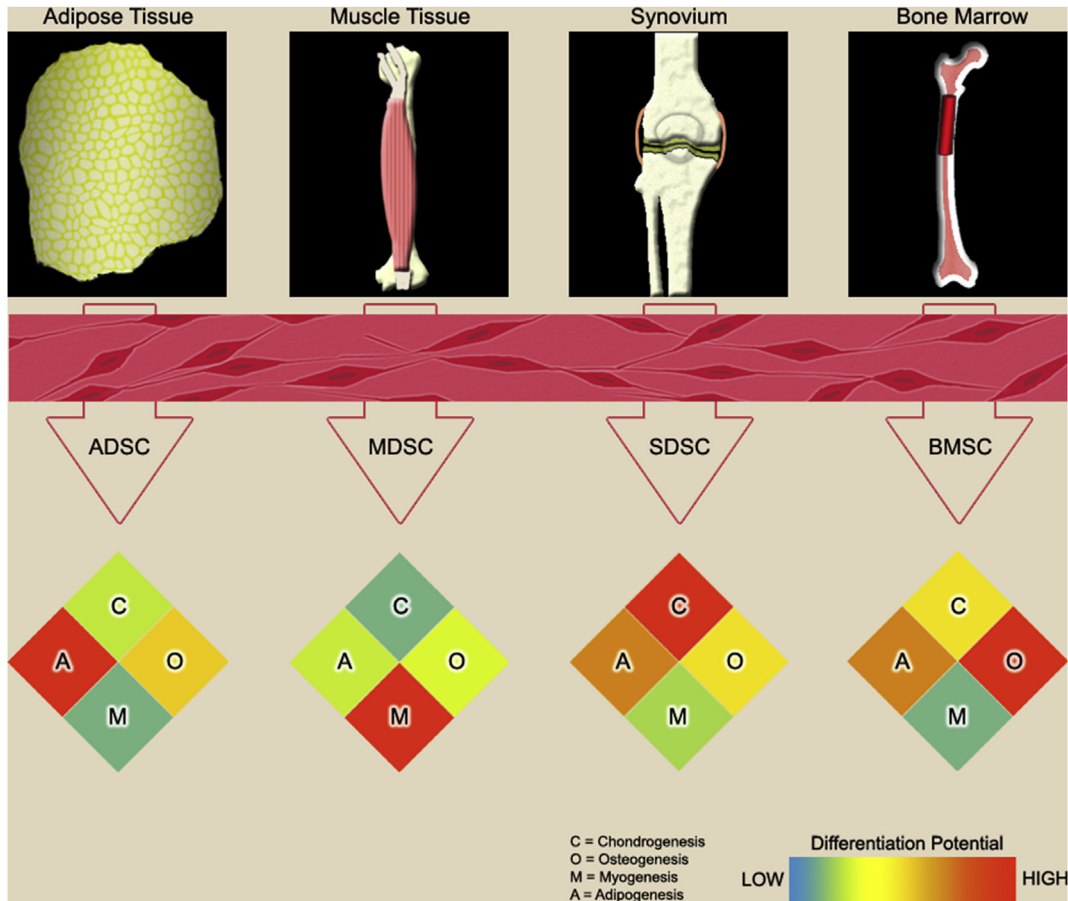
## Mesenchymal stem cells

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.<sup>8</sup> 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.<sup>7</sup>

Recent evidence indicates that tissue-specific stem cells exhibit lineage-specific differentiation potential (Fig. 2).<sup>26</sup> Compared to MSCs from adipose, bone marrow, and

muscle, synovium-derived stem cells (SDSCs) display superior chondrogenic capacity with a limited potential toward hypertrophy.<sup>27</sup> 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,<sup>48</sup> while decellularized matrix deposited by BMSCs promoted expanded BMSCs' endochondral bone formation.<sup>49</sup>

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.<sup>50</sup> 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",<sup>26</sup> 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).



important aspect to be considered when developing MSC-based cartilage repair technologies.<sup>51</sup>

## 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),<sup>52</sup> transforming growth factor beta1 (TGF- $\beta$ 1),<sup>53</sup> MMP13 inhibitor,<sup>54</sup> and extracellular signal-regulated protein kinases 1 and 2 (Erk1/2) inhibitor,<sup>55</sup> and at a gene level, such as NK3 homeobox 2 (*Nkx3.2*),<sup>56</sup> histone deacetylase 4 (*HDAC4*),<sup>57</sup> and Chondromodulin 1 (*ChM1*).<sup>58,59</sup>

### Protein-level intervention

**PTHrP.** During the development of cartilage, PTHrP, belonging to the family of parathyroid hormones (PTHs), maintained chondrocytes in a proliferative state and inhibited their terminal differentiation into hypertrophic chondrocytes.<sup>60</sup> The addition of PTHrP in a dose-dependent fashion increased DNA and GAG contents in the pellets of both human BMSCs and ADSCs with down-

regulation of *COL10A1* and *RUNX2* and up-regulation of SRY (sex determining region Y)-box 9 (*SOX9*) and *COL2A1*, suggesting PTHrP could promote chondrogenesis and suppress hypertrophy during *in vitro* chondrogenesis.<sup>61</sup> Mueller and coworkers found that PTHrP(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, PTHrP(1–40) could not diminish the induced enhancement of hypertrophy in the MSC pellets.<sup>52</sup> Kafienah and colleagues also found that the inclusion of PTHrP at a dose of 1  $\mu$ M or 10  $\mu$ M in chondrogenic induction medium resulted in significant suppression of *COL10A1* and ALP activity in cartilage constructs engineered from BMSCs of patients with osteoarthritis.<sup>62</sup> Of four PTHrP isoforms (1–34, 1–86, 7–34, and 107–139), Lee and Im found that PTHrP(1–34) most significantly enhanced chondrogenesis and suppressed hypertrophy in human BMSCs, supporting its use for cartilage tissue engineering.<sup>63</sup>

**TGF- $\beta$ .** Shintani and coworkers reported that TGF- $\beta$ 1 enhanced the bone morphogenetic protein 2 (BMP-2)-induced chondrogenesis of bovine synovial explants, improved the hyaline-like properties of the neocartilage, and arrested the downstream differentiation of cells at an early stage of hypertrophy.<sup>53</sup> Mello and Tuan found that TGF- $\beta$ 1 stimulated limb mesenchymal cell proliferation and suppressed hypertrophy from chick embryos, while the thyroid hormone triiodothyronine stimulated hypertrophy and apoptosis.<sup>64</sup> Intriguingly, Cals and coworkers found that human BMSC pellets cultured with TGF- $\beta$ 1 had significantly less mineralization than pellets cultured with TGF- $\beta$ 3.<sup>65</sup> Consistent with this finding, Pei and colleagues found that, despite a comparable chondrogenic capacity during *in vitro* induction, the supplementation of TGF- $\beta$ 3 up-regulated *COL10A1* and *ALP* in porcine SDSC pellets compared to TGF- $\beta$ 1.<sup>66</sup> However, Mueller and coworkers did not observe significant TGF- $\beta$  subtype-dependent differences in suppressing hypertrophy in chondrogenic induction of human BMSCs.<sup>67</sup> Interestingly, TGF- $\beta$ -depletion during expansion improved the re-differentiation capacity of chondrocytes and inhibited hypertrophy<sup>68</sup> while TGF- $\beta$ 1 administration during expansion of human articular chondrocytes in a serum-free medium redirected cell phenotype toward hypertrophy.<sup>69</sup>

**BMP-4/7/13 and Dorsomorphin.** Belonging to the superfamily of TGF- $\beta$ , BMP enhanced the TGF- $\beta$ -induced chondrogenesis of MSCs.<sup>70</sup> BMP-4 promoted MSC chondrogenic differentiation while suppressing hypertrophy.<sup>71</sup> 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).<sup>72</sup> BMP-13 inhibited osteogenic differentiation of human BMSCs *in vitro*.<sup>73</sup> Those studies indicated that BMP-4/7/13 can

**Table 1** Specific intervention to prevent hypertrophy during chondrogenic differentiation.

	Hypertrophy inhibitors	Target molecules	
Protein level	PTHrP	<i>Nkx3.2</i> /PKC/cAMP/CaMKII	
	TGF- $\beta$	Smad 2/3 or Smad1/5/8	
	BMP-4/7/13	<i>Bapx1</i> / <i>Nkx3.2</i>	
	GG86/2	MMP13	
	Dorsomorphin	BMPIR	
	PD98059	Erk1/2	
	NSC23766	Rac1	
	FK506	Calcineurin	
	Gene level	<i>Nkx3.2</i>	<i>RUNX2</i>
		<i>SOX9</i>	$\beta$ -catenin/PTHrP/ <i>RUNX2</i>
Smad6		Smad1/5/8	
<i>HDAC4</i>		<i>Runx2</i> /MEF2C	
<i>ChM1</i>		p21	
sFlt-1		VEGF	
C-1-1		<i>RUNX2</i> /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: Ca<sup>2+</sup>/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- $\beta$ 1: 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.<sup>74–76</sup> 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.<sup>77</sup>

**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.<sup>78</sup> 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.<sup>54</sup>

**Erk1/2 inhibitor.** Erk1/2 signaling, one of the mitogen-activated protein kinase (MAPK) pathways, increased chondrogenic hypertrophy.<sup>79</sup> 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.<sup>80</sup> 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- $\beta$ 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- $\beta$ 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- $\beta$ 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.<sup>55</sup>

**Rac1 inhibitor.** Rac1 belongs to the Rho family of small GTPases and can promote chondrocyte hypertrophy within the growth plate.<sup>81</sup> Rac1 inhibitor was also reported to decrease the expression of *COL10A1* in human ADSCs.<sup>82</sup> 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.<sup>83</sup> Furthermore, TGF- $\beta$ 3 promoted ADSC chondrogenic differentiation and NSC23766 prevented differentiated cells from hypertrophy *in vitro*. The combination of ADSCs, TGF- $\beta$ 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.<sup>84</sup>

**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).<sup>85</sup> van der Windt and colleagues found that inhibition of calcineurin activity by FK506 increased the expression of chondrogenic markers via endogenous TGF- $\beta$ 1 production in human articular chondrocytes.<sup>86</sup> Furthermore, FK506 at physiologic tonicity (380 mOsm) exerted a superior effect compared to the physiologic tonicity or FK-506 alone, increasing anabolic markers while suppressing hypertrophic and catabolic markers.<sup>87</sup>

### Gene-level intervention

**Nkx3.2.** Nkx3.2 is a transcription factor inhibitor promoting chondrogenesis<sup>88</sup> which acts as a negative regulator of chondrocyte maturation *in vivo*.<sup>89</sup> 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.<sup>56</sup> *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*.<sup>56</sup>

