The Effect of Mechanical Stimulation on PEG-Encapsulated Mesenchymal Stem Cells

by

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ABSTRACT OF THE THESIS

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Human mesenchymal stem cells (hMSCs) are multipotent cells capable of differentiating into any mesenchymal tissue, including bone, cartilage, muscle, and fat. hMSC differentiation can be influenced by a variety of stimuli, including environmental and mechanical stimulation, including scaffold physical properties or applied loads. Numerous studies have evaluated the effects of vibration or tensile strain on MSCs but these studies generally use MSCs on tissue culture plastic or scaffolds derived from natural sources. Tissue culture plastic forces cells into a 2D monolayer in which they behave differently than in their native tissues and naturally sourced scaffolds have inherent biochemical and microarchitectural cues that also influence MSC fate. To isolate the effects of vibration and strain on hMSCs, polyethylene glycol diacrylate (PEGDA), a bioinert synthetic polymer hydrogel, was used to 3D encapsulate cells in hydrogel sheets. This Masters’ thesis expands on previous results where microencapsulated hMSCs were subjected to vibrations. Microencapsulated cells are subjected to vortexing and the surface tension caused by forming emulsion-based microdroplets. Hydrogel sheets were selected to eliminate the confounding factors introduced by the fabrication method, to standardize the encapsulation efficiency, and to enable the performance of tensile tests. hMSCs were entrapped in 10 kDa PEGDA hydrogel sheets, then subjected to 10% cyclic tensile strain, or 100 Hz vibrations
at accelerations of 0.3, 3.0, or 6.0 g, for 24 hours. Following testing, entrapped cells were evaluated for viability and markers of differentiation at 1, 4, 7, 14, and 21 Days. Cells subjected to cyclic strain and cells subjected to accelerations of 0.3 g showed greater viability than control cells. hMSCs vibrated with accelerations of 3.0 g showed no change in viability compared to control while accelerations of 6.0 g were lethal to cells. Accelerations of 0.3 g also appeared to induce differentiation of encapsulated hMSCs along the osteogenic pathway. For vibration studies, these findings differed from previous findings with microspheres in that on day 4, 0.3 g microencapsulated cells exhibited alkaline phosphatase activity but cells in sheets did not. Additionally, 0.3 g cells encapsulated in hydrogel sheets exhibited mineral formation as early as day 7, while microencapsulated cells did not until day 14. These findings show the feasibility of using PEGDA as a scaffold for probing cell response to mechanical stimuli, and further demonstrate that the geometry of the scaffold selected can also influence hMSC fate.
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Chapter 1. INTRODUCTION

1.1 Tissue Engineering

The tissue engineering paradigm consists of cells, external signals, scaffolds, and interactions between these elements. Cells are isolated and proliferated, seeded on a scaffold, then stimulated to develop a tissue equivalent, and finally implanted in vivo. [1] Mesenchymal stem cells (MSCs) are a potential cell source for regenerative medicine and tissue engineering. While fully differentiated cells are often limited in supply, MSCs are relatively easy to obtain from fat or bone marrow, and unlike differentiated cells, are very prolific. MSCs can be harvested from a donor typically from the stroma of adult bone marrow (BMSCs). Cells of similar characteristics and differentiation capabilities have also been isolated from other mesenchymal tissues including adipose (ASCs), tendon, muscle, and skin. [2] Cells from a donor may be used to create tissue engineered products with limited or no immune response. [3] MSCs are multipotent cells capable of differentiating into adipocytes, osteocytes, myocytes, and chondrocytes, among others (Figure 1.1). In tissue engineering, one approach to growing target tissues requires guiding differentiation by exposing MSCs to a specific series of cues. MSC differentiation can be regulated by mechanical, chemical, and environmental signals. Chemical factors can be introduced in media and scaffolds. For the most part, media inducers are added to cell media to direct cell fate; however, there are benefits to directing cell fate via scaffold material properties. Materials can be designed to mimic niche in vivo topographical and chemical signals to guide cell fate. Shih et al. noted increased osteogenic activity in MSCs on hydrogels with a stiffness of 42 kPa compared to hydrogels with a stiffness of 7 kPa. [4] Stem cell responses to scaffold properties can offer insight to deterministic factors of the in vivo
environment. For instance, when MSCs are seeded on substrates approximating the elastic moduli of brain (0.1 to 1 kPa), pancreas (1.2 kPa), cartilage (3 kPa), muscle (8 to 17 kPa) and bone (25 to 40 kPa), those MSCs are directed to commit to neurons, beta cells, chondrocytes, myoblasts, and osteoblasts, respectively. [4-8] Scaffolds impart force propagation to cells. In general, this force propagation is often probed via a variety of mechanical testing, including tension/compression, shear, or vibration, and important parameters include stress/strain magnitude, stress/strain duration, and frequency of loading. Substrate composition and rigidity, in addition to loading type and duration can be specified to probe the cellular response to a variety of mechanical inputs.

A comprehensive understanding of the stem cell response to stimuli enables the development of stem cell use for clinical applications. Biomaterials can be developed to cultivate a desired stem cell response in vivo without the need for exogenous chemical factors that could diffuse into surrounding tissue. To best identify the mechanisms that direct cell fate, it is necessary to determine the cell response to one variable at a time, such as scaffold stiffness or topography, before the addition of other factors, such as chemical or mechanical signals. [2]

Figure 1.1 Mesenchymal lineage.
1.2 Cell Scaffolds

A tissue engineering scaffold is a structure that provides support to cells, and is ideally eventually replaced by an extracellular matrix secreted by the cells. Scaffolds may be composed of biocompatible metals, ceramics, glass, or polymers. [9] Biomaterials and fabrication method of scaffolds may determine properties such as stiffness, permeability, and degradation. Scaffolds can be derived from natural or synthetic sources. Synthetic materials with a natural coating, such as collagen, is a hybrid scaffold, have natural cues when the cells are seeded on the biological layer. Scaffolds can be used in a 2-dimensional (2D) manner where cells are seeded upon a flat surface, or a 3-dimensional (3D) configuration where cells are embedded or seeded throughout a 3D structure.

A natural scaffold can be composed of extracted and purified extracellular matrix (ECM) proteins such as fibrin and collagen or polysaccharides such as starches or alginate. [10] Natural polymers also are comparatively weaker mechanically than synthetic scaffolds. [11] Natural scaffolds often maintain biocompatibility, biodegradation, and bioactivity of their natural sources. [12] Natural scaffolds in general promote cellular adhesion and active biosignaling pathways without the need for modification. [12] Though advantageous in many respects, these properties are not desirable when attempting to isolate the impact of a single factor; the effects on cells of any single input cannot be isolated due to the confounding properties of natural scaffolds. Natural scaffolds such as collagen-derived scaffolds have biochemical and microarchitectural cues that can affect cell attachment, morphology, protein production, and thus differentiation. Using natural scaffolds to impart loads on cells incorporates these additional factors that make it impossible to separate the cellular response to the load from the cellular response to the scaffold. Force is propagated
from scaffolds to cells through cellular attachment sites and the cytoskeleton. [13] The microarchitecture of scaffolds can affect how cells attach, and thus how force is transmitted. [14] Additionally, the ligands and receptors present in scaffolds are cues for signaling pathways. MSCs in collagen/Mg doped hydroxyapatite scaffolds differentiated into mature osteoblasts without specific inducing factors, demonstrating the importance of scaffold type in eliciting osteogenic differentiation, supported by their expression of osterix, osteopontin, and osteocalcin. [10, 15] As previously mentioned, Shih et al. concluded increased stiffness of scaffolds increases osteogenesis of MSCs. [4] Thus, variations in scaffold material, or stiffness can impact cell response to loading. Identifying the cellular response to a variety of variables can inform the selection of chemical and mechanical factors to incorporate into scaffold design.

Scaffolds affect how mechanical load is distributed to cells. Scaffold selection can be used to better approximate mechanical loading in vivo. For example, chondrocytes, round in morphology when in situ, tend to assume a fibroblast-like morphology when cultured in 2D and demonstrate an associated change in the synthesis of matrix proteins. [16] Lawrence et al. found that after transferring pre-chondrocytes differentiated in 2D culture into a 3D scaffold, cells differentiated into rounded chondrocytes. [17] Cells in 3D cultures differ in shape to cells in 2D cultures, which can influence interactions between extracellular matrix and the cell membrane, resulting in the activation of different pathways of stem cell differentiation. [18] Indeed, hMSCs cultured in 3D matrices differentiate into different tissues than hMSCs cultured in 2D environments. [19-22]

Because matrix proteins offer attachment sites, they also determine how cells transmit forces, which may lead to greater effect during strain or vibration studies. Identical tensile
strain stimulation may result in differing results due to the use or lack of different matrix proteins. [22] Rathbone et al. found MSCs subjected to cyclic tension differentiate differently within three different cellular environments: two 3D scaffolds and one 2D scaffold. [22] Both 3D scaffolds were hydrogels that encapsulated cells, one scaffold provided attachment sites and the other did not. The cells within both 3D scaffolds demonstrated significant differences in gene expression compared to the 2D scaffold and compared to each other. Thus, the method of force transmission to cells impacts differentiation.

