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BIOMECHANICAL SIGNALS GUIDING STEM CELL CARTILAGE ENGINEERING: FROM MOLECULAR ADAPTION TO TISSUE FUNCTIONALITY

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Abstract

In vivo cartilage is in a state of constant mechanical stimulation. It is therefore reasonable to deduce that mechanical forces play an important role in cartilage formation. Mechanical forces, such as compression, tension, and shear force, have been widely applied for cartilage engineering; however, relatively few review papers have summarized the influence of biomechanical signals on stem cell-based neo-cartilage formation and cartilage engineering in both molecular adaption and tissue functionality. In this review, we will discuss recent progress related to the influences of substrate elasticity on stem cell chondrogenic differentiation and elucidate the potential underlying mechanisms. Aside from active sensing and responding to the extracellular environment, stem cells also could respond to various external mechanical forces, which also influence their chondrogenic capacity; this topic will be updated along with associated signaling pathways. We expect that these different regimens of biomechanical signals can be utilized to boost stem cell-based cartilage engineering and regeneration.

Keywords: Biomechanics, cartilage regeneration, extracellular matrix, stiffness, stem cell.

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Introduction

Damage to cartilage represents one of the biggest challenges in musculoskeletal therapeutics due to its limited aptitude for healing and regenerative capabilities (Karnes *et al*., 2014). Lack of treatment options to restore cartilage tissue function has prompted research on functional cartilage tissue substitutes in this rapidly emerging field (Athanasiou *et al*., 2015). Engineered tissue implants are attractive and offer a promising approach to cartilage restoration. Many tissue-engineering techniques have been attempted such as developing alternate cell sources, optimizing scaffolds and applying novel exogenous agents and mechanical stimulation regimens to produce cartilage constructs that could match native cartilage in biochemical and biomechanical properties (Tuan *et al*., 2013).

Although stem cell biologists have long appreciated the regulatory roles of soluble stem cell niche signals (*e.g*., growth factors and cytokines) in regulating stem cell fate, recent evidence demonstrated that biophysical information, such as mechanical cues that exist in the extracellular microenvironment, can also be sensed by cells. This information can be transduced into intracellular biochemical and functional responses, a process known as mechanotransduction (Chen, 2008; Geiger *et al*., 2009), which plays a critical role in determining stem cell fate. There are two different regimens of mechanotransduction: "inside-out" or "outside-in" sensing. For the active "inside-out" sensing, changes in extracellular matrix (ECM) stiffness and surface topography can be felt and responded to by cells through generating traction forces. For the passive "outside-in" sensing, the cell responds to a force imparted upon itself such as shear stress, compression, or pressure (Holle and Engler, 2011).

The report from Evans *et al*. (2009) suggests a fundamental role for mechanosensing in mammalian development and illustrates that the mechanical environment should be taken into consideration when engineering implantable scaffolds or when producing therapeutically relevant cell populations *in vitro*. Recently, there has been an increasing number of review papers regarding mechanical regulation of mesenchymal stem cell (MSC) chondrogenic differentiation *via* passive "outside-in" sensing (Huang *et al*., 2010; O'Conor *et al*., 2013) or from the cartilage development and maintenance standpoint (Responte *et al*., 2012; Rolfe *et al*., 2013). Biomechanics-driven approaches have also been summarized for their

Table 1. The influence of substrate stiffness on chondrogenic differentiation.

Substrate	Cell	Substrate stiffness	Chondrogenic effects	References
Hydrogel scaffold	Human BMSCs	PEG10 hydrogel (*E*, 4.231 kPa) *vs*. PEG-CMP hybrid hydrogel (*E*, 3.730 kPa)	More staining intensity of aggrecan and type II collagen as well as higher levels of *ACAN* and *COL2A1* when grown on softer hybrid hydrogel	Liu *et al*., 2010
	Human BMSCs	Type I collagen-coated PAM hydrogels with varied stiffnesses, "1 kPa" *vs*. "3 kPa" *vs*. "15 kPa"	Upregulation of *COL2A1* when expanded on soft hydrogels	Park *et al*., 2011
	Human BMSCs	PAM hydrogels with varied Young's modulus, "1.6 ± 0.3 kPa" *vs*. "40 ± 3.6 kPa"	Upregulation of *SOX9*, *ACAN*, and *COL2A1* when expanded on soft PAM hydrogel	Xue *et al*., 2013
	Mouse BMSCs	S-PAM hydrogels with varied elastic moduli, "1 kPa" *vs*. "15 kPa" *vs*. "150 kPa"	The highest mRNA levels of *SOX9*, *ACAN*, and *COL2A1* and the lowest mRNA level of *SCA1* when grown on the lowest stiffness hydrogels	Kwon and Yasuda, 2013
	Porcine chondrocytes	Type I collagen-coated PAM hydrogel with varied Young's moduli, "4 kPa" *vs*. "10 kPa" *vs*. "40 kPa" *vs*. "100 kPa"	The highest mRNA levels of *COL2A1* and *ACAN* and lowest levels of *COL1A1* when grown on the softest hydrogel	Schuh *et al*., 2010
	Porcine NP-SCs	Tetronic®1307 with two different shear storage moduli (G') of "1 kPa" *vs*. "2 kPa"	Promoted cell proliferation and increased expression of *SOX9* and *ACAN* genes and sulfated GAGs when grown on scaffolds with a low shear storage modulus	Navaro *et al*., 2015
	Primary murine chondrocytes and ATDC5 cells	PAM hydrogels with varied stiffnesses, "0.2 MPa" *vs*. "0.5 MPa" *vs*. "1.1 MPa"	The most proteoglycan deposition and mRNA levels of *SOX9*, *ACAN*, and *COL2A1* when expanded on 0.5 MPa PAM hydrogel	Allen *et al*.,2012
	Bovine articular chondrocytes	PEG hydrogels with different compressive moduli, "60 kPa" *vs*. "320 kPa" *vs*. "590 kPa"	The highest level of aggrecan and type II collagen when expanded on 60 kPa PEG hydrogel	Nicodemus *et al*., 2011
	Human ADSCs	Defined matrix hydrogels (CS/HA/HS) with varied stiffnesses, "3 kPa" *vs*. "30 kPa" *vs*. "90 kPa"	Increasing HA resulting in upregulation of *ACAN* and *COL2A1* in softer hydrogels, which was reversed in HA-containing hydrogels with higher stiffness	Wang *et al*., 2014
Porous/ fibrous scaffold	C3H10T1/2 murine embryonic MPCs	PCL fibers (7.1 MPa) *vs*. core-shell PES-PCL fibers (30.6 MPa)	Upregulation of chondrocytic *SOX9*, *ACAN*, and *COL2A1* genes and GAG production when grown on PCL fiber scaffold	Nam *et al*., 2011
	Wistar rat BMSCs	CG scaffolds with varied stiffnesses, "0.5 kPa" *vs*. "1 kPa" *vs*. "1.5 kPa"	Upregulation of *SOX9* genes when grown on soft scaffold	Murphy *et al*., 2012
	MEFs	RAD16-I with varied elastic moduli, low (~ 0.1 kPa) *vs*. high (~ 5 kPa)	A spontaneous process of cartilage-like formation when grown on scaffolds with a low elastic modulus	Fernández-Muiños *et al*., 2014
Acellular ECM scaffold	Human primary chondrocytes	DP treatment decreasing the linear modulus of pNSC (6.5 ± 2.3 MPa) to processed cartilage (1.92 ± 0.85 MPa)	Increased aggrecan and type II collagen at both mRNA and protein levels in pNSC constructs during chondrogenic induction	Schwarz *et al*., 2012
	Human ADSCs	Elastic modulus of PCL-CDM scaffolds approximately 1 % that of PCL scaffolds	Enhanced expression of sulfated GAG synthesis and *COL10A1* gene when grown on PCL-CDM compared to PCL	Garrigues *et al*., 2014
	Human SDSCs	FECM (58.26 ± 13.37 kPa) *vs*. AECM (64.82 ± 35.86 kPa) *vs*. Plastic flasks (infinite)	Expanded ASDSCs on FECM yielded 21-day pellets with the highest chondrogenic markers followed by AECM with plastic flasks being the least	Li *et al*., 2014
	Human SDSCs	dECM *vs*. Plastic flasks	Expanded ASDSCs on dECM yielded 35-day pellets with higher chondrogenic markers compared to plastic flasks	Zhang *et al*., 2015b

Abbreviations: ADSCs: adipose-derived stem cells; AECM: decellularized ECM deposited by adult SDSCs; ASDSCs: adult SDSCs; BMSCs: bone marrow stromal cells; CG: collagen-glycosaminoglycan; CDM: cartilage-derived matrix; CMP: collagen mimetic peptide; CS; chondroitin sulfate; dECM: decellularized ECM; DP: decellularization and sterilization process; ECM: extracellular matrix; FECM: decellularized ECM deposited by fetal SDSCs; HS: heparin sulfate; HA: hyaluronic acid; MPCs: mesenchymal progenitor cells; MEFs: mouse embryonic fibroblasts; NP-SCs: nucleus pulposus-derived stem cells; pNSC: porcine nasal septal cartilage; PAM: polyacrylamide; PES: poly(ether sulfone); PEG: poly(ethylene glycol); PCL: poly(ε-caprolactone); RAD16-I: PuraMatrix, self-assembling peptide nanofiber scaffolds; S-PAM: sulfonate-coated PAM; SCA1: stem cell antigen-1; SDSCs: synovium-derived stem cells; Tetronic®1307: synthetic tetrafunctional ethylene oxide/propylene oxide block copolymer

potential influence on MSC differentiation (Steward and Kelly, 2014); however, relatively few review papers have summarized the influence of biomechanical signals on stem cell-based neo-cartilage formation and cartilage engineering *via* cell-exerted forces. In this review, we will discuss recent progress related to the influence of substrate elasticity on stem cell chondrogenic differentiation and elucidate the potential underlying mechanisms. Aside from active sensing and responding to the extracellular environment, stem cells could respond to various external mechanical forces, which also influence their chondrogenic capacity; this topic will be updated along with associated signaling pathways.

