Review

Regenerative Medicine

Critical review on the physical and mechanical factors involved in tissue engineering of cartilage

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Articular cartilage defects often progress to osteoarthritis, which negatively impacts quality of life for millions of people worldwide and leads to high healthcare expenditures. Tissue engineering approaches to osteoarthritis have concentrated on proliferation and differentiation of stem cells by activation and suppression of signaling pathways, and by using a variety of scaffolding techniques. Recent studies indicate a key role of environmental factors in the differentiation of mesenchymal stem cells to mature cartilage-producing chondrocytes. Therapeutic approaches that consider environmental regulation could optimize chondrogenesis protocols for regeneration of articular cartilage. This review focuses on the effect of scaffold structure and composition, mechanical stress and hypoxia in modulating mesenchymal stem cell fate and the current use of these environmental factors in tissue engineering research.

Keywords: articular cartilage osteoarthritis • chondrogenesis • differentiation • mesenchymal stem cells • tissue engineering

Osteoarthritis represents the most common form of over 100 types of arthritis and affects more than 124 million people worldwide. Damage from trauma, infection, autoimmune disorders or age produces inflammation in articular cartilage (AC), which worsens over time causing pain, swelling of joints and diminished range of motion. Because it impairs function, it creates a significant burden on society. The impact stems from the cost of radiographic diagnosis, palliative treatment, surgical procedures, loss of productivity and co-morbid diseases. Current treatment of osteoarthritis focuses on alleviating the symptoms. After the onset of osteoarthritis, existing therapies are unable to reverse or prevent the progression of the disease. In advanced cases with severe pain that do not respond to symptomatic treatments, surgical procedures such as autologous chondrocyte implantation (ACI) or joint replacement may be recommended [1,2].

Because the spontaneous repair process of cartilage is temporary and inefficient, defects are often healed by the formation of fibrocartilage that is functionally inferior to the native hyaline cartilage [3]. Chondrogenesis, the processes by which cartilage is formed, is the result of a several steps orchestrated by signaling molecules [4–6], receptors, transcription factors [7], interaction of cells with the matrix [8] and other environmental factors. Mesenchymal stem cells (MSCs) are recruited and condense, beginning to proliferate and differentiate into a chondrogenic phenotype. This is followed by continued differentiation into mature chondrocytes during which they secrete cartilage-specific extracellular matrix (ECM) proteins such as type II collagen and aggrecan. Finally, unwanted terminal differentiation occurs when the chondrocytes become hypertrophic and bone tissue replaces the original cartilage. Control of the process of chondrogenesis is in damaged joints, these activities are not happening efficiently.

Encouraging advances in the field of stem cell research give hope for better options in

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treating cartilage damage. Researchers attempt to bioengineer cartilage for treatment of traumatic lesions where the healthy environment can support regeneration [9,10]. Stem cell or bioactive injectables are being tested for treating the more complicated pathogenesis of osteoarthritis [11–13]. For stem cell therapy to be effective and lasting, the aforementioned variables must be manipulated to achieve ideal conditions. Optimization of chondrogenesis relies on the appropriate combination of the different signals, the timing of those signals, concentration of required factors, mechanical stimulation, the upregulation and downregulation of specific genes through transcription factors and microRNAs [14], and the epigenetic modification of DNA via methylation and acetylation. The obstacle to effective stem cell transplantation therapies lies in getting the cells to differentiate and behave in the desired way, integrating and working with the host's other cells in a way that mimics native tissue. In order for stem cell-based tissue engineering of AC to be successful, a matrix surrounding the cells with the same mechanical and protective properties as native cartilage must be produced. The environment surrounding stem cells has a direct influence on how they differentiate, what signaling factors are utilized and which genes are activated or suppressed. Studies suggest that in addition to signaling molecules, scaffolding properties, mechanical loading and oxygen availability play a key role in the differentiation process [15–22]. This review focuses on how scaffold structure and composition, mechanical stress and modification of oxygen availability modulates the differentiation of MSCs to mature chondrocytes and also how these stimuli affect gene expression. Understanding of these environmental effects and how to control them will help optimize chondrogenesis protocols used in tissue engineering.

Important mechanical properties of cartilage

The difficulty in tissue engineering of cartilage lies in the complexity of the tissue. Although it constitutes a very thin covering on the surface of bones in the synovial joints (2.21 mm in the human knee, for example), AC is quite diverse. It varies in thickness, cell density, matrix composition and mechanical properties within the same joint, different joints and among species [23]. It is typically divided into four zones: superficial zone, transitional zone/middle zone, deep zone and calcified cartilage zone. Chondrocytes within each zone differ in shape, size and orientation relative to the articular surface. They also differ in metabolic activity and respond differently to mechanical loading.

Chondrocyte–matrix interactions are essential for the maintenance of AC throughout life. The matrix protects chondrocytes from mechanical damage and helps them maintain shape and phenotype. Passing through and stored in the matrix are nutrients, substrates, synthesized molecules, degraded molecules, metabolic waste, cytokines and growth factors [24]. The movement of fluid and molecules depends in large part on the presence of large aggregating proteoglycans and how they are organized within the ECM.