Hydrogels are hydrophilic cross-linked networks of polymers that can be used as cell scaffolds. [10] When exposed to liquids, hydrogels swell yet maintain viscoelastic properties because the polymers do not degrade while retaining a high fraction of water. [11] Hydrogels swell to an equilibrium volume while maintaining their shape. [12] Hydrogels can be categorized by gelation method (physical, ionic, or covalent interactions), physical structure (amorphous, semi-crystalline, or crystalline), and material (synthetic or natural). [10, 12]

Hydrogels are categorized as synthetic or natural to reflect the source polymers of which they are comprised. Synthetic hydrogels often do not have as high cell proliferation and viability as their natural counterparts, but their properties are highly reproducible with known chemical compositions and can be formed with tailored mechanical properties and degradation rates. Frequently used synthetic hydrogels include poly(hydroxyethyl methacrylate) (PHEMA), poly(ε-caprolactone) (PCL), poly(vinyl alcohol) (PVA), and poly(ethylene glycol) (PEG). [10] PHEMA and PVA are used in soft contact lenses. [12, 23] PCL has been used in controlled drug release devices and absorbable sutures. [24] The
The main application of PEG hydrogels is as drug carriers in controlled release of drugs, proteins, and biomolecules. [25]

1.3 Polyethylene glycol diacrylate (PEGDA)

Polyethylene glycol (PEG) is widely used as a cell scaffold or for cell encapsulation. PEG is a highly hydrophilic, tunable, bioinert polymer that can be photopolymerized with low toxicity to encapsulated cells. [10] PEG resists protein adsorption and thus has low cell adhesion. Furthermore, PEGDA has no attachment sites available for cells. Since cells transmit and exert force through their attachment sites, embedding them within a purely synthetic material with no attachment sites will alter the cells’ normal distribution of forces. PEG provides no biochemical or microarchitectural cues to cells. Combined, these properties result in PEG-based hydrogels being almost invisible to cells, including immune cells. Thus, they are often considered a “blank slate,” enabling researchers to add individual components to PEG hydrogels and investigate cellular responses to each component. In this thesis, PEG enables the analysis of the isolated effect of tensile strain or vibration.

PEG requires an additional group to enable crosslinking. PEG based derivatives include polyethylene glycol methacrylate (PEGMA), polyethylene glycol dimethacrylate (PEGDMA) and polyethylene glycol diacrylate (PEGDA). PEGDA is composed of PEG with an acrylate group at each end of the chain that act as a crosslinking chain between two or more polymers (Figure 1.2). [24]
There are three main methods of crosslinking PEG hydrogels: free radical photopolymerization (chain growth), step polymerization, and mixed mode polymerization, which combines the first two methods. PEGDA is photopolymerizable and follows chain growth polymerization. Visible light initiates the cleavage of initiator molecules that separate into free radical molecules. The free radical molecules initiate chain growth polymerization. The free radicals interact with the acrylate group to induce covalent bonding (Figure 1.3). [24]

PEGDA has been well researched and widely used for tissue engineering. The acrylate groups do not impact PEG properties described prior. As PEGDA is a bioinert, 3D scaffold, it can be used to isolate the effects of non-scaffold related inputs.
1.4 Effects of Loading on MSCs

A number of studies have evaluated the effects of vibration or uniaxial tensile strain on MSCs cultured on natural scaffolds, namely collagen based scaffolds. [2, 16, 18, 21] Studies have shown that mechanical loads on MSCs may trigger differentiation into desired cells, with or without additional chemical factors such as growth factors. [25] Chen et al. found that MSCs subjected to 3 % and 10 % strain committed to osteogenic and tenogenic differentiation, respectively, demonstrating the effect of different strain magnitudes on cell fate. [16] The physiological strain levels bones undergo is much less than the levels experienced by tendon; reproducing these ranges directed MSC differentiation. The type of loading required to elicit differentiation into a specific cell type depends on the function of the tissue of origin and physical conditions cells are subjected to in situ. [1]

1.5 Motivation

PEGDA hydrogels were used to isolate the effects of tensile strain and vibration on MSCs. Results from these studies will fill the existing gap in knowledge about the behavior of MSCs under mechanical loading within synthetic scaffolds. These studies are the first step in determining what components are necessary and sufficient for scaffold design for a variety of mesenchymal tissues and may pave the way to improve design parameters for synthetic scaffolds for tissue engineering.
In tissue engineering and regenerative medicine, mesenchymal stem cells (MSCs) are often preferable to fully differentiated cells, which are limited in supply and do not multiply as rapidly or to as great an extent. [5] MSCs can proliferate for numerous passages. MSCs’ response to tensile strain and vibration has been researched using various scaffolds and stimulation parameters. Typical MSC responses to various mechanical inputs include differentiation into osteocytes and chondrocytes, often guided by the presence of growth factors and calcium. Cell responses have also been guided by the microenvironment, whether cells are in their native environment, a transplanted in vivo environment, or cultured using tissue culture plastic, 2D scaffolds, or 3D scaffolds. Even the choice of scaffold material has an impact, whether scaffold is derived from natural or synthetic material. To the author’s knowledge, the present studies continue the only investigations of the effect of vibration on MSCs entrapped within synthetic 3D scaffolds.

2.1 Common Methods to Differentiate MSCs

Mesenchymal differentiation pathways of interest are osteogenesis, chondrogenesis, tenogenesis, myogenesis, and adipogenesis. When maintained in vitro, MSCs can be chemically and mechanically differentiated into a variety of tissues such as bone, cartilage, tendon, and ligament. [26] The biochemical factors that promote specific cell responses are well understood and thus enable researchers to successfully guide cell differentiation. Adding chemical factors such as ascorbate, transforming growth factor-β1 (TGF-β1), growth and differentiation factor 5 (GDF-5), PDGF, and IBMX to media promotes osteogenesis, chondrogenesis, tenogenesis, myogenesis, and adipogenesis for MSCs respectively. [2, 27, 28] Mechanical properties such as scaffold stiffness can be used to
guide differentiation of MSCs as well chemical factors. [4, 5] For instance, dexamethasone (dex) is widely used to promote MSC osteogenesis, and alternately, scaffolds with elastic moduli comparable to bone promote MSC osteogenesis. [2, 4] In situ, tissues are also subjected to a variety of biochemical signals, but they additionally experience a variation of forces that influence their development. Cell response to force is dependent on stress/strain magnitude, duration, loading type, and force propagation through attachment site geometry. Loading types include tension, compression, shear, bending, torsion, electromagnetic inputs, and vibration. Furthermore, loading can be separated into static or cyclic loading. All these loading types are experienced by cells in situ often in combination. For example, in bone marrow, tension, compression, and fluid-induced shear may all be present but the effects of these forces on stem cells are not well understood. [2, 29] A challenge of tissue engineering is identifying both the appropriate chemical and mechanical parameters to differentiate harvested MSCs into specific cell types in vitro. Once a scaffold is implanted, any biofactors it contains will eventually dissipate, thus the success of the scaffold will be maximized if its mechanical properties continue to influence cells.

2.2 The Effect of Vibration on Mesenchymal Stem Cells

Although not necessarily a loading condition experienced in nature, an extensive number of in vivo studies have been conducted with whole body vibration. [30-35] Whole body vibration studies have been used to model the cyclic tensile strain imparted on muscle or bone during physical actives such as walking, stair climbing, or weight lifting exercises. [31] The vibration stimulates the skeleton, in a manner similar to walking or running, and has been found to increase bone mass and bone strength. [30-32] Whole body vibration
stimulates osteogenesis of MSCs through mechanotransduction, resulting in bone mass increase. [32] Whole body vibration may also elicit a response from differentiated cells, which influence MSC differentiation. [32] The effect of whole body vibration on MSCs may be confounded by the effect of vibration on differentiated cells. The mechanism of mechanotransduction during vibration in MSCs and subsequent response is not fully understood. [33-35]

Stimulating cells in vitro allows the cell environment and loading factors to be controlled. Investigators specifically select loading parameters, biochemical additives, and cellular environment to observe or induce differentiation of cells.

### 2.2.1 Tissue Culture Plastic

Most in vitro vibration studies are performed with a cell monolayer cultured on tissue culture plastic (TCP). [21, 36-38] The MSC response to vibration depends on frequency, acceleration, and duration of stimulation.

Chen et al. investigated the effects of 0.3 g acoustic vibratory stimulation at 30, 400, and 800 Hz at on human MSCs (hMSCs) in cell culture plates. [36] Cells were stimulated 30 mins/day for 7 days. The 30 Hz frequency was selected because osteogenesis was promoted after whole body vibrations at <100 Hz. [32] The higher frequencies were selected because higher frequencies are more suited for localized body vibrations. [36] The authors found cell proliferation, calcium deposition, and Collagen 1 (Col I) gene expression to be highest after vibrations of 800 Hz at 0.3 g. At 800 Hz, adipogenic gene expression and lipid accumulation was decreased. The authors found adipogenesis to be promoted after 30 Hz
stimulation. Though acoustic vibration differs from direct mechanical vibration, both methods imparts physical vibration of the cells.