**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.<sup>90</sup> *SOX5* and *SOX6* knockout also can cause serious cartilage dysplasia, leading to death at birth in a mouse model.<sup>91</sup> Overexpression of *SOX9* promoted the ability for chondrogenesis in mouse BMSCs.<sup>92</sup> The *SOX5*, *SOX6*, and *SOX9* combination (the SOX trio) successfully induced chondrocyte differentiation of mouse ESCs.<sup>93</sup> 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.<sup>94</sup> 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. These effects were accompanied with decreased levels of  $\beta$ -catenin (an indicator of osteoblast lineage differentiation) and enhanced *PTHrP* expression via *SOX9* treatment.<sup>95</sup>

**SMAD6.** Smad6 and Smad ubiquitin regulatory factor 1 (Smurf1) blocked the signal transduction of the BMP-Smad1/5/8 pathway.<sup>96,97</sup> *SMAD6* transgenic mice showed postnatal dwarfism with osteopenia and inhibition of Smad1/5/8 phosphorylation in chondrocytes.<sup>96</sup> In cultured human articular chondrocytes, stimulation with interleukin 1 $\beta$  (IL-1 $\beta$ ) showed up-regulation of *SMAD7*, whereas *SMAD6*, aggrecan (*ACAN*), and *COL2A1* were down-regulated,<sup>98</sup> suggesting a potentially important role of IL-1 $\beta$  signaling in chondrocytes via indirect influencing of the BMP/TGF- $\beta$  signaling cascade. Histological analysis of appendicular bones revealed delayed onset of hypertrophic differentiation and mineralization at midgestation in *SMAD6*<sup>-/-</sup> mice.<sup>99</sup> 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.<sup>99</sup> Overall, those 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.<sup>100,101</sup> *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.<sup>102</sup> 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- $\beta$ 1, adenovirus-mediated *HDAC4* transduction in porcine SDSCs sped up and maintained a high level of chondrogenesis while down-regulating *COL10A1* expression.<sup>57</sup> 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.<sup>103</sup>

**Chondromodulin 1/soluble Flt-1.** ChM1 is significant in articular cartilage and could induce the chondrocyte phenotype and inhibit the invasion of vessel structures.<sup>104</sup> 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.<sup>105</sup> The underlying mechanism is unclear; some studies indicated that ChM1 promotes expression of the cell cycle inhibitor p21WAF1/Cip1,<sup>106</sup> preventing expression of *ALP* and *COL10A1*.<sup>58,59</sup>

VEGF increased the BMP-4-induced endochondral bone formation of muscle-derived stem cells (MDSCs) *in vitro*.<sup>107</sup> The soluble Flt-1 (sFlt1) gene, a VEGF antagonist, delayed the development of mouse osteoarthritis.<sup>108</sup> Kubo and coworkers found that sFlt-1 gene therapy improved BMP-4- and TGF- $\beta$ 3-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.<sup>109</sup> 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.<sup>110</sup> 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.<sup>111,112</sup>

**ERG/C-1-1.** The ETS-related gene (*ERG*) belongs to the family of erythroblast transformation-specific (ETS) transcription factors.<sup>113</sup> 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.<sup>114</sup> 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.<sup>115,116</sup> 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.<sup>117</sup> 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.<sup>118</sup>

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.<sup>119,120</sup> 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.<sup>121</sup> 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.<sup>122</sup> 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.<sup>123</sup>

### 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).<sup>124</sup> 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,<sup>125</sup> 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.<sup>126</sup> 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 great promise for tissue engineering of cartilage and disc tissues.<sup>127</sup>

### Chondroitin sulfate

Chondroitin sulfate (CS), a chemical that is normally found in articular cartilage, has been widely used in cartilage tissue engineering.<sup>128–130</sup> 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-moieties.<sup>131</sup> Similarly, a 3D alginate microbead 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.<sup>132</sup> 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.<sup>133</sup> 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.<sup>134</sup> 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,<sup>135,136</sup> but also the *in vitro* microenvironment for stem cell rejuvenation during *in vitro* expansion.<sup>48,49,137,138</sup>

**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.<sup>139</sup> 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.<sup>140</sup>

**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 chondrogenic induction, ECM expanded SDSCs exhibited a higher ratio of GAG to DNA than plastic expansion,  $39.37 \pm 3.05$  versus  $5.19 \pm 1.11$ )”, despite the pellets from the DSCM expanded SDSC group having half the level of *COL10A1*.<sup>48</sup> From the same laboratory, Li and Pei found that both 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.<sup>141</sup>

**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*.<sup>142,143</sup> From the same group, 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 *COL1* 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*.<sup>137</sup>

## Low oxygen

Cartilage is an avascular tissue and thus resides in a microenvironment with reduced oxygen tension, indicating that low oxygen might benefit *in vitro* chondrogenic differentiation of MSCs. Expansion under hypoxia was reported to enhance the preservation of “stemness” properties and inhibit osteogenic potential of marrow-isolated adult multilineage inducible (MIAMI) cells<sup>144</sup> as well as to increase chondrogenic differentiation of ADSCs while suppressing hypertrophy.<sup>145,146</sup> Similarly,

chondrogenic induction in a low oxygen environment would increase chondrogenic differentiation and suppress hypertrophy of MSCs cultured in both pellets and hydrogels used in tissue engineering strategies.<sup>147</sup> Hirao and coworkers found that hypoxia promoted BMP2-induced GAG production and suppressed ALP activity and mineralization of the pluripotent mesenchymal cell line C3H10T1/2. Thus, hypoxia promoted chondrocytic commitment rather than osteoblastic differentiation. In the mouse embryo forelimb organ culture, hypoxia increased cartilaginous matrix synthesis. These effects were primarily mediated by p38 MAPK activation, independent of SOX9. Hypoxia inhibited *COL10A1* expression via down-regulation of RUNX2 activity by Smad suppression and HDAC4 activation. These findings indicated that hypoxia promotes chondrocytic differentiation and cartilage matrix synthesis and suppresses terminal chondrocyte differentiation.<sup>148</sup> Furthermore, Kawato and colleagues demonstrated that Nkx3.2-dependent suppression of *RUNX2* was a crucial factor in hypoxia-dependent maintenance of chondrocyte identity.<sup>149</sup>

## Biomechanical stimulation

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*) in 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.<sup>150</sup> 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.<sup>151</sup>

Low-intensity ultrasound (LUS) was shown to enhance TGF- $\beta$ -mediated chondrogenic differentiation of human BMSCs in a pellet culture system.<sup>152</sup> 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.<sup>153</sup>

## 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

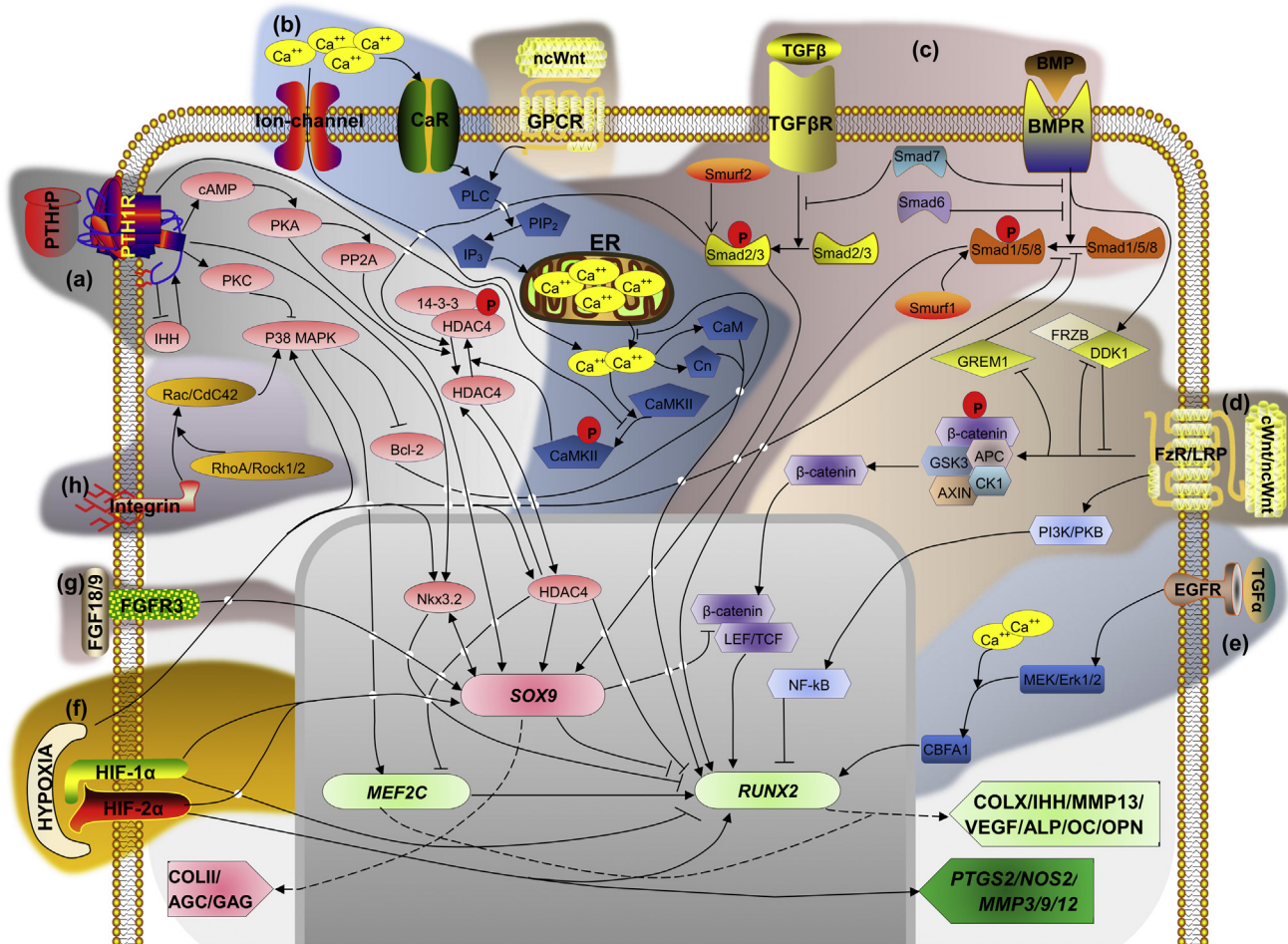
As one of the key anti-hypertrophy factors, PTHrP functions by activating protein kinase A (PKA) and, to a lesser extent, protein kinase C (PKC).<sup>154</sup> cAMP-dependent PKA phosphorylates SOX9 and activates protein phosphatase II (PP2A)<sup>155</sup> leading to the dephosphorylation of HDAC4 and the inactivation of MEF2C.<sup>156</sup> PKC inhibits the activity of p38 MAPK<sup>157</sup> reducing MEF2C phosphorylation<sup>158</sup> and, ultimately, hypertrophic gene expression.<sup>159</sup> 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.<sup>160</sup>

