The Role of Physiochemical Cues on MSC Differentiation and Tissue Regeneration in a Cartilage Biomimetic Hydrogel

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The role of physiochemical cues on MSC differentiation and tissue regeneration in a cartilage biomimetic hydrogel

by

Elizabeth A. Aisenbrey

B.S., Montana State University, 2011

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The role of physiochemical cues on MSC differentiation and tissue regeneration in a cartilage biomimetic hydrogel

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline
Abstract

Aisenbrey, Elizabeth A (Ph.D., Chemical and Biological Engineering)

The role of physiochemical cues on MSC differentiation and tissue regeneration in a cartilage biomimetic hydrogel

Thesis directed by Dr. Stephanie J. Bryant

Cartilage defects, whether caused by injury or disease, often lead to further degeneration and ultimately osteoarthritis. Current treatments such as autologous chondrocyte implantation (ACI) and microfracture have shown limited success at repairing and regenerating cartilage tissue, thus an alternative treatment is necessary. Cartilage tissue engineering using scaffolds as a vehicle to deliver cells, biochemical cues, and soluble factors presents a promising strategy to regenerate cartilage tissue. Photopolymerizable poly(ethylene glycol) (PEG) hydrogels that can be modified with different biochemical moieties, degradable and nondegradable peptides, and therapeutic agents are an attractive platform to design and develop scaffolds that can be tailored to enhance cartilage regeneration. Mesenchymal stem cells (MSCs) are a promising cell source for cartilage tissue engineering as they can chondrogenically differentiate. However, MSCs readily undergo hypertrophy in vitro, which can ultimately lead to endochondral ossification and bone formation during chondrogenesis. The interaction between MSCs and their surrounding environment plays a vital role in their differentiation. This begs the question as to whether the unique environment of cartilage is important for MSC chondrogenesis.
The goal of this thesis was to develop a PEG-based hydrogel that enhances chondrogenic differentiation of MSCs encapsulated within. Initial work focused on recapitulating the native cartilage environment within a PEG hydrogel. The incorporation of ECM analogs found in native cartilage tissue in combination with mechanical loading enhanced non-hypertrophic chondrogenesis. These physiochemical cues inhibited hypertrophy via Smad signaling. Scaffold degradation is necessary for macroscopic tissue elaboration, therefore, an enzymatically degradable peptide sequence was incorporated into the PEG hydrogel and allowed for cellular mediated degradation while enhancing cartilaginous matrix deposition. This degradable cartilage mimetic hydrogel was evaluated in vivo and the hydrogel alone showed successful results at repairing the chondral defects. Growth factors were tethered into the cartilage mimetic hydrogel and further enhanced the chondrogenesis of MSCs. Additionally, to maintain the health of the cartilage surrounding the defect, a hybrid hydrogel that incorporated a 3D printed structural support was developed. This thesis demonstrated that an enzymatically degradable cartilage mimetic hydrogel environment has the potential to promote tissue regeneration and chondral defect repair.
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Chapter 1
Introduction and Background

In the United States, over 6 million people visit the hospital each year due to various joint problems in the knee, wrist and ankle. Whether caused by traumatic injury or progressive wear and tear, injuries to the articular cartilage in joints results in significant pain and loss of mobility. If left untreated, further degeneration can occur and ultimately result in osteoarthritis. Osteoarthritis is the major cause of disability in the adult population and is projected to affect 67 million Americans by 2030. There is also a financial burden associated with osteoarthritis, where estimated costs for treatment in the United States are nearly $100 billion per year. Osteoarthritis has no cure, however, there is the potential to prevent it by treating cartilage injury early-on to restore biological and mechanical function.