AC demonstrates both viscous and elastic behavior when deformed. It affords a smooth, lubricated, low-friction surface during movement and assists in load transmission to the subchondral bone. AC can withstand high cyclic loads with little to no damage or degeneration [25,26]. The combination of frictional resistance to water flow and water pressurization within the matrix provides the two main mechanisms in which AC is able to endure substantial loads, often several times one's bodyweight. Joint motion and load are essential in maintaining proper AC structure, function and metabolism. Inactivity can actually lead to deterioration of cartilage [27].

Aggrecan, the largest and most abundant proteoglycan in AC, occupies the interfibrillar space of cartilage ECM and gives cartilage its osmotic properties – vital to its capacity to oppose compressive loads [27]. In an aqueous environment, proteoglycans have negatively charged sites that repel each other causing it to spread

out and take up more space in the ECM. Volume is limited by the entrapment of the aggregates in the collagen fiber network. When cartilage is compressed, the negatively charged sites on the aggregated proteoglycans are pressed together, which intensifies their repellant force and contributes to the compressive stiffness of cartilage [28].

Mechanical response of AC also depends on flow of fluid through the tissue and across the articular surface. Average surface pore size of AC is 6.0 nm, which allows water, oxygen, glucose and ions to move through, but hinders movement of the larger macromolecules such as hyaluronan and aggrecan. Under rapidly applied loads, there is not enough time for fluid flow and cartilage behaves as a single-phase, incompressible, elastic solid [28]. Under less rapid distortions, fluid can flow out of the AC and then back in once the force is removed. Under constant loads, the viscoelasticity of AC is time-dependent as fluid can initially move through the tissue more quickly followed by a slowing down as the force continues, the tissue stiffens and friction increases until the tissue reaches equilibrium [27]. The most compliant and viscous part of AC, the transitional zone, may be the most critical for dissipating energy and protecting the joint from damage [29]. Regardless of the type of load, fluid pressure is a major factor of total load support ability of AC because it reduces stress on the ECM.

Main components in cartilage tissue engineering

Tissue engineering has become synonymous with regenerative medicine. Broadly described as the manipulation of cells, biochemical factors, physical factors and biological engineering materials, the goal is to maintain, recover or replace biological functions. To repair cartilage defects like those found in osteoarthritis, tissue engineers must first determine and acquire the appropriate cell types for cartilage and bone regeneration. Embryonic stem cells are pluripotent and can form tissues from any of the three germ layers (endoderm, mesoderm and ectoderm). Ethical and safety debates over the use of ESCs caused a shift in attention to the use adult stem cells, which are limited to differentiating into the cell types of their origin tissue. MSCs are a type of multipotent adult stem cell with the ability to generate bone, muscle, cartilage, fat and other related tissues. Studies show MSCs improve the regeneration process mainly by releasing paracrine, or growth factors [30]. They can be isolated from a variety of tissues including peripheral blood, bone marrow, tooth pulp and adipose tissue [31–33]. In conjunction with their multipotency, they have a large capacity for self-renewal and therefore show huge promise for use in tissue repair. In addition, MSCs are nonimmunogenic and can be used in autologous or allogeneic transplant procedures. Induced pluripotent stem cells (iPSCs) are genetically reprogrammed adult stem cells, which behave like ESCs. They are currently useful tools in drug development and disease modeling and they are promising for tissue engineering. Cocultures of chondrocytes and MSCs are another possibility for regenerative efforts as stem cells may be able to catalyze chondrocytes to produce new tissue via paracrine signaling [34,35].

In addition to choosing a suitable cell type for tissue engineering of AC, scientists must decide how to encourage the proliferation and differentiation of the cells. During development, cartilage formation is strictly regulated by cellular interactions with the surrounding matrix, growth and differentiation factors, and environmental factors that activate or deactivate signaling pathways and spatiotemporal gene transcription. In adulthood, signaling factors regulate the various stages of chondrogenic differentiation. Certain bone morphogenic proteins help promote a chondrogenic phenotype [5,6]. TGF-β stimulates synthesis of ECM and begins the signaling cascade that activates gene expression of Sox9, a transcription factor known to be necessary for early chondrogenesis [7,36]. Conditioning with TGF-β1 and TGF-β3 enhances terminal differentiation of MSCs undergoing chondrogenesis [37]. Sox9 is necessary to activate gene promoters of collagen type II, aggrecan and other important cartilage-building proteins [38]. Sox9 inhibits activation of the Wnt/β-catenin pathway that drives hypertrophic differentiation and leads to calcification [39]. IGF and FGF are also necessary for proper differentiation to mature chondrocytes [36,40], enhancing type II collagen and glycosaminoglycan (GAG) production. Factors can be added individually; however, many times joint delivery of several factors is more beneficial. For example, delivering IGF-1 and FGF-2 together results in low expression of fibrocartilage and hypertrophic markers [41].