In addition, PTHrP blocks hypertrophy by stimulating Nkx3.2<sup>89</sup> and preventing RUNX2 expression.<sup>89,161</sup> Sonic Hedgehog (SHH) and BMP signals work in sequence to establish a positive regulatory loop between SOX9 and Nkx3.2.<sup>162</sup> 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.<sup>163,164</sup>

### Ion channels

Accumulation of cytosolic  $Ca^{2+}$  concentration is indispensable to proper chondrogenesis.<sup>165</sup> This cytoplasmic  $Ca^{2+}$



**Figure 3** Potential signaling pathways of chondrocyte hypertrophy, including but not limited to (a): PTHrP/IHH signaling, (b): calcium ion channel signaling, (c): TGF- $\beta$ /BMP signaling, (d): Wnt signaling, (e): MAPK (TGF- $\alpha$ ) signaling, (f): HIF signaling, (g) FGF signaling, and (h): integrin signaling. The main transcription factors regulating hypertrophy are SOX9, which is responsible for the expression of COLII and AGC, and RUNX2 which regulates transcription of COL10A1, IHH, MMP13, VEGF, ALP, OC, and OPN genes. SOX9 was shown to repress RUNX2<sup>162</sup> through Nkx3.2<sup>89</sup> and LEF/TCF/ $\beta$ -catenin complex.<sup>184,185</sup> Nkx3.2 and SOX9 mutually induce each other's expression.<sup>162</sup> Nkx3.2 is induced by PTHrP<sup>89</sup> and acts synergistically with SOX9<sup>243</sup> to inhibit RUNX2.<sup>56</sup> MEF2C is proposed to be the main regulator of RUNX2,<sup>15</sup> and drives the expression of the terminal differentiation markers.<sup>15</sup> 'P' depicts phosphorylation/dephosphorylation. ' $\rightarrow$ ' means 'increase', 'T' means 'inhibit'.

accumulation occurs through at least two distinct pathways. Extracellular  $\text{Ca}^{2+}$  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<sup>166</sup> that stimulate intracellular  $\text{Ca}^{2+}$  release from the endoplasmic reticulum (ER).<sup>167</sup> Activation of CaR could increase the expression of COLX. The  $\text{Ca}^{2+}$  release from the ER is regulated by phospholipase C (PLC) activation, prompting hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-triphosphate (IP3), which triggers the opening of ER ion-channels (IP3R).<sup>168</sup> A series of changes of signaling pathways are triggered by cytoplasmic calcium via CaM, CaMKII, and calcineurin.<sup>169</sup>

Phosphorylation of HDAC4 by CaMKII promotes nuclear export and prevents nuclear import of HDAC4 with consequent derepression of chondrocyte hypertrophy.<sup>170</sup> 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.<sup>171</sup> 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.<sup>160</sup> A synergistic relationship exists between the stimulatory effect of active CaMKII and RUNX2 on IHH expression.<sup>160</sup> Increasing  $\text{Ca}^{2+}$  concentration results in significant up-regulation of Erk1/2 phosphorylation promoting chondrocyte hypertrophy and mineralization.<sup>172</sup>

### TGF- $\beta$ /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.<sup>173</sup> TGF- $\beta$ -dependent Smad3 signaling pathways present a key role for the SOX9-dependent transcriptional activation in primary chondrogenesis<sup>174</sup> and also leads to RUNX2 inhibition through dephosphorylation of HDAC4.<sup>175</sup> 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.<sup>176,177</sup> Smurf1 can suppress terminal differentiation via blocking the Smad1/5/8 pathway<sup>178</sup> and Smurf2 can decrease chondrogenic differentiation by blocking the Smad2/3 pathway.<sup>179</sup> Thus, endochondral ossification can be stimulated by overexpression of Smurf2.<sup>180</sup>

### 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  $\beta$ -catenin via the Frizzled

receptor (FzR).<sup>181</sup> In the absence of cWnt,  $\beta$ -catenin is bound by a degradation complex consisting of glycogen synthase kinase 3 (GSK3), adenomatous polyposis coli (APC), AXIN, and casein kinase 1 $\alpha$  (CKI) which phosphorylate  $\beta$ -catenin initiating its ubiquitination and proteosomal degradation.<sup>182</sup> cWnt activation of the FzR and coreceptor low density lipoprotein receptor-related protein 5 and 6 (LRP5/6) interferes with the degradation complex;  $\beta$ -catenin can translocate to the nucleus where it binds to the lymphoid enhancer factor (LEF) and T cell factor (TCF) proteins. The LEF/TCF/ $\beta$ -catenin complex promotes RUNX2-expression inducing hypertrophy.<sup>183</sup> SOX9 inhibits this signaling through phosphorylation/degradation of  $\beta$ -catenin.<sup>184,185</sup> The inhibition of cWnt leads to an increase in *COL2A1* and *ACAN* expression but does not affect *COL10A1* expression in MSC pellet culture.<sup>186</sup> The ncWnts (such as Wnt5a) exhibit dual functions during chondrogenesis of MSCs. At early stages, Wnt5a induces chondrogenesis and hypertrophy through intracellular  $\text{Ca}^{2+}$  release via GPCR activation. Later, it acts as an inhibitor of hypertrophy by activating the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB or Akt)-dependent pathway which, in turn, activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B), an inhibitor of RUNX2.<sup>187</sup>

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.<sup>188</sup> GREM1, FRZB, and DKK1 mRNA expression levels are down-regulated in osteoarthritis.<sup>189,190</sup> 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.<sup>191</sup> The crosstalk between BMP and cWnt signaling might act as a feedback loop that balances the activity of both pathways.<sup>190</sup>

### MAPK signaling

MAPKs play a key role in a variety of cellular responses including proliferation, differentiation, and apoptosis of cells.<sup>192</sup> The Erk1/2 MAPK pathway is activated by growth factors and the JNK/p38 MAPK pathways are activated by cellular stress, cytokines, and hypoxia.<sup>193,194</sup> 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 prehypertrophic one, thereby preventing precocious chondrocyte hypertrophy.<sup>157,158</sup>

Activation of the Erk1/2 pathway promoted chondrocyte hypertrophy.<sup>79,195</sup> TGF- $\alpha$ , 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- $\alpha$ -induced deterioration of COLII and AGC.<sup>196</sup> Stimulation of EGFR signaling in articular chondrocytes by TGF- $\alpha$  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-1 $\alpha$ ), an oxygen-sensitive transcription factor, was reported to promote chondrogenesis of MSCs<sup>197</sup> in part by activating *SOX9* via a HIF-1 $\alpha$ -dependent mechanism.<sup>198</sup> 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-1 $\alpha$  inhibitor.<sup>199</sup> The exact role of HIF-2 $\alpha$  in chondrogenesis is currently undetermined. Hypoxic conditions enhanced robust chondrogenesis with an up-regulation of HIF-2 $\alpha$  but suppressed *COL10A1* in BMSC pellet cultures regardless of the oxygen tension during BMSC isolation and propagation.<sup>200</sup> Lafont and coworkers found that hypoxia promoted cartilage matrix synthesis specifically through HIF-2 $\alpha$ -mediated *SOX9* induction of key cartilage genes.<sup>201</sup> Interestingly, HIF-2 $\alpha$  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.<sup>202</sup> In addition, HIF-2 $\alpha$  caused cartilage destruction by regulating crucial catabolic genes, such as *MMPs*, *ADAMTS4*, nitric oxide synthase-2 (*NOS2*), and prostaglandin-endoperoxide synthase-2 (*PTGS2*).<sup>203</sup>

### 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.<sup>204,205</sup> The major ligands of FGFR3 in the growth plate include FGF18<sup>206,207</sup> and FGF9.<sup>208</sup> 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  $\beta$ -catenin levels and transcriptional activity in cultured mesenchymal cells. Since both *SOX9* overexpression and  $\beta$ -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.<sup>209</sup> 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.<sup>210</sup>

Xu and coworkers found that TGF- $\beta$  treated SDSCs illustrated the activation of the RhoA/ROCK pathway and concomitantly induced cytoskeletal reorganization and gene expression of *SOX9*, *COL2A1*, and *ACAN*.<sup>211</sup> RhoA overexpression in chondrogenic ATDC5 cells resulted in increased proliferation and a marked delay of hypertrophic differentiation.<sup>212</sup> Overexpression of Rac1/Cdc42 in ATDC5 cells activated the p38 MAPK pathway, which plays a crucial role in hypertrophy,<sup>158</sup> resulting in decreased proliferation and a marked acceleration of hypertrophic differentiation.<sup>81</sup> Integrin  $\alpha$ 1 $\beta$ <sup>213</sup> and integrin  $\alpha$ 5 $\beta$ 1<sup>214</sup> were demonstrated to induce GTP-bound transglutaminase 2 (TG2) mobilization to the cell surface, phosphorylation of p38 MAPK, and expression of MEF2C, indicating that blocking integrin  $\beta$ 1, 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'<sup>215</sup> 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.<sup>216,217</sup>

#### HDAC4

Up-regulation of HDAC4 increased chondrogenesis as well as suppressing hypertrophy by inhibiting RUNX2 and MEF2C.<sup>57,102,103,218</sup> 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.<sup>219</sup> 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.<sup>220</sup> HDAC4 nuclear localization was enhanced by TGF- $\beta$  and PTHrP,<sup>102,156,218</sup> whereas increased calcium concentration led to its phosphorylation and nuclear export.<sup>170</sup> In addition to HDAC4, HDAC5, and HDAC7 suppressed MEF2C and RUNX2, respectively.<sup>221,222</sup>

#### 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.<sup>223,224</sup> Previous studies found reduced miR-140 expression in human osteoarthritic cartilage,<sup>225,226</sup> which may contribute to the abnormal gene expression pattern characteristic of osteoarthritis. The expression of miR-140 shadows *SOX9* expression,<sup>227</sup> 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-1 $\beta$ -induced ADAMTS5 expression.<sup>226,228</sup> 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.<sup>229</sup>

### DNA methylation

DNA methylation of mammals occurs in the cytosine of the CpG dinucleotide via a reaction catalyzed by proteins called DNA methyltransferases.<sup>230</sup> 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.<sup>231</sup> 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.<sup>232</sup> These studies show that chondrogenic differentiation can be regulated by the level of DNA methylation.<sup>217</sup> 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.<sup>233,234</sup> The application of the nonspecific de-methylating agent 5-aza-deoxycytidine on chondrogenic differentiation yielded mixed results.<sup>235</sup> 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.<sup>26,88</sup> 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 rehabilitation; to our knowledge, SDSCs are the most appropriate stem cell source for cartilage engineering and regeneration.<sup>27</sup> Chondrogenic differentiation of MSCs is also influenced by specific intervention, such as growth factors and transduction signals, and the comprehensive environment, such as co-culture with articular chondrocytes, hypoxia, and biomechanical stimulation. Recently, culture substrates, such as decellularized ECM, have received intense attention in lowering hypertrophic potential during chondrogenic induction.