Articular Cartilage

Articular cartilage is a form of hyaline cartilage that covers the surfaces of long bones within synovial joints. It provides a smooth surface for articulation and facilitates the transmission of loads. It is also avascular, alymphatic, devoid of nerves, and has a low cell density. The cartilage cells, chondrocytes, are sparse and are embedded in a dense extracellular matrix composed of collagen (20% wet weight), proteoglycans (3-10% wet weight), and water (80% wet weight). Collagen II is the most abundant macromolecule in articular cartilage, and represents 90-95% of total collagen. It has a triple helix structure which forms highly crosslinked interconnected fibril matrices that help to stabilize the ECM and are responsible for the shear and tensile strength of cartilage. The second largest group of macromolecules in cartilage are proteoglycans. Aggrecan is
the most abundant proteoglycan in articular cartilage. The origin of its name stems from the aggregan molecules “aggregating” together along hyaluronic acid, a linear, non-sulfated glycosaminoglycan. As many as 100 aggregan molecules can be bound to a single hyaluronic acid molecule. Agragcan consists of a linear protein core and three globular domains. While one of the domains is responsible for binding to the hyaluronic acid, the space between the second and third globular domains binds to the linear, negatively charged sulfated glycosaminoglycan (sGAG) chains, chondroitin sulfate and keratin sulfate. Approximately 300 sGAG chains, predominately chondroitin sulfate, bind to a single agragcan molecules. The fixed negative charge associated with sGAGs interacts with mobile cations in the interstitial tissue fluid, which increases the local osmolarity (350-450mOsm) and draws water into the tissue. The increased swelling and osmotic pressure of the tissue is responsible for the high biomechanical properties of cartilage. The entrapment of the large, highly charged proteoglycans in the collagen matrix provides cartilage its ability to resist physiological strains.

*Cartilage defects, degeneration and osteoarthritis*

Due to the low cellular density and avascular nature, articular cartilage has a limited capacity for self-repair and regeneration. If damage occurs, whether due to injury or disease, it will only worsen without treatment and is at a major risk of developing osteoarthritis. During the progression of osteoarthritis, there are alterations in the molecular composition and organization of the cartilage matrix. This degeneration leads to deterioration in material properties and structural integrity of the articular surface and underlying hyaline cartilage. One of the earliest features of osteoarthritis is degeneration of the articulating surface of the joint as characterized by fibrillation of the articular cartilage. Degeneration of type II collagen, a decrease in the extracellular matrix, and a
loss of proteoglycans are observed. The loss of aggrecan in the ECM imposes an increased load on the collagen fibrils, and leads to subsequent breakdown. Initially, chondrocytes attempt repair by increasing cell proliferation and upregulating synthesis of ECM proteins, however, catabolic activity overwhelms and repair fails. An increase in catabolic activity is evident by enhanced production of degradative proteinases, including the matrix metalloproteinases (MMP) -1, -3, -9, -13, and 14, as well as the aggrecanases ADAMTS5, ADAMTS4, and ADAMTS9. This is also accompanied with an increase in the matrix protein collagen X. The degradation of ECM molecules reduce the articular cartilage resistance to compressive load and further degenerates. As degeneration progresses, the defects penetrate deeper into the cartilage until all cartilage is lost, and bone thickens.

Treatments for chondral defects and osteoarthritis

Although osteoarthritis has no cure, there are currently various forms of treatment in clinical use which range from pharmaceuticals for treating pain to full joint replacement. Many pharmaceutical treatments primarily aim to reduce pain and control inflammation which include anti-inflammatory and pain-relieving medications, steroids, and hyaluronic acid. These simply alleviate the symptoms but do not address treating the cartilage defect. The most dramatic treatment of chondral defects is full joint arthroplasty, where the damaged and disease joint is removed and fully replaced by a prosthetic. This method is typically done in older patients and should be considered as the last resort. Other surgical interventions such as microfracture and autologous chondrocyte implantation seek to repair the articular cartilage, but rarely restore the full function of articular cartilage.
Microfracture is a frequently used technique to repair focal chondral defects. During microfracture, small “micro” fractures are made into the subchondral bone underlying the damaged cartilage to allow for the infiltration of a clot containing blood, bone marrow, mesenchymal stem cells (MSCs), and growth factors that promote healing into the defect site. The marrow-derived MSCs produce a fibrocartilage repair tissue in the defect site with varying amounts of collagen II. This method is best used for small cartilage lesions, and has shown limited success in large, critical sized defects. Patient-reported outcomes are temporarily improved, however, the production of the fibrocartilage is often mechanically inferior and eventually leads to further degeneration. Best results are observed in young patients with low body mass index that have only experienced pain associated with cartilage damage for a short period preoperatively, which are a very small demographic of patients in need of treatment. Although success rates are low, this method is rather simple and does not require cells to be harvested from other sites, and is therefore often used as an initial treatment option.

