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Review Article

Role of Mechanical Stimulation in Stem Cell Differentiation

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Abstract

Mechanical forces are known to play a role in cell behavior and adaptation within their environment. In recent years, special attention has been paid to how these forces interact with various stem cell sources to direct stem cell differentiation. Embryonic, induced pluripotent, and adult or progenitor stem cells have all used in research involving mechanical stimulation. These various cell types have been exposed to numerous types of stimulation, such as tensile or compressive strain, fluid shear stress, or oscillatory vibration. Interestingly, despite the wide range of stem cell sources and types of mechanical stimulation used, the pathways activated under mechanical stimulation are very similar. Mechanical stimulation can impact numerous pathways, including TGF-β, Wnt, and MAPK. Forces can also affect cytoskeletal structure, osmolality of the cytoplasm, or affect nuclear pore size and permeability. This collective knowledge has provided great evidence for the field to use mechanical stimulation alone, or combined with biochemical stimulation, to promote differentiation towards various phenotypes. This differentiation is often associated with increased production of extracellular matrix proteins, such as collagens and glycosaminoglycans, which can greatly impact the mechanical properties of a tissue-engineered construct. Ultimately, the role of mechanical stimulation in stem cell differentiation and behavior is, and will continue to be, a vital component in countless tissue engineering applications.

INTRODUCTION

Tissue engineering and stem cell therapy developments always require determination of a viable cell source. Stem cells are being extensively studied for this purpose, with efforts focused on developing methods for differentiation into the desired phenotype. One important differentiation cue is mechanical stimulation, and despite its importance during development, it is often neglected. During the early stages of development, various forces and strains are directly responsible for some of the most important milestones of development. For example, fluid accumulation pushing the inner cell mass against the zona pellucida is necessary for the c of Oct 4, NANOG, and Sox-2 and thereby induction of pluripotency [1]. Mechanical cues also play a role in terminating pluripotency, as micro strains developed within the cell through actin filament alignment can significantly down regulate Sox-2, inducing differentiation [2]. Mechanical stimulation can also play a key role in embryonic and adult stem cell differentiation to various lineages [3]. Also important to note is mechanical stimulation's ability to increase production of key extracellular matrix proteins, which has significant impact on many tissue engineering attempts [4,5].

The vast majority of stem cell research focuses on the use of biochemical factors for differentiation, due to the vast existing knowledge of factors that influence gene transcription and protein production. However, gradually more information is emerging on how mechanical forces can directly influence the gene transcription of cells, much like biochemical factors. It has been established that molecular mechanosensing, like biochemical sensing, involves conformational changes to proteins or structures in the cell wall, generally resulting in a chain reaction resulting in changes to gene transcription [6]. During development, mechanical cues are necessary for proper development of several tissues [7]. Restriction of muscle contraction in the fetus inhibits growth of the synovial cavity, while increases in muscle contraction stimulates growth of the synovial cavity and allows for healthy development of articular joints [8].

Some of these changes are well studied, including the TGF-β, MAPK, and Wnt pathways [9,10]. These pathways can be activated directly through force-induced folding or unfolding of particular nuclear proteins or mechanical disassociation of previously associated proteins. Additionally, mechanical forces can affect the volume or shape of a cell, changing the osmolarity of the cell interior [11]. Forces can also affect ion channels in the cell membrane and/or nuclear pore sizes, altering which substances can enter the cell as well as the nucleus itself [6]. Even low forces can have a significant effect, with 0.8-1.7N forces resulting in rapid chromatin decondensation [12]. Perhaps most

importantly, the duration of these signals is crucial to the ultimate effect. In some cells, prolonged mechanical stimulation (>225s) can cause permanent change to histone structure. Interestingly, the same stimulation for shorter durations also results in histone structure modifications but the histone structures revert back to their original state once the stimulus is removed, creating a reversible effect [12].

The effects of mechanical forces on cells cannot be understated. Along with being capable of mimicking the effects of biochemical stimulation, mechanical signals can travel at speeds of 1-2 μm/s compared to 30 m/s for biochemical signals [6].

In this article, we review the role and influence of several types of mechanical stimulation on the differentiation of various stem cells. The most commonly studied types of mechanical stimulation are diagramed in Figure (1). This information is of importance as methods to direct stem cell differentiation are currently heavily studied, with the goal of creating more effective treatments for numerous diseases. As most of these efforts are focused on biochemical means of differentiation, it is vital to bear in mind the strong influence other factors have on differentiation *in vivo*, including mechanical stimulation. A summary of these studies can be found in Table (1).

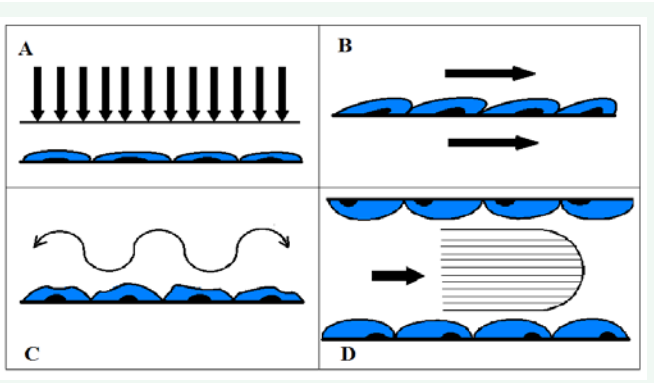

Figure 1 Diagram of various forms of mechanical stimulation: Black arrows represent direction of applied forces. Compression (A) can be applied directly to a cell-seeded construct or directly to the surrounding fluid as hydrostatic compression. Tension (B) can be applied biaxially or uniaxially, resulting in temporary structural deformation of cells. Oscillatory or vibrational stimulation (C) can be applied to a cell-seeded construct, or directly to the surrounding medium. Laminar shear stress (D) is applied through fluid flow, often to the interior of a cell-seeded lumen. The fluid velocity profile, shown by the curve and lines in the middle, demonstrates the distribution of fluid speed within a lumen experiencing pure laminar flow.

TENSION

The role of tensile strain in stem cell differentiation has been investigated in numerous organ systems and cell types. Feasibility for such experiments have been made more possible by the commercial availability of the Uniflex and Bioflex bioreactor models from Flexcell® International Corporation (Burlington, NC). These bioreactors subject cell cultures to uniaxial or biaxial strain through pneumonic pressure changes to a flexible membrane as shown in Figure (2). Along with commercially available devices, numerous custom-designed and custom-built devices have been constructed to use a wide variety of mechanisms to induce uniaxial and biaxial tensile strain.