In summary, to get adequate, high quality chondrocytes *in vitro*, the first challenge that needs to be overcome is

hypertrophy of chondrogenic MSCs in cartilage tissue engineering. 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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### References

1. Johnstone B, Alini M, Cucchiari M, et al. Tissue engineering for articular cartilage repair—the state of the art. *Eur Cell Mater.* 2013;25:248–267.
2. Mahmoudifar N, Doran PM. Chondrogenesis and cartilage tissue engineering: the longer road to technology development. *Trends Biotechnol.* 2012;30:166–176.
3. Falah M, Nierenberg G, Soudry M, Hayden M, Volpin G. Treatment of articular cartilage lesions of the knee. *Int Orthop.* 2010;34:621–630.
4. Ahmed TA, Hincke MT. Strategies for articular cartilage lesion repair and functional restoration. *Tissue Eng Part B Rev.* 2010;16:305–329.
5. Karnes J, Zhang Y, Pei M. Cell therapy for the creation of cartilage and related clinical trials. In: Templeton NS, ed. *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies.* 4th ed. Taylor & Francis/CRC Press; 2015:1123–1135.
6. Hubka KM, Dahlin RL, Meretoja VV, Kasper FK, Mikos AG. Enhancing chondrogenic phenotype for cartilage tissue engineering: monoculture and coculture of articular chondrocytes and mesenchymal stem cells. *Tissue Eng Part B Rev.* 2014;20:641–654.
7. Hennig T, Lorenz H, Thiel A, et al. Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6. *J Cell Physiol.* 2007;211:682–691.
8. Mueller MB, Tuan RS. Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. *Arthritis Rheum.* 2008;58:1377–1388.
9. Studer D, Millan C, Ozturk E, Maniura-Weber K, Zenobi-Wong M. Molecular and biophysical mechanisms regulating hypertrophic differentiation in chondrocytes and mesenchymal stem cells. *Eur Cell Mater.* 2012;24:118–135. discussion 135.
10. Bush PG, Parisinos CA, Hall AC. The osmotic sensitivity of rat growth plate chondrocytes *in situ*; clarifying the mechanisms of hypertrophy. *J Cell Physiol.* 2008;214:621–629.
11. Chao PH, West AC, Hung CT. Chondrocyte intracellular calcium, cytoskeletal organization, and gene expression responses to dynamic osmotic loading. *Am J Physiol Cell Physiol.* 2006;291:C718–C725.
12. Mackie EJ, Tatarczuch L, Mirams M. The skeleton: a multi-functional complex organ: the growth plate chondrocyte

- and endochondral ossification. *J Endocrinol.* 2011;211:109–121.
13. Wang F, Zhu Y. Aquaporin-1: a potential membrane channel for facilitating the adaptability of rabbit nucleus pulposus cells to an extracellular matrix environment. *J Orthop Sci.* 2011;16:304–312.
  14. Hellingman CA, Koevoet W, van Osch GJ. Can one generate stable hyaline cartilage from adult mesenchymal stem cells? A developmental approach. *J Tissue Eng Reg Med.* 2012;6:e1–e11.
  15. Arnold MA, Kim Y, Czubyrt MP, et al. MEF2C transcription factor controls chondrocyte hypertrophy and bone development. *Dev Cell.* 2007;12:377–389.
  16. Yoshida CA, Yamamoto H, Fujita T, et al. Runx2 and Runx3 are essential for chondrocyte maturation, and Runx2 regulates limb growth through induction of Indian hedgehog. *Genes Dev.* 2004;18:952–963.
  17. Kwon TG, Zhao X, Yang Q, et al. Physical and functional interactions between Runx2 and HIF-1 $\alpha$  induce vascular endothelial growth factor gene expression. *J Cell Biochem.* 2011;112:3582–3593.
  18. Fosang AJ, Last K, Knauper V, Murphy G, Neame PJ. Degradation of cartilage aggrecan by collagenase-3 (MMP-13). *FEBS Lett.* 1996;380:17–20.
  19. Shen G. The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. *Orthod Craniofac Res.* 2005;8:11–17.
  20. Anderson HC. Matrix vesicles and calcification. *Curr Rheumatol Rep.* 2003;5:222–226.
  21. van Donkelaar CC, Huiskes R. The PTHrP-Ihh feedback loop in the embryonic growth plate allows PTHrP to control hypertrophy and Ihh to regulate proliferation. *Biomech Model Mechanobiol.* 2007;6:55–62.
  22. Iannotti JP, Naidu S, Noguchi Y, Hunt RM, Brighton CT. Growth plate matrix vesicle biogenesis. The role of intracellular calcium. *Clin Orthop Relat Res.* 1994;222–229.
  23. Ortega N, Behonick DJ, Werb Z. Matrix remodeling during endochondral ossification. *Trends Cell Biol.* 2004;14:86–93.
  24. Ortega N, Behonick D, Stickens D, Werb Z. How proteases regulate bone morphogenesis. *Ann N Y Acad Sci.* 2003;995:109–116.
  25. Caplan AL. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. *Tissue Eng.* 2005;11:1198–1211.
  26. Pizzute T, Lynch K, Pei M. Impact of tissue-specific stem cells on lineage-specific differentiation: a focus on the musculoskeletal system. *Stem Cell Rev.* 2014. <http://dx.doi.org/10.1007/s12015-014-9546-8>.
  27. Jones BA, Pei M. Synovium-derived stem cells: a tissue-specific stem cell for cartilage engineering and regeneration. *Tissue Eng Part B Rev.* 2012;18:301–311.
  28. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *New Engl J Med.* 1994;331:889–895.
  29. Cournil-Henrionnet C, Huselstein C, Wang Y, et al. Phenotypic analysis of cell surface markers and gene expression of human mesenchymal stem cells and chondrocytes during monolayer expansion. *Biorheology.* 2008;45:513–526.
  30. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell.* 1982;30:215–224.
  31. Jakob M, Demarteau O, Schafer D, et al. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes enhance chondrogenesis and cartilaginous tissue formation in vitro. *J Cell Biochem.* 2001;81:368–377.
  32. Wolf F, Candrian C, Wendt D, et al. Cartilage tissue engineering using pre-aggregated human articular chondrocytes. *Eur Cell Mater.* 2008;16:92–99.
  33. Tew SR, Clegg PD. Analysis of post transcriptional regulation of SOX9 mRNA during in vitro chondrogenesis. *Tissue Eng Part A.* 2011;17:1801–1807.
  34. Peterson L, Minas T, Brittberg M, Nilsson A, Sjogren-Jansson E, Lindahl A. Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. *Clin Orthop Relat Res.* 2000;374:212–234.
  35. Roberts S, McCall IW, Darby AJ, et al. Autologous chondrocyte implantation for cartilage repair: monitoring its success by magnetic resonance imaging and histology. *Arthritis Res Ther.* 2003;5:R60–R73.
  36. Douthwaite GP, Bishop JC, Redman SN, et al. The surface of articular cartilage contains a progenitor cell population. *J Cell Sci.* 2004;117:889–897.
  37. Hattori S, Oxford C, Reddi AH. Identification of superficial zone articular chondrocyte stem/progenitor cells. *Biochem Biophys Res Commun.* 2007;358:99–103.
  38. Khan IM, Bishop JC, Gilbert S, Archer CW. Clonal chondroprogenitors maintain telomerase activity and Sox9 expression during extended monolayer culture and retain chondrogenic potential. *Osteoarthr Cartil.* 2009;17:518–528.
  39. Williams R, Khan IM, Richardson K, et al. Identification and clonal characterisation of a progenitor cell sub-population in normal human articular cartilage. *PLoS One.* 2010;5:e13246.
  40. Yu Y, Zheng H, Buckwalter JA, Martin JA. Single cell sorting identifies progenitor cell population from full thickness bovine articular cartilage. *Osteoarthr Cartil.* 2014;22:1318–1326.
  41. McCarthy HE, Bara JJ, Brakspear K, Singhrao SK, Archer CW. The comparison of equine articular cartilage progenitor cells and bone marrow-derived stromal cells as potential cell sources for cartilage repair in the horse. *Vet J.* 2012;192:345–351.
  42. Zhou C, Zheng H, Seol D, Yu Y, Martin JA. Gene expression profiles reveal that chondrogenic progenitor cells and synovial cells are closely related. *J Orthop Res.* 2014;32:981–988.
  43. Mathur D, Pereira WC, Anand A. Emergence of chondrogenic progenitor stem cells in transplantation biology-prospects and drawbacks. *J Cell Biochem.* 2012;113:397–403.
  44. Sokolove J, Lepus CM. Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. *Ther Adv Musculoskelet Dis.* 2013;5:77–94.
  45. Garcia-Armandis I, Guillen MI, Gomar F, Pelletier JP, Martel-Pelletier J, Alcaraz MJ. High mobility group box 1 potentiates the pro-inflammatory effects of interleukin-1 $\beta$  in osteoarthritic synoviocytes. *Arthritis Res Ther.* 2010;12:R165.
  46. Karlsson C, Emanuelsson K, Wessberg F, et al. Human embryonic stem cell-derived mesenchymal progenitors—potential in regenerative medicine. *Stem Cell Res.* 2009;3:39–50.
  47. Jukes JM, Both SK, Leusink A, Sterk LM, van Blitterswijk CA, de Boer J. Endochondral bone tissue engineering using embryonic stem cells. *Proc Natl Acad Sci USA.* 2008;105:6840–6845.
  48. He F, Chen X, Pei M. Reconstruction of an in vitro tissue-specific microenvironment to rejuvenate synovium-derived stem cells for cartilage tissue engineering. *Tissue Eng Part A.* 2009;15:3809–3821.
  49. Pei M, He F, Kish VL. Expansion on extracellular matrix deposited by human bone marrow stromal cells facilitates stem cell proliferation and tissue-specific lineage potential. *Tissue Eng Part A.* 2011;17:3067–3076.
  50. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science.* 1999;284:143–147.
  51. Taipaleenmaki H, Suomi S, Hentunen T, Laitala-Leinonen T, Saamanen AM. Impact of stromal cell composition on BMP-induced chondrogenic differentiation of mouse bone marrow

