Mechanical regulation of chondrogenesis
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Abstract
Mechanical factors play a crucial role in the development of articular cartilage in vivo. In this regard, tissue engineers have sought to leverage native mechanotransduction pathways to enhance in vitro stem cell-based cartilage repair strategies. However, a thorough understanding of how individual mechanical factors influence stem cell fate is needed to predictably and effectively utilize this strategy of mechanically-induced chondrogenesis. This article summarizes some of the latest findings on mechanically stimulated chondrogenesis, highlighting several new areas of interest, such as the effects of mechanical stimulation on matrix maintenance and terminal differentiation, as well as the use of multifactorial bioreactors. Additionally, the roles of individual biophysical factors, such as hydrostatic or osmotic pressure, are examined in light of their potential to induce mesenchymal stem cell chondrogenesis. An improved understanding of biomechanically-driven tissue development and maturation of stem cell-based cartilage replacements will hopefully lead to the development of cell-based therapies for cartilage degeneration and disease.

Keywords: Osteoarthritis, Cartilage defect, Scaffold, Tissue engineering, Mesenchymal stem cell, Adipose stem cell, Regeneration, Synovial joint, Ion channel, Mechanical signal transduction

Introduction
Musculoskeletal tissues are highly sensitive to their mechanical environment, allowing them to adapt to the physical demands of their environment. Mechanical factors can influence the structure and function of these tissues at all stages of life, including development, growth, remodeling, injury and disease, and repair [1-4]. In particular, chondrocytes and chondroprogenitors have been shown to transduce and respond to a wide array of mechanical stimuli both during development as well as throughout adulthood, including deformation, shear, fluid flow, streaming potentials, hydrostatic pressure, and osmotic pressure [1,2,5-7].

Similarly, there is growing interest in understanding the mechanobiology of multipotent stem cells, which are abundant, expandable, and available from various tissue depots including bone marrow, fat, and synovium [8-10]. The cells are capable of chondrogenic differentiation, and provide a potential cell source for the regeneration and replacement of damaged articular cartilage resulting from injury or diseases such as osteoarthritis [11-14]. However, an effective cell-based tissue replacement requires a stably differentiated cell population capable of producing and maintaining a functional neo-tissue. There is great interest in leveraging these native mechanical and biophysical cues to enhance the current strategies for stem cell-based cartilage tissue repair. This review summarizes recent progress on the use of mechanical and biophysical signals to influence the chondrogenic differentiation of stem cell-based cartilage replacements. Furthermore, we discuss several candidate transduction mechanisms that may play a role in the process of biomechanically-induced chondrogenesis, in the hope that a better understanding of the pathways behind the mechanosensitivity of stem cells will lead to more practical, reliable, and effective methods of leveraging these pathways for cartilage tissue engineering [15].

Mechanical stimulation of chondrogenesis
The current literature on in vitro mechanical stimulation of chondrogenesis encompasses a broad variety of scaffolds, cell types, and bioreactors, making it challenging to draw definitive conclusions across studies. This section focuses primarily on the most common model system of dynamic compressive loading of bone marrow-derived mesenchymal stem cells (MSCs) supported by synthetic foam and/or hydrogel scaffolds (summarized in Table 1). Of course,
future comparative analyses of studies involving other bioreactors and scaffold systems will probably be needed to further understand the mechanisms behind mechanical signals and chondrogenesis. Furthermore, investigations into the mechanical responses of stem cells sourced from other tissues of interest (adipose, synovium) will also be necessary to move them towards clinical applications, as it is currently unclear whether these stem cells utilize the same mechanisms of mechanical signal transduction as bone marrow-derived MSCs.

Mechanical stimulation can directly influence the fate of undifferentiated stem cells [1-3,5-7,26]. Dynamic compressive loading, specifically cyclic unconfined compression, has been one of the most highly utilized model systems of mechanical stimulation in cartilage tissue engineering and mechanobiology [7]. This system has also been used to investigate the potential of mechanical stimulation for use in MSC-based cartilage regeneration and repair; and the results of these studies can be summarized by four main findings: growth factor treatment is a more potent stimulus than mechanical stimulation for initiating MSC differentiation; provided there is a period of predifferentiation and other specific loading parameters, mechanical stimulation can be effective at enhancing growth-factor induced MSC differentiation and tissue neo-formation; the mechanoresponsiveness of differentiated MSC constructs appears to persist over time to direct matrix remodeling and maintain a stable chondrogenic phenotype; and bioreactors that impart multifactorial mechanical stimulation, such as compression–shear loading, further enhance mechanically-induced chondrogenesis.