Substrate elasticity influences stem cell/chondrocyte fate

It has long been known that stem cell microenvironment has significant influence on differentiation fate. Aside from biochemical and molecular factors, its physical properties, such as substrate elasticity, have been demonstrated to impact stem cell lineage differentiation (Mousavi and Doweidar, 2015). Landmark work by Engler *et al*. (2006) suggests that soft (elastic modulus, *E*, 0.1-1 kPa), medium (*E*, 8-17 kPa), and hard (*E*, 25-40 kPa) matrices can induce MSC lineage specification toward neurons, muscles, and osteoblasts, respectively. Despite still being in its infancy, studies on varied scaffolds, including hydrogels, porous/fibrous scaffolds, and acellular ECM, have provided robust evidence suggesting that stem cells/chondrocytes grown on soft substrates tend to chondrogenic differentiation compared to stiffer substrates. (Table 1)

Cells grown on less stiff hydrogel scaffolds showed enhanced chondrogenic potential

Synthetic hydrogels

Xue *et al*. (2013) found that, when using polyacrylamide (PAM) hydrogels with different stiffnesses as culture substrates, human bone marrow MSC (BMSC) aggregation was more predominant when cultured on soft matrix (*E*, 1.6 ± 0.3 kPa) rather than on hard matrix (*E*, 40 ± 3.6 kPa). BMSCs expanded on soft matrix exhibited a dramatic upregulation of chondrogenic marker genes, *SOX9* [SRY (sex determining region Y)-box 9], *ACAN* (aggrecan), and *COL2A1* (type II collagen). In contrast, the promotion of osteogenic marker expression on hard matrix was overridden by a high seeding density. Interestingly, Allen *et al*. (2012) reported that primary murine chondrocytes and ATDC5 cells grown on 0.5 MPa PAM hydrogel substrates deposited more proteoglycan and expressed more *SOX9*, *ACAN* and *COL2A1* mRNA relative to cells exposed to substrates of other stiffnesses (*E*, 0.2 MPa and 1.1 MPa). This finding suggests that the chondroinductive effect of this discrete stiffness (*E*, 0.5 MPa), which falls within the range reported for articular cartilage (Kiviranta *et al*., 2008), requires the stiffness-sensitive induction of transforming growth factor beta1 (TGFβ1).

Less stiff substrates favoring a chondrogenic lineage also apply to hybrid hydrogels. Kwon and Yasuda (2013) found that mouse BMSCs cultured on the high stiffness sulfonate-coated PAM (S-PAM) hydrogels (*E*, ~ 150 kPa) spread out with strong expression of stress fibers, while BMSCs cultured on the low stiffness S-PAM hydrogels (*E*, ~ 1 kPa) had a round shape with fewer stress fibers but more cortical actins. Importantly, even in the absence of chondrogenic induction, the lower stiffness S-PAM hydrogels led to higher mRNA levels of chondrogenic markers *COL2A1*, *ACAN*, and *SOX9* and lower mRNA levels of an undifferentiation marker *SCA1* (stem cell antigen-1), indicating that the mechanical properties of S-PAM hydrogels strongly influence chondrogenesis. Similarly, Park *et al*. (2011) found that human BMSCs on stiff substrates (type I collagen-coated PAM hydrogels) had higher expression of smooth muscle cell (SMC) markers *ACTA1* (α-actin) and *CNN1* (calponin 1); in contrast, BMSCs on soft substrates had a higher expression of chondrogenic marker *COL2A1* and adipogenic marker *LPL* (lipoprotein lipase). TGFβ increased SMC marker expression on stiff substrates; however, TGFβ increased chondrogenic marker expression and suppressed adipogenic marker expression on soft substrates, while adipogenic medium and soft substrates induced adipogenic differentiation effectively. Using a two-dimensional (2D) culturing system in which PAM gels with different concentrations of bis-acrylamide were coated with type I collagen, Schuh *et al*. (2010) found that, after porcine chondrocytes were cultivated on these hybrid hydrogels with a Young's modulus of 4, 10, 40 and 100 kPa for 7 d, cellular proliferation and actin organization were decreased on matrices of 4 kPa compared with stiffer substrates. The differentiated phenotype of the chondrocytes grown on matrices of 4 kPa was stabilized, evidenced by higher levels of *COL2A1* and *ACAN* and a lower level of *COL1A1*, indicating that chondrocytes sense the elasticity of the matrix and might be used for the design of scaffolds with mechanical properties specifically tailored to support the chondrogenic phenotype in tissue engineering applications.

Results from Liu *et al*. (2010) have shown that human BMSCs expanded on polyethylene glycol (PEG) 10-CMP (collagen mimetic peptide) hybrid hydrogel (*E*, 3.730 kPa) had significantly more staining intensity of aggrecan and type II collagen immunostaining than the cells grown on PEG10 hydrogels (*E*, 4.231 kPa), which was confirmed at mRNA levels by real-time PCR data, indicating that soft matrix induced a greater degree of chondrogenic differentiation. This finding in PEG hydrogels also applies to bovine articular chondrocytes. Nicodemus *et al*. (2011) found that the deposition of aggrecan and type II collagen was the highest at both protein and mRNA levels when chondrocytes were encapsulated in hydrogels with the lowest crosslinking 60 kPa compressive modulus while the catabolic gene expression of *MMP1* (matrix metalloproteinase 1) and *MMP13* was elevated about 25-fold in hydrogels with 590 kPa compressive modulus. This finding indicates that the ECM synthesis, degradation, and remodeling might be correlated with chondrocyte responses to hydrogel elasticity differences.

Natural hydrogels

Wang *et al*. (2014) synthesized 3D hydrogels with varied matrix stiffnesses (3, 30 and 90 kPa) by using hydrogels with different concentrations of matrix compositions such as chondroitin sulfate (CS), hyaluronic acid (HA), and heparin sulfate (HS). Results showed that, in softer hydrogels (~ 3 kPa), increasing HA concentrations resulted in substantial upregulation of *ACAN* and *COL2A1* expression in a dose-dependent manner in human adipose-derived stem cells (ADSCs), which was reversed in HA-containing hydrogels with higher stiffness (~ 90 kPa). This finding suggested that matrix stiffness and biochemical cues interact in a nonlinear manner to regulate chondrogenesis of ADSCs in a 3D environment.

Most recently, Navaro *et al*. (2015) developed a hydrogel biomaterial system that can encapsulate nucleus pulposus-derived stem cells (NP-SCs) in a 3D culture, sustain the cells' survival, and provide mechanical cues to

the cells based on variations in the shear storage modulus of the encapsulating milieu. This tunable hydrogel is a semi-synthetic material made from adducts of fibrinogen and poloxameric block copolymers called Tetronic 1307. NP-SCs expanded on matrices with a low shear storage modulus (G'= 1 kPa) significantly promoted more proliferation and chondrogenic differentiation, which was evidenced by higher expression of *SOX9* and *ACAN* genes and sulfated glycosaminoglycan (GAG), whereas matrices with a high modulus (G'= 2 kPa) promoted osteogenic differentiation, which was proven by enhanced expression of osteogenesis related markers such as *OCN* (osteocalcin) and *OPN* (osteopontin) genes and higher alkaline phosphatase (ALP) activity.

Cells grown on less stiff porous/fibrous scaffolds showed enhanced chondrogenic potential

Synthetic scaffolds

Nam *et al*. (2011) reported that pure poly(ε-caprolactone) (PCL) fibers with a lower modulus (7.1 MPa) provided a more appropriate microenvironment for chondrogenesis, evidenced by a marked upregulation of chondrocytic *SOX9*, *ACAN*, and *COL2A1* genes and chondrocyte-specific ECM GAG production in embryonic mesenchymal progenitor cells. In contrast, the stiffer core-shell poly (ether sulfone)-poly (ε-caprolactone) (PES-PCL) fibers (30.6 MPa) supported enhanced osteogenesis by promoting osteogenic *RUNX2* (runt-related transcription factor 2), *ALP*, and *OCN* gene expression as well as ALP activity. The findings demonstrate that the microstructure stiffness/modulus of a scaffold and the pliability of individual fibers may play a critical role in controlling stem cell differentiation.

Natural scaffolds

Murphy *et al*. (2012) investigated the influence of ECM elasticity on stem cell differentiation. They found that, in homogeneous collagen-GAG (CG) natural scaffolds with the same composition but different stiffnesses (0.5, 1.0 and 1.5 kPa), scaffolds with the lowest stiffness facilitated a significant upregulation of *SOX9* expression and that Wistar rat BMSCs were directed toward a chondrogenic lineage in more compliant scaffolds. In contrast, the highest level of *RUNX2* expression was found in the stiffest scaffolds and BMSCs were directed toward an osteogenic lineage in stiffer scaffolds. The results from the work of Fernández-Muiños *et al*. (2014) suggested that mouse embryonic fibroblasts (MEFs) which were cultured in RAD16-I (PuraMatrix, self-assembling peptide nanofiber scaffolds) at a low elastic modulus value (~ 0.1 kPa) expressed the chondrogenic inductor bone morphogenetic protein 4 (BMP4) and its antagonist Noggin. On the other hand, at a higher elastic modulus value (~ 5 kPa), the cells expressed Noggin but not BMP4 and did not engage in chondrogenesis, which suggested that the balance between BMP/Noggin could be implicated in the chondrogenic process.