Two methods dominate tissue engineering's use of MSCs:

- *Ex vivo* differentiation: after MSC isolation and expansion, cells are seeded onto scaffolds where they are matured using bioactive and chemical factors, as well as biomechanical stimuli. The cell-seeded scaffold could then be surgically implanted at the damage site [42–44]. This is a less than ideal approach due to the invasiveness of the implantation procedure. In addition, progress is limited by the tendency for chondrocytes to move toward terminal hypertrophy and produce calcified tissue [45–47];

Table 1. Scaffold biomaterials used for recent cartilage tissue engineering studies.

Natural scaffold materials	Ref.	Synthetic scaffold materials	Ref.
Collagen	[63,103,104]	Poly(ethylene) glycol	[61,105]
Chitosan	[106,107]	Gelatin-hydroxyphenylpropionic acid	[71]
Fibrin	[54,56,108,109]	Poly(caprolactone)	[110,111]
Alginate	[53,64]	Self-assembling peptides	[68,70,72,73]
Hyaluronic acid	[55,64,108]	poly(L-lactive acid)	[67,111]
Agarose and gellan gum	[56]	Polyurethane	[60,109,112]
Decellularized cartilage matrix	[57,58]	Polyvinyl alcohol-methacrylate	[59]

Various biomaterials have been used in attempts to optimize chondrogenesis and create tissue-engineered cartilage with the organization and mechanical properties of native articular cartilage.

- *In vivo* differentiation: a second approach is to inject harvested MSCs directly into the area of defect. There, the MSCs' regenerative functions (immune suppression, anti-inflammatory effects, soluble factors etc.) can work toward tissue restoration [48–50]. Intra-articular injections of MSCs by themselves provide short-term relief, however, they may or may not provide the long-term healing of cartilage defects desired in tissue repair [51,52] due to the migratory nature of the MSCs.

Effective scaffolding properties for tissue engineering of cartilage

For successful cartilage tissue engineering, biomaterial scaffolds must support the survival and differentiation of cells used. This is done via the basic properties of the material and also explicit cues built into the scaffold. Pore size and rigidity must be taken into account, as well as a 3D architecture that allows cell adherence, limits migration and encourages formation of hyaline-like tissue. Mechanical, lubricating and swelling properties must also be considered for cartilage tissue engineering. In an effort to provide all these properties, many different types of biomaterials have been tested as scaffolds: natural and synthetic polymers, hydrogels, decellularized scaffolds and nanofiber-based scaffolds. Table 1 lists some of the scaffolding biomaterials used in recent studies on cartilage tissue engineering. Efforts focus on providing an environment for cell attachment and growth that will encourage the cells to form a specific tissue type.

Natural & synthetic scaffolds

Natural biomaterial scaffolds include those made from proteins, such as collagen, fibrin and silk, as well as those made from polysaccharides such as alginate, hyaluronic acid (HA) and chitosan. Natural polymers are useful because they are biologically active and usually foster cell adhesion, proliferation and growth. In addition, they are biodegradable and, therefore, allow host cells to produce their own ECM to replace the scaffold as it degrades. MSCs seeded onto natural scaffolds tend to have high synthesis of cartilage structural components such as collagen II, aggrecan and GAGs, especially in the presence of cartilage-specific growth factors [53–55]. Although natural polymers do not interfere with differentiation and allow for high metabolic activity of host cells, it remains difficult to create the necessary macro- and microarchitecture that encourages formation of hyaline-like cartilage [56]. In addition, they do not provide the mechanical strength necessary to mimic native AC [54]. Another promising natural biomaterial used in tissue engineering is decellularized cartilage matrix. Although availability of this scaffold material is limited, use of cartilage grafts allow the collagen ultrastructure to remain intact and does not compromise the mechanical function or integrity of the tissue. Furthermore, it has a chondroinductive effect on cells *in vitro* [57,58].

Synthetic scaffolds afford an alternative to natural material scaffolds and include polymer-based and peptide-based biomaterials. Synthetic biomaterials as scaffolding provide the advantage of reproducibility and control over scaffold properties such as cross-linking density and degradation rate. The ability to adjust pore size and stiffness of the matrix allows for better stability and regulation of mechanical properties as well as better cell adhesion and motility [59–61]. Drawbacks to synthetic scaffolds include the risk of rejection once implanted as well as by-products formed during degradation that may negatively influence the cells and surrounding tissue [62].

Composite scaffolds

Scaffolds fabricated from two or more materials can help circumvent some of the problems found with single-phase biomaterials. Studies have combined natural polymers [63,64] as well as mixing natural and synthetic polymers [65]. Composite scaffolds allow for the creation of more complex geometries and better functional properties more representative of AC in order to support enhanced cell adhesion, proliferation and dif-

ferentiation [60,64]. Embedding fibers can reinforce the scaffold and improve mechanical strength and function [59]. Layered composite scaffolds have the potential to better mimic the zonal characteristics of native tissue [22,66]. 3D woven composite scaffolds may better recreate the biomechanical behavior of cartilage tissue [65]. More recently, nanostructured composite scaffolds are promising for tissue engineering due to their biomimetic mechanical features and physiochemical properties [60,67,68]. Because collagen fibers in the diameter range of 500–500 nm comprise the bulk of ECM, a nanotopographical approach to tissue engineering might better allow synthesis of tissues with complex geometric organizations. MSCs respond to changes in density of nanotopographical cues by regulating their internal cytoskeletal network and mechanical changes guide them to make cell fate decisions [69]. Composite synthetic scaffolds permit the creation of directionally dependent load support similar to native cartilage collagen ultrastructure and with an equilibrium coefficient of friction similar to native AC [65]. However, achieving the scale and organization of native AC has so far been unsuccessful.