**Table 1** Summary of recent dynamic compressive loading studies evaluating chondrogenesis in mesenchymal stem cells

| Bioreactor | Compressive loading regimen | Scaffold | Species (age) | Medium supplementation | Preculture (days) | Reference |
|------------|-----------------------------|---------|---------------|-----------------------|-------------------|-----------|
| Compression | 1 Hz, 10%, 1 to 20 hours    | Agarose | Bovine        | ± Dex ± TGFβ1      | 8 or 16          | [16]      |
| Compression | 0.33 to 3 Hz, 10%, 1 to 3 hours | Agarose | Bovine (3 to 6 months) | Dex ± TGFβ3 | 3          | [17]      |
| Compression (semipermeable) | 0.3 Hz, 7.5%, 45 minutes on/ 45 minutes rest | Agarose | Horse (2 to 5 years) | Dex ± TGFβ1 | 0          | [18]      |
| Compression | 0.01 to 1 Hz, 10%, 1 to 4 hour/day | Agarose | Bovine (3 to 6 months) | Dex ± TGFβ3 | 0 or 21    | [19]      |
| Compression + shear | 0.1 to 1 Hz, 5 to 20%, 1 hour/day | Fibrin/PU | Human | Dex ± TGFβ1 | 7          | [20]      |
| Compression | 1 Hz, 10%, 1 hour/day         | Agarose | Porcine (4 months) | Dex ± TGFβ3 | 0 or 21    | [21]      |
| Compression + shear | 1 Hz, 10% to 20%, 1 hour/day | Fibrin/PU | Human | None | 2 to 4     | [22]      |
| Compression | 1 Hz, 10%, 1 hour             | Agarose | Porcine (4 months) | Dex + TGFβ3 | 0, 7, 14, 21 | [23]      |
| Compression + shear | 0.1 Hz, 5 to 20%, 3 hours/day | Agarose | Bovine (3 to 6 months) | Dex ± TGFβ3 | 21         | [24]      |
| Compression | 1 Hz, 10%, 4 hours/day         | Hyaluronic acid | Human | Dex + TGFβ1 | 3          | [25]      |

Dex, dexamethasone; PU, polyurethane; TGFβ, transforming growth factor beta.
free loading of MSC-laden constructs. Loading of chondrocyte-laden disks in the presence of low levels (0.2 and 2%) of fetal bovine serum produced no change in sGAG production, but decreased functional properties of the constructs [31]. In contrast, a large positive effect on matrix accumulation and functional properties was observed in another study with chondrocyte-laden constructs loaded in serum-free conditions [30]. Interestingly, both bovine MSCs and chondrocytes embedded in agarose and precultured for 3 days in growth-factor-free medium were able to respond to dynamic loading with an increase in aggrecan promoter activity [17], suggesting that a similar mechanism of mechanotransduction in chondrocytes may be present, to some degree, in undifferentiated stem cells. Nevertheless, in the case of MSCs, dynamic compressive loading alone appears to be insufficient for inducing appreciable differentiation and matrix production in the absence of growth-factor stimulation, and therefore is not, as yet, a suitable substitute for growth-factor-induced stem cell differentiation.

Culture conditions that support mechanically-induced chondrogenesis

Dynamic loading in the presence of growth factors can significantly enhance MSC chondrogenic differentiation, particularly if a chondrogenic preculture period is provided. For example, while loading of MSCs after 8 days of preculture in TGFβ and dexamethasone-supplemented medium did not increase aggrecan or Col2α1 gene expression or sGAG or protein synthesis, loading after an additional 8 days (16 days in total) of preculture increased all of these measures [16]. Of note, the effects of loading were more limited when dexamethasone was not added. By preculturing constructs for 0, 7, 14, and 21 days in the presence of TGFβ and dexamethasone and then assessing the gene response immediate following 1 hour of loading, Haugh and colleagues observed that generally later time points (7 and 21 days) of preculture resulted in the greatest relative increases in core aggrecan and Col2α1 expression compared with unloaded controls [23]. Likewise, the annular cell population also demonstrated a dependence on 14 or 21 days of preculture to exhibit a loading-induced enhancement of Col2α1 and aggrecan expression. In a similarly designed study, loading was initiated both immediately following construct creation as well as after 3 weeks of preculture, all in the presence of TGFβ and dexamethasone. While continuous loading elicited negative effects on DNA, sGAG, and collagen content, 3 weeks of chondrogenic preculture completely abrogated these negative effects, as well as leading to improvements in functional properties and extracellular matrix distribution (Figure 1) [19]. Yet another study observed similar detrimental effects of loading without preculture on sGAG content and dynamic