- derived mesenchymal cells. *Exp Cell Res.* 2008;314:2400–2410.
52. Mueller MB, Fischer M, Zellner J, et al. Effect of parathyroid hormone-related protein in an in vitro hypertrophy model for mesenchymal stem cell chondrogenesis. *Int Orthop.* 2013;37:945–951.
  53. Shintani N, Siebenrock KA, Hunziker EB. TGF- $\beta$ 1 enhances the BMP-2-induced chondrogenesis of bovine synovial explants and arrests downstream differentiation at an early stage of hypertrophy. *PLoS One.* 2013;8:e53086.
  54. Bertram H, Boeuf S, Wachtors J, et al. Matrix metalloproteinase inhibitors suppress initiation and progression of chondrogenic differentiation of mesenchymal stromal cells in vitro. *Stem Cells Dev.* 2009;18:881–892.
  55. Lee JM, Kim JD, Oh EJ, Oh SH, Lee JH, Im GI. PD98059-impregnated functional PLGA scaffold for direct tissue engineering promotes chondrogenesis and prevents hypertrophy from mesenchymal stem cells. *Tissue Eng Part A.* 2014;20:982–991.
  56. Lengner CJ, Hassan MQ, Serra RW, et al. Nkx3.2-mediated repression of Runx2 promotes chondrogenic differentiation. *J Biol Chem.* 2005;280:15872–15879.
  57. Pei M, Chen D, Li J, Wei L. Histone deacetylase 4 promotes TGF- $\beta$ 1-induced synovium-derived stem cell chondrogenesis but inhibits chondrogenically differentiated stem cell hypertrophy. *Differentiation.* 2009;78:260–268.
  58. Ben-Eliezer M, Phillip M, Gat-Yablonski G. Leptin regulates chondrogenic differentiation in ATDC5 cell-line through JAK/STAT and MAPK pathways. *Endocrine.* 2007;32:235–244.
  59. Mikami Y, Asano M, Honda MJ, Takagi M. Bone morphogenetic protein 2 and dexamethasone synergistically increase alkaline phosphatase levels through JAK/STAT signaling in C3H10T1/2 cells. *J Cell Physiol.* 2010;223:123–133.
  60. Kronenberg HM. PTHrP and skeletal development. *Ann N Y Acad Sci.* 2006;1068:1–13.
  61. Kim YJ, Kim HJ, Im GI. PTHrP promotes chondrogenesis and suppresses hypertrophy from both bone marrow-derived and adipose tissue-derived MSCs. *Biochem Biophys Res Commun.* 2008;373:104–108.
  62. Kafienah W, Mistry S, Dickinson SC, Sims TJ, Learmonth I, Hollander AP. Three-dimensional cartilage tissue engineering using adult stem cells from osteoarthritis patients. *Arthritis Rheum.* 2007;56:177–187.
  63. Lee JM, Im GI. PTHrP isoforms have differing effect on chondrogenic differentiation and hypertrophy of mesenchymal stem cells. *Biochem Biophys Res Commun.* 2012;421:819–824.
  64. Mello MA, Tuan RS. Effects of TGF- $\beta$ 1 and triiodothyronine on cartilage maturation: in vitro analysis using long-term high-density micromass cultures of chick embryonic limb mesenchymal cells. *J Orthop Res.* 2006;24:2095–2105.
  65. Cals FL, Hellingman CA, Koevoet W, Baatenburg de Jong RJ, van Osch GJ. Effects of transforming growth factor- $\beta$  subtypes on in vitro cartilage production and mineralization of human bone marrow stromal-derived mesenchymal stem cells. *J Tissue Eng Reg Med.* 2012;6:68–76.
  66. Pei M, He F, Li J, Tidwell JE, Jones AC, McDonough EB. Repair of large animal partial-thickness cartilage defects through intraarticular injection of matrix-rejuvenated synovium-derived stem cells. *Tissue Eng Part A.* 2013;19:1144–1154.
  67. Mueller MB, Fischer M, Zellner J, et al. Hypertrophy in mesenchymal stem cell chondrogenesis: effect of TGF- $\beta$  isoforms and chondrogenic conditioning. *Cells Tissues Organs.* 2010;192:158–166.
  68. Narcisi R, Signorile L, Verhaar JA, Giannoni P, van Osch GJ. TGF $\beta$  inhibition during expansion phase increases the chondrogenic re-differentiation capacity of human articular chondrocytes. *Osteoarthr Cartil.* 2012;20:1152–1160.
  69. Narcisi R, Quarto R, Ulivi V, Muraglia A, Molfetta L, Giannoni P. TGF  $\beta$ -1 administration during ex vivo expansion of human articular chondrocytes in a serum-free medium redirects the cell phenotype toward hypertrophy. *J Cell Physiol.* 2012;227:3282–3290.
  70. Shen B, Wei A, Tao H, Diwan AD, Ma DD. BMP-2 enhances TGF- $\beta$ 3-mediated chondrogenic differentiation of human bone marrow multipotent mesenchymal stromal cells in alginate bead culture. *Tissue Eng Part A.* 2009;15:1311–1320.
  71. Miljkovic ND, Cooper GM, Marra KG. Chondrogenesis, bone morphogenetic protein-4 and mesenchymal stem cells. *Osteoarthr Cartil.* 2008;16:1121–1130.
  72. Caron MM, Emans PJ, Cremers A, et al. Hypertrophic differentiation during chondrogenic differentiation of progenitor cells is stimulated by BMP-2 but suppressed by BMP-7. *Osteoarthr Cartil.* 2013;21:604–613.
  73. Shen B, Bhargava D, Wei A, et al. BMP-13 emerges as a potential inhibitor of bone formation. *Int J Biol Sci.* 2009;5:192–200.
  74. Retting KN, Song B, Yoon BS, Lyons KM. BMP canonical Smad signaling through Smad1 and Smad5 is required for endochondral bone formation. *Development.* 2009;136:1093–1104.
  75. Shi Y, Massague J. Mechanisms of TGF- $\beta$  signaling from cell membrane to the nucleus. *Cell.* 2003;113:685–700.
  76. van der Kraan PM, Blaney Davidson EN, Blom A, van den Berg WB. TGF- $\beta$  signaling in chondrocyte terminal differentiation and osteoarthritis: modulation and integration of signaling pathways through receptor-Smads. *Osteoarthr Cartil.* 2009;17:1539–1545.
  77. Yu PB, Hong CC, Sachidanandan C, et al. Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. *Nat Chem Biol.* 2008;4:33–41.
  78. Wu CW, Tchetina EV, Mwale F, et al. Proteolysis involving matrix metalloproteinase 13 (collagenase-3) is required for chondrocyte differentiation that is associated with matrix mineralization. *J Bone Miner Res.* 2002;17:639–651.
  79. Bobick BE, Kulyk WM. Regulation of cartilage formation and maturation by mitogen-activated protein kinase signaling. *Birth Defects Res C Embryo Today.* 2008;84:131–154.
  80. Kim HJ, Im GI. The effects of ERK1/2 inhibitor on the chondrogenesis of bone marrow- and adipose tissue-derived multipotent mesenchymal stromal cells. *Tissue Eng Part A.* 2010;16:851–860.
  81. Wang G, Beier F. Rac1/Cdc42 and RhoA GTPases antagonistically regulate chondrocyte proliferation, hypertrophy, and apoptosis. *J Bone Miner Res.* 2005;20:1022–1031.
  82. Jungmann PM, Mehlhorn AT, Schmal H, Schillers H, Oberleithner H, Sudkamp NP. Nanomechanics of human adipose-derived stem cells: small GTPases impact chondrogenic differentiation. *Tissue Eng Part A.* 2012;18:1035–1044.
  83. Zhu S, Lu P, Liu H, et al. Inhibition of Rac1 activity by controlled release of NSC23766 from chitosan microspheres effectively ameliorates osteoarthritis development in vivo. *Ann Rheum Dis.* 2015;74:285–293.
  84. Zhu S, Chen P, Wu Y, et al. Programmed application of transforming growth factor  $\beta$ 3 and Rac1 inhibitor NSC23766 committed hyaline cartilage differentiation of adipose-derived stem cells for osteochondral defect repair. *Stem Cells Transl Med.* 2014;3:1242–1251.
  85. Nishigaki F, Sakuma S, Ogawa T, Miyata S, Ohkubo T, Goto T. FK506 induces chondrogenic differentiation of clonal mouse embryonic carcinoma cells, ATDC5. *Eur J Pharmacol.* 2002;437:123–128.
  86. van der Windt AE, Jahr H, Farrell E, Verhaar JA, Weinans H, van Osch GJ. Calcineurin inhibitors promote chondrogenic expression of dedifferentiated human adult chondrocytes via stimulation of endogenous TGF $\beta$ 1 production. *Tissue Eng Part A.* 2010;16:1–10.