Cells grown on less stiff acellular ECM scaffolds showed enhanced chondrogenic potential

The development of decellularized ECM (dECM) maintains the stemness and specific cell morphology desired for chondrogenesis (Pei *et al*., 2011) and allows dECM produced by the patient's own cells to be used thereby overcoming the issues of possible exogenous pathogen transfer (Benders *et al*., 2013).

Cartilage-derived dECMs

During the past decade, there has been increasing interest in creating biological scaffolds composed of ECM derived from the decellularization of tissues or organs for tissue engineering applications. Schwarz *et al*. (2012) found decellularization and sterilization process (DP) treatment made the linear modulus of the scaffolds decrease significantly from 6.5 ± 2.3 MPa in native porcine nasal septal cartilage (pNSC) to 1.92 ± 0.85 MPa in processed cartilage. Processed pNSC scaffolds seeded with human primary chondrocytes were cultured for 42 d in chondrogenic induction medium. Due to the decreased linear modulus in processed pNSC, scaffolds seemed to provide adequate substrate stiffness to support redifferentiation and maintenance of the chondrogenic phenotype. Garrigues *et al*. (2014) found that the predominant effects on human ADSCs of incorporation of cartilage-derived matrix (CDM) into electrospun PCL scaffolds were to stimulate sulfated GAG synthesis and *COL10A1* gene expression. Compared with single-layer scaffolds, multilayer scaffolds enhanced cell infiltration and *ACAN* gene expression. Interestingly, compared with single-layer constructs, multilayer PCL constructs had a much lower elastic modulus; PCL-CDM constructs had an elastic modulus approximately 1 % that of PCL constructs. These findings suggest the role of lower elastic modulus, particularly from CDM, in promoting chondrogenesis. Thus, decellularized cartilage (tissue/organ) scaffolds with controlled elasticity hold great potential in regulating the chondrogenesis of therapeutic cells and cartilage regeneration *in vivo*.

Cell-derived dECMs

Recent reports demonstrate the influence of cell-derived matrix stiffness on chondrogenic differentiation of adult stem cells. Zhang *et al*. (2015b) reported that human synovium-derived stem cells (SDSCs) expanded on stem cell-derived dECM greatly enhanced their proliferation and chondrogenic differentiation capacity. To determine the effect of mechanical properties of culture substrates on cultured cells, atomic force microscopy (AFM) was used to measure the elasticity of both substrates and expanded cells. They found that Young's moduli of the glutaraldehyde fixed cells were 91.22 ± 64.15 kPa after expansion on plastic flasks and 53.33 ± 53.47 kPa when expanded on dECM. The finding indicates that a lower stiffness substrate might be related to enhanced chondrogenic potential. Furthermore, an AFM study showed that adult SDSC (ASDSC) deposited dECM (AECM) exhibited a higher elasticity than fetal SDSC deposited dECM (FECM) (61.82 ± 35.86 *versus* 58.26 ± 13.37 kPa, $p = 0.000$); the elasticity of plastic flasks could be considered as infinite. Interestingly, Li *et al*. (2014) found that the elasticity of expanded ASDSCs was parallel to their substrate; the comparison between plastic flasks and AECM was 9.07 ± 4.56 *versus* 3.60 ± 1.79 (kPa) ($p = 0.000$), while the

comparison between AECM and FECM was 3.60 ± 1.79 *versus* 3.45 ± 2.29 (kPa) ($p = 0.024$). They also found that expanded ASDSCs on FECM yielded 21-day pellets with the highest chondrogenic markers including aggrecan and type II collagen at both the mRNA and protein levels followed by those grown on AECM; cells grown on plastic flasks had the lowest amount of chondrogenic markers. The fact that stem cells maintained better chondrogenic potential on dECM with lower elasticity suggested that, during *in vitro* expansion, dECM could be designed with an appropriate elasticity in order to better maintain its chondrogenic potential. However, the study of influences of mechanical properties of the *in vitro* microenvironment on chondrogenesis of stem cells is still in its infancy and many potential mechanisms need to be explored.

Potential mechanisms of substrate elasticity underlying chondrogenic differentiation

Even though the exact mechanisms underlying substrate stiffness mediated stem cell chondrogenic differentiation are still unknown, recent evidence indicates several signaling pathways might be involved, including integrin-mediated focal adhesion signaling, tension-sensitive protein pathway, stretch-activated channel pathway, TWIST1 signaling, and YAP/TAZ signaling (Fig. 1).

Integrin-mediated focal adhesion signaling

Integrins and other cell surface receptors provide external links to the ECM, but their functions at the cell-ECM interface require the participation of a multiprotein complex of adhesome components. The cytoskeleton includes microfilaments, microtubules and intermediate filaments. The main components of actin cytoskeleton are stress fibers and focal adhesion (FA), which have been reported to influence MSC shape, mechanical properties, and differentiation down osteogenic, adipogenic and chondrogenic pathways (Mathieu and Loboa, 2012). Focal adhesion is the attachment point of ECM and the actin cytoskeleton that could transmit the extracellular signals into cytoplasm (Geiger and Yamada, 2011). The aggregation of FA kinase (FAK) with integrins and other cytoskeletal proteins in focal contacts has been proposed to be responsible for FAK activation, whose phosphorylation is associated with Src activation. The activation of the FAK/Src complex serves as a potential substrate for tensin, vinculin, paxilin, p130cas (Guan, 1997) and talin (del Rio *et al*., 2009). Impaired functioning of vinculin in primary chondrocytes resulted in the reduced expression of chondrocyte-specific genes (Koshimizu *et al*., 2012). The round chondrocyte cytoskeletal structure was found to reduce vinculin expression compared with the fibroblastic chondrocyte cytoskeletal structure (Mahmood *et al*., 2004) (Fig. 1**A**).

Rho, Rac, and Cdc42 GTPase were reported to regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia (Nobes and Hall, 1995). Small GTPases impacted chondrogenic differentiation through regulating nanomechanics of human ADSCs; specifically, inhibition of Rac1 led to an increase of *COL2A1* gene expression (Jungmann *et al*., 2012) while Rac1 activation inhibited chondrogenesis (Gao *et al*., 2010). In mouse limb-bud MSCs, treatment with Y27632 to inhibit Rho-associated protein kinase (ROCK) increased GAG production of the cells and caused cortical actin organization. This treatment also reduced the number of actin fibers and caused cell rounding. Conversely, RhoA overexpression inhibited GAG synthesis and *SOX9* (Woods *et al*., 2005). Therefore, the RhoA/ROCK pathway plays a role in regulating the markers of chondrogenic differentiation. Cytochalasin also has been shown to increase SOX9 (Arnsdorf *et al*., 2009), strongly indicating that the mechanism for this regulation is likely related to the actin cytoskeleton. Therefore, decreased actin cytoskeletal organization appears to increase chondrogenesis in MSCs. A soft substrate can only withstand low levels of force exerted by cells. When cells generate traction forces higher than what the substrate can withstand, the matrix may yield and cells could adapt by decreasing the traction force, integrin binding and their own stiffness (Huebsch *et al*., 2010; Solon *et al*., 2007), which modulate cell spreading and thus differentiation (Chowdhury *et al*., 2010; McBeath *et al*., 2004). Limits of cell adhesion strength on a soft substrate can provide feedback to cells to decrease stress fiber formation, which also explains why Rho activation fails to induce stress fibers on soft substrates (Park *et al*., 2011) despite the fact that Rho GTPase plays an important role in actin assembly and focal adhesion formation (Fig. 1**A**).

Non-muscle myosin II (NM II)-mediated contraction sensed and transmitted through substrate elasticity by cells played a critical role in chondrogenesis. For instance, the co-culture of mouse BMSCs with blebbistatin, a potent NM II inhibitor, blocked NM II-mediated mechanical sensing and suppressed chondrogenesis induced by the low stiffness of S-PAM gels, suggesting the central roles of NM II-mediated mechanical sensing in inducing chondrogenic genes (Kwon and Yasuda, 2013). Similarly, Murphy *et al*. (2012) found that the treatment of Wistar rat BMSCs with blebbistatin hindered the ability of cells to sense the elasticity of the matrix and abolished the upregulation of transcription factor *SOX9,* as a result of cells sensing the elasticity of the scaffold. In addition, the actin structure also plays a critical role in chondrogenic differentiation; disruption of the actin cytoskeleton by cytochalasin D induced chondrogenic differentiation of limb bud mesenchymal cells (Zanetti and Solursh, 1984). Thus, the dependence of chondrogenic differentiation on substrate stiffness seems to be related to changes of cytoskeletal structures in stem cells (Fig. 1**A**).

The tension-sensitive protein pathway

The traction force generated by substrate elasticity difference might induce certain protein unfolding, which serves as 'molecular strain gauges' that expose binding domains of some proteins and signaling molecules. For example, stretch forces can unfold talin, which binds to integrins and actins, exposing its binding sites for vinculin, and the exposed sites on vinculin can activate mitogen-activated protein kinase (MAPK) leading to a series of cell signaling chain reactions (Holle and Engler, 2011).