Figure 1 Delayed dynamic compressive loading improves mechanical properties and extracellular matrix distribution without increasing biochemical content. Following 3 weeks of chondrogenic preculture, dynamic compressive loading was applied daily to human mesenchymal stem cell (MSC)–agarose constructs for 3 weeks. (A) The equilibrium modulus of MSC-seeded constructs was higher in medium containing transforming growth factor beta (TGFβ; CM+) compared with culture without TGFβ (CM−) at 3 and 6 weeks; dynamic loading (DL) in CM+ for 3 weeks further improved mechanical properties. (B) Biochemical content of dynamically loaded constructs at week 6 was not different compared with CM+ controls. (C) to (E) Alcian Blue staining at week 6 showed equal distribution of proteoglycans between CM+ controls and loaded constructs with weak staining in CM− controls. (F) to (H) Picrosirius Red staining and (I) to (K) collagen type II immunostaining showed more homogeneous distribution of collagen in loaded constructs compared with controls. Scale bar: 100 μm. *Greater than CM− controls (P <0.05). **Greater than CM+ controls (P <0.05). FS, free swelling. Reproduced from [19] with kind permission from eCM journal [32].
modulus, as well the attenuation of this negative effect with delayed loading [21].

Beyond the application of growth factors and the duration of preculture, the response of MSCs to dynamic loading has been found dependent on a number of other factors as well, including duty cycle and loading frequency. An extended loading regime totaling 12 hours of loading per day reduced sulfate and protein incorporation [18]. A separate study observed no effect of 20 hours of continuous loading on sGAG or protein synthesis after an 8-day preculture [16]. Interestingly, significant increases in these measures were observed after 16 days of chondrogenic preculture, indicating a dependence on differentiation status and construct maturation on the response to mechanical factors. The frequency of dynamic loading is also a critical parameter that may influence chondrogenic responses. For example, increases in the functional properties of MSC-laden constructs were observed with 1 Hz dynamic compressive loading, but were absent at lower frequencies of mechanical stimulation [19]. Given that chondrocytes also exhibit a high sensitivity to duty cycle and loading frequency [33,34], further optimization of loading parameters may be able to improve mechanically-driven MSC chondrogenesis and matrix accumulation.

**Mechanical regulation of neo-cartilage maintenance and turnover**

While current methods of mechanical stimulation do not appear to have an especially large effect on bulk matrix accumulation during growth factor-induced maturation of MSC-laden constructs, there have been stronger indications for the importance of mechanical signals on MSC-based neo-cartilage maintenance. Normal matrix metabolism, such as aggrecan turnover [35] and collagen reorganization and remodeling in response to loading, has probably been an underappreciated metric for cartilage tissue engineering. For example, a gene array analysis found that 413 genes associated with chondrogenesis were upregulated, versus 139 that were downregulated, with 3 weeks of dynamic loading of MSC constructs that had been precultured for 3 weeks [19]. Furthermore, mechanical loading differentially regulated genes specifically involved in matrix remodeling and organization, such as matrix metalloproteinases, tissue inhibitors of metalloproteinases, and cross-linking proteins, suggesting that this coordinated response may have led to the enhanced pericellular and extracellular matrix distribution and organization, as well as the improved functional properties, observed in the loaded constructs [19]. Again, we can compare this matrix remodeling response of MSCs with the native response of chondrocytes undergoing delayed, long-term loading, where functional property increases are elicited with delayed loading in the absence of any changes in bulk modulus, as well the attenuation of this negative effect with delayed loading [21].