Demiray et al. cultured mouse MSCs on glass cover slides within 6 well plates. [37] Plates were stimulated with low magnitude (<1 g), high frequency (20-90 Hz; LMHF) vibrations of 90 Hz at 0.15 g over 7 days for 15 mins/day. The authors hypothesized that low intensity vibrations would induce MSC differentiation into osteogenic cells. Following vibration, cells were tested for osteogenic markers Runx2 and osteocalcin (OC) to indicate osteogenic differentiation. While gene expression of vibrated and control cells was similar, the vibrated cells had increased proliferation and morphological changes. Vibrated cells had increased cellular height and increased molecular expression of focal adhesion kinase.

Lau et al. studied the effects of LMHF vibration on rat MSCs cultured on TCP while using osteogenic media. [38] Cells were stimulated with vibrations of 60 Hz at 0.3 g for six 1-hour bouts. The authors hypothesized the vibration would promote osteogenesis based on prior animal and human studies. [32, 39] Following vibration, cells were tested for osteoblast-specific transcription factor Osterix (Osx) to indicate osteoblastic differentiation. The MSCs displayed decreased Osx levels and inhibited mineralization, indicating that LMHF vibration did not enhance osteogenic differentiation. Further, LMHF vibration did not affect proliferation rate. As both the control and test groups contained osteogenic media, the study was more an investigation of the synergistic effect of LMHF and osteogenic media compared to osteogenic media alone.

Kim et al tested hMSCs with a wide array of vibrations. [21] MSCs were seed on TCP or a collagen sponge. The collagen sponge was prepared from a cross reaction of chondroitin-6-sulfate and type I collagen. [11] The cells were seeded within the pores of the sponge,
creating a multidimensional scaffold. Cells were subjected to varying accelerations of vertical vibration for 10 mins/day for 5 days using a custom platform on a shaker. Accelerations varied from 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 g and frequencies varied from 10, 20, 30, and 40 Hz. Vibration on TCP resulted in a minor increase of proliferation. At 0.2 g and 0.3 g accelerations, proliferation rates increased as frequency increased. For all frequencies, proliferation was significantly higher at 0.3 g compared to other accelerations. The highest proliferation was observed at 0.3 g for both 30 Hz and 40 Hz. Thus, their subsequent experiments were performed with 30 Hz vibrations delivering 0.3 g accelerations. In their differentiation assays, the authors found that osteoblastic differentiation markers alkaline phosphatase (ALP) and osteopontin (OPN) were upregulated in vibrated cells while OC and bone sialoprotein (BSP) were unaffected. Alizarin red staining was increased in MSC monolayers with vibration and osteogenic media compared to control, though staining was not increased for vibrated cells without osteogenic media. MSCs behaved differently on their scaffold compared to TCP. Specifically, OPG, Col I, VEGF expression showed significant increases in vibrated groups compared to non-vibrated groups; this effect was observed only in MSCs cultured on scaffolds. These differing results suggest additional factors, such as microarchitectural factors, may influence the mechanotransduction of vibration.

It is well known that cells in culture do not behave the same on TCP as they do in their natural environments. [40-44] The same is true with cells subjected to vibration. [11, 16, 21, 38, 40] In several LMHF studies, osteogenesis was increased significantly when MSCs on TCP were vibrated at accelerations of 0.3 g at 35 Hz or 45 Hz. [45, 46] Conversely, in other studies that vibrated MSCs on TCP with 0.3 g accelerations at 30 Hz or 60 Hz,
osteogenesis was inhibited. [16, 21] Scaffolds may provide a more accurate and consistent
*in situ* representation.

2.2.2 *Two Dimensional Scaffolds*

Scaffolds provide a more complex cellular interaction than a simple monolayer on TCP. A 2D scaffold has cells cultured on a flat surface while a 3D scaffold has cells embedded within or seeded on a multidimensional surface. Two dimensional scaffolds for vibration studies are often membranes coated with osteogenic proteins. Cell attachment and force transition varies with different scaffolds or bound matrix proteins.

Edwards et al. seeded hMSCs in gelatin coated 12 well plates. [47] Plates were stimulated with LMHF vibrations of 15, 30, 45, or 60 Hz at 0.02 g. Vibration occurred for 10 minutes or 45 minutes. The authors also investigated the effect of osteogenic media (dex). Alkaline phosphatase (ALP) activity was the only measurement for cell commitment. ALP activity was greatest following 45-minute vibrations of 60 Hz with osteogenic media. Forty-eight hours after stimulation, MSCs subjected only to vibration did not demonstrate a statistically relevant increase of ALP activity. ALP activity was greater in cells that had dex+ media compared to dex- media. For dex+ cells, 60 Hz stimulation had the statistically greater ALP activity compared to other frequencies.

Sen et al. investigated the inhibition of adipogenesis of mouse MSCs after vibration. [48] The authors subjected MSCs seeded on a collagen coated silicon membrane to low intensity vibrations of <10 microstrain, at 90 Hz. The MSCs were stimulated for 20 minutes twice a day. Following vibration, cells were tested for the adipogenic markers adiponectin, PPARγ2, and aP2. The MSCs expressed decreased levels for all markers and development
of lipid granules was inhibited. The results indicate that adipogenesis was inhibited in cells subjected to only vibration. The authors also investigated the synergistic effect of vibration and adipogenic media. The MSCs subjected to vibration and adipogenic media had a statistically insignificant expression of adipogenic markers.

Tong et al. subjected hMSCs to 200 Hz acoustic vibration to replicate vocal cord vibrations. [49] Cells were seeded on PCL scaffolds coated with fibronectin and subjected to vibrations for 12 hrs./day over 7 days continuously or discontinuously. All vibrated cells expressed enhanced F-actin and α5β1 integrin expression. Levels of vocal fold extracellular matrix components were significantly elevated. Myogenic differentiation in MSCs were indicated by elevated levels of nascin-C, collagen III, and procollagen I, while osteogenic markers were not expressed.

Edwards et al., like Lau et al., found the synergist effect of vibration and osteogenic media to promote osteogenesis. [38, 47] Sen et al. found adipogenesis to be inhibited after vibration, even with the addition of adipogenic media. Tong et al. found myogenetic differentiation of hMSCs seeded with fibronectin. [49] The biological components of the scaffolds could provide a compounding biological factor to the investigations, contributing the synergistic response.

2.2.3 Three Dimensional Scaffolds

Few studies of MSC response to mechanical stimulation in vitro have used three dimensional substrates. [21, 36-38, 48-50] Three-dimensional substrates translate mechanical force to cells via different mechanisms than 2D substrates. [51] Three dimensional substrates may better model in situ cell attachment and the resultant effects of
mechanical loading. [52] In 3D environments, there are increased cell-to-cell contact and cell-to-extracellular matrix interactions over 2D monolayers. [52] Due to these factors, cells within 3D scaffolds could better model the \textit{in vivo} response to stimulation than cells within 2D substrates.

2.2.3.1 \textit{Natural Scaffolds}
Kim et al., discussed in section 2.2.1, vibrated hMSCs after inoculation on a collagen sponge. [21] Cells were exposed to 30 Hz vibration at 0.3 g. In their differentiation assay, the authors found that the osteogenic markers OPG, Col I, and VEGF expression were increased after MSCs were vibrated. The difference in the response of MSCs within a 3D scaffold and MSCs in a monolayer suggest alternate factors, such as microarchitectural cues, may mechanotransduce vibration.

Zhou et al. subjected rat MSCs seeded on 3D bone derived scaffolds to LMHF vibration. [53] The hollow components of the scaffolds allowed cells to attach within the multidimensional matrix. Rat MSCS seeded on TCP were used as control groups. The authors vibrated cells with 40 Hz at 0.3 g for 6 hours. The vibrated MSCs demonstrated increased levels of osteogenic markers ALP, Coll I, and OC. ALP activity was significantly higher in cells vibrated within 3D scaffolds than cells vibrated on TCP. However, vibration resulted in lower proliferation after day 7. The increased response from vibrated MSCs within 3D scaffolds over those on TCP motivates further investigation of MSCs within 3D scaffolds.
2.2.3.2 Synthetic Scaffolds
To the author’s knowledge, no vibration studies have been conducted on MSCs entrapped in synthetic materials outside of this continuing work that extends the findings of vibration on microencapsulated cells to the effect of vibration on microencapsulated cells. [54] Cells entrapped within PEGDA microspheres were subjected to vibrations of 100 Hz at 0.3 g, 3.0 g, or 6.0 g for 24 hours. Cells were subsequently tested for adipocyte, chondrocyte and osteoblast differentiation. Osteogenic differentiation in MSCs was observed at 0.3 g accelerations. Chondrogenesis and adipogenesis was not observed.

Microspheres provide different matrix geometry and force transmission than hydrogel sheets. The polymerization conditions could result in variations in cellular response. Microencapsulated cells are subjected to vortexing and the surface tension caused by forming emulsion-based microdroplets of various radii. Hydrogel sheets were selected to eliminate the confounding factors introduced by the fabrication method, to standardize the encapsulation efficiency, and to enable the performance of tensile tests.