Hydrogels

Because hydrogels can be developed with mechanical, lubricating and swelling properties similar to that of native AC, they are often used in attempts to engineer cartilage. In addition, hydrogels are tunable – parameters such as polymer composition and cross-linking density can be manipulated [70,71]. Another benefit is that they are injectable. Hydrogels can be made with natural or synthetic polymers. Natural polysaccharide-based hydrogels, such as hyaluronan, chitosan, agarose and alginate, are intrinsically biocompatible but do not encourage cell attachment [56]. The lack of attachment makes these choices less attractive for 3D scaffolds used in cartilage repair efforts. In contrast, natural protein-based scaffolds, including collagen, fibrin and silk, promote cell-to-surface binding. Self-assembling peptide hydrogels can create precise arrangements approximating the micro- and nano-characteristics of natural ECM, which also allow for regulation of mechanical properties and stability. In addition, they can be use to dispense chondrogenic factors to encapsulated MSCs [72,73]. These properties, in turn, increase cell adhesion, proliferation and migration of cells. Synthetic hydrogels, such as poly(ethylene glycol) and poly(vinyl alcohol), can promote cell adhesion indirectly through surface modifications that improve cell seeding and anchorage [74,75]. Synthetic hydrogels are some of the most easily tunable biomaterial scaffolds allowing for research on macrostructure variations.

In order for a biomaterial scaffold to be useful for *in vivo* tissue engineering, cells must adhere to the scaffold surface and be able to survive, differentiate

Table 2. Effect of scaffold properties on mesenchymal stem cell chondrogenesis.

Property	Effects seen	Ref.
Pore size	300 μm mean pore size induced higher chondrogenic gene expression than smaller mean pore size (94 or 130 μm)	[113]
Geometry	Interpenetrating polymeric network scaffold has higher expression levels of chondrogenic markers	[64]
	Greater chondrogenic gene expression in polyurethane scaffolds with excellent porosity and good pore interconnectivity	[112]
	Scaffold alignment is sufficient to drive MSC differentiation without additional chemical stimuli	[15]
Adhesivity	Lower RGD densities enhance chondrogenesis	[114]
Rigidity	Lower stiffness gels lead to higher mRNA levels of chondrogenic markers (*Col2α1*, *Agc* and *Sox9*)	[115]
	Increased cross-linking promotes hypertrophic differentiation of chondrogenically induced MSCs	[116]
	Lower cross-linked matrix yields increase percentage of cells with chondrocytic morphology	[117]
Degradation rate	MSCs encapsulated in MMP-sensitive but hydrolytically stable hydrogels have a more spreaded morphology, express higher levels of chondrogenic marker genes and lower levels of hypertrophic genes compared with MMP-insensitive hydrogels	[118]

Various scaffolding properties can be manipulated to enhance chondrogenesis.
MSC: Mesenchymal stem cell.

and produce matrix. A functional biomaterial scaffold must provide a framework for the growth of the desired tissue and allow for organization and cellular communication. Table 2 summarizes the effect of various scaffold properties on chondrogenesis of MSCs. The biomaterial must also afford a smooth, lubricated low-friction surface during movement and assist in load transmission to the subchondral bone. Porous scaffolds engineered with bioactive factors and peptides common to the ECM of AC can help promote differentiation as well as improve cell attachment function. Engineered hydrogels can have similar mechanical properties to AC. Micro- and nanopatterned scaffolds are attractive options because of the high degree of control over geometry, adhesiveness and stiffness of the scaffold, which influences cell patterning and differentiation. 3D scaffolds tend to influence cell migration and lead to higher expression levels of differentiation-associated transcription factors, such as Sox9. While various scaffolds may be able to support healthy cartilage formation, mimicking the mechanical properties and resiliency of AC has eluded researchers thus far. Scaffolds will need to initially support joint loads and then gradually degrade as the developing cartilage forms. Future studies for tissue engineering of AC will most likely focus on unique, 3D, nanopatterned scaffolds that can be formulated to stimulate migration, differentiation and organization of MSCs in order to mimic the composition of the various layers in native AC. To prevent further damage from invasive implantation procedures, bioinjectable scaffolds are also at the forefront of tissue engineering research. For patients with articular defects, the eventual goal of cartilage tissue engineering is a return to normal joint function by redevelopment of healthy AC. The new cartilage must integrate with existing cartilage and the underlying bone. Cell-based techniques that utilize supportive biomimetic scaffolding along with growth factors and other environmental signals hold the greatest promise for advances in the clinical application of tissue engineering.

