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REVIEW

Designing the stem cell microenvironment for guided connective tissue regeneration

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Adult mesenchymal stem cells (MSCs) are an attractive cell source for regenerative medicine because of their ability to self-renew and their capacity for multilineage differentiation and tissue regeneration. For connective tissues, such as ligaments or tendons, MSCs are vital to the modulation of the inflammatory response following acute injury while also interacting with resident fibroblasts to promote cell proliferation and matrix synthesis. To date, MSC injection for connective tissue repair has yielded mixed results *in vivo*, likely due to a lack of appropriate environmental cues to effectively control MSC response and promote tissue healing instead of scar formation. In healthy tissues, stem cells reside within a complex microenvironment comprising cellular, structural, and signaling cues that collectively maintain stemness and modulate tissue homeostasis. Changes to the microenvironment following injury regulate stem cell differentiation, trophic signaling, and tissue healing. Here, we focus on models of the stem cell microenvironment that are used to elucidate the mechanisms of stem cell regulation and inspire functional approaches to tissue regeneration. Recent studies in this frontier area are highlighted, focusing on how microenvironmental cues modulate MSC response following connective tissue injury and, more importantly, how this unique cell environment can be programmed for stem cell–guided tissue regeneration.

Keywords: mesenchymal stem cell; stem cell microenvironment; connective tissue; regeneration; tendon/ligament

Introduction

Mesenchymal stem cells (MSCs) play important roles in tissue homeostasis and regeneration through their capacity for multipotent differentiation, their immunomodulatory characteristics, and their ability to promote healing through trophic signaling. Thus, MSCs are of increasing interest as a treatment modality for injury and disease in a number of tissue types. In particular, in connective tissues, MSCs are able to modulate the inflammatory microenvironment following acute injury and have been observed to interact with native tissue fibroblasts to promote cell proliferation and matrix synthesis.[1,2] Still, therapies that utilize delivery of MSCs as an adjuvant for tissue engineering approaches to connective tissue repair have yielded mixed results *in vivo*.[3–6] This is likely due to inadequate synergistic signaling to implanted MSCs by the surrounding microenvironment, which is largely disrupted following injury. In healthy tissues, stem cells reside within a microenvironment that promotes self-renewal, controls activation, and prevents depletion of the stem cell population. Following injury, this microenvironment undergoes a number of abrupt changes, resulting in differences in the types and densities of other cell types, the concentrations and combination of soluble signals, and the underlying matrix composition. These drastic changes likely alter MSC response, and there is tremendous interest in the field in designing an engineered stem cell microenvironment that can guide MSC differentiation, drive immunomodulation, stimulate trophic signaling, and promote functional healing.

Here, we highlight current approaches in engineering the stem cell microenvironment for

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connective tissue regeneration. First, the characteristics of MSCs and the various cues that compose the cell microenvironment under healthy and injured conditions are discussed. This is followed by a review of studies exploring the engineering of a stem cell microenvironment that is conducive to functional healing, concluding with a summary and future directions.

Mesenchymal stem cells and their microenvironment in connective tissues

MSCs are a heterogeneous population of nonhematopoietic, multipotent cells first discovered in the adult bone marrow[7–9] that form bone following heterotopic bone transplantation.[7] Exhibiting a fibroblast-like morphology and the ability to self-renew, these cells can differentiate toward mesenchymal lineages,[10] including bone, cartilage, and fat, as well as skin,[11] tendon/ligament,[12–14] muscle,[15] and bone marrow stroma.[16,17] In addition to those derived from bone marrow, stem cells have also been found to reside in other mesenchymal tissues, such as fat,[18] skin,[19] tendon,[20] periodontal ligament (PDL),[21] and dental pulp,[22] to name a few. These cells are believed to contribute to the ability of adult tissues to regenerate and repair following injury and aging.[23]

While there is currently no known gene expression profile for the definitive identification of MSCs, the Mesenchymal and Tissue Stem Cell Committee at the International Society of Cellular Therapy has devised a set of minimal criteria for defining these cells.[24] Cells must be substrate adherent; differentiate into osteocytes, adipocytes, and chondrocytes; and exhibit a specific expression profile of a subset of surface markers. Specifically, MSCs must express CD90/Thy-1, CD73, and CD105.[24] In addition to these, Stro-1 is the most widely accepted and well-known MSC marker, as this marker is correlated with the cell's ability to generate colony-forming units, a hallmark characteristic of MSCs *in vitro*.[25] However, the exact function of Stro-1 is not known, and its expression is not unique to MSCs, as it has been found in nucleated erythroid cells, limiting its use as a standalone MSC marker.[26] Furthermore, MSCs are observed to gradually lose Stro-1 expression during *in vitro* expansion, limiting its use beyond MSC isolation and early culture.

The markers that are absent from the surface of MSCs include CD34 (hematopoietic and endothelial cell marker), CD45 (leukocyte marker), CD11b (monocytes and macrophages), CD79-α or CD19 (B cell markers), and human leukocyte antigen class II surface molecules (antigen-presenting cells and lymphocytes).[24] Other markers, such as CD117 and CD31, are also commonly referred to as negative MSC markers.[24]

Stem/progenitor cells have been found in tendons and ligaments throughout the body, and the cell microenvironment comprises cellular, structural, and signaling cues (Fig. 1) that modulate stem cell participation in tissue maintenance, generation, and repair.[12,20,27–29] It has been proposed that these cells reside in the perivasculature of these tissues, close to blood vessels that can replenish depleted MSC populations by recruiting cells from the bone marrow. The perivascular microenvironment has also been implicated in the maintenance of stem cell populations in other tissues, such as neural tissue,[30–32] dental pulp,[33] and the bone marrow stroma.[16,17] It is likely that paracrine signaling and cellular interactions from the blood have important effects on stem cell maintenance and function.

More recent studies suggest that populations of stem/progenitor cells reside within the tissue proper, and cells rely more heavily on direct contact with the surrounding extracellular matrix (ECM) and inhabitant cell types for maintenance and regulation of function. In tendons, a population of stem cells was identified within the tissue proper through tracking the location of tendon stem cells within mouse patellar tendons.[20,34] It was observed that these cells reside between parallel collagen fibril chains, suggesting the potential importance of the ECM in maintaining a population of tendon stem/progenitor cells.[20] Similarly, cells capable of multilineage differentiation have been identified in ligaments.[21] Still, the various cues responsible for the maintenance of a population of multipotent stem cells within tendons and ligaments are not well understood. Consequently, there is a growing interest in developing models of the stem cell microenvironment that can be used to elucidate the mechanism of stem cell induction in both healthy and injured connective tissues.

Stem cells are attractive for clinical applications owing to their trophic capacity to promote tissue repair and remodeling and their ability to modulate the immune response following injury.[35] Specifically, MSCs actively respond to stress or injury

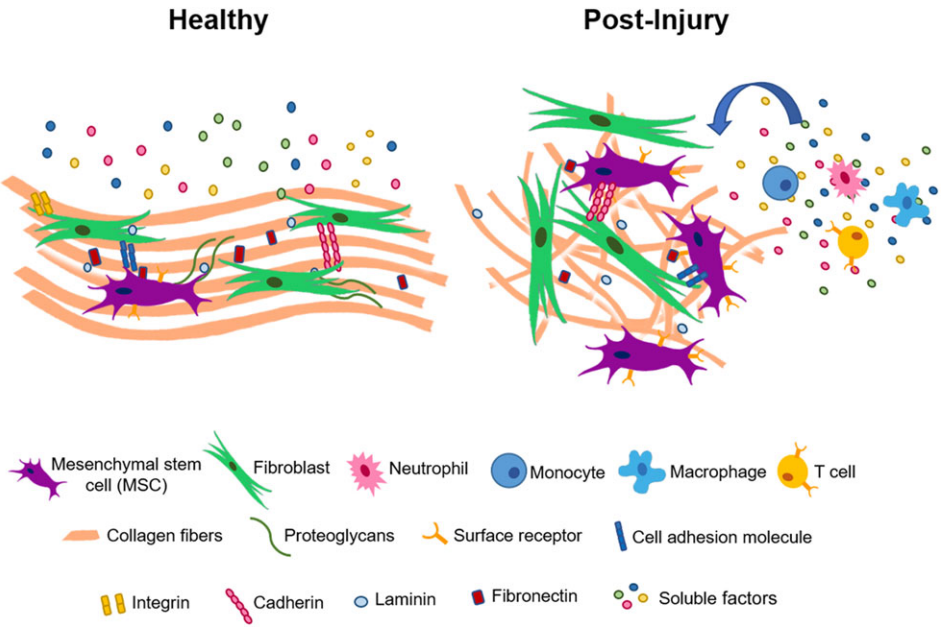


Figure 1. Schematic of the healthy versus injured microenvironment within connective tissue.