87. van der Windt AE, Haak E, Kops N, Verhaar JA, Weinans H, Jahr H. Inhibiting calcineurin activity under physiologic tonicity elevates anabolic but suppresses catabolic chondrocyte markers. *Arthritis Rheum.* 2012;64:1929–1939.
88. Demoor M, Ollitrault D, Gomez-Leduc T, et al. Cartilage tissue engineering: molecular control of chondrocyte differentiation for proper cartilage matrix reconstruction. *Biochim Biophys Acta.* 2014;1840:2414–2440.
89. Provot S. Nkx3.2/Bapx1 acts as a negative regulator of chondrocyte maturation. *Development.* 2006;133:651–662.
90. Wagner T, Wirth J, Meyer J, et al. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell.* 1994;79:1111–1120.
91. Smits P, Li P, Mandel J, et al. The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. *Dev Cell.* 2001;1:277–290.
92. Tsuchiya H, Kitoh H, Sugiura F, Ishiguro N. Chondrogenesis enhanced by overexpression of sox9 gene in mouse bone marrow-derived mesenchymal stem cells. *Biochem Biophys Res Commun.* 2003;301:338–343.
93. Lefebvre V, Li P, de Crombrughe B. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. *EMBO J.* 1998;17:5718–5733.
94. Ikeda T, Kamekura S, Mabuchi A, et al. The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. *Arthritis Rheum.* 2004;50:3561–3573.
95. Venkatesan JK, Ekici M, Madry H, Schmitt G, Kohn D, Cucchiari M. SOX9 gene transfer via safe, stable, replication-defective recombinant adeno-associated virus vectors as a novel, powerful tool to enhance the chondrogenic potential of human mesenchymal stem cells. *Stem Cell Res Ther.* 2012;3:22.
96. Horiki M, Imamura T, Okamoto M, et al. Smad6/Smurf1 overexpression in cartilage delays chondrocyte hypertrophy and causes dwarfism with osteopenia. *J Cell Biol.* 2004;165:433–445.
97. Nishihara A, Fujii M, Sampath TK, Miyazono K, Reddi AH. Bone morphogenetic protein signaling in articular chondrocyte differentiation. *Biochem Biophys Res Commun.* 2003;301:617–622.
98. Kaiser M, Haag J, Soder S, Bau B, Aigner T. Bone morphogenetic protein and transforming growth factor beta inhibitory Smads 6 and 7 are expressed in human adult normal and osteoarthritic cartilage in vivo and are differentially regulated in vitro by interleukin-1beta. *Arthritis Rheum.* 2004;50:3535–3540.
99. Estrada KD, Retting KN, Chin AM, Lyons KM. Smad6 is essential to limit BMP signaling during cartilage development. *J Bone Miner Res.* 2011;26:2498–2510.
100. McKinsey TA, Zhang CL, Olson EN. Signaling chromatin to make muscle. *Curr Opin Cell Biol.* 2002;14:763–772.
101. Thiagalingam S, Cheng KH, Lee HJ, Mineva N, Thiagalingam A, Ponte JF. Histone deacetylases: unique players in shaping the epigenetic histone code. *Ann N Y Acad Sci.* 2003;983:84–100.
102. Vega RB, Matsuda K, Oh J, et al. Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell.* 2004;119:555–566.
103. Shimizu E, Selvamurugan N, Westendorf JJ, Olson EN, Partridge NC. HDAC4 represses matrix metalloproteinase-13 transcription in osteoblastic cells, and parathyroid hormone controls this repression. *J Biol Chem.* 2010;285:9616–9626.
104. Shukunami C, Iyama K, Inoue H, Hiraki Y. Spatiotemporal pattern of the mouse chondromodulin-I gene expression and its regulatory role in vascular invasion into cartilage during endochondral bone formation. *Int J Dev Biol.* 1999;43:39–49.
105. Klinger P, Surmann-Schmitt C, Brem M, et al. Chondromodulin 1 stabilizes the chondrocyte phenotype and inhibits endochondral ossification of porcine cartilage repair tissue. *Arthritis Rheum.* 2011;63:2721–2731.
106. Mera H, Kawashima H, Yoshizawa T, et al. Chondromodulin-1 directly suppresses growth of human cancer cells. *BMC Cancer.* 2009;9:166.
107. Peng H, Wright V, Usas A, et al. Synergistic enhancement of bone formation and healing by stem cell-expressed VEGF and bone morphogenetic protein-4. *J Clin Invest.* 2002;110:751–759.
108. Afuwape AO, Feldmann M, Paleolog EM. Adenoviral delivery of soluble VEGF receptor 1 (sFlt-1) abrogates disease activity in murine collagen-induced arthritis. *Gene Ther.* 2003;10:1950–1960.
109. Kubo S, Cooper GM, Matsumoto T, et al. Blocking vascular endothelial growth factor with soluble Flt-1 improves the chondrogenic potential of mouse skeletal muscle-derived stem cells. *Arthritis Rheum.* 2009;60:155–165.
110. Matsumoto T, Cooper GM, Gharraieb B, et al. Cartilage repair in a rat model of osteoarthritis through intraarticular transplantation of muscle-derived stem cells expressing bone morphogenetic protein 4 and soluble Flt-1. *Arthritis Rheum.* 2009;60:1390–1405.
111. Haigh JJ, Gerber HP, Ferrara N, Wagner EF. Conditional inactivation of VEGF-A in areas of collagen2a1 expression results in embryonic lethality in the heterozygous state. *Development.* 2000;127:1445–1453.
112. Zelzer E, Mamluk R, Ferrara N, Johnson RS, Schipani E, Olsen BR. VEGFA is necessary for chondrocyte survival during bone development. *Development.* 2004;131:2161–2171.
113. Wasylyk B, Hagman J, Gutierrez-Hartmann A. Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. *Trends Biochem Sci.* 1998;23:213–216.
114. Iwamoto M, Higuchi Y, Koyama E, et al. Transcription factor ERG variants and functional diversification of chondrocytes during limb long bone development. *J Cell Biol.* 2000;150:27–40.
115. Acharya C, Adesida A, Zajac P, et al. Enhanced chondrocyte proliferation and mesenchymal stromal cells chondrogenesis in coculture pellets mediate improved cartilage formation. *J Cell Physiol.* 2012;227:88–97.
116. Lettry V, Hosoya K, Takagi S, Okumura M. Coculture of equine mesenchymal stem cells and mature equine articular chondrocytes results in improved chondrogenic differentiation of the stem cells. *Jpn J Vet Res.* 2010;58:5–15.
117. Aung A, Gupta G, Majid G, Varghese S. Osteoarthritic chondrocyte-secreted morphogens induce chondrogenic differentiation of human mesenchymal stem cells. *Arthritis Rheum.* 2011;63:148–158.
118. Bian L, Zhai DY, Mauck RL, Burdick JA. Coculture of human mesenchymal stem cells and articular chondrocytes reduces hypertrophy and enhances functional properties of engineered cartilage. *Tissue Eng Part A.* 2011;17:1137–1145.
119. Fischer J, Dickhut A, Rickhut M, Richter W. Human articular chondrocytes secrete parathyroid hormone-related protein and inhibit hypertrophy of mesenchymal stem cells in coculture during chondrogenesis. *Arthritis Rheum.* 2010;62:2696–2706.
120. Weiss S, Hennig T, Bock R, Steck E, Richter W. Impact of growth factors and PTHrP on early and late chondrogenic differentiation of human mesenchymal stem cells. *J Cell Physiol.* 2010;223:84–93.
121. Mo XT, Guo SC, Xie HQ, et al. Variations in the ratios of cocultured mesenchymal stem cells and chondrocytes regulate the expression of cartilaginous and osseous phenotype in alginate constructs. *Bone.* 2009;45:42–51.



122. Meretoja VV, Dahlin RL, Kasper FK, Mikos AG. Enhanced chondrogenesis in co-cultures with articular chondrocytes and mesenchymal stem cells. *Biomaterials*. 2012;33:6362–6369.
123. Streuli C. Extracellular matrix remodelling and cellular differentiation. *Curr Opin Cell Biol*. 1999;11:634–640.
124. Girard-Lauriault P-L, Mwale F, Iordanova M, Demers C, Desjardins P, Wertheimer MR. Atmospheric pressure deposition of micropatterned nitrogen-rich plasma-polymer films for tissue engineering. *Plasma Process Polym*. 2005;2:263–270.
125. Petit A, Demers CN, Girard-Lauriault PL, et al. Effect of nitrogen-rich cell culture surfaces on type X collagen expression by bovine growth plate chondrocytes. *Biomed Eng Online*. 2011;10:4.
126. Mwale F, Girard-Lauriault PL, Wang HT, Lerouge S, Antoniou J, Wertheimer MR. Suppression of genes related to hypertrophy and osteogenesis in committed human mesenchymal stem cells cultured on novel nitrogen-rich plasma polymer coatings. *Tissue Eng*. 2006;12:2639–2647.
127. Rampersad S, Ruiz JC, Petit A, et al. Stem cells, nitrogen-rich plasma-polymerized culture surfaces, and type X collagen suppression. *Tissue Eng Part A*. 2011;17:2551–2560.
128. Li Q, Williams CG, Sun DD, Wang J, Leong K, Elisseeff JH. Photocrosslinkable polysaccharides based on chondroitin sulfate. *J Biomed Mater Res A*. 2004;68:28–33.
129. Muzzarelli RA, Greco F, Busilacchi A, Sollazzo V, Gigante A. Chitosan, hyaluronan and chondroitin sulfate in tissue engineering for cartilage regeneration: a review. *Carbohydr Polym*. 2012;89:723–739.
130. Roughley P, Martens D, Rantakokko J, Alini M, Mwale F, Antoniou J. The involvement of aggrecan polymorphism in degeneration of human intervertebral disc and articular cartilage. *Eur Cell Mater*. 2006;11:1–7. discussion 7.
131. Varghese S, Hwang NS, Canver AC, Theprungsirikul P, Lin DW, Elisseeff J. Chondroitin sulfate based niches for chondrogenic differentiation of mesenchymal stem cells. *Matrix Biol*. 2008;27:12–21.
132. Wu YN, Yang Z, Hui JH, Ouyang HW, Lee EH. Cartilaginous ECM component-modification of the micro-bead culture system for chondrogenic differentiation of mesenchymal stem cells. *Biomaterials*. 2007;28:4056–4067.
133. Toh WS, Foldager CB, Pei M, Hui JH. Advances in mesenchymal stem cell-based strategies for cartilage repair and regeneration. *Stem Cell Rev*. 2014;10:686–696.
134. Badylak SF, Taylor D, Uygun K. Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds. *Ann Rev Biomed Eng*. 2011;13:27–53.
135. Benders KE, van Weeren PR, Badylak SF, Saris DB, Dhert WJ, Malda J. Extracellular matrix scaffolds for cartilage and bone regeneration. *Trends Biotechnol*. 2013;31:169–176.
136. Tottey S, Johnson SA, Crapo PM, et al. The effect of source animal age upon extracellular matrix scaffold properties. *Biomaterials*. 2011;32:128–136.
137. Choi KH, Choi BH, Park SR, Kim BJ, Min BH. The chondrogenic differentiation of mesenchymal stem cells on an extracellular matrix scaffold derived from porcine chondrocytes. *Biomaterials*. 2010;31:5355–5365.
138. Pei M, Li JT, Shoukry M, Zhang Y. A review of decellularized stem cell matrix: a novel cell expansion system for cartilage tissue engineering. *Eur Cell Mater*. 2011;22:333–343. discussion 343.
139. Cheng NC, Estes BT, Awad HA, Guilak F. Chondrogenic differentiation of adipose-derived adult stem cells by a porous scaffold derived from native articular cartilage extracellular matrix. *Tissue Eng Part A*. 2009;15:231–241.
140. Chang CH, Chen CC, Liao CH, Lin FH, Hsu YM, Fang HW. Human acellular cartilage matrix powders as a biological scaffold for cartilage tissue engineering with synovium-derived mesenchymal stem cells. *J Biomed Mater Res A*. 2014;102:2248–2257.
141. Li J, Pei M. Optimization of an in vitro three-dimensional microenvironment to reprogram synovium-derived stem cells for cartilage tissue engineering. *Tissue Eng Part A*. 2011;17:703–712.
142. Jin CZ, Choi BH, Park SR, Min BH. Cartilage engineering using cell-derived extracellular matrix scaffold in vitro. *J Biomed Mater Res A*. 2010;92:1567–1577.
143. Jin CZ, Park SR, Choi BH, Park K, Min BH. In vivo cartilage tissue engineering using a cell-derived extracellular matrix scaffold. *Artif Organs*. 2007;31:183–192.
144. D'ippolito G, Diabira S, Howard GA, Roos BA, Schiller PC. Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells. *Bone*. 2006;39:513–522.
145. Ronziere MC, Perrier E, Mallein-Gerin F, Freyria AM. Chondrogenic potential of bone marrow- and adipose tissue-derived adult human mesenchymal stem cells. *Biomed Mater Eng*. 2010;20:145–158.
146. Xu Y, Malladi P, Chiou M, Bekerman E, Giaccia AJ, Longaker MT. In vitro expansion of adipose-derived adult stromal cells in hypoxia enhances early chondrogenesis. *Tissue Eng*. 2007;13:2981–2993.
147. Sheehy EJ, Buckley CT, Kelly DJ. Oxygen tension regulates the osteogenic, chondrogenic and endochondral phenotype of bone marrow derived mesenchymal stem cells. *Biochem Biophys Res Commun*. 2012;417:305–310.
148. Hirao M, Tamai N, Tsumaki N, Yoshikawa H, Myoui A. Oxygen tension regulates chondrocyte differentiation and function during endochondral ossification. *J Biol Chem*. 2006;281:31079–31092.
149. Kawato Y, Hirao M, Ebina K, et al. Nkx3.2-induced suppression of Runx2 is a crucial mediator of hypoxia-dependent maintenance of chondrocyte phenotypes. *Biochem Biophys Res Commun*. 2011;416:205–210.
150. Wong M, Siegrist M, Goodwin K. Cyclic tensile strain and cyclic hydrostatic pressure differentially regulate expression of hypertrophic markers in primary chondrocytes. *Bone*. 2003;33:685–693.
151. Bian L, Zhai DY, Zhang EC, Mauck RL, Burdick JA. Dynamic compressive loading enhances cartilage matrix synthesis and distribution and suppresses hypertrophy in hMSC-laden hyaluronic acid hydrogels. *Tissue Eng Part A*. 2012;18:715–724.
152. Ebisawa K, Hata K, Okada K, et al. Ultrasound enhances transforming growth factor beta-mediated chondrocyte differentiation of human mesenchymal stem cells. *Tissue Eng*. 2004;10:921–929.
153. Cui JH, Park SR, Park K, Choi BH, Min BH. Preconditioning of mesenchymal stem cells with low-intensity ultrasound for cartilage formation in vivo. *Tissue Eng*. 2007;13:351–360.
154. Li TF, Dong Y, Ionescu AM, et al. Parathyroid hormone-related peptide (PTHrP) inhibits Runx2 expression through the PKA signaling pathway. *Exp Cell Res*. 2004;299:128–136.
155. Huang W, Zhou X, Lefebvre V, de Crombrughe B. Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase A enhances SOX9's ability to transactivate a Col2a1 chondrocyte-specific enhancer. *Mol Cell Biol*. 2000;20:4149–4158.
156. Kozhemyakina E, Cohen T, Yao TP, Lassar AB. Parathyroid hormone-related peptide represses chondrocyte hypertrophy through a protein phosphatase 2A/histone deacetylase 4/MEF2 pathway. *Mol Cell Biol*. 2009;29:5751–5762.
157. Zhen X, Wei L, Wu Q, Zhang Y, Chen Q. Mitogen-activated protein kinase p38 mediates regulation of chondrocyte differentiation by parathyroid hormone. *J Biol Chem*. 2001;276:4879–4885.