Autologous chondrocyte implantation (ACI) is a cellular therapy treatment method to improve cartilage repair. ACI has been used for decades to treat focal chondral defects, and outcomes have been similar to that of microfracture. ACI involves harvesting the patient’s own cartilage from a non-loadbearing area, isolating and expanding the chondrocytes, and injecting those cell into the defect site, and covering them with a periosteal patch or a collagen mesh. ACI has been successful in younger, active patients due to the increased chondrocyte anabolic activity, however, older patients have not seen similar results due to the dedifferentiation of the implanted chondrocytes into a fibroblast-like phenotype. A newer generation of ACI that is currently being
investigated is matrix-induced autologous chondrocyte implantation (MACI). MACI utilizes scaffolds to encapsulate the chondrocytes in 3D culture in order to prevent them from dedifferentiating. Scaffolds for MACI include scaffolds composed of collagen I, collagen III, or hyaluronic acid. MACI is the most promising treatment of chondral defects, however, there are limitations to this approach similar to ACI which include donor site morbidity, the need for multiple surgical procedures (chondrocyte harvest, isolation, expansion, and implantation), and the possibility of low cell numbers, and dedifferentiation of the chondrocytes. Therefore, an alternative approach is necessary.

_Tissue engineering approach to treating chondral defects_

Tissue engineering is a promising alternative solution for articular cartilage repair. The tissue engineering approach is attractive because it combines material science, biology, and chemistry to improve tissues and restore joint function. Cartilage tissue engineering employs the use of scaffolds, cells, bioactive and therapeutic factors, and mechanical stimuli to promote regeneration and repair.

_Scaffolds for tissue engineering_

Scaffolds are critical for cartilage repair and regeneration as they act as a carrier to deliver cells, biochemical moieties, and therapeutic factors to the defect site. They provide a 3D environment that supports cell viability, and extracellular matrix deposition for tissue regeneration, while supporting the surrounding cartilage tissue. The ideal scaffold for cartilage tissue engineering is biocompatible, biodegradable, promotes and encourages tissue production, and possesses the appropriate mechanical properties to support tissue growth under physiological mechanical loads.
Hydrogels have received widespread interest as a biomaterial for cartilage engineering\(^{38}\). Hydrogels are hydrophilic, bioinert, three-dimensional (3D) crosslinked networks \(^{39}\). Their high water content, increased swelling, and porosity allows for the diffusion of various solutes and nutrients to the encapsulated cells\(^{40}\). The microenvironment mimics that of the native ECM and creates an appropriate microenvironment to facilitate cell proliferation, adhesion and differentiation\(^{41}\). Hydrogels can be composed of natural or synthetic materials. Natural scaffolds are attractive for tissue regeneration because they are derived from biological tissue, making them both biocompatible and biodegradable\(^{42}\). Some examples of natural scaffolds are collagen\(^{43}\), fibrin\(^{44}\), agarose \(^{45,46}\), alginate\(^{47,48}\), and hyaluronic acid\(^{49}\). The biologically-derived materials have been successful \textit{in vivo} and are advantageous for cartilage repair because they naturally mimic the native ECM and encourage matrix production. However, these materials are often difficult to reproduce, are not mechanically robust, and degradation rates can be difficult to control \(^{50}\). Synthetic hydrogels are an attractive alternative as they are often easily fabricated, highly reproducible, and have a great degree of tunability in properties such as modulus and mesh size. Additionally, tissue analogs and degradable and nondegradable peptides can also be covalently incorporated to enhance the tissue produced by the encapsulated cells. Of the synthetic hydrogel systems, poly(ethylene glycol) (PEG) hydrogels are the most prevalent synthetic material used for cartilage tissue engineering. PEG hydrogels are relatively bioinert and have been shown to support cartilage tissue production of chondrocytes as well as MSCs\(^{51,52}\).