Use of mechanical stimulation in tissue engineering of cartilage

Everyday use puts joints under various types of stresses and strains. The motion and loading of synovial joints is necessary for the proper structure, function and metabolism of native AC. Mechanical stress includes hydrostatic pressure, dynamic and static compression, rotation and shear. AC possesses low permeability and as such, hydrostatic pressure in the interstitial fluid governs the natural environment of chondrocytes during joint loading [76]. Compression of AC tissue occurs naturally during everyday movements such as rising from a chair or climbing stairs. Rotation is the gyration of a bone around its central axis. Shear strain occurs when tissue layers are shifted laterally in relation to each other, such as when the knee is in a flexed position and then twisted. When present in the chondrocyte environment, these forces can affect cellular metabolism and help distribute nutrients and oxygen. *In vitro* mechanical stimuli encourage chondrogenic cells to differentiate and produce matrix, which enhances cartilage formation [77,78]. During joint loading, chondrocytes will deform, losing cellular height and volume, and then show a complete recovery after the compressive force is removed [79,80]. Forces that modify the cell and nucleus structure of chondrocytes correlate with local changes in aggrecan synthesis as well as type I and II collagen, proteoglycan 4 and collagen X [81,82]. Mechanical stimuli can induce calcium signaling, which triggers various metabolic and signaling activities [83]. Loading allows chondrocytes to regulate actin cytoskeleton dynamics and organization by changing gene expression for associated proteins [84,85].

There are multiple regulatory pathways chondrocytes use to detect and respond to stimuli. Upstream signaling pathways may lead to changes in transcription, translation, post-translational modification, extracellular assembly and ECM degradation [17,21,77,86–88]. The various forces and flows that occur during mechanical loading act in parallel with released cytokines and growth factors to regulate chondrocyte homeostasis [80]. Once in the ECM, these molecules can bind to receptors on cell surfaces in order to stimulate cellular activity or become trapped in the ECM. The activation and inhibition of these cascades and all the molecules involved are still under investigation and not completely understood; however, due to the nature of AC, a certain amount of mechanical stimulation is necessary for proper functioning of the tissue and cells. With that in mind, many studies have been undertaken *in vitro* to determine the types and frequencies of mechanical stimulation that will induce MSCs to produce functionally equivalent cartilage. Table 3 lists the effects of various loading protocols on matrix production and differentiation.

Several studies have looked into the signaling pathways involved in mechanical stimulation of stem cell proliferation and differentiation. A recent investigation discovered that the ERK1/2 pathway determines whether MSCs differentiate toward an osteogenic or chondrogenic fate. In the absence of these pathways, human bone marrow-derived stem cells (BMSCs) in a fibrin gel scaffold stimulated by dynamic compression experience a downregulation of aggrecan and *Sox9* and an upregulation of osteogenic markers such as osteo-

Table 3. Effect of loading protocols on matrix production and differentiation.

	Loading protocol	Environment	Effect	Ref.
Hydrostatic pressure	Cyclic, 10 MPa at 1 Hz, 4 h/day, 14 days	TGF-β3	Upregulation of Sox9 and sGAG synthesis; downregulation of prehypertrophy and terminal differentiation genes *Ihh* and type X collagen	[119]
	Intermittent, 5 MPa at 0.5 Hz, 4 h/day, 7 days	Chondrogenic medium	Higher expression of aggrecan, Sox9 and type II collagen	[120]
	Dynamic vs static, 14–36 kPa at 0.25 Hz, 1 h/day, up to 7 days	TGF-β1 prior to loading	Dynamic loading yielded stronger ECM and type II collagen staining; GAG secretion increased; elevated mRNA levels of type II collagen, aggrecan and Sox9	[121]
Compression	10% dynamic strain superimposed on static 2% tare strain, unconfined, 0.01–1 Hz, 1–4 h/day, 3–6 weeks	TGF-β3	Improved distribution of proteoglycans and collagens; modulation of many chondrogenesis-associated genes (413 upregulated and 139 downregulated)	[89]
	Dynamic, 10% strain at 1 Hz, 1 h/day for up to 21 days	TGF-β3 prior to loading	Gene expression of aggrecan and collagen type II in construct core increased at 7 and 21 days, but in the annular region at day 14 and 21	[90]
	Sinusoidal, 10–40 kPa at 0.5 Hz, 1 h/day, up to 14 days	Loading after chondrogenic induction	Increase in gene expression of *Col2α1* and *Ihh*	[93]
Pressureless	Cyclic, external magnetic field, 30 min every 1.5 h, 8 h/day, up to 3 weeks	With and without chondrogenic medium	GAG deposition; chondrogenesis confirmed by immunostaining of aggrecan, type II collagen and Sox9	[91]
Multifactorial bioreactors	Cyclic, compression and interfacial shear at 1 Hz, 1 h/day, 5 days/week, for 3 weeks	Fibrin-polyurethane scaffolds	Cellularity of scaffold increased; deposition of sulfate-rich GAG at top surface of scaffold	[109]
	Dynamic, compression and rotation for 2–4 weeks vs unloaded culture	Third passage cells seeded on fibrin-polyurethane scaffolds	Loading significantly higher in mRNA expression of type II collagen, aggrecan and COMP. Load after unload protocol also increased superficial zone protein.	[122]
	Dynamic, compression and shear for 2–4 weeks vs unloaded culture	Cultured within polyurethane scaffold rings	Loading maintained chondrogenic mRNA expression of collagen type II, aggrecan and COMP	[123]