**Mechanical stimulation and the maintenance of chondrogenesis**

Another critical issue of long-term MSC culture is the ossification and hypertrophic differentiation of chondrogenically-induced MSC constructs upon implantation [37]. Establishing a stable, articular chondrocytic phenotype is therefore another critical design goal of cartilage tissue engineering. Col1 gene expression is one marker of hypertrophic or osteogenic differentiation, and has been examined in response to loading [23,25]. For example, the accumulation of Col1 in the annulus of constructs was found to decrease with loading after 21 days of preculture [23], while other studies showed that dynamic compressive loading suppressed a number of other hypertrophic markers such as collagen type 10, matrix metalloproteinase-13, and alkaline phosphatase gene expression, as well as the calcium content of constructs exposed to hypertrophic factors [25]. Few studies have examined the effects of loading on terminal differentiation of chondrogenically-induced MSCs, so these preliminary findings will need to be followed up with more comprehensive studies.

**Multimodal bioreactors for enhancing mechanically-induced chondrogenesis**

Mechanical loading of the joint produces a complex environment in articular cartilage in vivo, consisting of a diverse array of tensile, shear, and compressive stresses and strains, in addition to other physicochemical effects [7,38]. Accordingly, bioreactors that impose additional components of mechanical stimulation have been examined for their potential to further induce and support chondrogenic differentiation. Adding a component of shear to compressive loading has been shown to be superior to single-factor loading regimens at inducing matrix biosynthesis in chondrocytes [39], and this multifactorial strategy has more recently been applied to the study of MSC chondrogenesis. For example, a compression–shear bioreactor consisting of a rotating ceramic ball in contact with the surface of a construct that also transverses perpendicular to the surface was shown to enhance both Col2a1 and aggrecan expression in the absence of exogenous growth factors, and also increased sGAG production, with or without exogenous TGFβ.
[20]. In addition, endogenous production of TGF-β was induced by this compression–shear loading in the absence of exogenous growth factors. Furthermore, a synergistic effect of compression and shear on Sox9 and Col2α1 upregulation was observed (Figure 2) when this system was used to compare the effects of compression–shear with each component individually [22].

In other studies, another compression plus shear bioreactor, utilizing a spherical indenter that travels across the surface of the construct, enhanced Col2α1 and aggregan expression, in the presence or absence of TGF-β, after 21-day chondrogenic preculture [24]. Furthermore, 21 days of additional slide-contact loading led to increases in sGAG and collagen content, a more uniform collagen distribution, and enhanced tensile properties. In addition, loaded constructs demonstrated a depth-dependent distribution of sGAG. In addition to their ability to promote anisotropy that is more similar to that of native cartilage, multifactorial bioreactors will help to further elucidate the roles of different mechanical stimuli, as well as their interactions. These systems also move a step closer to recreating the in vivo physical environment, and therefore may provide a system for predicting how cartilage tissue replacements will perform within a joint.

Mechanisms of mechanically-induced chondrogenesis

Physical transduction of mechanical loading

There are obvious practical limitations to mechanically conditioning anatomically-shaped tissue replacements, including the spatial constraints and inhomogeneity of the stress and strain fields. Furthermore, these inhomogeneities can also act to obscure the detailed understanding of the cellular effects on mechanical signals. As such, there is a growing interest in studying the individual biophysical components present during deformational loading, which practically are much simpler to deliver in a repeatable and uniform manner. A thorough understanding of the mechanisms by which individual components of mechanical stimulation leads to cartilage development, maintenance, and disease should also allow for more practical and predictable strategies for enhancing the maturation of stem cell-based cartilage replacement tissues.

Joint loading leads to complex tissue strains, including components of compression, tension, and shear, producing direct cellular and nuclear deformation [40]. In addition, indirect biophysical factors are also generated (Figure 3) as a result of the exudation of interstitial water and ions from cartilage, including streaming potentials, changes in local pH and osmolarity, and hydrostatic pressure [38]. While application of dynamic compression to isolated chondrocytes or MSCs seeded into hydrogels or polymeric scaffolds will recapitulate many of these biophysical changes that occur in native cartilage, it is important to appreciate that the amount of extracellular matrix relative to the original scaffold or hydrogel present within the constructs, as well as the mechanical properties of these scaffolds, will influence the range of biophysical stimuli generated by loading (for example [41]). A number of studies have examined the response of chondrocytes and MSCs to individual biophysical stimuli generated during loading of intact articular cartilage. In this respect, future studies comparing donor-matched responses among chondrocytes, undifferentiated MSCs, and differentiated MSCs to these stimuli would be highly useful in further elucidating the mechanisms involved in mechanotransduction in different cell types.