similarly to cells in the adaptive and innate immune systems following pathogen exposure or apoptosis.[36] While undifferentiated MSCs do not express major histocompatibility complex class II antigens, these molecules are observed to be upregulated on the cell surface following exposure to an inflammatory microenvironment.[37] MSCs have been shown to influence the immune system through the secretion of a variety of soluble factors, including indoleamine 2,3-dioxygenase (IDO),[38] nitric oxide,[39] transforming growth factor (TGF)-β,[40] prostaglandin E_2,[40,41] and tumor necrosis factor–stimulated gene (TSG)-6 protein.[42] Early studies on the immunosuppressive potential of MSCs found that cells derived from humans,[43–46] baboons,[47] and mice[48,49] are all capable of suppressing T cell proliferation and inflammatory cytokine secretion. Since then, it has been reported that MSCs are also able to suppress the proliferation and cytokine release of other inflammatory cell types, including B cells[50] and natural killer cells.[51] Specific to connective tissues, MSCs have also been shown to reduce the infiltration of inflammatory cells within the repairing tendon and the tendon-to-bone interface in animal models.[52]

In addition to their role in immunomodulation, MSCs have been reported to enhance fibroblast proliferation and collagen matrix synthesis via paracrine signaling.[1,2] These cells serve as a source of cytokines and proteinases essential to angiogenesis and tissue regeneration, including vascular endothelial growth factor (VEGF), matrix metalloproteinases, insulin-like growth factor-1 (IGF-1), hepatocyte growth factor, TGF-β, and basic fibroblast growth factor (bFGF).[53] Specific to connective tissues, MSCs promote functional healing within the tendon and tendon-to-bone interface through secretion of factors that stimulate fibroblast proliferation and angiogenesis, inhibit apoptosis, and minimize fibrosis.[52,54,55]

Given that stem cells have been identified in many tissues throughout the body, both mesenchymal and nonmesenchymal in origin, it is likely that common cell regulation features are shared among the MSC microenvironments. To understand the cues that are most critical for modulating stem cell response, novel model systems of the cell microenvironment have been developed and are highlighted in the following sections.

Engineering the stem cell microenvironment *in vitro*

The local delivery of MSCs to sites of injury is an attractive option to facilitate tissue healing. Early

MSC delivery methods have utilized bolus injection of cells either systemically via intravenous or intra-arterial delivery or locally via direct injection to the injury site.[56,57] Systemic delivery is the easiest of the two options and relies on MSC homing or migration to the site of injury and inflammation.[56] While MSC migration to the injury location is possible, the number of MSCs that reach the injured tendon/ligament tissue is limited.[58] Additionally, intravenous injection typically results in a buildup of MSCs in the lungs, limiting the number of stem cells available for homing.[59] Further complications, such as arterial thrombosis, have been reported in limbs where MSCs were delivered via the circulatory system.[59] Alternatively, a local intralesional injection offers direct delivery of MSCs to the injury site; however, stem cell survival can be compromised because of a lack of oxygen and nutrients to support viability. Specifically, it has been observed that, while equine embryonic stem cells persisted at the injury site for as long as 3 months following injection into an equine flexor tendon lesion, MSCs showed less than 5% survival within the first 10 days following injection.[60] Furthermore, in both instances, the inflammatory microenvironment has been shown to have a negative effect on MSC survival, as proinflammatory cytokines have been shown to diminish MSC proliferation and self-renewal and promote cell death. Despite promising effects of MSC delivery on tendon healing, transplantation of MSCs alone has resulted in ectopic bone formation within the tendon following delivery.[3,4]

These findings suggest that, while MSCs may be a valuable cell source for promoting tissue regeneration following injury, without proper stimuli from the surrounding microenvironment, the ability of MSCs to home to the injury site and participate in tissue regeneration is compromised. Therefore, engineering of an artificial microenvironment capable of directing MSC response following delivery to an injury is an appealing strategy for addressing these limitations. To this end, investigating the impact of each of the various components of the connective tissue microenvironment on MSC activity and tendon/ligament lineage commitment *in vitro* is vital. It has been shown that, through optimization of a number of these components, as shown in Figure 2, it may be possible to mimic the signals provided by the connective tissue microenvironment to control MSC response and promote stem cell–guided tissue regeneration. The current progress in engineering each of these individual aspects of the connective tissue microenvironment *in vitro* is described in more detail in the following sections, with the goal of discussing which environmental cues are critical for modulating stem cell response for guided connective tissue regeneration.

Cellular interactions

The cellular microenvironment within healthy ligaments and tendons largely consists of elongated fibroblasts, which lie parallel to the tissues' collagen fibrils, with multiple cell processes extended to aid in the synthesis of organized matrix and to allow for paracrine interactions and cell–cell communications via gap junctions between cells.[61,62] Following injury, the cellular microenvironment changes abruptly, as the tissue is invaded by neighboring fibroblasts, as well as inflammatory cells, recruited to the injury site via chemotactic signaling agents.[63] Specifically, neutrophils and proinflammatory and anti-inflammatory macrophages, as well as other immune cells, including T cells and mast cells, are found at the wound site.[64]

In vitro co- and tri-culture models with MSCs have been developed in order to (1) promote lineage-specific differentiation of MSCs and (2) elucidate the trophic signaling and immunomodulatory effects of MSCs on other cell types. As healthy fibrous connective tissues are largely composed of resident fibroblasts, the majority of co- and tri-culture models for ligament and tendon tissue engineering involve fibroblasts (Table 1). Segregated co-culture models provide a means for isolating the response of individual cell types to assess the impact of ligament/tendon cell paracrine signaling on MSC differentiation.[65–69] Lee *et al.* assessed the response of MSCs to co-culture with anterior cruciate ligament (ACL) fibroblasts using a Transwell co-culture model and found that expression of ligament-related markers, including types I and III collagen and tenascin-C, was upregulated by co-cultured MSC by day 7.[65] Similarly, Luo *et al.* found that both proliferation and expression of tenogenic markers were enhanced for MSCs in Transwell co-culture with Achilles tendon cells after 14 and 21 days.[68] In work by Lovati *et al.*, equine MSCs were co-cultured with fragments of digital flexor tendons using a segregated Transwell model. Results show that MSCs in co-culture expressed greater levels of decorin,