Various loading regimens have been used for the study of mechanical stress in tissue engineering efforts.
ECM: Extracellular matrix; GAG: Glycosaminoglycan.

calcin, alkaline phosphatase and collagen type I [92], demonstrating a tendency to move toward bone rather than cartilage formation. Another investigation explored the effect of dynamic compression on cell viability during chondrogenesis of rat BMSCs, as well as the underlying mechanisms involved. They found that inhibiting bone morphogenic protein signaling decreased cell viability, but application of dynamic stress was somewhat able to offset the reduction [93].

Transduction of mechanical stimulation into chemical signals seems to play an important role in the proliferation and differentiation of MSCs into a chondrogenic phenotype. Determining the optimal frequency, type and timing of mechanical loading on cultures for tissue engineering remains a significant challenge. Which signaling pathways are activated in response to mechanical loading is also important (Figure 1). Current research suggests several things. First, mechanical stimulation can be effective in the differentiation of MSCs after a time of preconditioning with exogenous growth factors. Second, the response of differentiated MSCs to mechanical stimulation has a positive effect on ECM remodeling. Finally, the development of bioreactors that can provide a combined, dynamic loading regimen more similar to the native stresses AC endures may lead to enhanced chondrogenesis in tissue-engineered constructs.

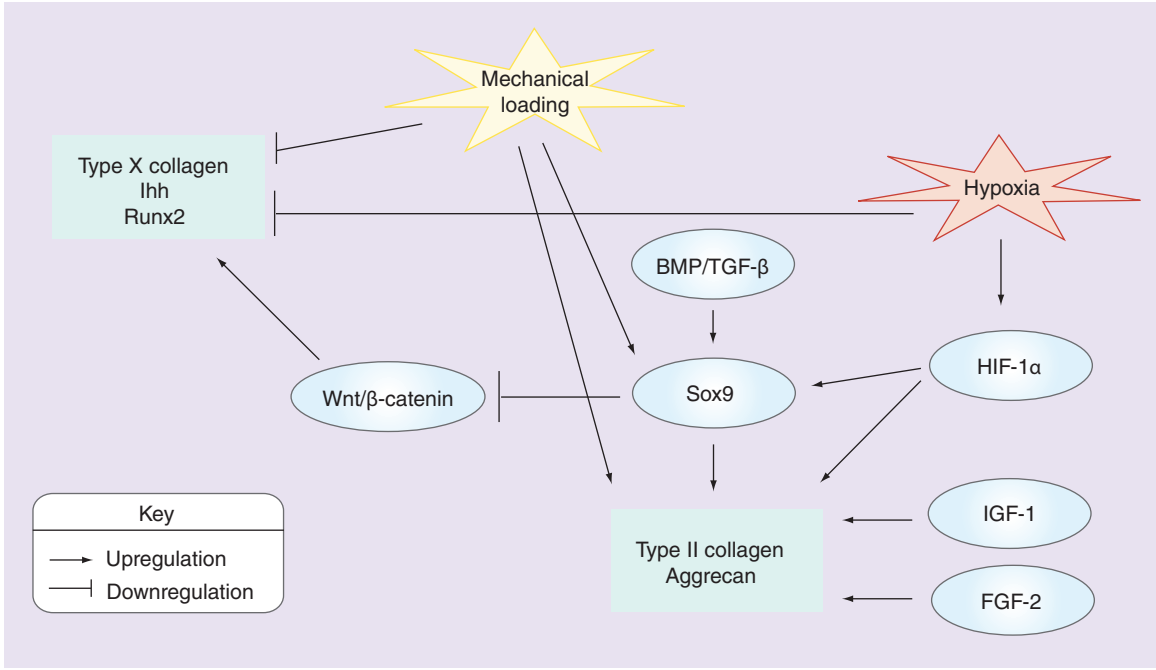

Figure 1. Signaling pathways modulating mRNA expression of chondrogenic genes. Both moderate hypoxia and mechanical loading inhibit pathways leading to hypertrophy and osteogenesis while stimulating the production of cartilage-building proteins.