Activated pathways and cellular effects

Tensile strain, like many types of mechanical strain, is thought to influence the TGF-β pathway, resulting in an accumulation of protein complexes in the nucleus that act as transcription factors [13]. This is thought to be of particular importance in fibrogenic, chondrogenic and osteogenic pathways [13-15]. Multiple studies have shown that combining TGF-β and tensile strain result can result in increased expression of collagen I, α-smooth muscle actinin, h1-calponin, along with other cytoskeleton markers [13,14]. An illustration of tension's role in increasing TGF-β, and hence directing differentiation, is shown in Figure (3). Further exploration has shown that uniaxial tensile strain can significantly increase the Young's Modulus of the cell after strain, owing to the increased alignment of cytoskeleton components, including F-actin fibers [14,16]. Additionally, cyclic strain, both uniaxial and biaxial, is thought to promote cell renewal and cell growth through activation of these pathways in both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) [15,16]. Tensile strain can be used to both direct differentiation towards cell pathways and inhibit differentiation towards others. Akt-induced inhibition of glycogen synthase kinase 3β (GSK3β) can be brought about through cyclic tensile strain [16,17]. This inhibition results in restructuring of the cell, with an increase in focal adhesions and increased RhoA activity [17]. In turn, increases in focal adhesions increase the sensitivity of the cell to mechanical stimulation, further amplifying the effects. This cellular activity is considered crucial in osteogenesis and directly leads to inhibition of adipogenesis [17].

Mechanical stimulation has also been used in tissue engineering to increase alignment of stem cells, even when not directly driving differentiation. Uniaxial tensile strain has been used to align numerous stem cell types, including adipose-derived stromal cells (ASCs), skeletal muscle cell line C2C12s, and murine skeletal muscle progenitor cells [18-20]. Despite the overall inconclusive results, some increase in myogenic markers have been noted in mesenchymal stromal cells (MSCs) when stretch is accompanied with myogenic media, specifically muscle markers myogenic factors 5 and 6 [20,21]. In all report cases reviewed, mechanical stimulation alone was insufficient to significantly increase gene expression, although inclusion of growth factors, such as IGF-1 showed significant increase in expression compared to either independent stimulus [21].

Fibrogenesis

Both osteogenesis and fibrogenesis in stem cells are thought to be at least partially dependent on the TGF-β pathway [13,15]. Cyclic, uniaxial strain has been shown to upregulate collagen I, fibronectin and versican among other key fibrogenic markers after as little as a few hours in MSCs and ASCs [22-24]. Strains as low as 3% have shown to induce an elongated morphology and alignment of actin fibers similar to that of fibroblasts [22]. Several attempts at obtaining a mixed fibroblast and chondrocyte phenotype have also investigated use of tensile strain. By using TGF-β along with tensile strain, potential for an apparently "mixed" phenotype has been shown [23,24]. With the seemingly

Table 1: Examples of mechanical stimulation in stem cell research.

Author	Cell Type	Mechanical Stimulation	Results
Absousleiman et al., 2009	Rat MSCs	2 weeks of 2% cyclic tensile strain at 0.00167Hz.	Increased cell and matrix alignment, 156% increase in ultimate tensile strength and 109% increase in elastic modulus.
Adamo et al., 2009	Murine ESCs	Fluid shear stress. 10 hour ramp period to 5 dyn/cm^2 then 26 hours at 5 dyn/cm^2.	Significant increase in Runx1, Myb, and Klf2 expression and formation of hematopoietic colonies.
Amin et al., 2014	Rabbit ASCs, MSCs	Equiaxial tensile strain of 10% at 1Hz for 24 hours.	Increase in GATA4 expression in cells subjected to strain. ASCs saw greater expression than MSCs.
Andersen et al., 2014	Human ASCs	Uniaxial tensile strain of 15% at 0.5Hz for 48 hours.	No significant changes in gene expression. Significant increase in stem cell alignment.
Arulmoli et al., 2015	Rat Neural stem cells	Static tensile strain 10%.	Static tensile strain increased axon length and width.
Baker et al., 2011	Bovine MSCs	Cyclic tension of 6% at 3Hz.	Significant increase in fibrogenic gene expression and collagen I production. Increase in tensile modulus of 16%
Boonen et al., 2010	Murine C2C12s, muscle progenitor cells.	Uniaxial tensile strain of 2%-6% at 1Hz. 3 hours on, 3 hours off for 48 hours MPa.	Decrease in expression of MRF and sarcomere markers. Delayed formation of cross striations.
Carroll et al., 2014	Porcine MSCs	10 Mpa cyclic hydrostatic pressure	Hydrostatic pressure suppressed calcification and increased chondrogenic marker expression.
Chang et al., 2013	Rat Neural stem cells	Uniaxial tensile strain of 5mm/5min static, dynamic, and 1mm/day dynamic for 1, 3, or 7 days.	Stretch increased axon length and diameter as well as increasing expression of MAP2 and βIII-tubulin.
Chen X et al., 2015	Human MSCs	Acoustic-frequency vibratory stimulation at 0, 30, 400, or 800Hz	Stimulation at 800Hz down regulated adipogenic genes and up regulated osteogenic markers. 30Hz showed up regulation of adipogenic markers.
Chowdhury et al., 2010.	Murine ESCs	Focal adhesion-induced stress	Substrate stiffness affects mechano transduction. Significant stress can alter Oct4 expression.
Connelly et al., 2010	Bovine MSCs	Cyclic tensile strain of 10% at 1 Hz for 24 hours or 1-2 weeks.	24 hours of stimulation increase proteogly can and protein synthesis, 2 weeks showed only net increase in protein synthesis. Increases were seen in collagen I gene expression, but no significant changes in collagen II, aggre can, or osteocalcin expression.
Correia et al., 2013	Human ASCs	Steady and pulsatile flow in varying combinations for 5 weeks with a flow rate of 400μm/s.	2 weeks of steady flow followed by 3 weeks of pulsatile flow showed greatest increase in osteogenic gene expression, histological changes, and increased equlibrium moduli.
Correia et al., 2012	Human ASCs	0.4MPA pulsatile and static loading for three weeks and 0.5MPA pulsatile and static loading for four weeks.	Pulsatile loading resulted in greatest gene expression and chondrogenic matrix production in both studies.
Egusa et al., 2013	Murine MSCs	10% uniaxial tensile strain at 0.17Hz	48 hours of strain increased cell alignment and actin fiber orientation. Up regulation of Myf5, myogenin, MRF4 was noted, but myocardin and α-SMA did not change.
Geuss et al., 2014	Murine ESCs	Paramagnetic beads encapsulated in EB exposed to 0.128, 0.2, or 0.4 Tesla magnetic field over seven days.	0.2 Tesla mediated strain activated PKA and increased pERK1/2 expression. Strain plus BMP4 induced cardiomyogenesis as indicated by increased contractility and α-actin expression.
Haghighipour et al., 2012	Human MSCs	10% cyclic uniaxial strain at 1 Hz for 24 hours.	Cyclic tension combined with IGF-I showed greatest expression of skeletal muscle markers Myf5, MyoD, MyoG, and Myf6, although significant increase was also noted with mechanical stimulation alone.
Huang CY et al., 2005	Rabbit MSCs	Cyclic compressive strain of 15% at 1 Hz for 4 hours for 2 days.	Compression promoted expression of c-Jun, Sox 9, and TGF-β.
Huang CH et al., 2009	Human MSCs	Cyclic tensile strain of 3% at 0.1 Hz for 1, 3, or 5 days.	Strain activated phosphorylation of FAK, Clba1, and increased ALP activity and matrix deposition.
Illi et al., 2003	HUVECs	Shear stresses of 10 dyn/cm^2	Shear stress induced histone H3 serine phosphorylation at (S10) and lysine acetylation at (K14). Shear stress also activated ribosomal S6 kinase-2 and mitogen- and stress-activated kinase-1 protein kinases and formation of a (CREB)/CREB-binding protein complex