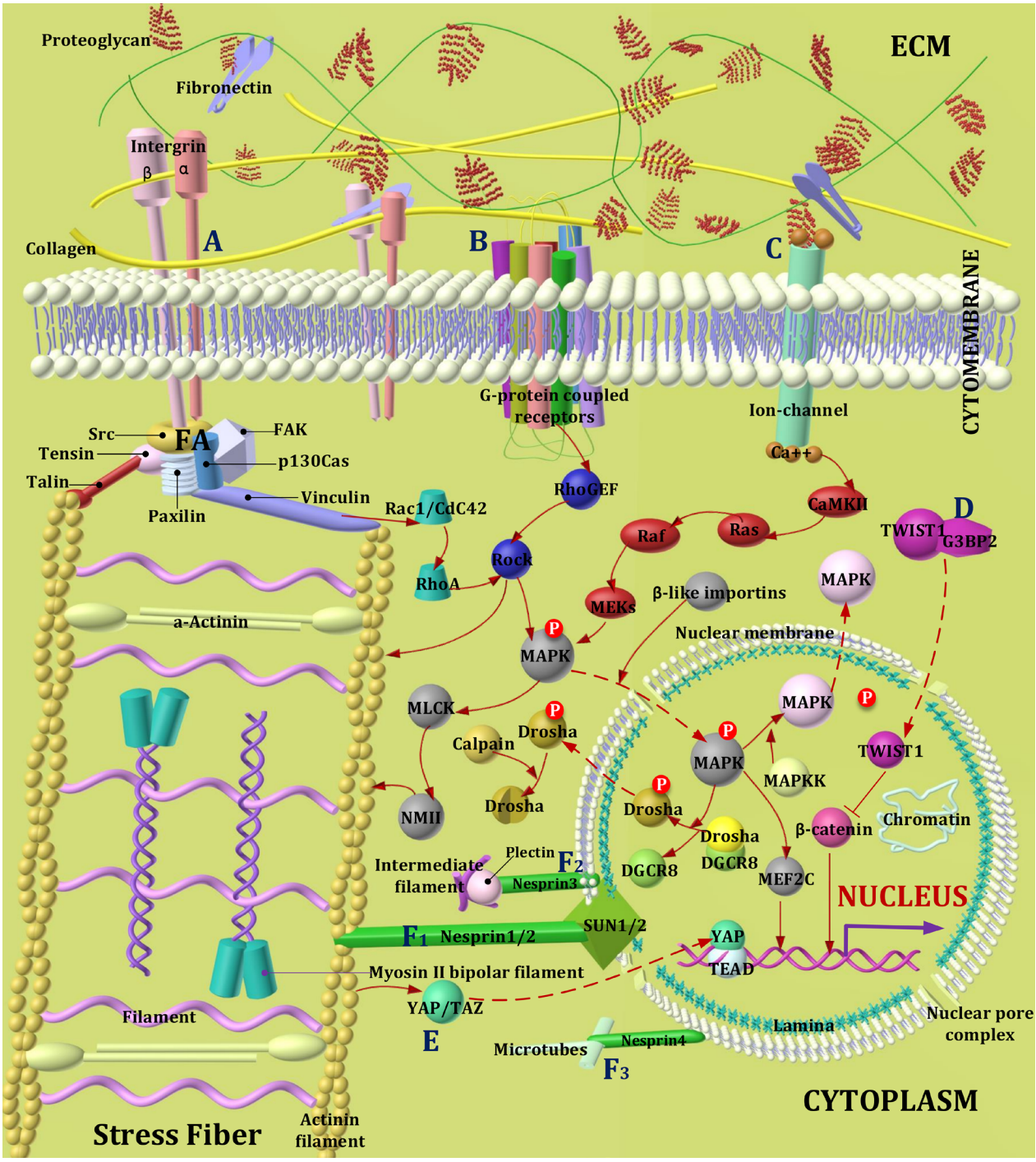

Fig. 1. Potential mechanisms of substrate stiffness on stem cell chondrogenic fate. (**A**) Integrin-mediated focal adhesion signaling; (**B**) The tension-sensitive protein pathway; (**C**) The stretch-activated channel pathway; (**D**) TWIST1 signaling; (**E**) YAP/TAZ signaling; and (**F**) Nuclear deformation and gene expression. Abbreviations: CaMK: Calcium/calmodulin-dependent protein kinase; CDC42: Cell division cycle 42; FA: Focal adhesion; FAK: Focal adhesion kinase; G3BP2: GTPase Activating Protein (SH3 Domain) Binding Protein 2; MEK: MAPK kinase; MLCK: Myosin light chain kinase; SAM: stretch activated channels; Src: Proto-oncogene tyrosine-protein kinase; TAZ: transcriptional coactivator with PDZ-binding motif; TF: Transcriptional factor; TWIST1: Twist-related protein 1; YAP: Yes-associated protein.

β-like importins mediate the nuclear translocation of MAPKs (Zehorai and Seger, 2014). p38 MAPK directly phosphorylates Drosha, which reduces its interaction with DGCR8, promotes its nuclear export and degradation by calpain, and increases cell death under stress (Yang *et al.*, 2015). On the other hand, the nuclear export of MAPK requires its dephosphorylation in a MAPK kinase (MAPKK)-dependent manner (Adachi *et al.*, 2000; Gong *et al.*, 2010). After phosphorylating MAPKK, nuclear localized MAPK is dephosphorylated by MAPK phosphatases (MKPs). Inactivated MAPK returns to the cytosol, partly through phosphorylated MAPKK acting as

a carrier, to receive the next stimulation. Tension forces may also influence the assembly, stability, and turnover of receptor-ligand complexes and functional module components. For example, the cell membrane tension and possible membrane fluidity might affect conformational dynamics of G protein-coupled receptor (GPCR); the activated GPCR might lead to cytoskeleton re-arrangement through regulating the RhoGEF protein and its downstream RhoA/ROCK activities (Siehler, 2009) (Fig. 1**B**).

The stretch-activated channel pathway

Stronger mechanical feedback on stiff substrates may also lead to the activation of stress-sensitive ion channels (Lee *et al*., 1999). Recently, it has been shown that cells on substrates of differing stiffness exhibit changes in the amplitude of calcium ion oscillations, proving that stretch activated channel permeability is related to active sensing of the substrate elasticity changes of the cell (Kobayashi and Sokabe, 2010). Substrate rigidity sensing was induced by intrinsic mechanical stimuli such as generation of traction forces onto the substrate *via* stress fiber/FA system. Endothelial cells cultured on stiffer substrates showed spontaneous Ca^{2+} oscillations with larger amplitude than those on soft substrates (Kobayashi and Sokabe, 2010). Intracellular Ca^{2+} plays a crucial role in the reorganization of the actin cytoskeleton through modulating actin-associating protein activities. Increases in cytosol Ca^{2+} induces stress fiber contractility through phosphorylation of myosin light chain kinase (MLCK) and/or activation of proteases (Ridley *et al*., 2003). Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) phosphorylates Raf-1 and mediates Ras-stimulated Raf activation, which might lead to activation of downstream signals (Salzano *et al*., 2012). Constitutive CaMKII signaling in premature maturation of chondrocytes was a cell-autonomous effect associated with downregulation of cell-cycle regulators and upregulation of chondrocyte maturation markers (Taschner *et al*., 2008) (Fig. 1**C**).

TWIST1 signaling

In the TWIST1-G3BP2 mechanotransduction pathway, increasing substrate stiffness was reported to induce integrin-dependent phosphorylation events and release of TWIST1 from its cytoplasmic anchor G3BP2 to enter the nucleus; TWIST1 is an essential mechano-mediator that promotes epithelial-mesenchymal transition (EMT) in response to increasing matrix stiffness (Wei *et al*., 2015) (Fig. 1**D**).

YAP/TAZ signaling

Dupont *et al*. (2011) reported the identification of YAP/TAZ as nuclear relays of mechanical signals exerted by ECM rigidity and cell shape. This regulation requires Rho GTPase activity and tension of the actomyosin cytoskeleton, but is independent of the Hippo/LATS cascade. Crucially, YAP/TAZ proteins are functionally required for differentiation of MSCs induced by ECM stiffness. This finding was mirrored by a recent report that YAP negatively regulated chondrogenic differentiation of MSCs (Karystinou *et al*., 2015). Finally, as Moore *et al*. (2010) put forward, how the short-term substrate rigidity sensing is translated into long-term sensing involved in cell differentiation remains unknown; although much is shared between the two processes, they may have radically different time constants and frequencies. It is still a key challenge to link the early rigidity mechanisms to the long-term processes involved in cellular differentiation (Fig. 1**E**).

Nuclear deformation and gene expression

Substrate elasticity variations induce integrin-mediated cell activities leading to actomyosin contraction, which is followed by transferring through nesprins to the nucleus. In addition, the chromosome is linked to the nuclear membrane through lamins, whose activities may lead to chromatin reorganization, DNA replication, and chondrogenesis-related gene changes. The plasma membrane is physically linked with the nuclear membrane (Ingber, 2003); it is possible that physical factors and nuclear deformation could regulate gene expressions through an as-yet-unknown mechanism. Molecular connections between integrins, cytoskeletal filaments, and nuclear scaffolds were supposed to provide a discrete path for mechanical signal transfer through cells and to produce integrated changes in cell and nuclear structure changes (Maniotis *et al*., 1997). Many related proteins are involved in this process, such as F-actins, intermediate filaments, Nesprin1/2, Nesprin3 and SUN1/2 as well as lamins. For example, nesprin-1 and nesprin-2 dimerise with nesprin-3 forms a meshwork composed of peri-nuclear cytoskeletal filaments around the nuclear periphery. The existence of the linker of the nucleoskeleton and cytoskeleton complex provides more evidence that the traction forces arising due to matrix rigidity can physically affect the structural organization of the nucleus (Fig. 1**F**).