2.3 The Effect of Cyclic Tensile Strain on MSCs
Cyclic uniaxial tensile strain can be applied to cells encapsulated in or seeded on a flexible scaffold. Rigid materials such as TCP cannot be used for tensile strain studies. Silicon scaffolds are regularly used as a synthetic, flexible scaffold. The effects of tensile strain on inducing tenogenic, osteogenic, and myogenic responses from MSCs have been investigated. [2]

2.3.1 Two Dimensional Scaffolds
2.3.1.1 Natural Scaffolds

The following studies investigate the effects of cyclic uniaxial tensile strain with magnitudes between 0.8 % and 15 % at 1 Hz on MSCs seeded on collagen membranes and other scaffolds. [16, 55-57] The strain magnitudes were selected to replicate the tensile strain experienced by bone, muscle, and tendon environments in situ.

Chen et al. seeded hMSCs on collagen type I coated scaffolds then subjected them to 3 % and 10 % cyclic tensile strain at 1 Hz for 8 or 48 hours. [16] The authors were investigating tensile strain and osteogenic or tenogenic commitment of MSCs following such strain. For all strains, organized cell alignment was noted. Cells for both strain rates became longer, slenderer in shape, and oriented perpendicular to the axis of strain. hMSCs subjected to 3 % strain for 8 hours demonstrated an upregulation of osteoblastic markers with increased levels of ALP and Cbfa1. hMSCs strained at 10 % for 48 hours demonstrated significant increases in type I collagen, type III collagen, and tenascin C, indicating tenogenic differentiation. The authors suggested strain amplitude and duration of straining may influence tenogenic and osteogenic commitment of MSCs.

Park et al. aimed to replicate the strain conditions of vascular smooth muscle on hMSCs seeded on elastin or collagen coated membranes. [55] MSCs were subjected to 10 % uniaxial tensile strain at 1 Hz for 24 hours. Smooth muscle cell (SMC) and osteogenic markers were investigated. After strain, cells in both scaffolds increased collagen I expression; however, markers of osteogenic differentiation were not significant. After being subjected to strain, levels of smooth muscle markers α-actin, SM-22α, and β-actin were transiently increased both scaffolds. Expression of α-actin and SM-22α decreased after cells aligned with the direction perpendicular to the strain. After 3 days, SM-22α decreased by 50 % on collagen coated scaffolds and 25 % on elastin coated membranes.
Levels of Col I also decreased after alignment. The authors suggest that uniaxial strain may promote MSC differentiation into SMCs if the cell orientation is fixed. The decrease in gene expression after alignment described by Park et al. is inconsistent with the stable gene expression described by Chen et al., who did not observe decreased gene expression from MSCs subjected to 10% strain. [16, 55]

Khani et al. studied the mechanical properties of hMSCs subjected to uniaxial strain with or without chondrogenic media (TGF-β1). [56] hMSCs were seeded on poly (dimethyl siloxane) (PDMS) with a collagen coating to enable cell attachment. hMSCs were subjected to uniaxial strain of 5% at 1 Hz, comparable to physiological levels within human arteries, for 24 hours. Strained cells without TGF-β1 had significantly increased Young’s Moduli (E) and elevated levels of smooth muscle markers ASMA, h1-Calponin, and SM22A. The strained hMSCs demonstrated increased myogenesis with or without TGF-β1. After stimulation, cells had aligned perpendicular to the axis of strain. The authors suggest the realignment of cells may reinforce the material, creating a stiffer composition, and impact mechanotransduction.

Koike et al. strained hMSCs seeded on collagen I coated membranes to 0.8%, 5%, 10%, and 15% cyclic strain at 1 Hz for 2 days. [57] Cell proliferation significantly increased at 5%, 10%, and 15% strain compared to unloaded controls. At 1 hour and 6-hour markers, Cbfa1/Runx2 increased at 0.8% and 5% strain but decreased at 15% strain. At 24 hours and 48 hours, cell proliferation and Col I increased at 5%, 10%, and 15% strain while Cbfa1/Runx2 expression, osteocalcin expression, and ALP activity was significantly decreased. ALP activity was increased at 0.8% strain. These results indicate high
magnitude mechanical strain will inhibit osteoblastic differentiation while low magnitudes may enhance osteoblastic differentiation.

All studies described above used a collagen scaffold to investigate the response of hMSCs to uniaxial tensile strain. [16, 55-57] On collagen scaffolds, MSCs subjected to low magnitude tensile strains (≤ 3 %) underwent osteogenic differentiation. [16, 57, 58] At greater tensile strains (5 %), myogenic differentiation was promoted. [56, 57] hMSCs subjected to high magnitude tensile strains (≥ 10 %) have indicated inhibition of osteogenic differentiation, transiently enhanced myogenic differentiation, and enhanced tenogenic differentiation. [16, 55, 57] The literature generally agrees that MSCs osteogenesis occurs at lower magnitudes of strain than MSC tenogenesis. [16, 55-58]

2.3.1.2 Synthetic Scaffolds
Park et al. noted a difference in MSC response to a synthetic scaffold compared to a collagen scaffold. [55] As mentioned in section 2.4.1, Park et al. subjected MSCs seeded on elastin coated or collagen coated scaffolds to 10 % uniaxial tensile strain at 1 Hz for 24 hours. The hMSCs transiently upregulated smooth muscle markers. Gene expression was less reduced in cells seeded on an elastic scaffold. The authors suggested that the MSCs sensed a difference in the mechanical loading of the two microenvironments.

Zhang et al. subjected rat MSCs (rMSCs) seeded on a silicone scaffold to a 10 % tensile strain at 1 Hz. [59] The authors investigated the effect of tensile strain on tenogenesis of hMSCs. The authors also investigated the effects of co-culturing hMSCs with ligament fibroblasts. Only strained cells had morphological changes. After tensile strain, rMSCs had a more elongated fibroblast-like cell type. The straining triggered an early up-regulation
of Col I and Col III. Tenascin-C expression was upregulated in strained cells as well. Strained cells demonstrated greater levels of tenogenic gene expression than cells co-cultured with fibroblasts.

Huang et al. subjected rMSCs seeded on elastic-silicone membrane to 5 %, 10 %, 15 %, and 20 % tensile strain at 1 Hz for 24 hours. [60] Huang et al. investigated the presence of cardiac related gene expression using negative controls and positive controls. Cells cyclically strained expressed GATA-4, β-MHC, NKx2.5 and MEF2c. Gene expression was greatest in cells stimulated with 10 % strain. Huang et al. then subjected rMSCs to 10 % strain at 1 Hz for 24, 48, and 72 hours. The expression of GATA-4, β-MHC, NKx2.5 and MEF2c was significantly increased for all durations of strain. Huang suggests cyclic mechanical strain of 10 % at 1 Hz induces cardiomyogenic differentiation of MSCs.

Jagodzinski et al. applied tensile strain to hMSCs seeded on a silicon scaffold. [61] Cells were strained six hours/day for three days at 1 Hz with 2 % or 8 % strain. hMSCs were cultivated with (dex+) or without (dex-) dexamethasone. For both strain magnitudes, cells had significantly increased ALP secretion and collagen III upregulation. Cells strained 8% significantly upregulated Col I and Cbfa1. The strained cells had significantly greater gene expression, with or without dexamethasone, for all markers of gene expression. Cyclic straining enhanced osteogenic commitment of hMSCs for low and high magnitudes, contrary to trends of hMSCs on collagen scaffolds.

After straining at 10 %, MSCs seeded on synthetic scaffolds demonstrated enhanced myogenic differentiation, resulting in fibroblasts, smooth muscle cells, and cardiac cells. [11, 55, 59] For other strain magnitudes, MSCs seeded on synthetic scaffolds respond differently to tensile strain than MSCs seed on natural scaffolds. [55, 59-61] Cells on a
synthetic scaffold had a greater expression of smooth muscle markers compared to cells on a natural scaffold. [55] Osteogenesis was induced at a magnitudes of strain in cells on silicone while osteogenesis is inhibited at high magnitudes of strain in cells on collagen. [61] Thus, demonstrating that the MSC response to strain differs on synthetic scaffolds compared to natural scaffolds.

2.3.2 Three Dimensional Scaffolds

Cells entrapped within a scaffold or seeded throughout a structure are subjected to a different microenvironment than cells seeded on a planar scaffold. Subtle differences in loading or cell-cell communication may impact cell response to strain.

2.3.2.1 Natural Scaffolds

The first study of hMSCs entrapped in 3D collagen matrix under cyclic strain was conducted by Sumanasinghe et al. [62] The authors subjected hMSCs entrapped within a collagen matrix to 10 % or 12 % uniaxial cyclic tensile strain at 1 Hz for 4hr/day. Strain was applied for 7 or 14 days. hMSCs remained highly viable for all strain conditions. hMSCs subjected to 10 % strain demonstrated a significant increase in BMP-2 expression. Cyclic strain of 12 % induced a significant increase in BMP-2 expression only after fourteen days. The authors suggested that strain alone can induce osteogenic differentiation without the addition of osteogenic supplements. Sumanasinghe et al. conducted a second study to investigate the expression of proinflammatory cytokines of hMSCs after the same strain conditions. [63] The authors also used osteogenic media to evaluate synergistic effect of cyclic strain and osteogenic
supplements. Initially, strained hMSCs had reduced viability. After day 6, hMSCs strained at 10 % had increased viability. Only strained cells with osteogenic media had increased levels of TNFα and IL-1β. The authors demonstrated that hMSCs entrapped within a collagen matrix maintains high viability after cyclic strain stimulation.