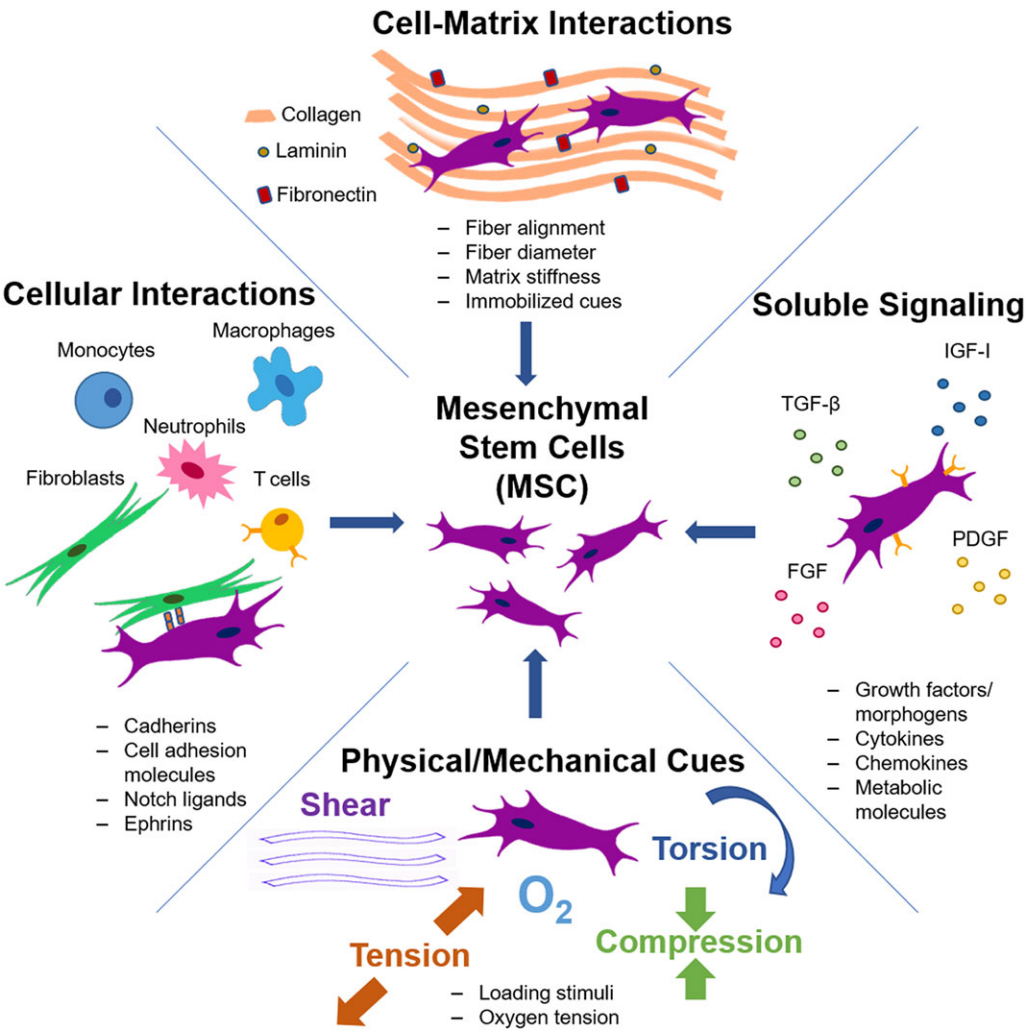


Figure 2. Schematic of the various cues within the microenvironment that guide stem cell response.

tenomodulin, and tenascin-C, and MSCs aggregated to form 3D tissue-like structures, which stained positively for type I collagen by day 15.[69] These results suggest that paracrine signaling between MSCs and cells within tendon tissue may be capable of inducing tenogenic differentiation of MSCs.

To assess the effects of direct contact between MSCs and ligament fibroblasts, mixed co-culture models have also been used (Table 1). Canseco *et al.* developed a mixed co-culture model in which autologous porcine ACL cells and MSCs were cultured in varying co-culture ratios (3:1, 1:1, 1:3 MSC:ACL fibroblasts). A co-culture ratio of 1:1 resulted in increased expression of type I collagen and tenascin-C at day 28, as well as increased tenascin-C staining compared with MSC controls, though histological staining was not different from fibroblast single-culture controls.[70] To assess the effects of co-culture on each cell type individually, Kramer *et al.* performed mixed co-culture of male human MSCs and female PDL cells at varying ratios (1:1, 2:1, 10:1 MSC:PDL cells) and were able to isolate individual cell types using Y chromosome labeling. Results show that co-culture increased MSC expression of PDL-related markers at day 7.[71]

Alternatively, co- and tri-culture models can also be used to analyze the effects of MSCs on tendon/ligament cell response (Table 1). To better understand the trophic effects of MSCs on fibroblasts, Proffen *et al.* used a mixed co-culture

Table 1. Cellular interactions

MSC co-culture models			
Study	Cell types	Co-culture model	Findings
Kramer *et al.*[71]	Human BMSCs and PDL cells	Mixed—1:1, 2:1, 10:1 BMSC:PDL cell	Increased expression of periodontal ligament–related markers by MSCs at day 7
Lee *et al.*[76]	Human BMSCs + murine skeletal myocytes	Mixed—1:5 myocyte:BMSC	MSCs incorporate into myotubes and express myogenic markers in co-culture; increased nestin expression in myotubes following MSC incorporation
Lee and Kemp[77]	Human ADSCs + murine skeletal myocytes	Mixed—1:5 myocyte:ADSC	ADSCs incorporate into myotubes and express myogenic markers in co-culture
Lee *et al.*[65]	Human BMSCs and ACL fibroblasts	Segregated—Transwell	Increased expression of ligament-related markers by MSCs by day 7
Mizuno *et al.*[66]	Human BMSCs and PDL cells	Segregated—Transwell, conditioned medium	Increased proliferation and decreased mineralization potential by MSCs in conditioned medium, upregulation in expression of 35 genes
Zhang *et al.*[67]	Rat BMSCs and ligament fibroblasts	Segregated—permeable membrane	Increased expression of collagen I, collagen III, and tenascin-C by BMSCs in co-culture
Luo *et al.*[68]	Rat BMSCs and tenocytes	Segregated—Transwell	Increased proliferation and expression of tenogenic markers for MSCs in co-culture compared with single-culture controls after 14 and 21 days
Beier *et al.*[78]	Rat BMSCs and myoblasts	Mixed	Upregulation of myogenic markers MEF2 (myogenic enhancer factor 2) and α-sarcomeric actin by MSCs
Canseco *et al.*[70]	Porcine BMSCs and ACL fibroblasts	Mixed—3:1, 1:1, 1:3	Increased expression of collagen I and tenascin-C and enhanced tenascin-C staining at day 28
Lovati *et al.*[69]	Equine BMSCs and tendon fragments	Segregated—Transwell	Positive collagen I staining, increased expression of decorin, tenomodulin, and tenascin-C after 15 days in co-culture
Proffen *et al.*[2]	Porcine ADSCs versus PBMCs and ACL fibroblasts	Mixed	Increased expression of collagen I and collagen III by day 14 by ADSCs in co-culture; proliferation and procollagen synthesis were increased for fibroblasts in co-culture with ADSCs at days 7 and 14
MSC tri-culture models			
Study	Cell types	Tri-culture model	Findings
Manning *et al.*[72]	Mouse ADSCs, macrophages, and tendon fibroblasts	Mixed versus Transwell	Macrophages switch from M1 to M2 phenotype in tri-culture, resulting in release of fewer proinflammatory factors
Wang *et al.*[73]	Bovine BMSCs, fibroblasts, and osteoblasts	Segregated on coverslips	BMSCs exhibited greater fibrochondrogenic potential than ligament fibroblasts in tri-culture; growth of BMSCs decreased while proteoglycan production and TGF-β3 expression increased by day 14
3D co-culture and tri-culture models			
Study	Cell types	3D matrix model	Findings
Fan *et al.*[74]	Human BMSCs and ACL fibroblasts	Segregated with MSCs on gelatin/silk hybrid scaffolds	Increased expression of ligament-related markers by MSCs in co-culture
Schneider *et al.*[79]	Canine ADSCs and tenocytes	Mixed in high-density pellet culture; conditioned media	Upregulation of tenogenic markers (collagen I, collagen III, decorin, tenomodulin, and scleraxis) in co-culture
He *et al.*[75]	Rabbit BMSCs, ligament fibroblasts, and osteoblasts	Mixed and segregated on hybrid fibrous silk scaffolds	Increased expression of fibrocartilage markers SOX9 and aggrecan after 21 days by MSCs in direct contact with fibroblasts while exposed to paracrine signaling from osteoblasts
Wang *et al.*[73]	Bovine BMSCs, ligament fibroblasts, and osteoblasts	Segregated with MSCs in agarose hydrogel	Greater collagen I and collagen II expression, increased collagen synthesis by MSCs in tri-culture

model of porcine ACL fibroblasts with either adipose-derived stem cells (ADSCs) or MSCs isolated from peripheral blood (PBMCs).[2] Co-culture of ADSCs with fibroblasts results in increased expression of both types I and III collagen by day 14, while no such effect of co-culture was found with PBMC co-culture. Additionally, proliferation and procollagen synthesis were increased for fibroblasts in co-culture with ADSCs at days 7 and 14.[2] Work by Manning *et al.* examined the combined trophic and immunomodulatory roles of MSCs using a tri-culture model with mixed and segregated culture of mouse ADSCs, macrophages, and tendon fibroblasts.[72] Results from this study show that, while macrophages induced an upregulation of proinflammatory and matrix degradation factors

by fibroblasts, contact of fibroblasts with MSCs during exposure to signaling from macrophages suppressed fibroblast expression of these markers, suggesting an immunomodulatory role of MSCs during inflammation and wound healing.