Effect of hypoxia on AC differentiation of MSCs

Since AC is avascular, oxygen levels within the AC are lower than many other tissues and oxygen levels vary by depth. The superficial zone receives oxygen from contact with the synovial fluid and maintains an oxygen level between 6 and 10%. The deep zone, which has minimal oxygen from the vascularized subchondral bone, has an oxygen level around 2% [94]. Chondrocytes are adapted to this oxygen-poor environment; in healthy AC, they maintain their phenotype and produce the necessary ECM and macromolecules for proper function. This is mainly achieved through the effects of HIF-1α, an oxygen-sensitive transcriptional activator. During embryogenesis when there are continuing changes in oxygen concentrations, HIF-1 is necessary for proper development and survival of the embryo. *In vitro* studies show it is a necessary transcription factor for the hypoxic induction of chondrogenesis [53]. Observations of cartilage that lacks HIF-1α show massive cell death in the central regions suggesting that HIF-1α is necessary for survival and differentiation of chondrocytes in hypoxic conditions by helping to maintain a balance between oxygen availability and handling in developing growth cartilage [95]. Adaptation may possibly also depend on the composition of the surrounding ECM [96]. For effective tissue engineering of cartilage, the effects of hypoxia on chondrogenesis must be better understood. Much research has been done to determine the influence that changing oxygen tension has on the proliferation and differentiation of MSCs from a variety of sources.

Studies suggest culturing MSCs *in vitro* under moderately hypoxic conditions (5% O_2) is beneficial to achieving a chondrogenic phenotype, expression of chondrogenic transcription factors, suppression of osteogenic transcription factors, synthesis of ECM and production of type II collagen (Figure 1). One study found that when compared with culturing in normoxic conditions (20–21% O_2), hypoxic conditions suppressed the osteogenic differentiation potential of human adipose-derived MSCs (ADSCs) without affecting overall protein production and cell viability. Calcium deposition decreases and there is a reduction in Runx2 and osteocalcin (two key osteoblast-related genes) mRNA levels in low oxygen. They observed that hypoxia supports earlier collagen II mRNA expression as well as its later production within the matrix [97], which is good for AC formation. Another study shows human ADSCs differentiated in 5% oxygen tension demonstrate increased cellularity and matrix deposition that organizes itself in a zonal manner and exhibits cartilage-like morphology [98]. Human BMSCs cultured under 5% oxygen conditions in alginate show downregulation of osteogeneic transcription factors, Cbfa1 and Runx2, stronger reduction of fibrocartilage and hypertrophic cartilage expression, as well as increased mRNA expression of collagen type II and aggrecan, increased protein level-expression of procollagen II protein and sulfated GAGs, as wells as upregulation of Sox transcription factors (L-Sox5, Sox6

and Sox9). They found this to be true *in vitro* and *in vivo* with no serum or growth factors used [53]. *In vitro* low-oxygen preconditioning of rabbit adipose stromal cells also leads to significantly greater expression of type II collagen and aggrecan [99]. Bovine articular chondrocytes and BMSCs cultured in 3D scaffolds under 5% hypoxia show an increased collagen type II to collagen type I ratio and decreased cellularity as well as increased matrix synthesis in early cultures [35]. When culturing in a 3D environment, hypoxia may significantly suppress human MSC hypertrophy and calcification [96].

Studies that considered both the MSC expansion process and the differentiation process, found that the timing of oxygen deprivation is important. According to one study, preconditioning BMSCs in 5% O_2 may enhance clonogenicity but impair differentiation. They recommend expansion in normoxia followed by hypoxia during differentiation to enhance *in vitro* chondrogenesis [100]. Another study using dermis MSCs confirmed that continuous hypoxic conditions (5% O_2) decreases the collagen type II to total collagen ratio; however, differentiation in hypoxic conditions following normoxic expansion increases production of collagen type II and GAGs. In that same study, expansion in hypoxia increased total matrix production [101]. Yet another study suggests that hypoxia alone has no effect on chondrogenesis or matrix synthesis, but that the effect is dependent on the hydrogel-scaffolding properties [96].

Culturing in extreme hypoxic conditions produces varying results. One study found that 1% O_2 conditions decrease mRNA levels of Runx2, decrease expression of type X collagen and enhance chondrogenesis in human BMSCs by yielding higher mRNA levels of Sox9, type II collagen and aggrecan, increasing proteoglycan synthesis and increasing type II collagen expression. The research reveals that extreme hypoxic culture activates the PI3K/Akt/FoxO pathway that suppresses capsase activation of chondrogenesis-induced apoptosis in MSCs [16]. Another study found that severe hypoxia decreases gene expression to the point that it halts all differentiation [102].

Because cartilage is naturally a low-oxygen environment, it makes sense that hypoxia might play a significant role in the development of tissue-engineered cartilage. Hypoxic-based culturing protocols fluctuate between the different research experiments. Dissimilarities in cell sources, culture conditions and oxygen-deprivation timing make a direct comparison of hypoxia studies difficult. These variations limit optimization of the expansion and differentiation process and may hinder rapid advancement of the field toward clinical applications. However, the majority of research supports the application of a low-oxygen culture environment around 5% during the *in vitro* differentiation process to promote chondrogenesis.