External forces on stem cell/chondrocyte chondrogenic differentiation

Biomechanical forces are key factors that keep mature cartilage healthy. During normal activity, *in vivo* articular cartilage undergoes cyclic compression (at a frequency of approximately 1 Hz during locomotion) and chondrocytes are exposed to a uniform compressive force within the range of 3 to 10 MPa (Elder and Athanasiou, 2009). With the emergence of tissue engineering as an independent research field and the understanding that chondrocytes are mechanically sensitive cells (Grodzinsky *et al*., 2000; Mow and Guo, 2002), great effort has been made to determine how external mechanical stimuli, such as compression and hydrostatic pressure (HP), may improve the development of cartilage tissue *in vitro* (Grad *et al*., 2011) (Table 2).

Compression

Direct compression has been used to modulate matrix composition and concomitantly influence neocartilage properties. Mauck *et al*. (2000) reported that dynamic loading yielded 28-day bovine chondrocyte-seeded agarose disks with a six-fold increase in the equilibrium aggregate modulus over free swelling controls; sulfated GAG content and hydroxyproline content were also found to be greater

Table 2. Influences of external mechanical forces on stem cell chondrogenic differentiation.

Forces	Cell type and culture conditions	Force conditions	Chondrogenic effects	References
Compression	Mouse limb bud mesenchymal cells in 3D type I collagen gel	20 %-30 % strain	Increased type II collagen and aggrecan expression, enhanced *SOX9* mRNA, down-regulation of IL-1β	Takahashi *et al*., 1998a
	Rabbit BMSCs in 2 % agarose gel (10^7 cells/mL)	Subjected to a cyclic, unconfined compression test	Increased aggrecan and type II collagen over non-loaded controls	Huang *et al*., 2004
	Porcine BMSCs embedded in 2 % agarose gel and grown in CIM	1 h/day, 1 Hz, 10 % strain	Increased *ACAN, COL1A1,* and *COL2A1* mRNA in a temporal and spatial-dependent manner	Haugh *et al*., 2011
	Porcine BMSCs seeded in 2 % agarose gel	1 Hz, 10 % strain, 1 h/d	The application of dynamic compression from day 0 inhibited chondrogenesis of MSCs	Thorpe *et al*., 2010
	Human BMSCs encapsulated in HA hydrogels in CIM	10 % strain, 1 Hz, 4 h/d, 5 d/week	Increased mechanical properties as well as the GAG and collagen contents but reduced hypertrophic markers	Bian *et al*., 2012
	Human BMSCs seeded in 3 % alginate	1.5 h on/4.5 h off, 8 d, 1 Hz, 15 % strain	Increased *SOX9*, *COL2A1*, *COL10A1*, and *ACAN* mRNA	Campbell *et al*., 2006
	Human BMSCs embedded in fibrin/ polyurethane gels and grown in CIM with 1 ng/mL TGFβ1	1 h/d, 7 d, 0.1 or 1 Hz, 10-15, 20 or 30 % strain	Higher load frequency and higher amplitude induced higher GAG synthesis, higher chondrocytic gene expressions	Li *et al*., 2010
	Human BMSCs embedded in fibrin/ polyurethane composites in defined medium w/o GFs	1 Hz with 0.4 mm strain and/or shear force (ball oscillation of ± 25 °C at 1 Hz)	Combined forces led to significant increases in chondrogenic gene expression and sGAG and type II collagen staining	Schätti *et al*., 2011
	Human BMSCs seeded onto the PLCL/chitosan scaffolds in CIM	5 % of strain, 1 Hz, 2 h/d, 7 d/week for 3 weeks	Enhanced cartilage formation and suppressed hypertrophy	Zhang et al., 2015a
Hydrostatic pressure	Porcine BMSCs and FPSCs embedded in agarose gel and cultured in CIM with 10 ng/mL TGFβ3	CHP at 10 MPa, 1 Hz, 4 h/d, 5 d/week for 5 weeks	Increased ratio of GAG to DNA and both dynamic and equilibrium moduli but suppressed calcification of BMSC constructs	Caroll *et al*., 2014
	Porcine SDSCs and FPSCs formed in pellets and cultured in CIM with 1/10 ng/mL TGFβ3	CHP at 10 MPa, 1 Hz, 4 h/d for 14 d	Increased and maintained chondrogenic differentiation only in the presence of a low dose of TGFβ3 (1 ng/mL) rather than none (0 ng/mL) or a high dose (10 ng/mL)	Vinardell *et al*., 2012
	Human ADSCs encapsulated in GG hydrogel and cultured in CIM with 10 ng/mL TGFβ3	HP at 0.4 and 5 MPa, 0.5 Hz, 4 h/d, 5 d/week for 4 weeks	Physiologic loading (5 MPa) increased chondrogenic differentiation and matrix deposition	Correia *et al*., 2012
	Human ADSCs embedded in collagen gel and cultured in CIM with 10 ng/mL TGFβ1	CHP at 0-0.5 MPa (4.93 bar), 0.5 Hz for 1 week	Increased matrix deposition and *COL2A1*, *ACAN*, and *SOX9* mRNA levels but decreased *COL1A1* level	Ogawa *et al*., 2015
	Human BMSCs formed in pellets and cultured in CIM containing 10 ng/mL TGFβ3	IHP at 10 MPa, 1 Hz, 4 h/d for 14 d	The treatment with IHP upregulated *SOX9*, *COL2A1*, and *ACAN*; the combined group yielded the most chondrogenic differentiation at both mRNA and protein levels	Miyanishi *et al*., 2006
	Human SDSCs embedded in agarose gel and cultured in a growth medium with 10 % FBS	CHP at 7.5 MPa, 1 Hz, 4 h/d, for 21 d	CHP initiated human ADSC chondrogenic differentiation; this effect did not last long in the absence of chondrogenic factors	Puetzer *et al*., 2013
Osmotic pressure	Bovine SDSCs and chondrocytes encapsulated in 2 % w/v agarose in 10 % FBS + GF cocktail	"300 mOsM" *vs*. "330 mOsM" *vs*. "400 mOsM" for 7 weeks	The application of hypertonic media to constructs led to increased mechanical properties and GAG contents.	Sampat *et al*., 2013
	ATDC5 and human BMSCs in a monolayer culture in CIM	ATDC5: 310 ± 5 mOsMBM-SCs: 285 ± 5 mOsM	An increase of 100 mOsM in medium enhanced levels of *COL2A1*, *ACAN*, *SOX9*, *RUNX2*, and *COL10A1*	Caron *et al*., 2013

Abbreviations: ADSCs: adipose derived stem cells; BMSCs: bone marrow stromal cells; CHP: cyclic hydrostatic pressure; CIM: chondrogenic induction medium; CPBTA: fiber mesh scaffolds, consisting of a blend of 50:50 chitosan:poly(butylene terephthalate adipate); ECM: extracellular matrix; FBS: fetal bovine serum; FPSCs: infrapatellar fat pad derived stem cells; GAG: glycosaminoglycan; GF: growth factor; GG: gellan gum; HA: hyaluronic acid; IHP: intermittent hydrostatic pressure; IL-1β: interleukin 1beta; PLCL: poly L-lactide-co-3-caprolactone; SDSCs: synovium-derived stem cells; TGFβ: transforming growth factor beta

in dynamically loaded disks after 21 d of loading. Kaviani *et al*. (2015) found that porcine chondrocyte viability was dependent on compression magnitude, duration, frequency, and amplitude in a way that increasing each parameter decreased viability in certain zones of the growth plate. More specifically, proliferative and hypertrophic chondrocytes were found to be more sensitive to the applied compression.

After human BMSCs were subjected to chondrogenic differentiation in either chitosan-coated poly L-lactide-co-3-caprolactone scaffolds (Zhang *et al*., 2015a) or HA hydrogel (Bian *et al*., 2012), dynamic compression enhanced cartilage formation and suppressed chondrocyte hypertrophy. Using a custom built loading device, compression, shear, or a combination of both stimuli were applied onto fibrin/polyurethane composites in which human BMSCs were embedded. While no exogenous growth factors were added to the culture medium (Schätti *et al*., 2011) found that either compression or shear alone was insufficient for the chondrogenic induction of human BMSCs. However, the application of shear superimposed upon dynamic compression led to significant increases in chondrogenic gene expression. Histological analysis detected sulfated GAG and type II collagen only in the compression and shear group.

Despite a positive effect on matrix deposition, compressive loading at the early stage of chondrogenic induction is not constructive. For instance, Mouw *et al*. (2007) found that the response to TGFβ1 treatment of bovine BMSCs seeded in agarose gel was determined by the timing of dynamic loading; application at the early stage (day 8 after chondrogenic induction) decreased the *ACAN* gene level while loading at the later stage (day 16 after chondrogenic induction) increased chondrogenic gene expression. Thorpe *et al*. (2010) also found that the application of dynamic compression from day 0 inhibited chondrogenesis of porcine BMSCs, indicating that MSC seeded constructs should first be allowed to undergo chondrogenesis *in vitro* prior to implantation in a load bearing environment. The influences of mechanical loading on stem cells' chondrogenic differentiation also depend on stem cell type. For example, dynamic mechanical compression significantly increased chondrogenic differentiation of goat BMSCs encapsulated in a hydrogel but decreased cartilage-specific gene expression in human embryonic stem cell-derived cells in the absence of TGFβ1 (Terraciano *et al*., 2007).

Hydrostatic pressure

As one component of the mechanical stresses present within diarthrodial joints (Hall *et al*., 1996), HP is the prevailing mechanical signal governing normal articular cartilage homeostasis (Grodzinsky *et al*., 2000) and thus has the potential to be applied in cartilage tissue engineering (Miyanishi *et al*., 2006).