Charoenpanich et al. entrapped hMSCs, specifically ASCs, within a collagen I gel sheet. [64] The entrapped cells were stimulated with 10 % cyclic tensile at 1 Hz for 4hr/day over 14 days. The authors were investigating the effects on osteogenic differentiation of a 3D scaffold and subjecting entrapped cells to tensile. The authors performed a microarray analysis of 847 genes and found 184 transcripts affected by tensile strain. Network analysis suggested strain may impact osteogenic differentiation by upregulation of proinflammatory cytokine regulator interleukin1 receptor antagonist (IL1RN) and angiogenic inductors including fibroblast growth factor 2 and vascular endothelial growth factor A. Cells subjected to strain and osteogenic media resulted in significantly increased calcium deposits, suggested synergistic effects towards osteogenic differentiation.

Qiu et al cyclically strained hMSCs seeded along collagen fibers to investigate fibroblastic differentiation. [65] The fibrous scaffold provided a nonplanar microenvironment. hMSCs were subjected to 10 % tensile strain at 1 Hz for 12 hrs/day over 14 days. Collagen I, collagen III, tenascin-C, and fibroblastic transcription factor scleraxis were all found to be significantly upregulated in cyclically strained hMSCs compared to unstrained control cells. Thus, cyclic strain of 10% significantly promoted tenogenic differentiation of hMSCs.

Juncosa et al. strained rabbit MSCs seeded within collagen sponges. [66] The authors were investigating the effect of tensile cyclic strain on MSCs and tenogenic differentiation.
MSCs were cyclically strained 2.4% at 0.003 Hz for 8 hrs/day over 12 days. Strained MSCs showed three or 4 times greater collagen I and collagen III production compared to unstrained controls. Gene expression of fibronectin or decorin were not significantly increased in strained MSCS. Tenogenic differentiation was not conclusively promoted by cyclic strain, but significant gene expression was induced by cyclic strain. Not many studies investigate both the effects of 3D scaffolds and cyclic tensile strain on hMSC differentiation without osteogenic supplements. However, while a collagen coating or collagen based scaffold is not an osteogenic supplement, it still provides a biological factor that induces a cell response. [10, 15]

2.3.2.2 Synthetic Scaffolds
Rathbone et al investigated the response of hMSCs to cyclic tensile strain entrapped within 3D hydrogels with either tripeptide Arg-Gly-Asp (RGD) or RGE. [22] Cells were either entrapped within a hydrogel with direct cell attachment (RGD), or entrapped within a hydrogel without direct attachment (RGE). The authors’ peptide hydrogel was composed of Fmoc-FF:Fmoc-RGD/RGE. The hMSCs were subject to 3 % strain 1 Hz for 1 hour or 24 hours and evaluated 2 hours or 24 hours post straining. Cells within hydrogels demonstrated high viability. The authors investigated CCNL2, WDR61 and BAHCC1 as potentially important mechanosensitive genes. After straining for 1 hour, hMSCs on monolayers significantly downregulated CCNL2, WDR61 and BAHCC1. After 24 hours of straining, hMSCs on monolayers significantly upregulated BAHCC1. BAHCC1 was not expressed by hMSCs in either 3D scaffolds. WDR61 was significantly upregulated by hMSCs in both 3D scaffolds after 1hr of strain. CCNL2 was upregulated in hMSCs only
in scaffolds with RGD. The cells’ response differed when in a monolayer or in a 3D scaffold. The cells’ response within 3D scaffolds when attached or unattached. Indicating different microenvironments impact mechanotransduction and thus cell response.

Kreja et al. strained hMSCs seeded throughout a novel texture PLA scaffold to investigate fibroblastic differentiation. [67] Cells were strained at 2 % or 5 % at 1 Hz for 1 hr/day over 15 days. The authors analyzed the gene expression of ligament matrix markers: collagen I, collagen III, fibronectin, tenasin C, decorin, MMP-1, MMP-2 and inhibitors TIMP-1 and TIMP-2. Strained cells did not demonstrate significant gene expression except in the downregulation of both MMP-1 and TIMP-2 in 5 % strained cells. For both strain parameters, tenogenic differentiation was not promoted in hMSCs.

Yang et al. strained hMSCs entrapped within fast and slow degrading MMP-sensitive PEG hydrogels to investigate tenogenic differentiation. [68] Cells were subjected to 10 % strain at 1Hz for 12 hrs/day over 14 days. Cell realignment was not observed. Strained hMSCs within the slow degrading hydrogel upregulated collagen III 3.8-fold and tenasin-C 2.5-fold while hMSCs within the fast degrading hydrogel upregulated collagen III 2.1-fold and tenasin 1.7-fold. The authors suggested cyclic straining promoted tenogenic differentiation and the presence of strain had a greater influence on cell differentiation than the difference between the hydrogels.

Doroski et al. expanded Yang et al.’s investigation of cyclically strained hMSCs entrapped with PEG based hydrogels. [68, 69] Doroski et al. entrapped hMSCs within oligo(poly(ethylene glycol) fumarate) (OPF). Cells were subjected to 10 % strain at 1Hz for 12 hrs/day over 21 days. By Day 21, cyclic strain significantly upregulated the tenogenic markers collagen I, collagen III, and tenasin-C while osteogenic, chondrogenic,
and adipogenic markers were not increased. Thus, the cyclic straining promoted tenogenic differentiation.

2.4 Summary

Summary tables of hMSC response to vibration and cyclic tensile strain across various cellular environments are provided. (Table 1, Table 2, Table 3) The response of strained and vibrated MSCs on tissue culture plastic varies with the response of MSCs on scaffolds. Even more intriguing, cells entrapped within 3D scaffolds have demonstrated differences with cells seeded on 2D scaffolds. Additionally, differing elastic moduli between cellular environments may affect cell response when exposed to similar mechanical parameters. There is no consensus on the effects of vibration or strain on MSCs in vitro, likely due to the varying cellular environments. Thus, further work is required to understand the effect of mechanical stimulation on cells within a 3D environment, devoid of biochemical and microarchitectural cues. In this thesis, the “blank state” of the PEGDA hydrogel is utilized to investigate the effects of vibration and tensile strain on encapsulated hMSCs. The hMSCs are encapsulated within 10kDa PEGDA hydrogel sheets then vibrated or subjected to cyclic tension.
Table 1. Effect of vibration on MSCs

<table>
<thead>
<tr>
<th>Environment</th>
<th>Ref.</th>
<th>Cell</th>
<th>Acceleration</th>
<th>Frequency [Hz]</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TCP</strong></td>
<td>[36]</td>
<td>hMSC</td>
<td>0.3 g</td>
<td>30, 400, 800</td>
<td>Osteogenic</td>
</tr>
<tr>
<td></td>
<td>[37]</td>
<td>mMSC</td>
<td>0.15 g</td>
<td>90</td>
<td>Osteogenic</td>
</tr>
<tr>
<td></td>
<td>[38]</td>
<td>rMSC</td>
<td>0.3 g</td>
<td>60</td>
<td>Inhibition (synergist)</td>
</tr>
<tr>
<td></td>
<td>[21]</td>
<td>hMSC</td>
<td>0.1 - 0.6 g</td>
<td>10, 20, 30, 40</td>
<td>Cell proliferation</td>
</tr>
<tr>
<td><strong>2D</strong></td>
<td>[47]</td>
<td>hMSC</td>
<td>0.02 g</td>
<td>15, 30, 45, 60</td>
<td>Inconclusive (synergist osteogenic)</td>
</tr>
<tr>
<td>Gelatin</td>
<td>[48]</td>
<td>mMSC</td>
<td>10 µstrain</td>
<td>90</td>
<td>Adipogenesis inhibited</td>
</tr>
<tr>
<td>Collagen</td>
<td>[49]</td>
<td>hMSC</td>
<td>Acoustic</td>
<td>200</td>
<td>Myogenic</td>
</tr>
<tr>
<td><strong>3D</strong></td>
<td>[21]*</td>
<td>hMSC</td>
<td>0.3 g</td>
<td>30</td>
<td>Osteogenic</td>
</tr>
<tr>
<td>Collagen Sponge</td>
<td>[53]*</td>
<td>rMSC</td>
<td>0.3 g</td>
<td>40</td>
<td>Osteogenic</td>
</tr>
<tr>
<td>Bone derived</td>
<td>[54]*</td>
<td>hMSC</td>
<td>0.3, 3, 6 g</td>
<td>100</td>
<td>Osteogenic</td>
</tr>
<tr>
<td>PEGDA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Results significantly different compared to TCP controls
<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Ref.</th>
<th>Cell</th>
<th>Strain (%)</th>
<th>Time (hrs)</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D Natural</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>[56]</td>
<td>hMSCs</td>
<td>5</td>
<td>24</td>
<td>Myogenic</td>
</tr>
<tr>
<td></td>
<td>[55]*</td>
<td>hMSCs</td>
<td>10</td>
<td>24</td>
<td>Myogenic</td>
</tr>
<tr>
<td></td>
<td>[16]</td>
<td>hMSCs</td>
<td>3, 10</td>
<td>8, 48</td>
<td>Tenogenic (10 %)</td>
</tr>
<tr>
<td></td>
<td>[57]</td>
<td>hMSCs</td>
<td>0.8, 5, 10, 15</td>
<td>48</td>
<td>Osteogenic (&lt;5 %) Inhibition (&gt;5 %)</td>
</tr>
<tr>
<td>2D Synthetic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elastin</td>
<td>[55]**</td>
<td>hMSCs</td>
<td>10</td>
<td>24</td>
<td>Myogenic</td>
</tr>
<tr>
<td>Silicone</td>
<td>[59]</td>
<td>rMSCs</td>
<td>10</td>
<td>N/A</td>
<td>Tenogenetic</td>
</tr>
<tr>
<td>Elastin-silicone</td>
<td>[60]</td>
<td>rMSCs</td>
<td>5, 10, 15, 20</td>
<td>24</td>
<td>Myogenic (10 %)</td>
</tr>
<tr>
<td>Elastin-silicone</td>
<td>[60]</td>
<td>rMSCs</td>
<td>10</td>
<td>24, 48, 72</td>
<td>Myogenic</td>
</tr>
</tbody>
</table>