Figure 2 Mechanical loading using a multimodal bioreactor enhances mesenchymal stem cell chondrogenesis. (A) The bioreactor can apply both compression and shear to the cell-seeded construct through rotation of the ceramic hip ball in contact with the surface of the construct and through vertical movement of the ball perpendicular to the construct surface. Relative (B) Sox9 and (C) Col2 mRNA expression of human mesenchymal stem cells after culture for 21 days in fibrin/polyurethane constructs without exogenous growth factors. Although either compression or shear loading alone increased these chondrogenic markers above free swelling levels, the combination of shear and compression loading further enhanced the response. *P < 0.05, **P < 0.01, ***P < 0.001. Reproduced from [22] with kind permission from eCM journal [32].
Cell deformation

Mechanical loading of hydrogel scaffolds results in the transmission of strains to cells embedded within such constructs [28,42]. The relationship between ECM and cell-level strains in agarose-laden chondrocytes is also comparable with that of in situ chondrocytes in loaded cartilage explants [43] once a pericellular matrix has been elaborated. Lee and Bader observed that a 20% strain of day 0 chondrocyte-laden agarose constructs led to supraphysiological cell strains in chondrocytes cultured with or without serum, but that after 3 and 6 days of preculture the constructs cultured specifically with serum exhibited reduced cell strains, which were more similar with what occurs in situ [28]. They attributed this finding to enhanced pericellular matrix accumulation in the constructs cultured with serum, which was associated with increased pericellular sGAG staining with preculture. The requirement of a preculture period as well as growth factor treatment to facilitate loading-induced enhancement of MSC chondrogenesis suggests that the pericellular matrix is also an important transducer of deformational compressive loading in stem cells.

Dynamic compressive loading of MSCs in agarose and similarly compliant scaffolds would also be expected to induce cellular and nuclear deformation, and further investigation into the mechanical properties of stem cells, stem cell nuclei, and the neo-pericellular matrix prior to,
during, and after stem cell differentiation may help elucidate the roles of cell and nuclear deformation in the transduction of compressive loading. Unconfined compression also allows for radial expansion, and generates a degree of radial and circumferential tensile strains. Tensile loading also modulates stem cell differentiation, although this loading regime in isolation appears to stimulate a fibrochondrocyte phenotype [44-46].

**Hydrostatic pressure**

While peak physiologic levels of fluid pressurization in articular cartilage are in the order of 10 to 20 MPa [47,48], compression of agarose hydrogels is predicted to generate only around 0.5 kPa of hydrostatic pressure, due to the scaffold’s high porosity [17]. With sufficient matrix elaboration, however, the fluid pressurization of agarose cylinders subjected to dynamic compressive loading [49] is predicted to resemble the increase in native loaded cartilage [50]. Nonetheless, fluid pressurization, even at high levels, is expected to induce little or no deformation of tissues and cells, due to the intrinsic incompressibility of water and the cartilage extracellular matrix [51]. To evaluate fluid pressurization as an isolated mechanical stimulus, in vitro bioreactors have been developed that directly pressurize the culture medium surrounding cell-seeded constructs, in the absence of cell or tissue deformation [6]. Alternatively, bioreactors can pressurize the gas phase above the culture medium, but one limitation of these types of bioreactors is that the partial pressures of gas in the culture medium are affected, and these bioreactors are less commonly used. Studies using systems that directly pressurize the culture medium have shown that isolated chondrocytes are responsive to hydrostatic pressure. Acute application of static hydrostatic pressure at 5 MPa for 4 hours enhanced Col2α1 and aggrecan expression by chondrocytes in agarose gels [52], while application of both dynamic and static hydrostatic pressure at 10 MPa to scaffoldless chondrocyte constructs for 1 hour/day on days 10 to 14 of culture led to increased sGAG production and compressive stiffness at day 28 [53].