While the above studies are important for understanding cellular communications between MSCs and native connective tissue cell types, other researchers have also studied these interactions on physiologically relevant matrices to understand the role that cell–matrix interactions play in modulating communications among cell types (Table 1). Wang *et al.* assessed the effects of a 3D microenvironment on MSC fibrochondrogenic differentiation through the use of a tri-culture model in which bovine MSCs were seeded in a 3D agarose hydrogel and cultured with osteoblasts and fibroblasts to promote ligament-to-bone interface regeneration.[73] It was noted that MSCs in hydrogels in both mono-culture and tri-culture exhibited greater expression of types I and II collagen compared to monolayer controls, suggesting that 3D culture facilitates MSC differentiation *in vitro*.[73] In work by Fan *et al.*, human MSCs were seeded on fibrous hybrid gelatin/silk fibroin scaffolds and cultured in segregated co-culture with ACL fibroblasts.[74] In this study, MSC proliferation and collagen production were increased at both 7 and 14 days compared with single-culture of MSCs on gelatin/silk fibroin scaffolds. In addition, expression of types I and III collagen was increased at days 7 and 14, with increased expression of tenascin-C at day 14 in co-culture.[74] He *et al.* similarly used knitted silk scaffolds for tri-culture of MSCs with fibroblasts and osteoblasts for ligament-to-bone regenerative applications.[75] In this study, rabbit MSCs, osteoblasts, and fibroblasts were seeded on individual scaffolds and cultured separately for 7 days, at which point the three scaffolds were sutured together to achieve a scaffold composed of an osteoblast-only region, an overlapping osteoblast–MSC region, an MSC-only region, an overlapping MSC–fibroblast region, and a fibroblast-only region. Results from this study show that MSCs in direct contact with fibroblasts while also exposed to paracrine signaling from osteoblasts undergo differentiation toward a fibrocartilage lineage on the basis of increased expression of SOX9 and aggrecan after 21 days in tri-culture.[75]

Collectively, these studies highlight the importance of studying cellular interactions on a physiologically relevant 3D matrix *in vitro* and show that the underlying matrix plays an important role in modulating MSC response for connective tissue regeneration.

Cell–matrix interactions

The matrix microenvironment within fibrous connective tissues is composed of mostly aligned type I collagen, as well as elastin, in a proteoglycan-rich matrix that functions to lubricate the tissue, as well as organize collagen fibril assembly.[80,81] Type I collagen fibrils are crosslinked to one another in a staggered fashion to form fibers, the primary unit of tendons and ligaments. These fibers are aligned along the direction of load bearing, separated by type III collagen fibrils.[80]

Following injury, cells within the tissue are induced to synthesize a dense mat of largely collagenous fibrotic scar tissue.[63] During scar formation, fibroblasts are triggered to form not only type I collagen but also an increased amount of type III collagen.[63,82–85] These collagen fibers are initially disorganized and randomly oriented, as opposed to the aligned fibrillar bundles observed in healthy tissue, with an increased presence of defects.[81,86] Furthermore, owing to its disorganized structure, this newly formed tissue is classically weaker than healthy connective tissues, unable to withstand physiological levels of loading.[82,87]

Extensive research through the years has focused on designing biomaterials and forming 3D ECM analogues, which serve to mimic the collagenous fibers within connective tissues in order to direct MSCs toward tendon/ligament lineages. To this end, matrices derived from either natural materials, such as silk and collagen, or synthetic materials, including the poly-α-hydroxyester family, have been developed and studied extensively for their effects on native tissue fibroblasts and MSCs.[88–90] Since fibrous connective tissues are largely made up of type I collagen, collagen-based gels have been used extensively as an engineered tendon/ligament matrix.[88] However, owing to the low mechanical properties of type I collagen hydrogels, fibrous matrices that can be woven to produce scaffolds with enhanced material properties have since been developed. Naturally derived silk fibers have been explored for tendon/ligament tissue engineering, as the fibers can be woven into braids or ropes with mechanical properties similar to native tissue.[91] Stem cells seeded on

these substrates have been shown to proliferate and secrete collagen matrix.[92] Similarly, synthetic materials such as poly-L-lactic acid (PLLA), polylactide-*co*-glycolide (PLGA), and polycaprolactone (PCL) have been used to produce fibrous meshes. These matrices are ideal for mimicking the structure of connective tissues, as they can be tuned with reference to fiber alignment[93] and diameter,[94] as well as matrix mechanical properties.[95] Current strategies to evaluate these matrices for promotion of tenogenic differentiation of MSCs include optimization of matrix topography, matrix mechanical properties, and ECM components.

A number of studies have evaluated the effects of matrix topography on tenogenic differentiation of MSCs through fabrication of unaligned and aligned fibrous matrices that mimic the architecture of the native tissue (Table 2). Yin *et al.* reported that tendon-derived MSCs upregulated their expression of tendon-related markers, with decreased expression of osteogenic markers, on aligned PLLA fibers compared with unaligned fibers.[96] These results suggest that aligned fibers are best suited for mimicking the structure of healthy connective tissues, such as tendons and ligaments.

In addition to the effects of matrix topography on cell response, the mechanical properties of the underlying substrate can also be optimized to modulate MSC response (Table 2). It is well established that tissue-adherent cells are capable of sensing and responding to the stiffness of the tissue microenvironment.[97] Foundational work by Engler *et al.* has shown that the stiffness of the underlying matrix can control MSC lineage commitment without the addition of chemical factors.[98] Specific to fibrous connective tissues, MSCs on polyacrylamide gels with mechanical properties similar to those of muscle have been shown to undergo differentiation toward a muscle lineage, while cells on softer substrates differentiate toward nerve cells, and stiffer substrates result in osteogenic lineage commitment.[99] Alternatively, it has also been shown that human MSCs can be kept quiescent by growing them on polyacrylamide substrates that mimic the properties of marrow.[100] Similarly, work by Sharma and Snedeker shows that, for human MSCs on acrylamide–bisacrylamide electrophoresis gels of varying stiffnesses, tenogenic marker expression is upregulated on matrices with mechanical properties similar to the native tendon but not on stiffer substrates.[101] Rehmann *et al.*, however, observed a combination of tenogenic and osteogenic marker upregulation for MSCs on polyethylene glycol (PEG)–tetranorbornene with increasing stiffnesses.[102] It has been speculated that intracellular changes resulting from alterations in matrix stiffness are a result of changes in integrin expression. Activation of these integrins results in the activation of mitogen-activated protein kinases, which have a downstream effect on the activation of Rho GTPases, such as RhoA and ROCK, a key pathway in MSC differentiation.[103]

In addition to the structure of the underlying ECM, ECM-bound factors and cell–ECM interactions in response to surface molecules are critical drivers of stem cell activity and homeostasis.[104,105] Interactions between stem cells and the surrounding ECM are mediated through a number of cell receptors, including integrins, and the extent of interaction between MSCs and the matrix has an effect on MSC spreading and shape, which have been shown to be important for MSC response and lineage commitment. Surface functionalization with matrix ligands, such as type I collagen and fibronectin, has been used to modulate cell spreading and integrin expression, both of which have been shown to affect MSC response and lineage commitment (Table 2). Sharma and Snedeker assessed the effects of surface functionalization on MSC response by coating the surface of acrylamide–bisacrylamide gels with varying densities of collagen and fibronectin.[101] The results show that MSC attachment is greater on collagen-coated surfaces than on fibronectin within 1 h, with increased cell spreading on collagen at 24 hours. Additionally, tenogenic differentiation was achieved on collagen substrates but not fibronectin, as MSCs on collagen coatings also exhibited an increase in the expression of scleraxis, tenomodulin, tenascin-C, and type III collagen, while fibronectin coatings resulted in enhanced RUNX2 and ALP expression, suggesting differentiation toward an osteogenic lineage.[101] This work indicates the importance of integrin-driven cell signaling in modulating cell response.