To date, many studies have focused on the effects of HP stimuli on chondrocyte-mediated synthesis and degradation of cartilage matrix macromolecules (Jortikka *et al*., 2000; Toyoda *et al*., 2003; Sakao *et al*., 2008). Articular chondrocytes, usually from an animal source, respond positively to pulsatile (0.0125-1 Hz) HP loadings ranging from 0.3-5 MPa by increasing GAG synthesis and deposition as well as expression of healthy articular cartilage markers (Mizuno *et al*., 2002; Jortikka *et al*., 2000; Toyoda *et al*., 2003). However, when high HP magnitudes were applied, on the order of 20 MPa, incorporation rates of $^{35}SO_4$ and [^{3}H] proline into adult bovine articular cartilage slices decreased but were reversible (Hall *et al*., 1991); on the order of 30 MPa, stress response related genes such as heat shock protein 70 (*HSP70*), *HSP45*, growth arrest and DNA damage-inducible 45 (*GADD45*), and *GADD153* were significantly increased in immortalized human chondrocyte cell lines (Sironen *et al*., 2002). At 50 MPa, inflammatory factors such as interleukin 6 (*IL6*) and tumor necrosis factor alpha (*TNFA*) mRNAs were significantly increased in a human chondrosarcoma cell line (Takahashi *et al*., 1998b) and apoptosis was observed in rabbit chondrocytes cultured in alginate beads (Nakamura *et al*., 2006). The same outcome was observed when culturing human osteoarthritic chondrocytes under physiologically normal pressure magnitudes (5 MPa) (Wenger *et al*., 2006). Duration and magnitude of applied intermittent HP were also reported to differentially alter human chondrocyte matrix protein expression (Ikenoue *et al*., 2003). Continuous high HP could cause structural alterations in normal human chondrocytes, which adopted similar, if not identical, characteristics to those typical of osteoarthritic chondrocytes (Fioravanti *et al*., 2005).

Recently, more attention has focused on the influence of HP on stem cell chondrogenic differentiation. In the absence of chondrogenic factors, such as TGFβ, Miyanishi *et al*. (2006) found that treatment with intermittent HP alone upregulated *SOX9*, *COL2A1*, and *ACAN* in human BMSC pellets, and co-treatment with intermittent HP and TGFβ greatly enhanced chondrogenic differentiation. Puetzer *et al*. (2013) found that cyclic HP initiated human ADSCs' chondrogenic differentiation; this effect did not last long in the absence of chondrogenic factors. In the presence of chondrogenic factors, HP was found to increase chondrogenic differentiation in human ADSCs (Correia *et al*., 2012; Ogawa *et al*., 2015), porcine SDSCs, and infrapatellar fat pad derived stem cells (FPSCs) (Vinardell *et al*., 2012). Using porcine BMSCs and FPSCs embedded in agarose gel, Caroll *et al*. (2014) found that cyclic HP not only increased the ratio of GAG to DNA and both dynamic and equilibrium moduli but also suppressed calcification of BMSC constructs.

Osmotic pressure

Cartilage matrix contains a large amount of negatively charged aggrecans entangled within the collagen matrix, which can attract a high concentration of cations relative to other physiologic fluids, leading to increased interstitial osmolarity. The interstitial osmolarity range of healthy articular cartilage lies within 350 to 450 mOsm, depending on the zone (Urban *et al*., 1993). The loss and gain of water exposes the chondrocytes to dynamic changes in osmotic pressure, thereby exposing chondrocytes to both hyper- and hypo-osmotic stresses (Mow *et al*., 1992). The production of ECM of articular chondrocytes is also greatly influenced by the osmolarity of the culturing medium. For instance, chondrocyte alginate beads cultured in 550 mOsm medium for 48 h increased sulfated GAG synthesis compared to culture in 380 mOsm medium, while culture in 270 mOsm medium decreased sulfated GAG synthesis (Hopewell and Urban, 2003). The effects of osmolarity on ECM production may be due in part to regulation of the chondrocyte transcription factor Sox9. Treatment of freshly isolated chondrocytes from osteoarthritic human articular cartilage with hyperosmotic medium led to an

increase in the levels of Sox9 mRNA and protein, an effect mediated in part by an increase in the half-life of *SOX9* mRNA with hyperosmotic exposure (Tew *et al*., 2009). A similar study in equine articular chondrocytes showed that hyperosmotic treatment had varying effects on *SOX9* mRNA levels depending on chondrocyte sources and whether treatment was applied in a static or cyclic manner (Peffers *et al*., 2010).

Growth and chondrogenic differentiation of MSCs are also influenced by culture medium osmolarity. High-osmolarity medium (485 mOsm) reduced proliferation of human ADSCs (Liang *et al*., 2012). Caron *et al*. (2013) found that increasing the osmolarity of chondrogenic induction medium by 100 mOsm resulted in significantly enhanced chondrogenic marker expression such as *COL2A1* and *ACAN* in two chondroprogenitors, ATDC5 and human BMSCs. Sampat *et al*. (2013) found that the application of hypertonic media (400 mOsM) to constructs comprised of SDSCs or chondrocytes led to increased mechanical properties as compared to hypotonic (300 mOsM) or isotonic (330 mOsM) media. Constant exposure of SDSC-seeded constructs to 400 mOsM media from day 0 to day 49 yielded a Young's modulus of 513 ± 89 kPa and GAG content of 7.39 ± 0.52 %ww on day 49, well within the range of values of native, immature bovine cartilage. Primary chondrocyte-seeded constructs achieved a Young's modulus almost as high, reaching 487 ± 187 kPa and 6.77 ± 0.54 %ww (GAG) for the 400 mOsM condition (day 42). These findings suggest hypertonic loading as a straightforward strategy for 3D cultivation with significant benefits for cartilage engineering strategies.

Transient receptor potential vanilloid 4 (TRPV4) is a Ca^{2+}-permeable ion channel that is highly expressed by articular chondrocytes and can be gated by osmotic and mechanical stimuli (Kang *et al*., 2012). Human TRPV4 mutations that alter channel function are known to disrupt normal skeletal development and joint health (Everaerts *et al*., 2010; Kang *et al*., 2012; Nilius and Voets, 2013). Similarly, targeted deletion of TRPV4 leads to a lack of osmotically induced Ca^{2+} signaling in articular chondrocytes, accompanied by progressive, sex-dependent increases in bone density and osteoarthritic joint degeneration (Clark *et al*., 2010). TRPV4-mediated Ca^{2+} signaling has also been shown to enhance chondrogenic gene expression in chondroprogenitor cell lines (Muramatsu *et al*., 2007), as well as increase matrix synthesis in chondrocyte-based self-assembled constructs (Eleswarapu and Athanasiou, 2013). TRPV4-mediated Ca^{2+} signaling plays a central role in the transduction of mechanical signals to support cartilage extracellular matrix maintenance and joint health, indicating the possibility of therapeutically targeting TRPV4-mediated mechanotransduction for the treatment of diseases such as osteoarthritis, as well as to enhance matrix formation and functional properties of tissue-engineered cartilage as an alternative to bioreactor-based mechanical stimulation (O'Conor *et al*., 2014).

Shear force

Chondrocytes in articular cartilage are subjected to synovial fluid flow-induced shear force. Shear force is known to affect chondrocyte homeostasis and induce catabolic and anabolic pathways. Fluid flow-induced shear force (1.6 Pa) caused individual chondrocytes to elongate and align tangential to the direction of cone rotation, stimulated GAG synthesis two-fold, and increased the length of newly synthesized chains in human and bovine chondrocytes. However, it also increased prostaglandin E2 and nitric oxide synthesis and caused higher rates of apoptosis and downregulation of *COL2A1* and *ACAN* mRNA (Smith *et al*., 1995). These results are consistent with reports from Mohtai *et al*. (1996), showing that high shear stress (1 Pa) elicits the release of pro-inflammatory cytokines such as IL-6 and mediates matrix degradation and chondrocyte cell death. Goodwin *et al*. (1993) have shown that shear force level as low as 0.092 Pa could have an adverse effect on cells.

Not only for chondrocytes, caution should also be taken when applying flow-perfusion in stem cell chondrogenic induction. Alves da Silva *et al*. (2011) found that 28-day flow-perfusion dramatically enhanced ECM deposition and type II collagen production in human BMSCs seeded onto fiber mesh scaffolds in chondrogenic induction medium. This finding is inconsistent with a recent report. After 18-day chondrogenic induction of human BMSCs in pellets mounted into 3D-printed porous scaffolds followed by a continuous flow for 10 d, Kock *et al*. (2014) found that decreased GAG release, in combination with diminished type II collagen staining, indicated reduced chondrogenesis in response to flow-perfusion. Although flow-perfusion could possibly influence the differentiation of chondrogenic differentiated MSCs, the effects are inconsistent and strongly donor-dependent.

In the context of cartilage engineering, high or even moderate levels of shear are still undesirable in the formation of hyaline cartilage; the fluid flow-induced convective transport and low shear force have been manipulated using a rotating-wall bioreactor. Many experiments have been proven effective at producing better cartilage constructs in rotating-wall bioreactors (Freed and Vunjak-Novakovic, 1995; Vunjak-Novakovic *et al*., 1999; Pei *et al*., 2002a; Pei *et al.,* 2002b). Although successfully applied in cartilage engineering, the effects seen in studies applying fluid shear force might be due to more than the physical stimuli of the fluid-induced shear, because fluid flow through engineered cartilage constructs also undoubtedly increases nutrient transport and oxygen diffusion.

Multiaxial mechanical load

Articular motion is a combination of compressive, tensile, and shear deformations; one single external mechanical stimulus alone is unlikely to provide a sufficient mechanical signal to generate a cartilage-like tissue *in vitro*. There is an increasing body of work that has focused on multiaxial mechanical loading for cartilage tissue engineering, despite most work focusing on chondrocytes.