Applying dynamic hydrostatic pressurization (3 to 10 MPa, 1 Hz) to human MSCs either seeded within scaffolds or in pellet culture in the presence of TGFβ increased expression of cartilage extracellular matrix genes and enhanced biochemical content compared with TGFβ alone [54-56]. In these studies, hydrostatic pressure was applied by directly pressurizing the culture medium for 1 to 4 hours/day beginning in the first week of culture, indicating that the MSC response to hydrostatic loading does not require a preculture period. Miyanishi and colleagues examined the dose dependency of hydrostatic loading with TGFβ supplementation, and found that while 0.1 MPa was sufficient to increase Sox9 expression, upregulation of Col2α1 expression only occurred with loading at 10 MPa [57]. Hydrostatic pressure also transiently increased cartilage-associated genes in the absence of TGFβ [55,58,59]. Recent studies with rat MSCs cultured in alginate applied hydrostatic pressure following an initial 8-day preculture in chondrogenic medium including TGFβ. Dynamic hydrostatic pressure applied by pressurization of the gas phase above the culture medium for 7 days at 13 to 36 kPa and 0.25 Hz – parameters lower than in previous studies – increased expression of Col2α1 and aggrecan, as well as sGAG accumulation, both in the absence and presence of TGFβ [60,61]. Furthermore, in the absence of exogenous TGFβ, hydrostatic pressure increased expression and secretion of TGFβ1, as well as the phosphorylation of Smad2/3 and p38 mitogen-activated protein kinase. However, pharmacologic inhibition of TGFβ signaling only modestly reduced the upregulation of Col2α1 by loading and had no influence on the upregulation of aggrecan by loading, suggesting the involvement of other signaling pathways in mediating the response to hydrostatic pressure [61].

**Osmotic pressure**

Healthy articular cartilage has an interstitial osmolarity ranging from 350 to 450 mOsm due to the high concentration of negatively charged proteoglycans in the tissue, which attracts counterions [62]. Extracellular matrix production by articular chondrocytes has been shown to be sensitive to the medium osmolarity. Culture for 48 hours in 550 mOsm medium increased sGAG synthesis by chondrocytes in alginate beads relative to culture in 380 mOsm medium, while culture in 270 mOsm medium decreased sGAG synthesis [63]. Chondrocytes cultured in medium at 370 mOsm for 6 days exhibited the greatest sGAG accumulation and sGAG synthesis by chondrocytes in alginate compared with culture in medium with either higher or lower osmolarity [64]. Recent longer-term studies have indicated that neo-tissue formation by articular chondrocytes in hydrogel systems is influenced by osmolarity of the culture medium, but the results have been contradictory [65,66]. Freshly isolated chondrocytes in alginate accumulated less sGAG at 270 mOsm compared with osmolarities ranging from 380 to 550 mOsm [65], while culture-expanded chondrocytes produced neo-tissue with superior mechanical properties when cultured in agarose at 300 mOsm compared with 400 mOsm [66].

Effects of osmolarity on extracellular matrix 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 (550 mOsm vs. 380 mOsm) 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 [67]. However,
the level of Col2α1 mRNA and its half-life were decreased by exposure to hyperosmotic conditions. Hyperosmotic medium also increased phosphorylation of p38 mitogen-activated protein kinase, and induction of Sox9 mRNA by hyperosmotic treatment was disrupted in the presence of a pharmacologic inhibitor to p38 mitogen-activated protein kinase. A similar study in equine articular chondrocytes showed that hyperosmotic treatment had varying effects on Sox9 mRNA levels dependent on whether treatment was applied in a static or cyclic manner and whether chondrocytes were from normal or osteoarthritic cartilage [68].

In these studies with isolated chondrocytes, the osmolarity of the culture medium was kept constant. However, articular chondrocytes in situ are exposed to cyclic changes in osmolarity due to joint loading and unloading during normal daily activity. Compression of articular cartilage causes extrusion of water relative to solutes due to fixed charges on the sulfated GAG chains, which leads to an increase in tissue osmolarity. High-frequency loading, such as walking, as well as prolonged joint loading resulting in diurnal strains [69], will produce a dynamic osmotic environment on the time scale ranging from seconds to hours. Similar to hydrostatic pressure, the osmotic changes in chondrocyte and MSC-laden constructs in response to dynamic compressive loading should be minimal initially, but should increase with sGAG accumulation. Although there is evidence that dynamic hypotonic loading at 0.1 Hz may enhance cartilage matrix gene expression in chondrocytes in monolayer culture after 2 hours of loading [70], little is known regarding the long-term effects of dynamic or repetitive daily osmotic loading on neo-tissue matrix content and mechanical properties.