Illi et al., 2005	Murine ESCs	Shear stresses of 10 dyn/cm^2	Shear stress induced expression of cardiac markers, including SMA, smooth muscle protein 22-alpha, platelet-endothelial cell adhesion molecule-1, VEGF receptor 2, myocyte enhancer factor-2C (MEF2C), and alpha-sarcomeric actin.
Ji et al., 2014	Human dental pulp stromal cells	Compressive loading 3x30 minutes daily at 1Hz for 1-3 weeks.	Compression increased cell viability and alkaline phosphatase staining. Significant increase in collagen I content.
Kearney et al., 2010	Rat MSCs	Cyclic tensile strain of 2.5% at 0.17Hz for 0-14 days.	Cbfα1, collagen I, osteocalcin, and BMP2 were temporally expressed. Strain induced synthesis of BMP2 was inhibited by ERK, p38 and PIK inhibition.
Khani et al., 2015	Human MSCs	Cyclic uniaxial tensile strain of 5% at 1Hz for 24 hours.	Stretch with or without TGF-β increased elastic modulus of cells, drop in creep compliance curve, and formation of f-actin bundles.
Koike et al., 2005	ST2 Stromal cells	0.8-15% tensile strain at 1 Hz for 2 days.	Cbfa1 and Run 2 expression increased at 0.8 and 5% strains, but decreased at 10 and 15% strain. Type I collagen and osteocalcin increased at higher strains at later time points.
Kong et al., 2012	Rat Endothelial progenitor cells	5% compressive strain at 1Hz, four hours daily for 7 days.	Compression increased cell proliferation and formation of vascular-like tubes.
Kreutzer et al., 2014	Human iPSCs	Cyclic biaxial tensile strain of 5% at 1 Hz, ramped from 1% at 0.2Hz, for 21 days.	Stretching was not found to significantly affect expression of cardiac markers.
Kuo et al., 2015	Human MSCs	Oscillatory shear stress of 0.5+/- 4 dyn/cm^2 for 0.25 to 24 hours.	Oscillatory shear stress for 30 minutes or greater led to activation of β-catenin pathway and reorganization of f-actin. Up regulation of Wnt inhibition factors was also observed.
Li J et al., 2012	Rabbit ASCs	Cyclic compression of 5% strain at 1Hz, four hours daily for 7 days.	Activation of calcium signaling pathways and up regulation of Sox9 was noted. Inclusion of IGF-I displayed increased expression of collagen II, Sox9, and aggrecan.
Li YJ et al., 2004	Human MSCs	Oscillatory fluid flow	Increased calcium mobilization, and osteocalcin and osteopontin expression were noted.
Li Z et al., 2010	Human MSCs	Up to 20% compression at 1 Hz for one hour daily for 7 days.	Up regulation of TGF-β with compression, blocking TGF-β pathway prevent chondrogenesis.
Lim et al., 2014	Human MSCs	Shear stresses of 0.86-1.51 dyn/cm^2 for 10, 30, 60, 120, or 180 min.	Shorter durations resulted in an increase in mineralized nodules. Longer durations resulted in increased quantities of BMP2 and VEGF protein.
Liu et al., 2012	Human MSCs	Cyclic compression of 10% strain at 0.5Hz for 2 hours on/4 hours off for 2 weeks.	After 2 weeks, equilibrium modulus increased 185% and tensile modulus increased 202%. Procollagen I also increased.
Lohberger et al., 2014	Human MSCs	10% continuous cyclic strain at 0.5Hz for 7 or 14 days.	Significant increase in mRNA for collagen I, BMP2, osteocalcin, and osteopontin. Stretched groups also had greater calcium deposits.
Lucitti et al., 2007	Murine Ebs	Characterization of flow in embryonic development.	Vascular remodeling and expression of eNOS is dependent on fluid flow and fluid viscosity.
Luo et al., 2011	Rat MSCs	Laminar shear stresses of 15 dyn/cm^2 for 4, 12, or 24 hours.	Shear stress suppressed apoptosis in MSCs and significant increases in Bcl-2 and Bcl-2/Bax ratio were noted.
Matziolis et al., 2011	Human MSCs	Cyclic loading of 4kPa at 0.05Hz for 24 hours MPa hydrostatic.	Significant increase in expression of SMAD5, osteopontin, TGF-β-R1, PDGF-α, annexin-V, and ITGβ1.
Meyer et al., 2011	Human MSCs	10 MPA hyrdostatic pressure applied at 1 Hz for 1 hour/day for 5 days per week for 6 weeks.	Upregulation of TGF-β and chondrogenic markers, but two donors had different responses to loading.
Nguyen et al., 2014	Chick ESC-CMs	Cyclic tensile strains of 8-15% at 2Hz for 4 days. Bioreactor had internal pressure of 10mmHg.	Stimulated cells had higher beat rate and contractility response to isoproterenol. Significant increase in total protein levels as well as SERCA2a and TnT expression.
Ogawa et al., 2009	Human ASCs	Cyclic pressure at 0-0.5MPA at 0.5 Hz for 2, 3, or 4 weeks.	Cell number increased until week 2, then decreased until week 4. SRY box9, collagen II, and aggrecan increased in culture through week 4.
Pelaez et al., 2009	Human MSCs	Cyclic compressive strain of 10% at 0.1, 0.5, or 1.0Hz for 4 hours for 2 days MPa.	Higher frequencies (1.0Hz) resulted in highest cell viability. Lower frequencies saw significant cell death.
Puetzer et al., 2013	Human ASCs, MSCs	7.5 MPa CHP for 4 h per day at a frequency of 1 Hz for up to 21 days.	mRNA expression peaked at 7 days. Collagen II expression up regulated at day 14, with Sox 9, aggrecan, and COMP at day 7.
Qi et al., 2009	Rat MSCs	Cyclic tensile strains of 2000με at 0.5Hz.	Significant increase in ALP activity and upregulation of TGF-β, Ets-1, bFGF, IGF-II, and Cbfa-1.