To compare and contrast with previous studies using cyclic axial compression, Frank *et al*. (2000) developed a biaxial system capable of applying both large and small cyclic shear and compression to articular cartilage explants in tissue culture and studied the biosynthetic response of chondrocytes to dynamic tissue macroscopic

shear deformation. Application of sinusoidal macroscopic shear deformation to articular cartilage explants resulted in a significant increase in the synthesis of proteoglycan and proteins (uptake of ^{35}S-sulfate and ^{3}H-proline) over controls held at the same static offset compression. Schätti *et al*. (2011) investigated the potential enhancing effect of surface shear, superimposed on cyclic axial compression, on chondrogenic differentiation of human BMSCs. Results showed that compression and shear alone were insufficient for stem cell chondrogenic induction. However, application of shear superimposed upon dynamic compression led to significant increases in chondrogenic gene expression. Histology detected sulfated GAG and collagen II only in the compression and shear group.

Potential mechanisms of external forces underlying chondrogenic differentiation

Previous attempts to decipher signaling pathways that may be involved in chondrocyte mechanotransduction were primarily focused on plasma membrane proteins with putative mechanosensor functions (*i.e.* receptors and ion channels that are primarily exposed to mechanical stimuli), including purinergic P2 receptors (Garcia and Knight, 2010), α5β1 integrins (Orazizadeh *et al*., 2006), TRPV4 (Guilak *et al*., 2010), N-methyl-D-aspartate type glutamate receptors (NMDAR) (Salter *et al*., 2004), annexin V (Lucic *et al*., 2003) and stretch-activated Ca^{2+} channels (Mobasheri *et al*., 2002; Wright *et al*., 1996). As far as signaling events downstream of plasma membrane receptors are concerned, the involvement of the Src and FAK, the extracellular signal-regulated kinase (ERK), and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways have been reported in various models (Muhammad *et al*., 2012). Of note, the majority of the above studies were performed on mature articular chondrocytes. This section will focus on potential mechanisms of external forces underlying stem cell chondrogenic differentiation. (Fig. 2)

The PKA/PP2A-CREB-SOX9 pathway

Since the Sox transcription factors, Sox9 in particular, are pivotal elements of chondrogenesis, many signaling events regulate their expression and/or function in chondrogenic cells. Among other factors, the Sox9 promoter is known to be regulated by the cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) that binds to a CRE site upstream of Sox9 (Piera-Velazquez *et al*., 2007). The cAMP-dependent Ser/Thr protein kinase A (PKA) is known to phosphorylate the Sox9 protein (at Ser 211), which induces its translocation into the nucleus and enhances its transcriptional activity (Huang *et al*., 2000). Indeed, there is a complex synergism between Sox9 function and the cAMP-PKA-CREB pathway in both mature and differentiating chondrocytes (Zhao *et al*., 2009). Juhász *et al*. (2014) applied a uniaxial intermittent cyclic load (0.05 Hz, 600 Pa; for 30 min) which provided HP and fluid shear on chondroprogenitor cells isolated from limb buds of four-day-old chicken embryos. They found that the applied mechanical load significantly augmented cartilage matrix production and elevated mRNA expression of several cartilage matrix constituents, including *COL2A1* and *ACAN*, as well as matrix-producing hyaluronan synthases through enhanced expression, phosphorylation, and nuclear signals of the main chondrogenic transcription factor Sox9. Along with increased cAMP levels, a significantly enhanced PKA activity was also detected and CREB, the archetypal downstream transcription factor of PKA signaling, exhibited elevated phosphorylation levels and stronger nuclear signals in response to mechanical stimuli. The involvement of PKA in the regulation of *in vitro* chondrogenesis was also confirmed in earlier reports (Lee and Chuong, 1997; Zákány *et al*., 2002). All of the above effects were diminished by the PKA-inhibitor H89. Meanwhile, protein phosphatase 2A (PP2A) activity was reduced following mechanical loading and treatments with the PP2A-inhibitor okadaic acid were able to mimic the effects of the intervention, which confirm an earlier report in that PP2A could counterbalance the chondrogenesis promoting effect of PKA *via* Sox9 and CREB phosphorylation (Zákány *et al*., 2002). These findings indicate that proper mechanical stimuli augment *in vitro* cartilage formation by promoting both differentiation and matrix production of chondrogenic cells; the opposing regulation of the PKA/CREB-Sox9 and the PP2A signaling pathways is crucial in this phenomenon (Fig. 2**A**).

Calcium signaling

Mechanotransduction of HP has been proposed to differ from other mechanical loads, due to the fact that HP generates a state of stress with little deformation (Elder and Athanasiou, 2009), which might be partially explained by a proposed element of HP mechanotransduction with fluctuations in intracellular ion concentrations (Browning *et al*., 2004; Hall, 1999; Mizuno, 2005). Stretch activated calcium channels (SACCs) have been demonstrated to be required for mechanotransduction of a variety of loading types in chondrocytes and MSCs (McMahon *et al*., 2008; Mizuno, 2005; Mobasheri *et al*., 2002; Wright *et al*., 1996). Voltage gated calcium channels (VGCCs) are activated by membrane depolarization and mediate Ca^{2+} influx (Catterall, 2011). Ca^{2+} influx *via* SACCs and/or VGCCs increases Ca^{2+} concentrations directly and also indirectly through calcium-induced calcium release *via* calcium-sensitive receptors in the sarcoendoplasmic reticulum calcium stores (SERCS) (Catterall, 2011). VGCCs and SERCS were both found to be required for chondrogenesis in high-density chicken MSC culture (Fodor *et al*., 2013) and they have both been implicated in mechanotransductive pathways (Riddle *et al*., 2006; Shakibaei and Mobasheri, 2003; Valhmu and Raia, 2002) (Fig. 2**B**).

In an *in vitro* study using porcine BMSCs seeded into agarose hydrogels, Steward *et al*. (2014) found that chelating free Ca^{2+}, inhibiting VGCCs, and depleting intracellular calcium stores suppressed the beneficial effect of HP on chondrogenesis, indicating that Ca^{2+} mobility may play an important role in the mechanotransduction of HP. However, inhibition of SACCs in the current experiment yielded similar results to the control group, suggesting that mechanotransduction of HP is distinct from loads that generate cell deformation. Inhibition of the downstream targets calmodulin, CaMKII and calcineurin, all knocked