*Myogenic expression ↓ 50% in collagen scaffold

**Myogenic expression ↓ 25% in elastin scaffold

Osteogenic (≤ 3 %), Myogenic (~5-10 %), Tenogenic (≥ 10 %)
Table 3. Effect of cyclic tensile strain on MSCs within 3D scaffolds.

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Ref.</th>
<th>Cell</th>
<th>Strain (%)</th>
<th>Time (hrs)</th>
<th>Differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D Natural Collagen</td>
<td>[62]</td>
<td>hMSCs</td>
<td>10, 12</td>
<td>28, 56</td>
<td>Osteogenic</td>
</tr>
<tr>
<td></td>
<td>[63]</td>
<td>hMSCs</td>
<td>10</td>
<td>56</td>
<td>Osteogenic</td>
</tr>
<tr>
<td></td>
<td>[65]</td>
<td>hMSCs</td>
<td>10</td>
<td>168</td>
<td>Tenogenic</td>
</tr>
<tr>
<td></td>
<td>[66]</td>
<td>rbMSCs</td>
<td>2.4</td>
<td>96</td>
<td>Inconclusive*</td>
</tr>
<tr>
<td>3D Synthetic Hydrogel (RGD/RGE)</td>
<td>[22]</td>
<td>hMSCs</td>
<td>3</td>
<td>2, 24</td>
<td>Gene expression ↑ (RGD)</td>
</tr>
<tr>
<td>PLA</td>
<td>[67]</td>
<td>hMSCs</td>
<td>2, 5</td>
<td>15</td>
<td>Inconclusive</td>
</tr>
<tr>
<td>PEG</td>
<td>[68]</td>
<td>hMSCs</td>
<td>10</td>
<td>168</td>
<td>Tenogenic</td>
</tr>
<tr>
<td>OPC</td>
<td>[69]</td>
<td>hMSCs</td>
<td>10</td>
<td>252</td>
<td>Tenogenic</td>
</tr>
</tbody>
</table>

Inconclusive (≤ 3 %), Osteogenic (~10 %), Tenogenic (~10 %)
MATERIALS AND METHODS

3.1 Experimental Design

Human MSCs (hMSCs) were encapsulated into non-degradable PEGDA hydrogel sheets and then exposed to uniaxial cyclic tension or vibration (Figure 3.1) to probe the response of MSCs, which were evaluated for viability and differentiation. All stains and assays were performed in triplicate.

Figure 3.1 Experimental design. a. Cells are combined with PEGDA prepolymer. b. Cells and PEGDA prepolymer are polymerized into hydrogel sheets. c. Cell-laden hydrogel sheets are subjected to vibration or uniaxial cyclic strain in a material testing device. [70]

For vibration, hydrogels placed in a 12 well plate and secured to the upper motorized fixture. For strain, the hydrogels are placed within a bag. The bag is fixed to the upper fixture and stationary lower fixture. As the upper fixture moves, the cells experience strain or vibrational loading.

3.2 hMSC Culture
Cryopreserved hTERT-MSCs were obtained as a gift (Glackin Lab; City of Hope, Duarte, CA). The hTERT-MSCs were defrosted then seeded in 75 cm² corning cell culture flasks at a density of 2500 cells/cm². Cells were incubated with MSC culture medium: Alpha-Minimum Essential Medium (containing nucleosides, Phenol Red and L-glutamine), 15% fetal bovine serum (Sigma), 1% penicillin-streptomycin (Sigma) and were maintained at 37°C and at 5% CO₂ in a humidified incubator. After 2-3 days, hTERT-MSCs were harvested using 0.25% trypsin-EDTA solution (Sigma) and expanded in 225 cm² culture flasks at 4000 cells/cm² until reaching 70-80% confluency.

3.3 Cell Encapsulation

Hydrogel precursor solution was prepared by combining 0.1 g/mL 10 kDa PEGDA (10% w/v), 37 mM 1-vinyl-2-pyrrolidinone, and hydrophilic photoinitators (1.5% (v/v) thriethanolamine and 0.1 mM eosin Y) in HEPES-buffered saline (pH 7.4). hTERT-hMSCs with a final concentration of 5000 cells/µl were then mixed with the hydrogel precursor. Hydrogel sheets (vibration sheets = 1 cm x 1 cm, or tension sheets = 2.5 cm x 3 cm) were formed by aliquoting the prepolymer/cell solution into glass molds formed by clamping two glass slides separated by 0.5 cm thick Teflon spacers and photopolymerizing the solution by exposing it to white light for 30 seconds and 50 seconds, respectively, using a Metal Halide Illuminator (MH-100, Edmund optics). The resulting hydrogel sheets were removed from molds with a flat spatula, placed in a 6 well plate containing MSC culture medium, and allowed to swell in a humidified incubator (37°C and at 5% CO₂) 24 hours. The small sheets for vibration contained 450,000 encapsulated hMSCs per cm³ and the larger sheets for tensile strain contained 480,000 encapsulated hMSCs per cm³.
3.4 Mechanical Stimulation

Hydrogel sheets were kept in media while being subjected to 24 hours of vibration or strain at room temperature using a Bose ElectroForce material tester.

3.4.1 Vibration

Sheets exposed to vibration were placed in 12 well plates for testing. A custom clamp was built to secure flasks and 12 well plates for vibration (Figure 3.2). This was attached to the machine’s moving actuator, which applied the vibration. The frequency and displacement parameters were specified in the ElectroForce’s WinTest software. Hydrogels were vibrated at 100 Hz for 24 hours at room temperature. Vibrated plates were divided into three different accelerations: 0.3 g, 3 g, and 6 g, with displacement amplitudes of 0.00745 mm, 0.0745 mm, and 0.149 mm, respectively. (Table 4) The acceleration (g) imparted was determined by the relation:

$$a = \frac{D(2\pi f)^2}{9.8},$$

where $a$ is acceleration, $f$ is the frequency, $D$ is peak-to-peak displacement, and 9.8 is the conversion factor between m/s$^2$ and g-force.
Figure 3.2 Vibration set up. Overall view of the Bose ElectroForce 3100 device (left) and enlarged area where well plates were clamped (right). hTERT-hMSCs encapsulated in sheets were placed in 12-well plates, clamped in a custom-built holder, and mounted on the machine.

<table>
<thead>
<tr>
<th>Frequency (Hz)</th>
<th>Peak Displacement (mm)</th>
<th>Peak-to-Peak Displacement (mm)</th>
<th>Acceleration (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.00745</td>
<td>0.0149</td>
<td>0.3</td>
</tr>
<tr>
<td>100</td>
<td>0.0745</td>
<td>0.149</td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>0.149</td>
<td>0.298</td>
<td>6</td>
</tr>
</tbody>
</table>

Control encapsulated cells were not vibrated and kept on an isolated bench at room temperature for 24 hours. After 24 hours of vibration, test and control sheets were placed in an incubator with humidified, 5% CO₂ atmosphere.

3.4.2 Cyclic Tensile Strain
Sheets subjected to tension were encased in a sterile, sealed plastic bag with 80 MGA 120 grit sand paper placed around the ends of the hydrogel where they would be gripped by the device clamps (Figure 3.3). The sandpaper prevented slip between the clamp and the hydrogel. Sand paper of was sterilized in an autoclave prior to testing. The plastic bags encasing the hydrogels were exposed to UV light for 4 hours to sterilize them. Tweezers and spatulas were sterilized in an autoclave. All sterilized materials were gathered in the hood.