Riddle et al., 2006	Human MSCs	Oscillatory shear stress of 5, 10, 20 dyn/cm^2 MPa	Activation of calcineurin and phosphyorylation of ESR kinase 1 and 2.
Safshekan et al., 2012	Human ASCs	Hydrostatic pressure of 5 Mpa at 0.5 Hz four hours daily for 7 days.	Mechanical stimulation and biochemical stimulation resulted in greatest expression of Sox9, collagen II, and aggrecan.
Saha et al., 2006	Human ESCs	10% cyclic biaxial strain at varying frequencies.	Mechanical strain maintained pluripotency.
Saha et al., 2008	Human ESCs	Cyclic biaxial strain (flexcell) of 10% at 10 cycles/ min for up to 12 days MPa.	Strain induced phosphorylation of Smad 2 and 3. TGF-β1 and Activin A inhibition promoted differentiation.
Sakao et al., 2008	Rabbit MSCs	Hydrostatic pressure of 1-5 Mpa	Proteoglycan, collagen II, and Sox 9 mRNA expression increased at 5 Mpa. Protein content of Sox 9 and GAGs also increased at high pressures.
Salameh et al., 2010	Neonatal rat CMs	Cyclic tensile strains of 0, 10, or 20% for 0, 24, or 48 hours at 1 Hz.	10% strain at 24 hours induced elongation and reorganization of Cx43 at the induced poles. Upregulation of Cx43 mRNA and protein was noted as well as upregulation of ERK 1/2, GSK 3β, and AKT.
Schatti et al., 2011	Human MSCs	Shear (25º rotation) and compression (0.4mm, 1Hz) 15 loading cycles over 3 weeks.	mRNA expression increased under shear, compression, and shear + compression. Shear and shear + compression had significantly greater expression than compression.
Sen et al., 2011	MSCs, unspecified	1-2% cyclic tensile strain applied 10x/min for 24 hours.	Cyclic strain activates AKT phosphorylation and GSK3β inhibition. Increase in focal adhesion quantity.
Shen et al., 2014	Human Periodontal ligament stem cells	12% cyclic tension at 1Hz applied for 6, 12, or 24 hours.	Runx2, ALP, and OCN mRNA and protein quantities were all upregulated.
Steinmetz et al., 2011	Human MSCs	Cyclic compressive loads of 15% strain at 0.3Hz for 4 hours daily for 14 days.	Loading over stimulated the cells, resulting in downregulation of osteogenic and chondrogenic markers.
Tao et al., 2007	Endothelial Progenitor Cells	Shear stresses of 5, 15, or 25 dyn/cm^2 for 5, 15, or 25 hours.	Shear stress proportionally upregulated Cu/Zn SOD activity.
Teramura et al., 2012	Human iPSCs	FX-3000 Flexcell used to apply 15% strain at 12 cycles/min for 12 hours.	Small GTPase Rho was activated and AKT phosphorylation was decreased. Rho/ROCK pathway affected by mechanical stress.
Terraciano et al., 2007	Human MSCs, Human ESCs	10% compressive strain at 1 Hz for 1, 2, 2.5, or 4 hours.	MSCs showed upregulated Sox-9, collagen II, aggrecan and increased matrix proteins. EB saw decreased expression of chondrogenic genes under compression alone.
Tsai et al., 2014	Human MSCs	Compressive strain of 10% at 1 Hz for 1 hour for 21 days.	Compressive effects on chondrogenesis were significant, but dependent upon scaffold structure.
Wang X et al., 2013	Human ESCs	Application of a wide range of micro forces.	Less than 12pN is required to activate Notch receptors. 40pN is the peak force required in integrin attachments between cells.
Wang Y et al., 2013	Rat MSCs	Sinuosoidal compression of 10-40 kPa for one hour daily at 0.125, 0.25, 0.5, and 1 Hz. Testing was completed for 1, 3, 5, 7, 10, 12, and 14 days.	Dynamic compression increased cell proliferation and survival. Ihh, cyclin DI, CDK, and collagen II were significantly upregulated.
Wolfe et al., 2012	Murine ESCs	Varying levels of shear stress (1.5-15 dyn/cm^2)	By day 4, a sustained increase in T-BRACHY and decrease in AFP were noted, shear stress influenced pluripotency markers.
Wu et al., 2013	Rat MSCs	2x4 hours daily tensile strains of 10% at 0.5Hz for 7 days.	RANKL/OPG ratios increased until day 5, after which a steady decline was noted.
Yamamoto et al., 2003	Endothelial Progenitor Cells	Varying levels of shear stress (1.5-15 dyn/cm^2)	Shear stress increased expression of kinase insert domain-containing receptor and fms-like tyrosine kinase-1, and vascular endothelial-cadherin, at both the protein and mRNA levels
Yanagisawa et al., 2007	C2C12	Continuous compression of 0.5-2.5 g/cm^2 for 0.5- 24 hours.	Loading significantly increased Runx2, Msx2, Osterix, Sox5, and Sox9 expression. Activated phosphorylation of p38 MAPK was also noted. AJ18, MyoD, and PPARγ were downregulated.
Yang P et al., 2012	Human MSCs	10% sinusoidal cyclic tension applied at 1 Hz for 3 hours, followed by 3 hours rest for 1, 7, or 14 days.	Tensile strain upregulated key ligament/tendon genes, including tenascin-C and collagen III.
Yang Z et al., 2006	Endothelial Progenitor Cells	Varying levels of shear stress.	Shear stress proportionally increased t-PA expression in EPCs.
Youngstrom et al., 2015	Equine MSCs	Cyclic tension of 3 or 5% at 0.33Hz for 1 hour daily for 11 days.	Constructs at 3% strain doubled the failure strength of controls and increased the elastic modulus 2.56x (within 25% of native values).
Zeng et al., 2006	Human ESCs	Laminar shear stresses of 12 dyn/cm^2	Shear activated histone HDAC3 through the Flk-1-PI3K-Akt pathway, deacetylated p53, leading to p21 activation