Soluble signaling cues

Besides matrix-driven cell signaling, soluble signaling cues are also involved in driving cell response following connective tissue injury. Specific to tendons/ligaments, research has shown that, following

Table 2. Cell–matrix interactions

Matrix topography *Fiber alignment* Study	Cell type	Scaffold	Results
Yin *et al.*[96]	Human TDSCs	PLLA nanofibers—aligned and unaligned	Increased expression of tendon markers on aligned fibers, increased expression of osteogenic markers on unaligned fibers
Tang *et al.*[106]	Human GFP-expressing BMSCs	Achilles tendon blocks of different angles (0°, 12°, 20°, 30°, 45°, 75°, and 90°), collagen I gel	0° and 12° sections result in increased tenomodulin expression at day 3
Zhang *et al.*[107]	Human iPSC-derived MSCs	Aligned and unaligned chitosan-based ultrafine fibers	Increased ALP expression and collagen staining on random fibers; increased tendon marker expression on aligned fibers
Popielarczyk *et al.*[108]	Equine BMSCs	Polystyrene fibers—parallel versus perpendicular	No major differences in gene expression or matrix synthesis between groups
Fiber diameter			
Cardwell *et al.*[109]	Murine BMSCs	Aligned and unaligned poly(ester urethane urea) mats with small (<1 μm), medium (1–2 μm), and large (>2 μm) diameters	Increased scleraxis expression on large fibers compared with medium fibers; increased collagen I expression on large fibers compared with small and medium fibers at day 14
Matrix mechanical properties			
Engler *et al.*[98]	Human BMSCs	Polyacrylamide gels	Muscle cells can be generated using medium stiffness (20 kPa) gels

injury, there is an increase in local concentrations of a number of growth factors, including bFGF, TGF-β, IGF-1, and platelet-derived growth factor (PDGF), all of which are active at multiple stages of the injury and healing process.[110] To this end, there is interest in using growth factor supplementation *in vitro* as a means of controlling MSC function and promoting differentiation (Table 3).

Both *in vitro* and *in vivo* studies have shown the potential of bFGF to act as a mitogen, as well as an angiogenic stimulator, and it has been proven to maintain MSC differentiation potential, stimulate proliferation, and induce fibroblastic differentiation. Specifically, at low doses, Hankemeier *et al.* showed that bFGF is capable of increasing MSC proliferation as observed at day 7, and promotes a tenogenic phenotype through increased expression of types I and III collagen, as well as fibronectin and smooth muscle actin, at days 14 and 21.[111] Sahoo *et al.* later incorporated bFGF into hybrid silk/PLGA fiber meshes and observed increased proliferation and matrix synthesis by MSCs cultured on these substrates, resulting in enhanced scaffold mechanical properties within 3 weeks.[112]

Another mitogenic factor, TGF-β, is produced by tendon and ligament fibroblasts and has been shown to be active in all stages of fibrous connective tissue healing.[113] In work by Holladay *et al.*, stimulation with TGF-β1 results in synthesis of fibrocartilaginous matrix by equine tendon-derived stem cells (TDSCs), which is undesirable for fibrous connective tissue repair and regeneration.[114] Jenner *et al.* also assessed the effects of TGF-β1 on human MSCs on PLGA fibers and saw enhanced proliferation in all TGF-β1–containing groups at day 12, as well as increased total collagen and synthesis per cell of both types I and III collagen.[115]

Bone morphogenetic proteins (BMPs) have also been used to induce tenogenic differentiation (Table 3). BMP-7, -12, -13, and -14 have been implicated in the neoformation and repair of tendons, and BMP-12 has specifically been shown to promote tendon differentiation and formation *in vitro* and *in vivo*.[116–118] Multiple studies have shown that BMP-12 alone is sufficient to promote tenogenic differentiation of MSCs *in vitro*, as observed through increased expression of tendon markers, including tenomodulin, decorin, and scleraxis.[118,119]

Table 3. Soluble factors

2D culture

Study	Cell type	Growth factor	Results
Hankemeier *et al.*[111]	Human BMSCs	bFGF	Low doses of bFGF stimulate MSC proliferation and upregulate expression of collagen I, collagen III, fibronectin, and α-SMA
Violini *et al.*[118]	Equine BMSCs	BMP-12	Increased tenomodulin and decorin expression by MSCs in BMP-12 on day 20
Park *et al.*[123]	Rat ADSCs	GDF-5	Increased cell number at 100 ng/mL at days 3–12; increased expression of scleraxis and tenomodulin in 100 ng/mL, increased tenascin-C expression in 1000 ng/mL
Zhang *et al.*[130]	Human TDSCs	Dexamethasone	All concentrations of dexamethasone result in suppressed collagen I expression and increased PPAR-γ and SOX9 expression at day 7
Mohanty *et al.*[119]	Equine umbilical cord blood (UCB) MSCs	BMP-12	Increased expression of scleraxis, tenomodulin, decorin, Mohawk, and collagen-1α1
Reed and Johnson[131]	Equine UCB-MSCs, ADSCs	FGF-2	FGF-2 results in increased proliferation by ADSCs only
Holladay *et al.*[114]	Equine TDSCs	IGF-1, GDF-5, and TGF-β1	IGF-1 preserves multipotency; GDF-5 supplementation results in increased tenogenic gene expression and decreased adipogenic and chondrogenic expression by day 28; TGF-β1 results in fibrocartilage/scar matrix formation

3D culture

Study	Cell type	Growth factor	Scaffold	Results
Moreau *et al.*[132]	Human BMSCs	FGF versus EGF + TGF-β1	RGD-modified silk fibers	Increased matrix synthesis for bFGF+TGF-β1 compared with EGF
Jenner *et al.*[115]	Human BMSCs	GDF-5	Braided PLGA fibers (Panacryl 2.0 suture material)	Increased collagen production and collagen synthesis per cell by day 12. No effect due to GDF5
Sahoo *et al.*[112]	Rabbit BMSCs	bFGF	bFGF-releasing silk/PLGA fibers	Increased proliferation, total collagen production, and enhanced mechanical properties after 3 weeks
Lee *et al.*[120]	Rat BMSCs	BMP-12	Collagen sponges	12-h treatment w/ BMP results in increased scleraxis and tenomodulin expression at day 14 *in vitro*; increased cell number, matrix synthesis, and expression of tendon markers at day 21 *in vivo*
James *et al.*[133]	Rat ADSCs	GDF-5	poly(D,L-lactide-*co*-glycolide) (PLAGA) fiber scaffolds and films	Dose-dependent increase in cell proliferation and expression of tenogenic markers and ECM markers beginning at day 7 on fibers
Bottagisio *et al.*[121]	Rabbit BMSCs	BMP-12, BMP-14, TGF-β, and VEGF	Fibrin-based constructs	Combination of factors results in tenogenic differentiation in monolayer and in 3D culture

Interestingly, for rat MSCs on collagen sponges, increases in the expression of scleraxis and tenomodulin were observed over 14 days, after only 12 h of exposure to BMP-12 on day 1. This 12-h stimulation resulted in increased cell number, matrix synthesis, and expression of tendon markers after 21 days *in vivo*.[120] Alternatively, Bottagisio *et al.* reported that BMP-12 or BMP-14 alone was

insufficient for inducing tendon lineage commitment and required the addition of TGF-β1 and VEGF to the culture medium to achieve tenogenesis.[121] Clearly, additional studies are needed to elucidate the effect of BMPs on MSC-mediated connective tissue response.

Growth differentiation factors (GDFs) are also members of the TGF-β superfamily and are closely related to BMPs. Factors such as GDF-5, -6, and -7 are believed to act as signaling molecules during tendon, ligament, and muscle development and have been shown to induce neotendon/ligament formation *in vivo*.[122] Adipose-derived stem cells exposed to GDF-5 *in vitro* have been shown to undergo enhanced proliferation, with a dose-dependent increase in scleraxis and tenomodulin expression. High doses of GDF-5 also result in increased tenascin-C expression, suggesting tenogenic lineage commitment in these groups.[123] Similar effects were observed for equine TDSCs, in which there was an upregulation in the expression of tendon-related markers by day 28 in culture and a concomitant downregulation in adipogenic and chondrogenic markers.[114] However, when MSCs were cultured on PLGA fibers, GDF-5 did not have any observable effects on MSC response compared with untreated controls.[115]

IGF-1 has also been shown to be highly expressed during early inflammation,[124] as it plays a significant role in the inflammatory and proliferative phases of wound healing.[110,125] Additionally, IGF-1 has been applied to damaged tendons and was observed to mitigate inflammation and accelerate the functional recovery of the tissue.[126] When used to stimulate MSC response, IGF-1 was observed to preserve the multipotency of equine TDSCs over 28 days *in vitro*.[114]

PDGF is a chemotactic agent, as well as a mitogen, and has been shown to promote protein synthesis by MSCs.[127] PDGF has been observed to be elevated in the healing canine digital flexor tendon[128] and is thought to play a role in connective tissue healing by inducing the synthesis of other growth factors, including IGF-1.[127] In work that assesses the response of ADSCs on aligned collagen fibers doped with PDGF-containing nanoparticles, PDGF stimulation resulted in enhanced cell proliferation for up to 7 days and increased expression of tendon lineage markers, including tenomodulin and scleraxis, at days 3, 7, and 14.[129] These observations suggest that not only can PDGF be used to promote tendon lineage commitment by MSCs, but it can also be incorporated directly into scaffold materials to provide multiple cues to cells simultaneously.