Growth and chondrogenic differentiation of MSCs are also influenced by culture medium osmolarity. High-osmolarity medium (485 mOsm) reduced proliferation of both rat MSCs and human adipose-derived stem cells [71,72]. Increasing the osmolarity of chondrogenic differentiation medium containing TGFβ by 100 mOsm enhanced Sox9, Col2α1, and aggrecan expression, as well as expression of the hypertrophic chondrocyte markers Col10 and Runx2, in day 21 monolayer cultures of human MSCs [73]. Whether osmolarity influences matrix accumulation or functional properties of MSC-laden constructs remains to be investigated.

**Fluid flow**

Mechanical loading of the cartilage layer results in large gradients in hydrostatic pressure, which subsequently induce flow of the interstitial fluid within the extracellular matrix. One way that mechanical loading is predicted to enhance tissue maturation is through this flow-mediated nutrient and growth factor exchange, as well as through physical activation of growth factors [74]. Loading may also influence tissue maturation through direct transduction of fluid shear stress across the cellular membrane. Fluid flow in response to joint loading is complex and challenging to recapitulate for isolated chondrocytes in three-dimensional cultures. However, controlled medium flow has been used to culture chondrocyte-seeded constructs, as culture with dynamic fluid flow provides several advantages over static culture including enhanced mass transport, a more controlled biochemical environment, and the application of hydrodynamic stimuli. Perfusion and rotating wall bioreactors have been shown to enhance extracellular matrix accumulation by chondrocytes seeded in porous polymeric scaffolds [75-77]. Perfusion bioreactors have similarly been found to enhance the biochemical content of MSC-seeded constructs grown in chondrogenic medium including TGFβ [78,79]. In another study, culture in an oscillating bioreactor that delivered slow, directional perfusion to MSC-woven poly(ε-caprolactone) constructs improved functional properties, increased type II collagen content, and supported more homogeneous matrix deposition (Figure 4) [80]. These studies indicate the importance of fluid flow on construct maturation, although it remains unclear whether flow-induced nutrient transport, growth factor activation, and/or direct cellular sensing are influencing cellular behavior.

**Molecular mechanisms of transduction**

The molecular mechanisms of mechanical signal transduction in chondrocytes and MSCs are not fully understood and represent an area of growing investigation. Ion channels, the primary cilium, the nucleus, and the cytoskeleton have all been proposed as candidate mechanical signal transducers in articular chondrocytes (Figure 3). Ion channels in chondrocytes include potassium channels, sodium channels, transient receptor potential (TRP) nonselective cation channels, and chloride channels [81]. Various ion channels in chondrocytes appear to be regulated by changes in osmolarity, as well as different forms of mechanical stimulation. For example, the TRPV4 ion channel has been identified as the major sensor of osmolarity in chondrocytes [82], and activation of this channel leads to an influx of calcium ions. Calcium signaling has also been observed in chondrocytes in response to hydrostatic pressurization [83] and compressive loading [84]. Changes in intracellular calcium downstream of ATP secretion and binding to purinergic receptors have also been proposed as a mechanical signaling pathway in chondrocytes [85]. Calcium signaling has been linked to the propagation of mechanical effects on gene expression in cartilage explants [86]. Treatment with nifedipine, a calcium channel inhibitor, or 4-aminopyridine, a potassium channel inhibitor, disrupted mechanical stimulation of sGAG synthesis by chondrocytes in agarose, suggesting possible roles for calcium and potassium signaling in mediating this loading
A recent study showed that altering the intracellular sodium and calcium concentrations using the pharmacologic agents oubain and ionomycin for 1 hour daily on days 10 to 14 of culture increased the tensile modulus of neo-tissue produced by chondrocytes from young bovine donors in a scaffoldless culture system at 4 weeks of culture, providing evidence that ion channel regulation can also influence functional properties of neo-cartilage [88].