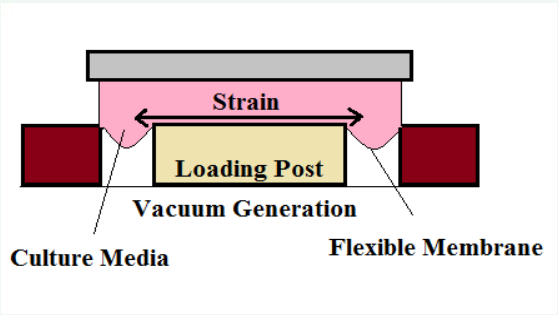

Figure 2 Diagram of the flexcell tensile strain mechanism: Generation of vacuum results in stretching of a flexible membrane, resulting in tension applied to cells cultured on a loading post.

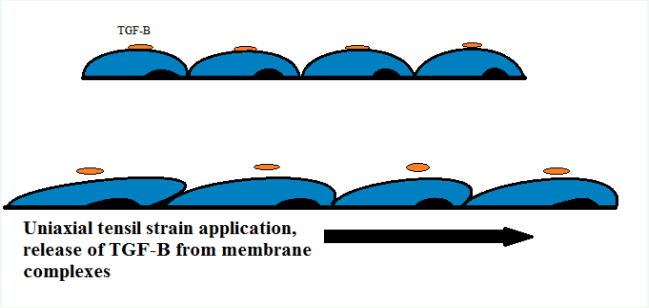

Figure 3 Illustration of the effects of applied tension on TGF-β release: TGF-β is initially adhered to latent complexes in cell membrane (top). Application of tensile strain results in physical deformation of complexes, resulting in TGF-β release into the surrounding environment, where it may act upon local cells (bottom).

simple method of obtaining alignment and, at minimum, premature differentiation of MSCs and ASCs to a fibroblast-like phenotype, it seems intuitive that applying tensile stain to tissue constructs is a popular approach for ligament and tissue engineering. In 2009, Abousleiman et al., seeded decellularized umbilical veins with rat MSCs in a collagen I hydrogel and subjected the constructs to 2% tensile strain for one hour daily for two weeks. Following the two week period, the constructs exhibiteda 156% increase in strength and 109% increase in elastic modulus [4]. Cell population increased fourfold, as did gene expression of collagen I, while collagen III exhibited a threefold increase in expression after two weeks [4]. A similar approach was taken by [5], with MSCs seeded onto aligned collagen I fibers and loaded under 6% tensile strain for 3 hours per day for two weeks. This approach also yielded significant changes, with a two-fold increase in collagen I and fibronectin gene expression and a significant increase in lysl oxidase expression [5]. A significant increase in collagen I protein was also noted in the constructs, which is thought to have contributed to the 16% increase in elastic modulus. Another tendon tissue engineering attempt involved the use of decellularized horse tendons and applications of 0, 3, or 5% strain to seeded horse MSCs [25]. Gene expression of collagen I, III, biglycan, decorin, and scleraxis showed upregulation or no change at 3% strain, however the failure strength was greatest at 3% strain after 7 days, with the hypothesis that cells did not fully adhere in the 5% strain group [25]. To address cellular adhesion and mechanotransduction, Yang et al., utilized a PEG-RDG hydrogel enhanced with matrix metalloproteinases to create a biodegradable hydrogel capable of mechanical transduction. This hydrogel was then seeded with human MSCs and subjected to 10% tensile strain for 3 hours per day for 1, 7, and 14 days [26]. Cells showed significant increases in scleraxis, decorin and collagen III expression by day 14, however collagen I and tenascin-C, prominent tendon markers, peaked at day 7 [26].

Osteogenesis

It is unsurprising that fibrogenesis and osteogenesis can be induced through similar stimulation mechanisms given the similarities in structure and function of the tissues in which these cell types are found. MSCs and ASCs are the most common stem cells studied in these applications, although several types of progenitor cells, including human periodontal ligament stem cells have also been researched and display similar responses to tensile strain as MSCs and ASCs [27]. In general, lower strains for longer duration's appear to encourage osteogenesis, along with various growth factors [28-32]. Rat MSCs express high levels of osteocalcin, runx2, ALPase, collagen 1 and cbfa1 mRNA at lower stains over a period of 1-10 days [28,31,32]. Cell proliferation was also significantly increased [31]. This is similar to human MSC behavior, when exposed to 2.5% strain for 1-14 days, hMSCs showed osteocalcin and BMP2 expression increasing through day 14 and collagen I increasing through day 12 [29]. A similar study also showed increased mRNA expression of osteopontin and osteocalcin along with collagen I and BMP2 [30]. High amounts of deposited calcium were also noted. Strains above 5% however, tend to show dramatic decreases in expression of osteopontin, osteocalcin, and BMP2 [28].

Cardiogenesis

The inclusion of tensile strain is increasingly being included in cardiac stem cell differentiation research. Several studies have shown that its inclusion may help mimic radial and longitudinal strains during contraction. A 2014 study by Amin et al., compared the differentiation of both rabbit bone marrow mesenchymal stem cells and rabbit adipose-derived stem cells under the influence of both 10% biaxial strain and 10μM 5-azacytidine. After 4 and 7 days of continuous strain at 1Hz, significant increases in GATA-4 were noted. Perhaps most interesting was that the ASCs showed a significantly greater change in GATA-4 expression over MSCs as well as the control. It was also noted that inclusion of 5-azacytidine was necessary for greatest gene expression [3].