Physical stimuli

In addition to matrix-guided cues, mechanical stimulation is an important factor in modulating stem cell behavior within the tissue environment, especially in orthopedic tissues. The primary modes of stimulation experienced by fibrous connective tissues, such as tendons, ligaments, and muscles, are tensile and torsional loading,[88,134,135] the magnitudes of which have been shown to vary among tissue types and anatomic locations.[136] Specifically, tendons typically undergo greater levels of loading compared with ligaments, likely due to the forces generated by contracting muscles, and consequently tendons have been shown to have greater mechanical strength.[136] A number of models have been developed to elucidate the effects of both tensile and torsional loading on the response of MSCs in relation to cell proliferation, alignment, matrix synthesis, and organization, as well as expression of tendon- and ligament-related genes, and optimized loading regimens have shown promise for modulating stem cell metabolic activity and promoting MSC differentiation toward tendon and ligament fibroblasts (Table 4).

The simplest approach to mechanical stimulation of cells is the application of static loads. While these methods have proven effective for guiding cell orientation and organized matrix synthesis, little effect on MSC differentiation or proliferation has been observed (Table 4). Awad *et al.* seeded MSCs in collagen gels at varying densities and observed that contraction occurs to a greater extent in collagen gels with high cell densities compared with lower density gels.[137] Additionally, for gels with higher cell densities and consequently greater contraction, cells appeared more aligned with elongated nuclei compared with cells in less-contracted gels.[137] In another instance, van Eijk *et al.* tested the effect of varying the timing of static load application, and found that, by loading MSCs during seeding onto PLGA fibers, cell number was increased by day 5 compared with unloaded groups.[138] Still, there were no observable differences in cell proliferation or differentiation after 23 days in culture for any loading regimen.[138]

Table 4. Physical stimulation

Static loading Study	Cells	Scaffold	Regimen	Findings
Awad *et al.*[137]	Rabbit BMSCs	Collagen gels	Static stretch (contraction of collagen gels)	Greater contractions result in more aligned cells and elongated cell nuclei
Van Eijk *et al.*[138]	Goat BMSCs	Braided PLGA	Static tension by spring wire	Greatest number of cells after 5 days on loaded scaffolds, no effect by 23 days
Kawasaki *et al.*[145]	Human PDL cells	Tissue culture plastic	Oxygen tension—hypoxia versus anoxia	Upregulation of stem cell markers at hypoxic and anoxic conditions after 6 h; anoxic: increased scleraxis expression
Dynamic tensile loading				
Noth *et al.*[146]	Human BMSCs	Collagen I gel	Cyclic stretch: stretching frequency of 1 Hz and amplitude of 3 mm was performed for 14 days (continuously for 8 h/day)	Increased collagen I, collagen III, elastin, and fibronectin expression and enhanced matrix production by loaded MSCs by day 14
Juncosa-Melvin *et al.*[142]	Rabbit BMSCs	Collagen sponge	Dynamic stretch	Improved biomechanics following tendon repair in a rabbit model
Juncosa-Melvin *et al.*[143]	Rabbit BMSCs	Collagen sponge	Dynamic stretch	Increased collagen I and collagen III expression
Butler *et al.*[147]	Rabbit BMSCs	Collagen gel/collagen gel–sponge composite	Tensile strain—bioreactor before implantation	Improved mechanical properties after 12 weeks *in vivo*
Shearn *et al.*[148]	Rabbit BMSCs	Collagen sponges	1 Hz to produce a 2.4% post-to-post strain once every 5 min for 8 h/day for 12 days	No difference in mechanical properties following *in vitro* culture, but improved mechanical properties for loaded scaffolds after implantation
Lee *et al.*[65]	Human BMSCs	Flexcell®	1 Hz with 10% elongation for 2 days	Cells align perpendicular to strain; increased expression of collagen I, collagen III, and tenascin-C
Chen *et al.*[149]	Human BMSCs	Collagen I-coated Flexcell®	Stretching of 3% or 10% surface elongation at 1 Hz for 8 or 48 h	Increased MMP3 expression at 48 h for 3% strain; increased MMP3 expression for 10% strain group, but to a lesser extent; downregulation of MSCP (stem cell differentiation marker) in both stretch groups
Kuo and Tuan[150]	Human BMSCs	3D collagen I gels atop Flexcell®	Static tension: MSC-contracting collagen gel OR dynamic tension: 7 days cyclic uniaxial strain at 1 Hz for 30 min/day at 1% elongation	Collagen fiber alignment observed in stretched groups, increased collagen content following loading
Zhang *et al.*[151]	Rat BMSCs	Silicon membrane	Cyclic strain (10% at 1 Hz) for 3, 6, 12, 24, and 36 h	Increased collagen I and collagen III synthesis in loaded groups
Zhang *et al.*[67]	Rat BMSCs	Silicon membrane	Cyclic strain (10%, 1 Hz) was applied for different durations: 3, 6, 12, 24, and 36 h	Increased collagen III, collagen I, and tenascin-C expression
Nirmalanandhan *et al.*[152]	Rabbit BMSCs	Collagen sponge	Varied peak strain, cycle number, and cycle repetition at 1 Hz for 8 h/day for 12 days	Ideal loading regimen consists of 2.4% strain, 3000 cycles/day, and one cycle repetition
Nirmalanandhan *et al.*[153]	Rabbit BMSCs	Collagen sponge	2.4% strain, 3000 cycles/day, and one cycle repetition	Increased stiffness of scaffolds via crosslinking results in decreased mechanical properties after implantation *in vivo*

Continued

Table 4. *Continued*

Static loading Study	Cells	Scaffold	Regimen	Findings
Abousleiman *et al.*[154]	Rat BMSCs	Human umbilical veins	2% strain for 1 h/day at a frequency of 0.0167 Hz	Increased proliferation and collagen I/collagen III expression in loaded groups
Chokalingam *et al.*[155]	Mouse BMSCs	Collagen sponge	Tensile—2.4% peak strain for 20 s at 1 Hz followed by a rest period at 0% strain for 100 s (5 h/day)	Increased collagen I expression and increased linear stiffness for loaded groups
Song *et al.*[139]	Rat BMSCs	Silicon membrane	Dynamic stretch—cyclic uniaxial	Detectable tenascin-C and scleraxis, with increased collagen I and III expression in stretched samples
Zhang and Wang[141]	Rabbit TDSCs	Silicon dish with microgrooves	Cyclic stretching of 4% or 8% at 0.5 Hz was applied to silicone dishes for 12 h	Loading results in enhanced proliferation; increased collagen I expression for loaded samples, with no change in expression of fat or cartilage-related markers at 4% strain; increased expression of fat, cartilage, bone, and ligament markers with 8%
Doroski *et al.*[156]	Human BMSCs	PEG hydrogel	10% strain, 1 Hz, 2 h strain/3 h rest	Upregulation in tendon/ligament marker gene expression for loaded samples
Thomopoulos *et al.*[157]	Rat BMSCs	Collagen matrix	Dynamic cyclic loading 1.5 mm amplitude and 1 Hz for 7 days	Increased scleraxis, collagen I, and aggrecan expression under compressive and tensile loading
Issa *et al.*[158]	Rat BMSCs	Human umbilical vein	2% strain, 0.0167 Hz, 1 h/day	Lowest seeding density results in greatest tensile strength after 7 days
Kreja *et al.*[159]	Human BMSCs	Fibrous PLA scaffolds	Cyclic tensile—1 Hz 2% or 5% strain, 1 h/day for 15 days	No effect on gene expression, decreased MMP1, TIMP-2 expression with stretch
Xu *et al.*[103,160]	Human BMSCs	Silicon chamber	Stretch treatment at an amplitude of 10% and a frequency of 1 Hz for 48 h	Cells align perpendicular to strain, RhoA/ROCK, cytoskeletal organization, and FAK compose a signaling network that drives mechanical stretch–induced tenogenic differentiation
Morita *et al.*[140]	Human BMSCs	Silicon rubber chamber	1-Hz uniaxial cyclic stretching of 5%, 10%, or 15% elongation over 24 or 48 h	Expression of collagen I, collagen III, tenomodulin, and scleraxis is greatest for 10% strain group
Zhang and Wang[161]	Mouse TDSCs	Patellar + Achilles tendons	*In vivo*—treadmill running; *in vitro*—4% or 8% strain	Increased expression of both tenocyte (collagen I and tenomodulin) and nontenocyte (LPL, SOX9, and RUNX2) markers in the high-stimulation group (at day 5?)
Xu *et al.*[162]	Rabbit TDSCs	P(LLA-CL)/collagen scaffolds	4% elongation in length and 0.5 Hz, 2 h/day for a total of 14 days	Increased proliferation with loading; increased expression of tendon markers and decreased chondrogenic marker expression with stretch; loading promotes healing in rabbit patellar tendon injury model
Dynamic torsional loading				
Altman *et al.*[144]	Bovine BMSCs	Collagen gel	Dynamic tensile (10%) and torsional (25%) strains applied at 1 cycle/minute and	Observable collagen I, collagen III, and fibronectin synthesis by day 14, no detection of bone or cartilage markers
Chen *et al.*[163]	Human BMSCs	Silk fibers	45° rotation at 1.39×10^4 Hz	Cell response is dependent on temporal application of mechanical stimulation