The primary cilium is a nonmotile organelle that extends from the cell surface and has been implicated in both sensory and signaling functions in a variety of cells [89]. Primary cilia were identified on articular chondrocytes more than three decades ago [90,91], and recent work indicates that the primary cilium may have an important role in chondrocyte mechanotransduction [92,93]. Primary cilia on sternal chondrocytes were shown to have α2, α3, and β1...
integrins on their surface [94], allowing a direct linkage between the cilium and collagens in the surrounding pericellular matrix. As such, tissue compression during joint loading could lead to deformation of the cilium. Ion channels, including TRPV4, are also found on primary cilia. Interestingly, chemical disruption of the primary cilium on articular chondrocytes blocked the increase in intracellular calcium caused by exposure to hypo-osmotic stress or a TRPV4 channel agonist [82]. Acute compressive loading of sternal chondrocyte–agarose disks has been shown to induce an increase in calcium signaling, upregulation of aggrecan expression, and higher sGAG accumulation; these loading effects were absent in IFT88 (orpk) mutant chondrocytes that lack primary cilium [95]. Together, these studies suggest that the primary cilium may contribute in multiple and complex ways to mechanical signal transduction in chondrocytes, and further investigations are needed to clarify the contributions of this proposed mechanosensory organelle.

Deformation of the nucleus in chondrocytes may be important in propagating the cellular response to biophysical stimuli [96]. The connections between the extracellular matrix, integrins, cytoskeleton, LINC complex, and nuclear lamina allow for direct transmission of biophysical forces from the cell exterior to the nucleus and potentially to subnuclear structures. The nucleus in chondrocytes deforms in response to compression of articular cartilage explants [40] and chondrocyte/agarose constructs [97]. Application of osmotic stress to chondrocytes also influences nuclear volume and structure [98], with changes in the nucleus probably reflecting alterations in intracellular macromolecular concentrations [99]. Studies are needed to define how these direct pathways by which biophysical stimuli influence the nucleus contribute to regulation of gene expression by mechanical loading in chondrocytes and stem cells.

The cytoskeleton in articular chondrocytes is primarily composed of actin microfilaments, microtubules, and vimentin intermediate filaments [100]. Disruption of actin microfilaments with cytochalasin D was found to decrease viscoelastic mechanical properties of chondrocytes [101] and to alter chondrocyte nuclear deformation in response to compression of cartilage explants [40]. The actin cytoskeleton in articular chondrocytes has also been shown to undergo reorganization with osmotic stress [70,102], as well as compressive loading and hydrostatic pressure [103]. These studies suggest that the cytoskeleton is involved in the response of chondrocytes to mechanical loading, yet studies directly implicating the cytoskeleton are lacking. Prior work has shown that integrins are involved in responses of chondrocyte–hydrogel constructs to dynamic compressive loading [104,105]. A recent study demonstrated that, when chondrocytes were suspended in agarose and pretreated with a blocking antibody for αv or β1 integrin, the increases in sGAG synthesis and sGAG accumulation induced by 24 hours of dynamic compression were disrupted [106]. Linkages between integrins and cytoskeletal components are thought to be integral to mechanotransduction in various cell types [107], but such linkages in chondrocytes have not been well defined. How early signaling events downstream of changes in ion channels, the primary cilium, the nucleus, and the cytoskeleton are propagated into changes in gene expression and matrix synthesis that support chondrogenic differentiation and neo-tissue formation remain open questions for future investigations.

**Conclusion**

Mechanically-generated signals appear to play a critical role in the differentiation and maturation of MSCs into a chondrogenic phenotype. Compressive deformational loading of MSC-laden constructs produces a pro-chondrogenic and biosynthetic response that is advantageous for developing MSC-based neo-tissues for cartilage regeneration and repair, and this system can also be used as a model to better understand the mechanisms of MSC mechanotransduction. Use of more advanced bioreactors, such as those that also incorporate shear and other components of loading, further enhances the chondrogenic response of MSCs to mechanical loading, and better mimics the *in vivo* environment in which these cartilage neo-tissues are designed to reside. Knowledge about the mechanisms that transduce macroscopic mechanical forces into intracellular events is increasing with respect to both chondrocytes and chondrogenically-induced MSCs. Further delineations about these mechanisms will probably lead to controllable strategies for rapid and effective preconditioning of anatomically shaped MSC-based cartilage replacements.

**Note:** This article is part of a thematic series on Physical influences on stem cells edited by Gordana Vunjak-Novakovic. Other articles in the series can be found online at http://stemcellres.com/series/physical.

**Abbreviations**

MSC: Mesenchymal stem cell; sGAG: Sulfated glycosaminoglycan; TGFβ: Transforming growth factor beta; TRP: Transient receptor potential.

**Competing interests**

The authors declare that they have no competing interests.
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