A similar approach has been taken with H7 hESCs, in which the cells were exposed to equiaxial strain via a custom pneumatic strain device [33]. ESCs required gradual increase in strain, a daily increase of 1% strain and 0.2Hz was used up to 5% at 1Hz to upregulate myosin heavy chain 6 and 7 (MYH6,MYH7), troponin I and Connexin 43 (Cx43) after 10 days of maximum stimulation [33]. Beating was also noticed in these cells, although the authors could not conclusively determine that the beating was a direct result of the tensile strain. Likewise, increased beating was also noted in day 4 embryoid bodies exposed to 10% cyclic strain [34]. The cardiomyogenic effects of cyclic tension can also have an opposite effect. Saha et al., demonstrated that 10% cyclic

strain at 0.5Hz applied to hESCs resulted in 85% SSEA-4 + cells after two days. Reduction of this strain to 8% at 0.166Hz led to a decrease to 36% SSEA-4 + cells [35]. However, cells exposed to higher strains for too long, or exposed to high strains too quickly have a high occurrence of cell detachment and death [33]. Strain can also be applied using fluid force, such as in the work by Correia et al., in which murine iPSCs were seeded in a 3D bioreactor that rotated at 90 Hz, inducing fluid shear strain on the seeded cells [36]. Cells cultured in this manner showed increased cardiac gene expression and protein markers, particularly when combined with hypoxic conditions [36].

Nearly every approach involving hESCs or hiPSCs results in immature cardiomyocytes, which are more similar to fetal cells than mature, adult cells [37]. Methods to address this issue are currently being studied. For example, Chun et al., evaluated the behavior of iPSC-CMs under static 5% strain and cyclic 5% strain in a fibronectin network for 48 hours [38]. Interestingly, static strain resulted in the greatest increase in cardiac markers; most notably cardiac troponin T, indicating that mechanical stimulation can also encourage maturation [38]. Importantly, mechanical stretch is also known to help orient differentiated cells and form gap junctions. Salameh et al., discovered that 10% cyclic tensile strain applied over 24 hours upregulated Cx43 and oriented and elongated cells along the tensile axis [39]. The strain also resulted in accumulation of Cx43 and N-cadherin at the induced cell poles, priming the cells for junction formation [39]. These formations are crucial for mature cell behavior and function.

Exposure to tensile strain can also be applied on the micro scale. Geuss et al., 2013 used magnetic beads and exposure to magnetic fields. RGD-conjugated beads were incorporated into the interior of embryoid bodies (EBs) during formation and exposed to BMP-4 [40]. After confirmation that bead attachment did not influence embryoid body behavior, the EBs was exposed to 0.128, 0.2, and 0.4 Tesla magnetic forces. The results showed that integrin-mediated forces can induce differentiation, and specifically, 0.2 Tesla exposure for one hour daily over 3 days leads to an increase in cardiomyogenesis, specifically, a 20% increase in the production of cardiomyocytes [40]. Interestingly, this confirmed the findings of [41], who proposed that approximately 12pN of force was required to induce changes in Notch signaling, and that forces over 40pN transduced through integrin attachments could induce formation of stress fibers [41]. The 0.2 Tesla exposure resulted in forces of approximately 20pN applied to the RGD-integrin complex [40].

Neurogenesis

Mechanical stimulation can be used to promote maturity of progenitor cells. Neural progenitor cells (NPCs) stretched at 1 mm/day experienced a 30% increase in axon diameter compared to unstretched cells, as well as upregulation of BIII tubulin and MAP2 [42]. Similarly, another study identified that 10% static tensile strain applied to NPCs could decrease differentiation to oligodendrocytes, although differentiation to astrocytes and neurons was not affected [43].

COMPRESSION

Like tensile loading, the effect of compressive strain on stem cell differentiation is one of the more commonly studied types of mechanical stimulation. Also like tensile loading, a great deal of focus is placed on deriving cells whose tissue regularly experience compressive loads, such as cartilage and bone. Compressive loading has been shown to activate the TGF-β pathway, indicated by increases in expression of TGF-β1, SMAD-5, and other markers indicative of pathway activation [13,44,45]. Some research has also shown that compressive loads affect the phosphorylation of 38 MAPK, which can also directly affect chondrogenesis or osteogenesis [46,47].

Compressive loads ranging from 5-15% strain are commonly investigated for chondrogenesis applications [13,44,48,49]. While nearly all studies show some similar results, including the upregulation of Sox 9, collagen II and aggrecan, among other chondrogenic genes, the ideal duration of the stimulation is undetermined, with daily stimulation ranging from 1-24 hours per day for 1-21 days [13,44,47-49]. Similarly, relatively little attention has been paid to determining the ideal frequency for stimulation, although those that have looked at this variable have concluded that the commonly used 1Hz may just be the most effective frequency, as it has been suggested that frequencies below 1Hz are ill-suited for collagen II expression (Peleaz et al., 2009). Overall, exposing MSCs to compressive strains of approximately 10% for 2-4 hours per day for 2-14 days seems to be the most common approach, with similar results between rabbit, rat, and human MSCs as well as human ESCs [13,44]. The effects of the compressive protocols could also be affected by scaffolds and substrates, as the mechanical properties and mechanotransduction potential of these scaffolds can vary widely. Agarose gel, fibrin gel, 10% PEGDA, polyurethane, and gelatin/chitosan scaffolds have all been used with success [13,44,48,49]. Specific attention has also been paid to the attachment mechanism, noting that PEG scaffolds containing RDG (Arg-Gly-Asp; a component of fibronectin) showed that the presence of the attachment components of the protein can greatly increase the chondrogenic gene expression for a given compressive strain, indicating that cell-extracellular matrix (ECM) interactions are at least partially responsible for the effects of compression [50]. Particularly interesting is that these effects were only seen under compression, with no change in chondrogenesis observed in the absenceof mechanical strain. It is also important to note that greatest increases in chondrogenic gene expression are coupled with inclusion of TGF-β in the cell media. This is appropriate, as the TGF-β pathway has been shown to be the regulatory process through which chondrogenesis occurs [13,44]. Compressive strain has been proven to activate this pathway independent of the presence of TGF-β in the culture media, although gene expression levels were not as high as with the combined mechanical and biochemical stimulation [13]. Groups that have evaluated cell survival have indicated that compression increases cell survival and overall population compared to controls without compression (Peleazet al., 2009).