PEG monomers (and other synthetic hydrogels monomers) can be crosslinked with a variety of chemical modifications, such as acrylate, methacrylates and norbornenes
which contain a vinyl group that is amenable to free-radical polymerization\textsuperscript{53,54}. Photopolymerization, a method that uses light to initiate free-radical polymerization, is promising for cartilage tissue engineering as it allows for spatial and temporal control of the polymerization reaction with relatively fast reaction times and limited cellular damage. Photopolymerization can also occur at physiological temperature and pH, making it usable for \textit{in situ} polymerization\textsuperscript{53–55}. Both chain-growth and step-growth polymerization mechanisms have been used with PEG-based monomers. The photopolymerization of PEG-acrylate and PEG-dimethacrylate hydrogels through chain-growth polymerization form relatively heterogeneous networks and are suitable for cell encapsulation, and tissue production\textsuperscript{56–58}. The step-growth polymerization of PEG-based thiol and norbornene monomers form more homogenous network structures. These PEG thiol-ene hydrogels have shown successful MSC and chondrocyte cartilaginous tissue production\textsuperscript{59–61}. Both of these PEG-based scaffolds will be investigated in this thesis. The combination photopolymerization with the injectable PEG hydrogel, with and without suspended cells, offers a simple technique to deliver the hydrogel and cells directly into the chondral defect for cartilage repair.

In order for the scaffold to be effective for cartilage tissue engineering long-term, scaffold degradation is necessary. Non-degradable hydrogels restrict matrix deposition to the pericellular region, limiting the elaboration of macroscopic tissue production\textsuperscript{56}. In order to maintain overall structural integrity of the scaffold to allow for macroscopic ECM deposition, the degradation rate must match the rate of matrix production\textsuperscript{42}. Multiple methods of degradation have been investigated. Hydrolytically degradable hydrogels, which contain hydrolytically degradable esters such as poly(lactic acid) (PLA) have shown
promising results to enhance extracellular matrix production by encapsulated cells\textsuperscript{42,52}. The rate of degradation of hydrolytically degradable hydrogels can be finely tuned and controlled by manipulating the ratio of PLA to PEG\textsuperscript{56,62,63}. These hydrogels undergo bulk degradation which results in a significant drop in compressive modulus, increase in swelling and loss of synthesized matrix constructs, making it undesirable for chondral defect treatments\textsuperscript{58,64}.

As an alternative, cellular-mediated degradation mechanisms are promising because they permit spatial and temporal control over degradation by the encapsulated cells. During cellular-mediated degradation, enzymes that are secreted from the encapsulated cells can diffuse and react with the crosslinks of the hydrogel scaffold, often resulting in local degradation. Peptide crosslinks that are sensitive to cell secreted enzymes can easily be incorporated into PEG hydrogels. Matrix metalloproteinase (MMPs) sensitive peptides are of interest, as they have shown increased collagen II and aggregan matrix deposition by encapsulated MSCs and chondrocytes\textsuperscript{61,65–68}. There are over 25 MMPs that are secreted by cells to regulate biological processes such as differentiation, proliferation, and maturation\textsuperscript{69}. Different MMPs are active throughout different developmental stages, and they have a range of affinities for different peptide sequences. Although some MMPs are markers of hypertrophy or disease (ie. MMP-13) others are involved in matrix remodeling and tissue homeostasis (MMP-2, MMP-7)\textsuperscript{70–73}. The temporal activation of MMPs make MMP-sensitive peptides a promising candidate for cellular mediated degradation. This method of degradation will be investigated in this thesis.
Cell Source-Chondrocytes

Chondrocytes have been used clinically to repair chondral defects with promising results\textsuperscript{74}. However, the use of chondrocytes \textit{in vivo} is limited by the low number of cells harvested from non-load bearing areas of the joints\textsuperscript{75}, their low expansion capacity and their tendency to dedifferentiate into fibroblast-like cells\textsuperscript{76–78}. The harvesting of chondrocytes from a donor site can result in morbidity and tissue degeneration\textsuperscript{79}. This technique also requires multiple surgeries, resulting in pain and discomfort, and is inconvenient for the patient. The majority of patients in need of a cartilage repair are often older adults, whose chondrocytes have reduced proliferation and matrix production potential\textsuperscript{80,81}. Additionally, the patients are often already experiencing signs of osteoarthritis and the aged and diseased phenotype of the cells can inhibit their ability to form functional cartilage tissue\textsuperscript{82}.