Because of the limited effects of static loading on MSC response, more physiologically relevant dynamic tensile stimulation regimens have been developed to guide cell response (Table 4). Two-dimensional cyclic strain has been shown to not only encourage MSC alignment, but also to increase the expression of tendon- and ligament-related markers.[65,139,140] In work by Xu *et al.*, MSCs grown on silicone substrates coated in fibronectin were exposed to 10% strain at a frequency of 1 Hz for 48 hours.[103] Following mechanical stimulation, cells showed increased expression of tendon-related markers, including types I and III collagen, tenascin-C, and scleraxis. To better understand the mechanism behind these changes, the phosphorylation of focal adhesion kinase (FAK) was assessed, and it was shown to increase about twofold within 30 min of the application of tensile loading. To determine whether this pathway was responsible for the observed changes in MSC differentiation, the FAK pathway was blocked using Y-27632 (a RhoA/ROCK inhibitor), cytochalasin-D (an inhibitor of actin polymerization), and PF-228 (a PAK inhibitor). In all three groups, FAK activation was significantly decreased compared with the loaded positive control, with attenuated expression of all four tenogenic markers, suggesting that this pathway plays a role in MSC mechanotransduction and differentiation.[103]

Still, overstimulation of MSCs with mechanical stimuli can result in undesired cell response (Table 4). By loading human MSCs with varying degrees of strain, Morita *et al.* showed that MSCs stimulated with 10% strain at a frequency of 1 Hz for 24 h upregulated their expression of tendon-related markers compared with MSCs that underwent 5% or 15% strain.[140] Zhang and Wang similarly showed that, for rabbit tendon-derived MSCs, cyclic stretching at 4% strain at a frequency of 0.5 Hz for 12 h resulted in increased type I collagen expression, while increasing the strain to 8% resulted in upregulation of type I collagen, as well as cartilage-, bone-, and fat-related markers, such as type II collagen, SOX9, RUNX2, and peroxisome proliferator-activated receptor (PPAR)-γ.[141]

Extensive work by Butler *et al.* shows that mechanical stimulation of MSCs can be used for both tendon and ligament tissue engineering applications (Table 4). Initial work evaluated the effects of mechanical stimulation on MSCs on type I collagen sponges. Scaffolds were loaded under cyclic tension to a maximum strain of 4% once every 5 min for 8 h/day for 2 weeks.[142,143] Stimulated scaffolds exhibited increased mechanical properties compared with unstimulated scaffolds. Additionally, mechanically loaded sponges that were implanted into rabbit patellar tendon defects showed enhanced mechanical properties after harvest compared with unstimulated controls,[142] as well as increased expression of types I and III collagen by MSCs on loaded scaffolds.[143]

Increasing the complexity of applied loading regimens, Altman *et al.* seeded collagen gels with bovine MSCs and subjected scaffolds to 10% tensile strain and 25% torsional strain at a rate of 1 cycle per minute. By day 14, cells showed increased expression of types I and III collagen and fibronectin, with no observable increases in bone or cartilage markers. Following these initial studies, human MSCs were seeded on silk fiber matrices and exposed to 45° rotation at a rate of 1.39×10^4 Hz, which was applied 1, 3, 6, or 9 days after cell seeding to assess the impact of temporal application of torsional strain on stem cell response. Results show that MSC metabolic activity was greatest on samples loaded 9 days after seeding.[144]

Synergistic effects: combined cues

While *in vitro* results suggest that MSC metabolic activity and differentiation can be controlled through optimization of individual microenvironment components, *in vivo* studies using these models have yielded mixed results. Therefore, several groups have begun testing the effects of combining multiple environmental cues on the ability to control stem cell behavior (Table 5). In works by Nirmalanandhan *et al.*, rabbit MSCs were seeded on both type I collagen sponges and gels and exposed to uniaxial tension. Mechanical stimulation of MSCs on sponges resulted in enhanced mechanical properties, while loading MSCs on gels did not improve the elastic modulus of gels, suggesting that the combination of mechanical and matrix-based cues affects MSC response.[164] Subramony *et al.* also assessed the combined effects of matrix and mechanical cues on MSCs through application of uniaxial tension to MSCs on unaligned and aligned PLGA nanofibers. It was determined that, while mechanical stimulation resulted in increased cell proliferation and collagen synthesis on both

Table 5. Combined microenvironmental cues

Study	Cells	Scaffold	Combined cues	Findings
Nirmalanandhan *et al.*[164]	Rabbit BMSCs	Collagen I sponge and gel	Matrix properties + mechanical stimulation	Loading results in increased mechanical properties for collagen sponges but not collagen gels
Nirmalanandhan *et al.*[168]	Rabbit BMSCs	Collagen I sponge and gel	Matrix properties + mechanical stimulation	Longer sponge constructs result in higher *in vitro* linear stiffness
Petrigliano *et al.*[165]	Rat BMSCs	bFGF-coated PCL	Growth factors (bFGF) + mechanical stimulation	Upregulation of collagen I, collagen III, and tenascin-C expression over 21 days
Moreau *et al.*[166]	Human BMSCs	Silk fiber matrix	Growth factors (bFGF, EGF) + mechanical stimulation	Rotation at 0.5 cycles/h is optimal when combined with bFGF
Rowlands *et al.*[169]	Human BMSCs	Collagen I-, collagen IV-, laminin-, and fibronectin-coated gels	Matrix components + matrix mechanical properties	Myogenic differentiation is achieved on all gel–protein combinations with stiffnesses > 9 kPa
Farng *et al.*[170]	Mouse BMSCs	Porous PCL scaffolds	Growth factors (GDF-5) + mechanical stimulation	Combined mechanical and chemical stimulation enhanced mRNA production of collagen I, collagen II, and scleraxis
Sharma and Snedeker[101]	Human BMSCs	4–12% acrylamide–bisacrylamide gels (10–110 kPa)	Matrix components (varying concentration of collagen, fibronectin) + matrix mechanical properties	Increased osteogenic differentiation on fibronectin-coated substrates, with decreased osteogenic marker expression with decreasing stiffness; tenogenic marker expression enhanced on softer and collagen-coated substrates
Beier *et al.*[78]	Rat BMSCs	N/A—tissue culture plastic	Myoblast co-culture + growth factors (bFGF, dexamethasone)	Upregulation of myogenic markers (MEF2 and α-sarcomeric actin) in co-culture with medium supplementation
Kishore *et al.*[171]	Human BMSCs	Collagen fibers	Fiber alignment + BMP-12	Increased cell adhesion, decreased proliferation, increased expression of tendon-related markers, and decreased expression of bone-related markers on aligned fibers; no effect of BMP-12
Subramony *et al.*[13]	Human BMSCs	Unaligned and aligned PLGA nanofibers	Matrix alignment + mechanical stimulation	Loaded MSCs on aligned fibers produce both collagen I and collagen III, while collagen I is predominantly synthesized by loaded MSCs on unaligned fibers; upregulation of fibroblast marker expression on loaded aligned fibers only
Raabe *et al.*[167]	Horse ADSCs	Collagen I gels	Growth factors (GDF-5, -6, -7) + oxygen tension + mechanical stimulation	GDF-5/GDF-7 supplementation results in enhanced expression of collagen I, collagen III, and scleraxis
Subramony *et al.*[14]	Human BMSCs	Unaligned and aligned PLGA	Matrix alignment + mechanical stimulation + bFGF supplementation	bFGF results in increased proliferation, while mechanical stimulations led to increased matrix synthesis and upregulation in ligament-related gene expression
Cheng *et al.*[129]	Rat ADSCs	Aligned and unaligned collagen fibers	Matrix alignment + PDGF release	Increased proliferation up to day 7 and increased expression of tendon markers on aligned PDGF-eluting fibers
Czaplewski *et al.*[95]	Human iPSC-derived MSCs	Braided submicron fibrous scaffolds—PLLA versus PCL	Matrix composition/mechanical properties + mechanical stimulation	Increased expression of both ligament- and bone-related markers on PLLA compared with PCL at day 3
Banks *et al.*[172]	Human ADSCs	Collagen gel—crosslinked membranes (2.5–5 MPa)	Matrix mechanical properties + growth factors (PDGF-BB) (BMP-2, PDGF-BB)	Increased osteogenic differentiation and decreased adipogenic differentiation with increasing stiffness; PDGF-BB decreased ALP expression by ADSCs on stiff substrates, while BMP-2 increased ALP expression on soft substrates