158. Stanton LA, Sabari S, Sampaio AV, Underhill TM, Beier F. p38 MAP kinase signalling is required for hypertrophic chondrocyte differentiation. *Biochem J.* 2004;378:53–62.
159. Han YS, Bang OS, Jin EJ, Park JH, Sonn JK, Kang SS. High dose of glucose promotes chondrogenesis via PKC $\alpha$  and MAPK signaling pathways in chick mesenchymal cells. *Cell Tissue Res.* 2004;318:571–578.
160. Li Y, Ahrens MJ, Wu A, Liu J, Dudley AT. Calcium/calmodulin-dependent protein kinase II activity regulates the proliferative potential of growth plate chondrocytes. *Development.* 2011;138:359–370.
161. Zhang M, Xie R, Hou W, et al. PTHrP prevents chondrocyte premature hypertrophy by inducing cyclin-D1-dependent Runx2 and Runx3 phosphorylation, ubiquitylation and proteasomal degradation. *J Cell Sci.* 2009;122:1382–1389.
162. Zeng L, Kempf H, Murtaugh LC, Sato ME, Lassar AB. Shh establishes an Nkx3.2/Sox9 autoregulatory loop that is maintained by BMP signals to induce somitic chondrogenesis. *Genes Dev.* 2002;16:1990–2005.
163. Kobayashi T, Soegiarto DW, Yang Y, et al. Indian hedgehog stimulates periarticular chondrocyte differentiation to regulate growth plate length independently of PTHrP. *J Clin Invest.* 2005;115:1734–1742.
164. Mak KK, Kronenberg HM, Chuang PT, Mackem S, Yang Y. Indian hedgehog signals independently of PTHrP to promote chondrocyte hypertrophy. *Development.* 2008;135:1947–1956.
165. Matta C, Fodor J, Sziogyarto Z, et al. Cytosolic free Ca<sup>2+</sup> concentration exhibits a characteristic temporal pattern during in vitro cartilage differentiation: a possible regulatory role of calcineurin in Ca-signalling of chondrogenic cells. *Cell Calcium.* 2008;44:310–323.
166. Chang W, Tu C, Chen TH, et al. Expression and signal transduction of calcium-sensing receptors in cartilage and bone. *Endocrinology.* 1999;140:5883–5893.
167. Wu S, Palese T, Mishra OP, Delivoria-Papadopoulos M, De Luca F. Effects of Ca<sup>2+</sup> sensing receptor activation in the growth plate. *FASEB J.* 2004;18:143–145.
168. D'Andrea P, Calabrese A, Capozzi I, Grandolfo M, Tonon R, Vittur F. Intercellular Ca<sup>2+</sup> waves in mechanically stimulated articular chondrocytes. *Biorheology.* 2000;37:75–83.
169. Steward AJ, Kelly DJ, Wagner DR. The role of calcium signalling in the chondrogenic response of mesenchymal stem cells to hydrostatic pressure. *Eur Cell Mater.* 2014;28:358–371.
170. Backs J, Song K, Bezprozvannaya S, Chang S, Olson EN. CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. *J Clin Invest.* 2006;116:1853–1864.
171. Bassik MC, Scorrano L, Oakes SA, Pozzan T, Korsmeyer SJ. Phosphorylation of BCL-2 regulates ER Ca<sup>2+</sup> homeostasis and apoptosis. *EMBO J.* 2004;23:1207–1216.
172. Orfanidou T, Malizos KN, Varitimidis S, Tsezou A. 1,25-Dihydroxyvitamin D(3) and extracellular inorganic phosphate activate mitogen-activated protein kinase pathway through fibroblast growth factor 23 contributing to hypertrophy and mineralization in osteoarthritic chondrocytes. *Exp Biol Med.* 2012;237:241–253.
173. Hellingman CA, Davidson EN, Koevoet W, et al. Smad signaling determines chondrogenic differentiation of bone-marrow-derived mesenchymal stem cells: inhibition of Smad1/5/8P prevents terminal differentiation and calcification. *Tissue Eng Part A.* 2011;17:1157–1167.
174. Furumatsu T, Tsuda M, Taniguchi N, Tajima Y, Asahara H. Smad3 induces chondrogenesis through the activation of SOX9 via CREB-binding protein/p300 recruitment. *J Biol Chem.* 2005;280:8343–8350.
175. Kang JS, Alliston T, Delston R, Derynck R. Repression of Runx2 function by TGF- $\beta$  through recruitment of class II histone deacetylases by Smad3. *EMBO J.* 2005;24:2543–2555.
176. Konrad L, Scheiber JA, Bergmann M, Eickelberg O, Hofmann R. Identification of a new human Smad6 splice variant. *Andrologia.* 2008;40:358–363.
177. Scharstuhl A, Diepens R, Lensen J, et al. Adenoviral overexpression of Smad-7 and Smad-6 differentially regulates TGF- $\beta$ -mediated chondrocyte proliferation and proteoglycan synthesis. *Osteoarthr Cartil.* 2003;11:773–782.
178. Ying SX, Hussain ZJ, Zhang YE. Smurf1 facilitates myogenic differentiation and antagonizes the bone morphogenetic protein-2-induced osteoblast conversion by targeting Smad5 for degradation. *J Biol Chem.* 2003;278:39029–39036.
179. Lin X, Liang M, Feng XH. Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in transforming growth factor- $\beta$  signaling. *J Biol Chem.* 2000;275:36818–36822.
180. Wu Q, Chen D, Zuscik MJ, O'Keefe RJ, Rosier RN. Overexpression of Smurf2 stimulates endochondral ossification through upregulation of beta-catenin. *J Bone Miner Res.* 2008;23:552–563.
181. Angers S, Moon RT. Proximal events in Wnt signal transduction. *Nat Rev Mol Cell Biol.* 2009;10:468–477.
182. Ma B, Landman EB, Miclea RL, et al. WNT signaling and cartilage: of mice and men. *Calcif Tissue Int.* 2013;92:399–411.
183. Dong YF, Soung do Y, Schwarz EM, O'Keefe RJ, Drissi H. Wnt induction of chondrocyte hypertrophy through the Runx2 transcription factor. *J Cell Physiol.* 2006;208:77–86.
184. Topol L, Chen W, Song H, Day TF, Yang Y. Sox9 inhibits Wnt signaling by promoting beta-catenin phosphorylation in the nucleus. *J Biol Chem.* 2009;284:3323–3333.
185. Yano F, Kugimiya F, Ohba S, et al. The canonical Wnt signaling pathway promotes chondrocyte differentiation in a Sox9-dependent manner. *Biochem Biophys Res Commun.* 2005;333:1300–1308.
186. Im GI, Lee JM, Kim HJ. Wnt inhibitors enhance chondrogenesis of human mesenchymal stem cells in a long-term pellet culture. *Biotechnol Lett.* 2011;33:1061–1068.
187. Bradley EW, Drissi MH. WNT5A regulates chondrocyte differentiation through differential use of the CaN/NFAT and IKK/NF- $\kappa$ B pathways. *Mol Endocrinol.* 2010;24:1581–1593.
188. Leijten JC, Emons J, Sticht C, et al. Gremlin 1, frizzled-related protein, and Dkk-1 are key regulators of human articular cartilage homeostasis. *Arthritis Rheum.* 2012;64:3302–3312.
189. Gelse K, Ekici AB, Cipa F, et al. Molecular differentiation between osteophytic and articular cartilage—clues for a transient and permanent chondrocyte phenotype. *Osteoarthr Cartil.* 2012;20:162–171.
190. Leijten JC, Bos SD, Landman EB, et al. GREM1, FRZB and DKK1 mRNA levels correlate with osteoarthritis and are regulated by osteoarthritis-associated factors. *Arthritis Res Ther.* 2013;15:R126.
191. Gazzzerro E, Smerdel-Ramoya A, Zanotti S, et al. Conditional deletion of gremlin causes a transient increase in bone formation and bone mass. *J Biol Chem.* 2007;282:31549–31557.
192. Stanton LA, Underhill TM, Beier F. MAP kinases in chondrocyte differentiation. *Dev Biol.* 2003;263:165–175.
193. Pearson G, Robinson F, Beers Gibson T, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocr Rev.* 2001;22:153–183.
194. Xiao G, Gopalakrishnan R, Jiang D, Reith E, Benson MD, Franceschi RT. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. *J Bone Miner Res.* 2002;17:101–110.
195. Provot S, Nachtrab G, Paruch J, Chen AP, Silva A, Kronenberg HM. A-raf and B-raf are dispensable for normal