Figure 3.3 Tensile strain stimulation set-up. Left image shows overall set-up in the Bose Electroforce 3100 device. Right shows close-up of hydrogel clamped within its hydration bag. hTERT-MSCs encapsulated in sheets are placed in sterile bags and clamped on the mechanical testing machine. The ends of cell-seeded hydrogel are surrounded by sand paper in a sterile plastic bag. The plastic bag is gripped and the sandpaper does not allow slip between the hydrogel, the bag, and the clamp.
Once in the hood, the tweezers and spatulas were used to move the hydrogel into the plastic bag and place the sandpaper in the proper configuration. Ten mL of MSC media was added to each bag. After bags were sealed, they were removed from the hood and gripped in the clamps of the material tester. The bottom region of the hydrogel was gripped first and moved to the appropriate height. Then the top grip was tightened around the top region of the hydrogel sheet within the bag. Slack was created in the bags before the top grip was tightened to ensure tension was applied to the hydrogel and not the bag. The clamps were attached to the machine’s moving actuator. Preloading of 0.01 N was applied to ensure nonzero loading. The frequency and displacement parameters were specified in the machine’s WinTest software. Cyclic strains cycled from 0-10% strains at 1 Hz for 24 hours. All tensile testing was performed in the plastic bag to ensure hydrogels remained hydrated and cells maintained constant access to media for the 24-hour period.

After straining, hydrogel sheets were cut in thirds. The gripped hydrogel region (0.6 cm on either end) was not analyzed, leaving a 0.8 cm x 1.7 cm area of interest. The center 1 cm (within the 1.7 cm width) was analyzed to minimize edge effects. Controls were kept in 12 well plates with the same media at room temperature for 24 hours.

3.5 Cell Viability Assays

hTERT-MSC in both test and control hydrogel sheets were assessed for cell viability on Days 1, 4, 7, 14, and 21. The larger hydrogel sheets used in tensile tests were cut into thirds and stained at different time points. The sheet was cut immediately before staining as to minimize artifact. The hydrogel sheets were incubated with media and 2 mM calcein acetoxyemethyl ester and 4 mM ethidium homodimer (LIVE/DEAD Viability/Cytotoxicity
Kit for mammalian cells, Life technologies) for 10 minutes in a 37°C, 5% CO₂ incubator. Hydrogel sheets were then observed under an epifluorescent microscope (Axio Observer Z1, Zeiss) to image labeled live (green; ex/em ~495 nm/~515nm) and dead (red; ex/em ~495 nm/~635 nm) hTERT-MSCs. For each hydrogel, 3 sets of pictures were taken in the two fluorescent channels and in phase contrast. Z stacks were taken in 25 nm increments through the thickness of the hydrogel sheet.

Images of control and vibrated cells are taken at 10x magnification. Images of cells subjected to cyclic tensile strain were taken at 2.5x magnification, in addition to 10x magnification, to analyze trends across axis of tension.

3.6 Histochemistry

Several stains were used to detect encapsulated hTERT-MSC differentiation. Alkaline Phosphatase (ALP) was used to directly visualize MSC differentiation into osteoblasts. Alizarin red stain was used to stain calcium deposits, which indicate that mineralization has occurred. Mineralization indicates the presence of active osteoblasts, which are in turn the result of osteogenic differentiation. Oil red O staining was used to identify mature adipocytes by staining the lipid droplets they produce. Finally, Safranin O staining was used to identify chondrocytes by staining their glycosaminoglycans (GAGs). Formalin was used to fix cells prior to histochemical staining, except where otherwise noted. All formalin solutions were composed of 10% formalin and 90% distilled water. All test and control hydrogel sheets were placed in 12 well plates to perform assays.

3.6.1 Alkaline Phosphatase Assay
ALP assays were conducted four days after the initial vibration using a Fluorescence Alkaline Phosphatase Detection Kit (Sigma). The hydrogel sheets were washed with phosphate buffered saline (pH 7.4, containing TWEEN) (PBST) followed by fixing entrapped cells with a proprietary fixation buffer for 5 min, another PBST wash, and finally staining the sheets with the staining solution for 30 min. ALP positive cells stain purple and the stained cells were imaged in phosphate buffered saline (PBS) in a 12 well plate under bright field microscopy.

3.6.2  *Alizarin Red S Assay*

Briefly, hydrogel sheets were washed with PBS and fixed with 10% neutral buffered formalin solution for 15 minutes, and then washed with distilled water. Hydrogel sheets were then stained with 2% Alizarin Red S stain (Sigma) for 20 minutes, followed by five distilled water washes. Calcium deposits stain red in color and these were observed in distilled water under color microscopy. Representative images of positively stained sheets were taken. Images were taken at three depths at three randomly chosen locations along the hydrogel sheet.

3.6.3  *Oil red O Stain*

Hydrogel sheets were washed with PBS and fixed with 10% formalin solution for 15 min, washed twice with distilled water, and then later washed with 60% isopropanol. A 0.3% Oil red O solution (Sigma) was made with 300 mg of Oil red O powder and 100 mL of isopropanol. Hydrogel sheets were then stained with 0.3% Oil red O solution for 5 minutes,
followed by 2-3 washes with distilled water. The lipid droplets if stained, appear red in color. These were imaged in distilled water under color microscopy.

3.6.4 Safranin O Stain

Hydrogel sheets were washed with PBS and fixed with 10% formalin solution for 15 minutes and then later washed with distilled water. They were then stained with 0.6% Safranin O solution (Sigma) for 10 minutes and then following staining underwent two distilled water washes. The sulphated GAGs, if stained, appear orange. Stained sheets were observed in distilled water under color microscopy.
Chapter 4.  Results

4.1 Mechanical Loading

The load cell of the MTM captured the force propagating through the hydrogel. The uniaxial tension imparted a 10% strain equating to 0.7 N. Sheet maintained tension.

4.2 Cell Viability

On Day 1, all cells within hydrogel sheets had high viability. Qualitative assessment was performed by ranking level of viability from 1-5 (Table 5 and Figure 4.3). Values of 5 indicates mostly alive cells while values of 1 indicates mostly dead cells. All evaluations of image viability were conducted at the same time by the author. The viability assessments were performed on hTERT-MSCs encapsulated within test (vibrated or cyclically strained) and control hydrogel sheets (Figure 4.1, Figure 4.2). After strain, there was no apparent cell alignment. Cyclic tensile strain cells demonstrated greater viability than control cells. All cells accelerated at 0.3 g demonstrated greater viability compared to control. Cells accelerated at 3.0 g demonstrated greater viability than control after seven days. Cells accelerated at 6 g had the lowest viability for all cells on all days. The greatest viability for all days was that of 0.3 g cells on Day 1. On Day 21, cells accelerated at 0.3 g demonstrated the highest viability.
Figure 4.1 Cell viability of cyclically strained and control cells. Viability of from Day 1 and Day 7, stained with live (green; ex/em ~495 nm/~515 nm) and dead (red; ex/em ~495 nm/~635) cells. Control cells, at 10 x magnification, are compared to strained cells at 10x magnification. Cyclic tensile strain images were also taken at 2.5 x to visualize any cell alignment along the axis of tension.
Strained cells had qualitative averages of 4.00 ± 0 and 4.17 ± 0.41 on Days 1 and 7, respectively, while control cells had averages of 4.14 ± 0.69 and 2.50 ± 0.55 on the same days. Indicating viability for strained cells remained the same over time while viability for control cells decreased over time.

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>Vibration 0.3 g</th>
<th>Vibration 3 g</th>
<th>Vibration 6 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
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<tr>
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<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
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<tr>
<td>7</td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
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<td><img src="image13.png" alt="Image" /></td>
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<td><img src="image19.png" alt="Image" /></td>
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</table>

Figure 4.2 Cell viability of vibrated and control cells. Viability from Day 1 to Day 21, stained with live (green; ex/em ~495 nm/~515 nm) and dead (red; ex/em ~495 nm/~635) cells. Images taken at 2.5x magnification. An unidentified issue with 0.3 g hydrogels on Day 14 occurred, resulting in images with no stain.
Table 5. Cell viability of control, vibrated, and strained hMSCs.

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>0.3 g</th>
<th>3.0 g</th>
<th>6.0 g</th>
<th>Cyclic Tension</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.14 ±0.69</td>
<td>5.00±0</td>
<td>3.33±0.71</td>
<td>2.00±0.71</td>
<td>4.00±0</td>
</tr>
<tr>
<td>4</td>
<td>3.83±0.75</td>
<td>3.83±0.75</td>
<td>3.67±0.50</td>
<td>1.17±0.39</td>
<td>unavailable</td>
</tr>
<tr>
<td>7</td>
<td>2.50±0.55</td>
<td>3.60±0.55</td>
<td>2.33±0.52</td>
<td>1.00±0</td>
<td>4.17±0.41</td>
</tr>
<tr>
<td>14</td>
<td>1.78±0.83</td>
<td>unavailable</td>
<td>3.33±0.58</td>
<td>1.25±0.46</td>
<td>unavailable</td>
</tr>
<tr>
<td>21</td>
<td>1*</td>
<td>4.33±0.58</td>
<td>3.00±1.00</td>
<td>1.13±0.50</td>
<td>unavailable</td>
</tr>
</tbody>
</table>

Images were ranked on a scale from 1-5. The mean and standard deviation is presented. Unavailable indicates an experiment was performed, but the data was corrupted. Blacked out boxes indicate that these time points were not included in the study.

* Indicates only one image was obtained.

Figure 4.3 Average cell viability of control, vibrated, and cyclically strained hMSCs over time. Error bars show standard deviation.
For control cells, viability decreased over time. Cells accelerated at 0.3g demonstrated the greatest number of live cells every time point. For 3 g cells, viability was comparable to control on Days 1-7. In the later time points, there is a greater amount of calcein AM staining in 3.0g cells than in control cells. For 6 g accelerations, there are fewer live cells compared to control for every time point.