While conceptually similar to compressive strain, application of hydrostatic pressure has several advantages, such as reduced risk of injury or damage to the cells or scaffolds. Additionally, in the case of cartilage and meniscus tissue engineering, application of hydrostatic compression better reflects the developmental *in vivo* loading mechanism. Overall, most research involves use of hydrostatic pressures between 1-10MPa and investigation

of key chondrogenic genes- Sox9, collagen II and aggrecan [51-55]. Long term studies (>3 days) generally take note of ECM production as well, checking for protein expression of collagen II and glycosaminoglycans (GAGs) [53,56]. Safshekan et al., was able to demonstrate that human ASCs experiencing cyclic hydrostatic pressures of 5MPa at 1Hz expressed key chondrogenic genes at near-*in vivo* levels [57]. Similarly, Ogawa et al., noted that addition of TGF-β to hASC culture induced chondrogenesis, but that the effects were significantly increased by inclusion of 0.5MPa hydrostatic pressure at 0.5Hz. Also of note was that after four weeks, pericellular and extracellular matrix proteins accumulated significantly in the hydrostatic pressure samples [58]. In the interest of identifying the role of hydrostatic compression independent of growth factors, Puetzer et al., applied cyclic hydrostatic pressure of 7.5MPa at 1Hz for 4 hours per day to hMSCs and hASCs over a period of 21 days. By day 7, Sox 9 and COMP were significantly upregulated in hASCs, with significant collagen II expression at day 14. No other genes showed increased expression and measurable mRNA ceased for both cell types after 21 days, indicating limited cell vitality [59]. This indicates that while hydrostatic pressure can have a significant impact on chondrogenesis, inclusion of growth factors is highly recommended for progression towards the desired phenotype.

Osteogenesis

There is a great deal of overlap in osteogenesis and chondrogenesis research, and effects of compressive strain are no exception. Due to the developmental relationship of articular cartilage and bone, it is logical that inducing osteogenesis in stem cells relies, in part, on the same pathways as chondrogenesis [13,45]. Much like chondrogenesis, gene expression of osteogenic markers can be greatly increased under compressive strain. Osteopontin, procollagen 3, collagen and procollagen I are among the markers shown to increase under < 10% strain [45,46,60]. Lower frequencies (0.05-0.5 Hz) are shown to increase this expression as well, although similar durations as chondrogenesis have been investigated. While definitive ranges, magnitudes, and frequencies of stimuli have not yet been determined for osteogenesis and chondrogenesis, it is clear that strain, frequency, and duration must be carefully balanced as overstimulation can occur. Steinmetz and Bryant determined that 15% compressive strain at 0.3Hz for 4hours per day for 14days was sufficient to overload human mesenchymal stem cells as down regulation of key chondrogenic and osteogenic genes was noted, along with significant cell death [61]. This is in stark contrast to their previous work, which limited mechanical stimulation to 2days, which showed significant proteoglycan production and other indications of a chondrogenic phenotype [50]. These observations are similar to those made by Meier et al., in which hASCs exhibited low RNA expression and poor morphology when exposed to tensile strains greater than 20% [23].

Other Tissue Types

Along with inducing osteogenesis, there is some evidence that cyclic compressive strain can stimulate endothelial progenitor cells (EPCs) to multiply, organize and terminally differentiate into vascular networks [62].

Nucleus pulposus research, like cartilage and meniscus research, has taken a prime focus on mechanical stimulation. A coculture of hMSCs and human nucleus pulposus cells in a bioreactor subjected to cyclic compression and perfusion lead to significant increases in osteochondrogenic markers after 1 day of stimulation [63]. These results are supported by the findings that nucleus pulposus cell phenotype is regulated by cellular interactions regulated by N-cadherins (Hwang et al., 2015). As other referenced sources have indicated, mechanical stimulation is known to affect cellular behavior and transcription through numerous pathways, including integrin interactions [13,41].

While not nearly as commonly researched as MSCs, dental pulp stromal cells have also been used with compressive strain. Specific to its purpose, loading was conducted for 30 minutes, 3 times per day to mimic chewing [64]. While gene expression changes were not investigated, cell population and density were significantly increased, and variance in cell polarity greatly decreased, indicating that compressive loading of stromal cells could increase cell maturity and function [64].

SHEAR STRESS

Another commonly investigated source of mechanical stimulation is shear stress. Perhaps most frequently associated with vascular tissue engineering research, shear stress has been shown to impact differentiation of stem cells in multiple applications, such as promoting differentiation of embryonic stem cells, MSCs, and other cell types to endothelial cells and osteoblasts, among others [65]. Shear stress is induced early in embryogenesis after initiation of the heartbeat in vertebrates and directly influences the expression of Runx1 [66]. Runx1 regulates hematopoiesis and leads to development of hematopoietic cells [66]. Later in development, shear stress has also been shown to suppress apoptosis and help maintain quiescence of bone marrow MSCs [67]. Shear stress has been shown to mediate cell differentiation through activation of the B-cadherin/Wnt pathways and can also help orient actin fibers and mediates cell polarity in as little as 1 hour. Perfusion of stem-cell seeded scaffolds can induce similar effects to pure shear stress as shown in Figure (3). ASCs seeded in silk fibroin scaffolds underwent pulsatile media perfusion at 0.5Hz or steady flow for five weeks [68]. After two weeks of steady flow and three weeks of pulsatile flow, osteogenic markers were significantly increased as was equilibrium modulus [68]. Although exact shear stresses were not measured, it was apparent that the shear stress had a significant impact on osteogenesis of ASCs. Bone marrow-derived stem cells exposed to a narrow range of shear stress (0.86-1.51 dyn/cm) for 30-180 minutes per day was adequate to activate Cx43 and significantly upregulate osetopontin, osteocalcin, vascular endothelial growth factor (VEGF), BMP-2, and also significantly increase mineralization, intercellular calcium, and ALP activity in the absence of any biochemical stimulation [69,70]. However, inclusion of biochemical stimulation (VEGF, BMP-2) additionally increased gene expression and mineralization as well. Similarly, shear stresses of 0.575 and 0.70 Pa have been shown to induce osteogenesis in MSCs [70,71]. Interestingly, these levels of mechanical stress seem to have similar effects *in vivo*, with shear stresses experienced within trabecular bone having a direct correlation to the bone formation balance [71]. Not only do *in*

vitro and *in vivo* forces seem to correlate, there also seems to be a correlation between intensity of the stimulus and its effects with Riddle et al., demonstrating that as shear stress increases from 5-20 dyn/cm^2, cell proliferation and intercellular calcium also increased proportionally [72]. Increased activation of Erk1, Erk2, and calcineurin were also noted, although these increases were not uniformly proportional [72].