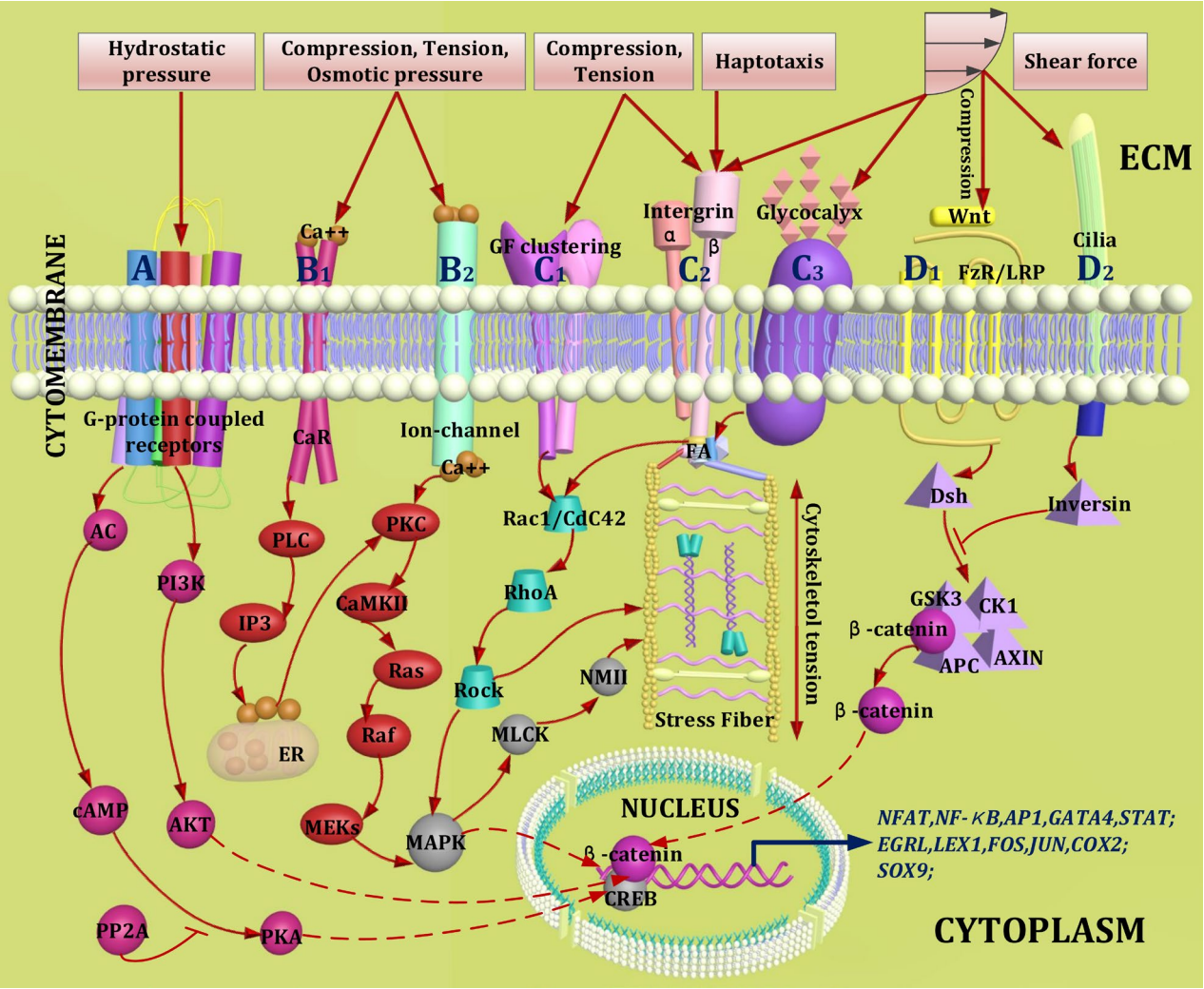

Fig. 2. Potential mechanisms of external forces on stem cell chondrogenic fate. (**A**) The PKA/PP2A-CREB-SOX9 pathway; (**B**) Calcium signaling; (**C**) The RhoA/Rac1-ROCK pathway; and (**D**) Other related pathways. Abbreviations: AC: adenylate cyclase; AKT: Protein Kinase B (PKB); CaMK: Calcium/calmoduin-dependent protein kinase; FAK: Focal Adhesion Kinase; GATA4: Transcription factor GATA-4; GR: Growth Factors; IP3: Inositol 1,4,5-triphosphate; Mechano-sensitive genes: *EGRL, LEX1, FOS, JUN, COX2;* MLCK: Myosin Light Chain Kinase; NFAT: Nuclear Factor of Activated T cells; PI3K: Phosphatidylinositol 3-Kinase; PKC: Protein Kinase C (PKC); PLC: Phospholipase C; STATs: Signal transducers and activators of transcription.

down the effect of HP on chondrogenesis, implicating these targets in MSC response to HP. All of the pharmacological inhibitors that abolished the chondrogenic response to HP also maintained a punctate vimentin organization in the presence of HP, as opposed to the mechanoresponsive groups where the vimentin structure became more diffuse. These results suggest that Ca^{2+} signaling may transduce HP *via* vimentin adaptation to loading. Calmodulin, CaMKII, and calcineurin also have been implicated in mechanotransduction of fluid flow and compression in MSCs as well as chondrocytes (Riddle *et al*., 2006; Valhmu and Raia, 2002). Moreover, transient receptor potential (*TRP)* channels have received the most attention because of their calcium ion permeability (Clapham, 2003) and ability to permit transient calcium influx in stretched cells (Follonier *et al*., 2008). Functional gene screening has identified that TRPV4, a mechanically and osmotically sensitive ion channel (Gao *et al*., 2003), is a regulator of the chondrogenic differentiation of C3H10T1/2 MSCs (Muramatsu *et al*., 2007).

The RhoA/Rac1-ROCK pathway

Rho GTPases not only function as key regulators of stress fiber assembly and FA formation (Darling and Guilak, 2008) but also play a central role in the regulation of actin cytoskeleton (Shifrin *et al*., 2009). Rho GTPases are involved in integrin-mediated mechanotransduction (Burridge and Wennerberg, 2004; Matthews *et al*., 2006) and regulate stress fiber formation in adult stem cells under mechanical stimulation (Discher *et al*., 2009). By activating Rho GTPase, the MAPK/ERK cascade is connected with the regulation of integrin-β signaling (Saleem *et al*., 2009). Chondrocytes have been found to predominantly express β1 subunits (Aszodi *et al*., 2003). Along with binding a subunits, the β1 subunits serve as receptors for collagen which further facilitate cellular cytoskeletal interaction

with ECM components (Millward-Sadler and Salter, 2004). Formation of stress fibers, along with activation of integrin β1, FAK and ERK, was involved in tension stretch-mediated inhibition of chondrogenesis (Takahashi *et al*., 2003), highlighting the role of the integrin/FAK/ERK signaling axis in mediating external physical stimulation (Fig. 2**C2**).

Connective tissue growth factor (CTGF), a mechanosensitive gene which provides unique binding domains for growth factors (*i.e.* TGFβ) and integrins, is involved in cytoskeletal tension modulated by the RhoA/ROCK signaling pathway (Xu *et al*., 2010) (Fig. 2**C1**). CTGF gene expression is known to be induced through mechanical stimulation and, in particular, through mechanical stretch (Kadota *et al*., 2004; Wong *et al*., 2003). Since CTGF does not work independently, other growth factors, integrin family proteins, and possible regulatory mechanisms linking this signaling cascade remain to be investigated. Moreover, after glycocalyx is damaged, the response of cytoskeleton to shear stress is suppressed; however, the FA is almost unaffected (Thi *et al*., 2004) (Fig. 2**C3**).

Rac1 plays an important regulatory role in cytoskeletal assembly during lamellipodia formation in cells under mechanical stimulation (Hu *et al*., 2002; Masuda and Fujiwara, 1993a; Masuda and Fujiwara, 1993b). The activation of Rac1 has also been shown to promote the expression of N-cadherin and ultimately enhance the transcription of *SOX5*, *SOX6*, *SOX9*, *COL2A1*, and *ACAN* mRNAs, Woods *et al*. (2007) and Zhao *et al*. (2015) found that RhoA played a positive and Rac1 had a negative role in the HP-induced F-actin stress fiber assembly; RhoA and Rac1 play central roles in the pressure-inhibited ERK phosphorylation, and Rac1, but not RhoA, was involved in the pressure-promoted c-Jun N-terminal kinase (JNK) phosphorylation. Pressure promoted the expression of osteogenic marker genes in BMSCs at an early stage of osteogenic differentiation through the upregulation of RhoA activity while pressure enhanced the expression of chondrogenic marker genes in BMSCs during chondrogenic differentiation *via* the upregulation of Rac1 activity. This finding is corroborated by other studies that showed that the inhibition of RhoA-ROCK activity induced rounded cell morphologies and promoted the expression of chondrogenic marker genes in BMSCs (Woods *et al*., 2005; Woods and Beier, 2006) (Fig. 2**C**).

Primary cilium mediated signaling

Another possible mechanism by which mechanical stimuli may exert their positive role during *in vitro* chondrogenesis is *via* the primary cilium. Ultrastructural studies have confirmed that mature chondrocytes possess an immotile primary cilium that has a microtubule axoneme core made up of a ring of nine microtubule doublets, but devoid of the central pair of microtubules (thus designated as 9 + 0) that motile cilia always have (called a 9 + 2 axoneme) (Muhammad *et al*., 2012). Interestingly, Wann and Knight (2012) found that primary chondrocytes treated with IL-1 exhibited a 50 % increase in cilia length after 3 h exposure and this elongation occurred *via* a PKA-dependent mechanism; inhibition of IL-1-induced cilia elongation by PKA inhibition also attenuated the chemokine response. Primary cilia have been well documented to serve as a nexus that integrates chemical and mechanical signals. In particular, they are considered as mechanosensors of fluid flow in many cell types including human MSCs, arthritic chondroprogenitor cells (Muhammad *et al*., 2012), and chondrocytes where they are unique sensory organelles that project into the pericellular matrix and interact with their close environment (*i.e.* types II and IV collagen) *via* integrins, G proteins and various Ca^{2+} channels, many of which have been implicated as mechanoreceptors (Wann *et al*., 2012). Studies show that the canonical Wnt/β-catenin signaling pathway is closely related to the primary cilia. Glycogen synthase kinase 3 (GSK3) regulates the assembly and maintenance of flagella in *Chlamydomonas reinhardtii* (Wilson and Lefebvre, 2004). Corbit *et al*. (2008) demonstrated that the primary cilium restricts the activity of the canonical Wnt pathway in mouse embryos, primary fibroblasts, and embryonic stem cells. Meanwhile, the primary cilium may act as a detour to the nucleus, thereby segregating cytosolic components to keep the Wnt pathway in check (Lancaster *et al*., 2011) (Fig. 2**D**).

Concluding remarks

The prevalence and severity of articular cartilage degeneration have stimulated potential methods of cartilage regeneration inspired by biomechanical approaches. Although multiple mechanical factors such as compression, hydrostatic pressure, and shear force have been introduced in cartilage engineering, the traditional method often produces fibrocartilage rather than hyaline cartilage. It appears that mechanotransduction is not an isolated event but rather the integration of a number of concurrent signals and pathways. For example, compression could cause osmotic pressure changes and this change might activate the tension-sensitive protein pathways in which the calcium signaling pathways might also be involved. Influenced by internal or external forces, the cytoskeletal changes might be reorganized and re-arranged to change the overall configuration of cell structure, which might trigger a chondrogenesis-related signaling pathway. Future prospects for cartilage engineering should focus more on optimization of cell niches such as adjusting ECM matrix stiffness to activate cell signals toward chondrogenic differentiation and introduction of multiaxial external forces such as superimposing shear upon compression. The differentiated phenotype of the chondrocytes was stabilized in a certain elasticity range (Schuh *et al.*, 2010) indicating that chondrocytes sense the elasticity of the matrix that might be used for the design of scaffolds with mechanical properties specifically tailored to support the chondrogenic phenotype in tissue engineering applications. For external forces, future work is required to identify which type of mechanical stimuli or combinations are the most appropriate as well as the load magnitude, duration, and frequency, and when to initiate the loading during culture (O'Conor *et al*., 2013). By doing so, stem cells could respond to a changing environment in a desirable mechanotransductive way, together with many other potent

growth factors to regulate stem cell chondrogenic fate. Biomechanics research from tissue to molecular levels has driven recent developments in musculoskeletal medicine and will continue to be at the frontier of cartilage biology and regeneration.

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Editor's Note: All questions/comments by the reviewers were answered by text changes. There is hence no Discussion with Reviewers section. Scientific Editor in charge of the paper: Martin Stoddart.