4.3 ALP Assay

Positive ALP staining is denoted by purple stain. No positive ALP was detected in any cells (Figure 4.4).

<table>
<thead>
<tr>
<th>Control</th>
<th>Vibration 0.3 g</th>
<th>Vibration 3 g</th>
<th>Vibration 6 g</th>
</tr>
</thead>
</table>

Figure 4.4 Alkaline phosphatase staining of vibrated and control cells was performed on Day 4. The color was adjusted for contrast, white balance, and saturation. No positive staining was observed. Images were taken at 10x magnification.

4.4 Differentiation

4.4.1 Osteogenesis

The images were stained with Alizarin red S (ARS) and representative images are provided in Figure 4.5. Positive Alizarin red stain bright red and stains calcium deposits. Positive staining was present in control, 0.3 g, and 3.0 g cells. Day 1 was used as a baseline reference
for all staining at later points. There was a small amount of positive staining noted on Day 1 and Day 7 for 0.3 g cells. Day 14 images showed widespread positive Alizarin red S stain in control, 0.3 g, and 3.0 g cells. Day 21 showed highly positive Alizarin red S staining in control and 0.3 g cells. No positive Alizarin red staining was present at any time in cells accelerated at 6 g.

<table>
<thead>
<tr>
<th>Day</th>
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<th>Vibration 0.3 g</th>
<th>Vibration 3 g</th>
<th>Vibration 6 g</th>
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Figure 4.5 Representative Alizarin red S staining for vibrated and control cells. Low levels of positive stain was marked on Day 1 and Day 7 for 0.3 g. Positive stains present on Day
14 for control, 0.3 g, and 3.0 g cells. Highly positive stain present on Day 21 for control and 0.3 g cells. Images were taken at 10x magnification.

4.4.2 Adipogenesis

Oil Red O (ORO) stains adipocytes a bright red color. No positive Oil Red O staining was observed in any vibrated hydrogels.

<table>
<thead>
<tr>
<th>Control</th>
<th>Vibration 0.3 g</th>
<th>Vibration 3 g</th>
<th>Vibration 6 g</th>
</tr>
</thead>
</table>

Figure 4.6 Oil Red O staining on Day 21 for vibrated and control cells. Images were taken at 10x magnification. Staining did not indicate the presence of adipocytes.

4.4.3 Chondrogenesis

Safranin-O (SO) is used to stain articular cartilage stains an orange-red color. There was no positive Safranin O staining observed in either vibrated or control groups. The background of the sheet is reddish in the control image but the cells are not stained.
Figure 4.7: Safranin O stain on Day 21 from vibrated and control cells. Staining did not show the presence of chondrocytes.

4.4.4 Summary

Table 3 provides a summary of negative (-), positive (+), and highly positive staining (++) for ALP, ARS, ORO, SO for the latest respective time points. Highly positive ARS was noted in control and 0.3 g cells while positive ARS was noted for 0.3 g. All other stains were negative.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Control</th>
<th>0.3 g</th>
<th>3.0 g</th>
<th>6.0 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALP (osteogenic)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ARS (osteogenic)</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ORO (adipogenic)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SO (chondrogenic)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6. Cell differentiation of control and vibrated cells.
Chapter 5. Discussion

This study investigated the effect of vibration and cyclic tensile strain on hTERT-hMSCs encapsulated within bioinert PEGDA hydrogels. Cyclic strain, accelerations at 0.3 g, and accelerations at 3.0 g promoted cell viability compared to static controls. In addition, 0.3 g and 3.0 g accelerations promoted osteogenic differentiation demonstrated by positive Alizarin red staining. The lower acceleration, 0.3 g, showed more pronounced staining than all other groups.

In this investigation, cyclic tensile strain of 10% at 1 Hz for 24 hours increased cell proliferation by Day 7 over non-strained controls. Although several studies that investigated the effect of 10% cyclic tensile strain did not analyze cell proliferation [36, 55, 59, 60, 62, 64], a few did. While Koike et al. observed increased cell proliferation, in agreement with the results of this thesis [57], Qiu et al. and Sumanasinghe et al. did not observe an effect on cell proliferation by Day 7 [63, 65]. It is possible that their findings were different due to their use of collagen derived scaffolds.

In prior studies, cells aligned after cyclic tensile strain along the axis perpendicular to tensile strain. [36, 55, 57, 59, 60, 67] Cellular realignment in response to tensile strain was not present here, consistent with the findings of Yang et al., Rathbone et al., and Doroski et al. who also strained cells encapsulated in PEG based hydrogels. [22, 68, 69] It is worthy of note that lacking attachment sites, the cells in this thesis likely were unable to reorient themselves in any way, thus unable to align with any axes of strain. Furthermore, even if attachment sites were provided, hMSCs encapsulated within PEG based hydrogels may be unable to reorient because the scaffold is nondegradable.
The positive Alizarin red staining indicates that 100 Hz vibration at 0.3 g acceleration alone is a powerful osteogenic stimulus. This corresponds to the findings of Kim et al., Zhou et al., Mehta, and Chen et al., who used vibrations of 30, 40, 100 and 800, Hz respectively at 0.3 g and also saw evidence of osteogenesis. [21, 36, 53, 54] The results differ, however, from Lau et al., potentially due to the authors’ use of osteogenic media for both vibrated and control MSCs. [38]

Although the results of Alizarin staining correspond with previous findings by Mehta, [54] who used PEGDA microspheres rather than hydrogel sheets, the onset of differentiation differs. In this thesis, no alkaline phosphatase (ALP) activity was detected on Day 4 while Mehta found positive ALP staining in control, 0.3 g, and 3.0 g hMSCs on Day 4. Sigma recommends ALP 3-5 days after cell culturing to identify the osteogenic commitment of cells. ALP testing was performed on Day 4, potentially missing the period of osteogenic commitment.

Accelerations of 3.0 g were found to mildly promote MSC proliferation and osteogenic differentiation while accelerations of 6.0 g were lethal to MSCs. These results also confirm with the findings of Mehta. [54] Accelerations of these magnitudes are not extensively researched potentially due to the low occurrence of these accelerations in vivo as accelerations of 3.0 g are experienced by fighter pilots and astronauts during liftoff. [71] Oil red O staining and Safranin O staining did not show any evidence that vibration could induce chondrogenesis or adipogenesis. It is the consensus in the literature that vibration does not induce chondrogenesis or adipogenesis. [21, 36, 53, 54]

Taken together, the data demonstrate that hMSCs encapsulated within PEGDA hydrogels, then subjected to cyclic tensile strain or vibration, respond differently than MSCs under
the same load in different scaffolds. Vibration promoted viability and osteogenic differentiation in entrapped hMSCs, similar to the reported effects of vibration on MSCs seeded on 2D scaffolds or TCP at comparable parameters. Strained hMSCs entrapped within PEGDA based scaffolds did not change in orientation, while MSCs strained on natural scaffolds aligned perpendicular to the axis of tension. The goal of this thesis was to isolate the effect of tensile strain from the choice of scaffold and thereby identify the effect of that strain on hMSCs. The effect of tensile strain at magnitudes between 5% and 10% on hMSCs conflict in the literature, as the data widely varies. Results from this study provide a step in determining a sufficient scaffold design and experimental design to investigate hMSCs with a variety of mechanical stimulus. This may provide improved design of synthetic scaffolds for tissue engineering.
Chapter 6. **Conclusions and Future Directions**

In this study, the effects of uniaxial cyclic tensile strain and vibration on PEGDA-encapsulated hTERT-MSCs were investigated. Experiments were designed to isolate the effects of the described mechanical stimulation absent any biochemical or microarchitectural cues. Differences in cell viability and fate were observed in these studies compared to studies that used natural scaffolds, suggesting that the scaffold may have influenced the results rather than the mechanical stimulation. Similarities were also observed, suggesting that the cell response is independent of scaffold selection. Vibration and tensile strain promoted cell proliferation in hMSCs. Vibration promoted osteogenic differentiation of PEGDA entrapped hMSCs, confirming the findings of prior studies that vibrated MSCs seeded within 3D scaffolds. Prior investigations of MSCs that utilized 3D scaffolds demonstrated significantly greater osteogenic differentiation compared to MSCs seeded on monolayers. hMSCs realigned after uniaxial tensile strain except MSCs entrapped within synthetic 3D scaffolds. Further use of PEGDA hydrogels as 3D scaffolds could provide a greater depth of information on hMSC response to isolated mechanical stimuli. The inclusion of a variety of cell adhesion peptides could expand the description of these cells’ response to tension or vibration, specific to a defined peptide sequence. In addition, with the described encapsulation process and mechanical stimulation set up, further ranges of vibration and cyclic tensile strain, as well as ranges of static tensile strain, can be investigated.

Such studies could lead to a catalogue or database containing adhesion peptide sequences, vibration and/or strain parameters with their corresponding effects on MSCs. From there,
synthetic hydrogels could be designed with known and reproducible responses from the cells within. This could lead to cell-laden scaffolds that are “primed” mechanically prior to implantation. Alternately, it could lead to scaffolds that are designed for a known environment within the body. This thesis is a step in that direction.
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