Cell Source-Mesenchymal stem cells

Bone marrow-derived mesenchymal stem cells (MSCs) are an attractive alternative to chondrocytes for cartilage tissue repair. MSCs are multipotent, meaning they can differentiate into many tissue types including fat (adipogenesis), bone (osteogenesis) and cartilage (chondrogenesis)\textsuperscript{83}. Unlike chondrocytes, MSCs can be harvested from the patient in a less invasive manner which does not require an additional surgery\textsuperscript{84}. MSCs obtained from the bone marrow can be passaged multiple times and be expanded to a large number of cells while maintaining their chondrogenic potential\textsuperscript{83,84}. This is advantageous because cartilage tissue engineering approaches often require a large cell concentration to achieve macroscopic tissue deposition\textsuperscript{85}. The use of MSCs as a cell-based therapy for cartilage repair has shown some success clinically, where
injections of autologous MSCs into a diseased knee joint have resulted in decreased pain and increased joint mobility\textsuperscript{86,87}.

In addition to bone marrow MSCs, MSCs can also be derived from other cells including embryonic stem cells and induced pluripotent stem cells. Embryonic mesenchymal progenitor cells are attractive because they can be expanded multiple times and maintain their differentiation potential\textsuperscript{88,89}. However, the ethical and political controversy of embryonic stem cells makes their future in clinical applications unknown\textsuperscript{90}. Additionally, embryonic stem cells used for cell therapy are often allogenic which can lead to an inflammatory response and rejection of the cells\textsuperscript{90}. Induced pluripotent stem cells (IPSCs) are an alternative cell source that are phenotypically similar to embryonic stem cells but lack the controversy associated with them\textsuperscript{91}. IPSCs are embryonic-like stem cells that are derived from reprogrammed cells such as skin fibroblasts. They have similar differentiation capabilities as ESCs, and maintain their pluripotency after many passages\textsuperscript{91}. IPSCs may be advantageous over BM-MSCs because unlike BM-MSCs, proliferation and differentiation potential is maintained regardless of patient’s age or health\textsuperscript{92}. For cartilage tissue engineering, IPSCs are first differentiated into mesenchymal progenitor cells and are then differentiated into cartilage tissue similar to BM-MSCs. The investigation of BM-MSCs and IPSCs for cartilage tissue engineering applications is evaluated in this thesis.

\textit{The Hypertrophic phenotype}

While promising results have been reported from MSC-based cellular treatments of chondral defects, the tissue formed is often fibrous and lacks the biological and mechanical properties of articular cartilage needed to restore joint function\textsuperscript{3}. This is due
to the inability to control the differentiation of MSCs. MSCs readily undergo terminal differentiation resulting in a hypertrophic phenotype that can progress into endochondral ossification, mineralization and bone formation$^{93-95}$. Hypertrophy is characterized by an increase in cell volume, and the expression of the hypertrophic markers Runx2, collagen X, alkaline phosphatase and matrix metalloproteinase 13 (MMP13)$^{72,96}$. Similar to bone development in vivo by endochondral ossification, collagen II in the extracellular matrix of the cartilage tissue begins to be replaced by collagen X which can interact with soluble calcium, and results in the calcification and mineralization of cartilage tissue, and potentially turn into bone$^{97,98}$. Hypertrophic cartilage is both mechanical and biologically inferior to healthy hyaline cartilage, and therefore would not be a promising treatment for chondral defects$^{93}$.