Conclusion

Osteoarthritis causes a huge societal burden and there is no known cure. Pharmaceuticals can help alleviate pain and delay deterioration, but cannot restore the damaged tissue. AC is a complex layered environment and the spontaneous repair process is inefficient resulting in production of inferior fibrocartilage without the strength and resiliency of native hyaline cartilage. Advances in regenerative medicine, specifically stem cell knowledge and tissue engineering techniques, have made progress in repairing and replicating AC. The goal in stem cell chondrogenesis is to find a cell source that can be directed to produce new cartilage capable of functioning efficiently in stressful joint environments. The use of MSCs in tissue engineering efforts shows great promise due to their availability, immunosuppressive characteristics and paracrine signaling.

Promotion of MSC chondrogenesis is achieved using many different strategies. Addition of chondrogenic growth factors individually or in combination helps direct the cell toward a chondrogenic fate. However, growth factors alone are not enough to recreate AC tissue. The stem cell environment has a direct influence on how they differentiate. The employment of 3D scaffolds provides sites for cell attachment, helps direct migration and allows for control of pores size, geometry, adhesivity and stiffness, which help mimic the mechanical properties of AC. Mechanical stimulation in conjunction with exogenous growth factors directs MSC differentiation and assists with ECM remodeling. The use of multifactorial bioreactors capable of creating the combined stresses similar to normal joint loading seems promising. Oxygen being sensed by the cells depends on fac-

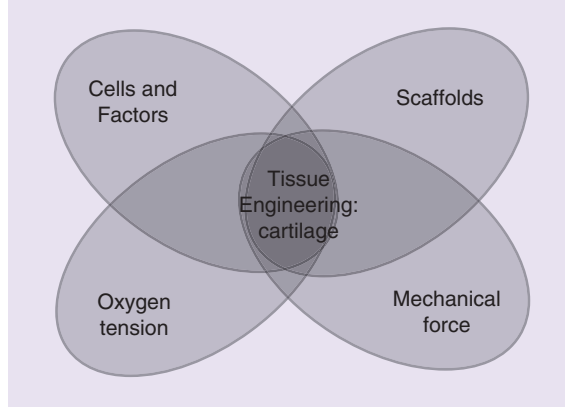

Figure 2. Important variables to consider for tissue engineering of cartilage. Placing cells in an environment with the appropriate signals and a scaffold on which to grow may hold the most promise articular cartilage regeneration.

tors that are hard to control, most importantly, level of medium and density of cells. However, hypoxic culture conditions of around 5% O_2 promote chondrogenesis of MSCs and should be considered when conducting future *in vitro* tissue engineering research.

Current technology has been unable to successfully regenerate cartilage with the appropriate structure, viscoelastic properties, ECM organization and biological activity. However, to date, no studies have dealt with all of these factors together (Figure 2). Increased experimentation with various biomaterials and cues, knowledge of how mechanical factors influence stem cell fate and manipulation of hypoxia-induced pathways will enhance AC tissue engineering efforts. To avoid surgical implantation issues, it would be ideal to develop injectable biomaterials that form a scaffold internally and provide a place for cells and factors to be implanted at the defect site. Mechanical stimulation and oxygen deprivation could then occur naturally in the joint.

Future perspective

For advancement of cartilage engineering, future studies cannot focus on only one or two variables, but must look at the bigger picture. Noninvasive stem cell techniques that trigger the body into healing more completely an effectively are the wave of the regenerative future. Many stem cell procedures are available internationally, but the US FDA currently limits how they are used in the USA. Autologous stem cell procedures require a significant amount of cells, which necessitates amplification of harvested cells. This requires a current Good Manufacturing Practice facility. Allogenic sources such as amniotic membrane may be the safest and simplest way to improve joint function in the future. Already used for wound healing and in the treatment of plantar fasciitis, cryopreserved chorion-free amniotic membrane preserves all tissue components, including the 3D tissue matrix architecture, growth factors and MSCs. Much progress has been made in the field of regenerative medicine. Well-designed clinical trials, rigorous evaluation of engineered tissue and methods for assessing successful outcomes will be very important in the quest for engineered cartilage that truly mimics healthy AC and restores damaged joints.

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Executive summary

Background
- When engineering cartilage with stem cells, scaffold structure and composition, mechanical loading and oxygen availability play a key role in the differentiation process.

Important mechanical properties of cartilage
- The diverse composition and organization of articular cartilage make it difficult to reproduce with current tissue engineering strategies.

Main components in cartilage tissue engineering
- Current cartilage tissue engineering efforts are not totally successful and the various methods using mesenchymal stem cells (MSCs) still have several drawbacks.

Effective scaffolding properties for tissue engineering of cartilage
- For successful cartilage tissue engineering, biomaterial scaffolds must support the survival and differentiation of the cells used via the basic properties of the material and also explicit cues built into the scaffold.

Use of mechanical stimulation in tissue engineering of cartilage
- Transduction of mechanical stimulation into chemical signals plays an important role in the proliferation and differentiation of MSCs into a chondrogenic phenotype.

Effect of hypoxia on articular cartilage differentiation of MSCs
- Mimicking the low-oxygen environment of articular cartilage during the *in vitro* MSC differentiation process promotes chondrogenesis.

Conclusion & future perspective
- In the future, allogenic sources of stem cells that can be directly injected may be the safest and simplest way to improve joint function.

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