Shear stresses also play a major role in the formation and function of the circulatory system. The majority of research focuses on the impact of shear stresses on the formation of the vasculature; however some attention has also been paid to the mesodermal commitment pathways, which lead to cardiogenesis. It is apparent that shear stress plays a key role in cardiomyogenesis as shear stresses of 10 dyn/cm^2 can trigger expression of vascular endothelial growth factor 2 and myocyte enhancer factor 2c [73]. Additionally, halving the shear stress over four days resulted in the doubling of Brachyury expression, indicating that the ESCs were committing towards a mesoderm lineage [74]. These results are supported by the observations from Davies et al., in 1986, which suggest that shear stresses up to 15 dyn/cm^2 can affect the cytoskeleton and increase expression of cardiac markers [75]. Interestingly, zebrafish embryos that are unable to experience shear stress through blocked blood flow are unable to form functional hearts [76,77]. This suggests that the blood flow within the developing embryo is critical not only to the development of vasculature, but to the developing heart as well.

The effects of shear stress on MSCs have been studied in attempts to obtain a viable source of endothelial cells for vascular tissue engineering. Frequently, these studies utilized a frequency of 1Hz to mimic the cyclic strains of pulsatile blood flow (Keung et al., 2009). Illi et al., showed that mechanical stimulation through fluid shear stress on embryonic stem cell monolayers caused histone modifications that lead to protein expression typical of cardiovascular tissue [78,79]. The sensitivity of embryonic stem cells was made quite apparent when Zeng et al., showed that an increase of 2 dyn/cm^2 could up regulate Flk1, a VEGF receptor, and endothelial nitric oxide synthase to reflect endothelial cell precursors, and that these precursor cells self-assembled into tube-like structures in matrigel [80]. Similar findings have been discovered with MSCs, including increased RNA expression of VE-cadherin and CD31 and increased protein expression of CD34 when exposed to 1-15 dyn/cm^2 shear stress over 4days [81]. Perhaps most encouraging, is that this upregulation took place with no inclusion of growth factors in the culture media. When these and similar approaches are applied to endothelial progenitor cells, a functional endothelial cell phenotype can be obtained [79]. Exposure to low levels of shear stress (<1 dyn/cm^2) can induce opposite morphological changes as well as increased gene and protein expression of endothelial markers Flk1, Flt-1, VE-cadherin, and PECAM-1. These changes enabled the resulting cells to readily form vessel-like tubular structures [82]. Increasing the stress to 10 dyn/cm^2 within a tubular scaffold, not only resulted in similar RNA and protein upregulation, but resulted in secretion of tissue-type plasminogen activator (tPA) [83]. Similar studies have also shown secretion of prostacyclin, another antithrombotic molecule, and mild inhibition of prothrombic factor plasminogen activator inhibitor-1 (PAI-1) [84].

OSCILLATION/VIBRATION

Mechanical stimulation through vibration is another medium that is gaining traction as a viable option for inducing differentiation in stem cell lines, particularly mesenchymal stem cells. Chen et al., investigated the effect of acoustic-frequency vibratory stimulation on the osteogenesis and adipogenesis of bone marrow MSCs. Cell cultures were placed on an industrial shaker under vertical sinusoidal vibration at 0, 30, 400, and 800Hz. Their results showed that lower levels of stimulation (both frequency and duration) experienced greater adipogenesis, while 800Hz showed inhibition of adipogenesis and induction of osteogenesis [38]. This work indicates that mechanical stimulation can not only induce differentiation, but can be adjusted to direct differentiation to various pathways.

COMBINED APPROACHES

All of the tissues and cell types mentioned thus far experience a wide array of mechanical stresses and it is rare that a given group of cells experiences only one type of mechanical stimulation. For this reason, more and more research is focusing on the effects of combined mechanical stimuli, to better simulate native tissue conditions.

All joints experience multiple types of forces through movements. For example, movements in the knee have both a compressive element as the knee is loaded, and a shear element, as the tibial surface moves relative to the femoral surface. Therefore, it is logical that differentiation of cells such as chondrocytes could be directed by combining shear and compressive stimulation. Both Schatti et al., and Li et al., investigated the effects of compressive loading (10-20% strain) and shear stress (rotations of ± 25°) on the differentiation of hMSCs [13,85]. Results showed not only differentiation towards a chondrogenic phenotype, but that combined stimulation had significantly greater expression of Sox9, COMP, collagen II, and aggrecan than controls, but no changes in collagen I, alkaline phosphatase, or collagen X were observed [85]. Interestingly, combined stimulation resulted insignificantly greater expression of Sox9 and collagen II than both shear and compression independently [85]. Application of shear stress did have slightly higher GAG production and total mRNA than compression alone, but neither was deemed statistically significant.

Cardiovascular research has also begun to take advantage of the benefits of combining types of mechanical stimulation. As both the heart and vasculature are constantly undergoing changes in hemodynamic pressure, shear stress, and tensile loading, it is unsurprising that combining these types of stimuli have shown great promise in cardiac stem cell research. Combining hemodynamic pressures of 10 mmHg with 8-15% tensile strain has shown to significantly increase protein synthesis of key proteins SERCA2A; α- and β- Myosin Heavy Chain; α-actinin; and cardiac troponin T in 3D culture of chick embryonic-derived CMs in as little as 4 days [86]. The contractility and calcium handling capabilities were also significantly increased compared to 2D cultured cells and 3D cultures without the mechanical stimulation.

CONCLUSION

Mechanical stimulation is an undisputedly crucial part of development of numerous tissues and can thusly be applied to stem cell differentiation techniques. Key cellular pathways are activated and inhibited through mechanical stimulation both *in vivo* and *in vitro* research. Many tissues that depend on some form of mechanical stimulation are tissues that depend on specific mechanical properties to function properly, including bone, cartilage, ligaments, and cardiac tissues. Other cell types, such as neurons, can also benefit from mechanical stimulation, with increased cell size and alignment commonly observed as direct effects of this stimulation. Stimulation approaches can include tensile or compressive strain, shear stress and perfusion, vibratory stress, or a combination of multiple types of stimulation. These techniques shed light on the potential approaches to utilizing stem cells to their full potential. Whether mechanical stimulation is used to direct stem cell differentiation, progenitor cell maturity, or adjust cytoskeletal dynamics, it is clear that many stem cell differentiation protocols could benefit from inclusion of a type, or several types, of mechanical stimulation.

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