- endochondral bone development, and parathyroid hormone-related peptide suppresses extracellular signal-regulated kinase activation in hypertrophic chondrocytes. *Mol Cell Biol.* 2008;28:344–357.
196. Appleton CT, Usmani SE, Mort JS, Beier F. Rho/ROCK and MEK/ERK activation by transforming growth factor- $\alpha$  induces articular cartilage degradation. *Lab Invest.* 2010;90:20–30.
  197. Kanichai M, Ferguson D, Prendergast PJ, Campbell VA. Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1 $\alpha$ . *J Cell Physiol.* 2008;216:708–715.
  198. Robins JC, Akeno N, Mukherjee A, et al. Hypoxia induces chondrocyte-specific gene expression in mesenchymal cells in association with transcriptional activation of Sox9. *Bone.* 2005;37:313–322.
  199. Strobel S, Loparic M, Wendt D, et al. Anabolic and catabolic responses of human articular chondrocytes to varying oxygen percentages. *Arthritis Res Ther.* 2010;12:R34.
  200. Adesida AB, Mulet-Sierra A, Jomha NM. Hypoxia mediated isolation and expansion enhances the chondrogenic capacity of bone marrow mesenchymal stromal cells. *Stem Cell Res Ther.* 2012;3:9.
  201. Lafont JE, Talma S, Murphy CL. Hypoxia-inducible factor 2 $\alpha$  is essential for hypoxic induction of the human articular chondrocyte phenotype. *Arthritis Rheum.* 2007;56:3297–3306.
  202. Saito T, Fukai A, Mabuchi A, et al. Transcriptional regulation of endochondral ossification by HIF-2 $\alpha$  during skeletal growth and osteoarthritis development. *Nat Med.* 2010;16:678–686.
  203. Yang S, Kim J, Ryu JH, et al. Hypoxia-inducible factor-2 $\alpha$  is a catabolic regulator of osteoarthritic cartilage destruction. *Nat Med.* 2010;16:687–693.
  204. Colvin JS, Bohne BA, Harding GW, McEwen DG, Ornitz DM. Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat Genet.* 1996;12:390–397.
  205. Deng C, Wynshaw-Boris A, Zhou F, Kuo A, Leder P. Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell.* 1996;84:911–921.
  206. Liu Z, Xu J, Colvin JS, Ornitz DM. Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. *Genes Dev.* 2002;16:859–869.
  207. Ohbayashi N, Shibayama M, Kurotaki Y, et al. FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. *Genes Dev.* 2002;16:870–879.
  208. Hung IH, Yu K, Lavine KJ, Ornitz DM. FGF9 regulates early hypertrophic chondrocyte differentiation and skeletal vascularization in the developing stylopod. *Dev Biol.* 2007;307:300–313.
  209. Millward-Sadler SJ, Salter DM. Integrin-dependent signal cascades in chondrocyte mechanotransduction. *Ann Biomed Eng.* 2004;32:435–446.
  210. Amano M, Chihara K, Kimura K, et al. Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science.* 1997;275:1308–1311.
  211. Xu T, Wu M, Feng J, Lin X, Gu Z. RhoA/Rho kinase signaling regulates transforming growth factor- $\beta$ 1-induced chondrogenesis and actin organization of synovium-derived mesenchymal stem cells through interaction with the Smad pathway. *Int J Mol Med.* 2012;30:1119–1125.
  212. Wang G, Woods A, Sabari S, Pagnotta L, Stanton LA, Beier F. RhoA/ROCK signaling suppresses hypertrophic chondrocyte differentiation. *J Biol Chem.* 2004;279:13205–13214.
  213. Johnson KA, Rose DM, Terkeltaub RA. Factor XIIIa mobilizes transglutaminase 2 to induce chondrocyte hypertrophic differentiation. *J Cell Sci.* 2008;121:2256–2264.
  214. Tanaka K, Yokosaki Y, Higashikawa F, Saito Y, Eboshida A, Ochi M. The integrin  $\alpha$ 5 $\beta$ 1 regulates chondrocyte hypertrophic differentiation induced by GTP-bound transglutaminase 2. *Matrix Biol.* 2007;26:409–418.
  215. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet.* 2003;33(suppl):245–254.
  216. Barter MJ, Bui C, Young DA. Epigenetic mechanisms in cartilage and osteoarthritis: DNA methylation, histone modifications and microRNAs. *Osteoarthr Cartil.* 2012;20:339–349.
  217. Furumatsu T, Ozaki T. Epigenetic regulation in chondrogenesis. *Acta Med Okayama.* 2010;64:155–161.
  218. Wang AH, Bertos NR, Vezmar M, et al. HDAC4, a human histone deacetylase related to yeast HDA1, is a transcriptional corepressor. *Mol Cell Biol.* 1999;19:7816–7827.
  219. Guan Y, Chen Q, Yang X, et al. Subcellular relocation of histone deacetylase 4 regulates growth plate chondrocyte differentiation through Ca<sup>2+</sup>/calmodulin-dependent kinase IV. *Am J Physiol Cell Physiol.* 2012;303:C33–C40.
  220. Nishino TG, Miyazaki M, Hoshino H, Miwa Y, Horinouchi S, Yoshida M. 14-3-3 regulates the nuclear import of class IIa histone deacetylases. *Biochem Biophys Res Commun.* 2008;377:852–856.
  221. Backs J, Backs T, Bezprozvannaya S, McKinsey TA, Olson EN. Histone deacetylase 5 acquires calcium/calmodulin-dependent kinase II responsiveness by oligomerization with histone deacetylase 4. *Mol Cell Biol.* 2008;28:3437–3445.
  222. Jensen ED, Gopalakrishnan R, Westendorf JJ. Bone morphogenic protein 2 activates protein kinase D to regulate histone deacetylase 7 localization and repression of Runx2. *J Biol Chem.* 2009;284:2225–2234.
  223. Ambros V. microRNAs: tiny regulators with great potential. *Cell.* 2001;107:823–826.
  224. Stefani G, Slack FJ. Small non-coding RNAs in animal development. *Nat Rev Mol Cell Biol.* 2008;9:219–230.
  225. Iliopoulos D, Malizos KN, Oikonomou P, Tsezou A. Integrative microRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. *PLoS One.* 2008;3:e3740.
  226. Miyaki S, Nakasa T, Otsuki S, et al. MicroRNA-140 is expressed in differentiated human articular chondrocytes and modulates interleukin-1 responses. *Arthritis Rheum.* 2009;60:2723–2730.
  227. Nakamura Y, He X, Kato H, et al. Sox9 is upstream of microRNA-140 in cartilage. *Appl Biochem Biotechnol.* 2012;166:64–71.
  228. Miyaki S, Sato T, Inoue A, et al. MicroRNA-140 plays dual roles in both cartilage development and homeostasis. *Genes Dev.* 2010;24:1173–1185.
  229. Buechli ME, Lamarre J, Koch TG. MicroRNA-140 expression during chondrogenic differentiation of equine cord blood-derived mesenchymal stromal cells. *Stem Cells Dev.* 2013;22:1288–1296.
  230. Quina AS, Buschbeck M, Di Croce L. Chromatin structure and epigenetics. *Biochem Pharmacol.* 2006;72:1563–1569.
  231. Ezura Y, Sekiya I, Koga H, Muneta T, Noda M. Methylation status of CpG islands in the promoter regions of signature genes during chondrogenesis of human synovium-derived mesenchymal stem cells. *Arthritis Rheum.* 2009;60:1416–1426.
  232. Zimmermann P, Boeuf S, Dickhut A, Boehmer S, Olek S, Richter W. Correlation of COL10A1 induction during chondrogenesis of mesenchymal stem cells with demethylation of two CpG sites in the COL10A1 promoter. *Arthritis Rheum.* 2008;58:2743–2753.
  233. Cheung KS, Hashimoto K, Yamada N, Roach HI. Expression of ADAMTS-4 by chondrocytes in the surface zone of human osteoarthritic cartilage is regulated by epigenetic DNA demethylation. *Rheumatol Int.* 2009;29:525–534.

234. Roach HI, Yamada N, Cheung KS, et al. Association between the abnormal expression of matrix-degrading enzymes by human osteoarthritic chondrocytes and demethylation of specific CpG sites in the promoter regions. *Arthritis Rheum.* 2005;52:3110–3124.
235. El-Serafi AT, Oreffo RO, Roach HI. Epigenetic modifiers influence lineage commitment of human bone marrow stromal cells: differential effects of 5-aza-deoxycytidine and trichostatin A. *Differentiation.* 2011;81:35–41.
236. Houston B, Stewart AJ, Farquharson C. PHOSPHO1-A novel phosphatase specifically expressed at sites of mineralisation in bone and cartilage. *Bone.* 2004;34:629–637.
237. Roberts S, Narisawa S, Harmey D, Millan JL, Farquharson C. Functional involvement of PHOSPHO1 in matrix vesicle-mediated skeletal mineralization. *J Bone Miner Res.* 2007;22:617–627.
238. Balcerzak M, Hamade E, Zhang L, et al. The roles of annexins and alkaline phosphatase in mineralization process. *Acta Biochim Pol.* 2003;50:1019–1038.
239. Kirsch T, Harrison G, Golub EE, Nah HD. The roles of annexins and types II and X collagen in matrix vesicle-mediated mineralization of growth plate cartilage. *J Biol Chem.* 2000;275:35577–35583.
240. Terkeltaub RA. Inorganic pyrophosphate generation and disposition in pathophysiology. *Am J Physiol Cell Physiol.* 2001;281:C1–C11.
241. Addison WN, Azari F, Sorensen ES, Kaartinen MT, McKee MD. Pyrophosphate inhibits mineralization of osteoblast cultures by binding to mineral, up-regulating osteopontin, and inhibiting alkaline phosphatase activity. *J Biol Chem.* 2007;282:15872–15883.
242. Xiao Z, Blonder J, Zhou M, Veenstra TD. Proteomic analysis of extracellular matrix and vesicles. *J Proteomics.* 2009;72:34–45.
243. Yamashita S, Andoh M, Ueno-Kudoh H, Sato T, Miyaki S, Asahara H. Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes. *Exp Cell Res.* 2009;315:2231–2240.