Continued

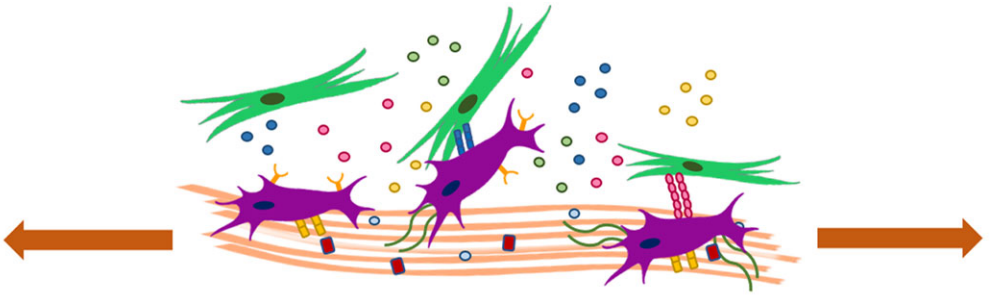
Table 5. *Continued*

Study	Cells	Scaffold	Combined cues	Findings
Durant *et al.*[173]	Human BMSCs	Fibrin gels	Growth factors (TGF-β) + oxygen tension	TGF-β supplementation and low oxygen tension results in increased cell number; increased collagen I and III expression with addition of TGF-β regardless of oxygen tension
Rehmann *et al.*[102]	Human BMSCs	PEG–tetranorbornene (10–90 kPa)	Matrix components (varying concentration of collagen, fibronectin) + matrix mechanical properties + BMP-13, ascorbic acid	Increasing modulus and collagen content results in increased ligamentogenic/tenogenic gene expression and protein production in the presence of BMP-13 and ascorbic acid

aligned and unaligned fibers, the expression of ligament-related markers, including scleraxis and tenascin-C, was increased on aligned fibers only.[13] Czaplewski *et al.* studied the impact of matrix composition and mechanical properties on the response of MSCs to mechanical loading by seeding cells on braided fibers composed of PLLA, PCL, or blends of the two polymers, braided using a range of braid angles. While matrix composition was observed to affect MSC attachment and spreading, braiding angle was shown to impact tendon- and ligament-lineage commitment, as fibers with large braid angles resulted in increased expression of tendon and ligament markers and downregulation of bone markers by day 10 compared with day 3.[95] These studies show that, while mechanical stimulation is known to influence MSC commitment toward tendon and ligament lineages, matrix-based cues, such as matrix organization or mechanical properties, can be used as a means of further enhancing these observed effects.

In addition to synergistic matrix and mechanical cues, others have combined either matrix microenvironment or mechanical stimulation with chemical stimuli to promote MSC differentiation (Table 5). Petrigliano *et al.* incorporated bFGF into PCL nanofibers and exposed human MSCs seeded on fibers to uniaxial tensile loading.[165] These combined stimuli led to an upregulation in the expression of tendon-specific markers, including types I and III collagen and tenascin-C, by day 21. Similarly, in work by the Altman group, MSCs seeded on fibrous silk scaffolds were exposed to either FGF or epidermal growth factor (EGF), followed by cyclic torsional loading. Sequential exposure to chemical and mechanical stimulation while in contact with a physiologically relevant matrix resulted in increased matrix production and cellular ingrowth into scaffolds, as well as enhanced differentiation toward ligament fibroblasts.[166] In work by Subramony *et al.*, human MSCs on unaligned and aligned PLGA fibers were exposed to uniaxial tensile loading in the presence of bFGF. This combinatorial approach showed that exposure to bFGF enhances MSC proliferation, while mechanical stimulation results in increased collagen synthesis and the upregulation of ligament-specific genes, including types I and III collagen, tenascin-C, and tenomodulin, suggesting a synergistic effect due to MSC exposure to a combination of these cues on a physiologically relevant aligned fibrous substrate.[14] Raabe *et al.* also developed an *in vitro* model that combines mechanical and chemical stimulation to promote differentiation of equine ADSCs in type I collagen gel scaffolds.[167] It was observed that high oxygen tension combined with exposure to GDF-5 or GDF-7, as well as cyclic tensile strain, could promote tenogenic differentiation, as observed through increased expression of types I and III collagen, cartilage oligomeric protein, and scleraxis by day 21.

Therefore, each of these studies highlights the potential synergistic effects of exposing MSCs to a combination of cues mimetic of the surrounding connective tissue microenvironment, either sequentially or simultaneously. While these studies have been successful for promoting MSC differentiation toward connective tissue lineages *in vitro*, future work will need to focus on determining whether these cues will be sufficient to promote MSC regenerative capabilities *in vivo*. To this end, development of an implantable artificial microenvironment to control stem cell response and promote stem cell–guided tissue regeneration is an attractive option to augment stem cell–based treatments for connective tissue repair.



Microenvironmental Component	Optimal Cues	Effects on Regeneration
Cellular Cues	Fibroblast–MSC interactions	Increased proliferation, collagen I synthesis, MSC differentiation toward a fibroblastic lineage
Matrix-Based Cues	Aligned, microfibers	Increased expression of tendon and ligament markers
Soluble Signaling	bFGF, BMP-12, GDF-5	Increased proliferation and collagen synthesis
Mechanical Cues	Cyclic tensile loading +/– torsion	Increased alignment, expression of ligament/tendon markers, collagen synthesis

Figure 3. Critical cues for promoting stem cell–guided tissue regeneration.

Summary

Adult MSCs are a powerful candidate cell type for regenerative medicine because of their capacity for self-renewal and multipotent differentiation, as well as the critical role they play in trophic signaling and immunomodulation. However, the key to harnessing the regenerative potential of stem cells lies in the design of a cell microenvironment that is conducive to stem cell lineage commitment, biomimetic tissue regeneration, and, ultimately, restoration of physiological function. We highlighted current strategies in designing an optimal microenvironment for connective tissue healing, including cellular interactions, soluble factors, mechanical stimulation, and/or features of the ECM that direct stem cell–mediated connective tissue regeneration (Fig. 3). It is clear that there has been significant progress in our understanding of how individual aspects of the microenvironment can guide stem cell differentiation and mediate their regeneration potential.

The frontier of the field resides in elucidating the effects of combined cues from the microenvironment and distilling the opportune timing for implementing these cues as current understanding of the biology of connective tissue healing advances. In order to drive this area of research forward, methods for standardizing experimental conditions *in vitro* and *in vivo*, including optimization of cell seeding density and cell source, are critical to the success of stem cell–guided tissue regenerative therapies. Additional challenges in engineering the healing connective tissue microenvironment include determining the relative importance of the environmental cues and how best to strategically guide and accelerate stem cell–mediated healing and restore tissue function. Finally, the translation of these exciting discoveries must be pursued in parallel in order to restore mobility and improve the quality of life for millions of patients worldwide.

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Competing interests

The authors declare no competing interests.

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