**Controlling the differentiation of MSCs**

Strategies to control MSC differentiation to maintain a hyaline like phenotype and inhibit hypertrophy have been investigated extensively. MSC interactions with the surrounding microenvironment plays a critical role in the terminal differentiation process through different mechanisms such as cell-cell and cell-matrix interactions, growth factors, and mechanical stimulation. Controlling these interactions in a tissue engineering scaffold may be necessary to promote non-hypertrophic chondrogenesis of MSCs.

One method of controlling MSC differentiation is by recapitulating the native cartilage environment. Biomimetic scaffolds used for stem cell differentiation contain some aspects that resemble the native tissue. The premise of using biomimetic materials is that cues found in native tissue may provide the necessary stimuli to control the differentiation of the encapsulated MSCs. One way to recapitulate the native environment
is to incorporate moieties found in the extracellular matrix of cartilage tissue into a hydrogel scaffold. Cartilage tissue is water swollen, highly negatively charged, and the chondrocytes embedded within have a rounded morphology. PEG-based hydrogels work as the perfect platform as a biomimetic material for cartilage tissue as they provide the encapsulated cells with the rounded morphology, have a high water content, and negative charge and other ECM analogs can easily be incorporated in the matrix. For instance, the negatively charged glycosaminoglycan, chondroitin sulfate (ChS), has been incorporated into a PEG hydrogel and results showed an upregulation of cartilage specific genes and matrix production. Other ECM analogs such as cell adhesion peptides and hyaluronic acid have also been incorporated into synthetic hydrogels and, similar to the ChS, have led to an increase in chondrogenesis of MSCs.

Cartilage tissue is highly mechanically dynamic and mechanical factors play a vital role in the development of articular cartilage in vivo. In this regard, recapitulating the mechanical stimulation in tissue engineered constructs might also have a crucial impact on MSC chondrogenesis. Chondrocytes and chondroprogenitor cells have been found to respond to a variety of mechanical stimuli such as deformation, fluid flow, shear stress, hydrostatic pressure and osmotic pressure. Similarly, mechanical stimulation can directly influence the differentiation of MSCs. Unconfined dynamic compression has been one of the most utilized model systems of mechanical stimulation in cartilage tissue engineering. Studies have found that mechanical loading can suppress hypertrophic markers such as collagen X, MMP13, and alkaline phosphatase. Although mechanical loading is a promising mode of controlling the differentiation of MSCs, the affect mechanical loading has on MSC chondrogenesis has varied. The
success of mechanical loading on chondrogenesis is dependent on the presence of growth factors, the predifferentiation culturing period and loading parameters, and type of mechanical stimulation imparted by the bioreactors.

In addition to recapitulating the extracellular matrix and mechanically dynamic environment, soluble factors may have the most significant effect on cell differentiation. The range of soluble factors that have been investigated include the growth factors transforming growth factor beta (TGFβs), bone morphogenic proteins (BMPs), insulin like growth factor (IGF), and fibroblast growth factor (FGF). These soluble cues, especially TGFβs and BMPs, have been found to be the most potent inducers of chondrogenesis. Studies suggest that there is a relationship between mechanical loading and soluble growth factors and their effectiveness at promoting chondrogenesis of MSCs. Mechanical stimulation can enhance growth factor-induced MSC differentiation and tissue production, and vice versa, growth factors can enhance chondrogenesis induced by mechanical loading. For instance, when combined with dynamic compression, exposure to TGFβ resulted in a much greater increase than dynamic compression alone. The combination of soluble cues, with mechanical loading, in a biomimetic hydrogel construct may be the necessary cues to enhance non-hypertrophic chondrogenesis.

**MSC cellular signaling during chondrogenesis and hypertrophy**

As previously mentioned, the differentiation of MSCs is complex and there are no known “master” genes that direct the differentiation down specific lineages. For chondrogenesis, in vitro differentiation is similar to in vivo cartilage development. TGF-β BMP, growth and differentiation factor (GDF) and Wnt ligands have been identified as differentiation promotors of MSCs. TGFβs and BMPs bind to their receptors and signal
through the intracellular mediators Smad proteins and mitogen-activated protein kinase (MAPK) which induces a cascade of signaling mechanisms. Smad proteins are expressed in chondrocytes during the entire process of chondrogenesis. The activation of Smad2 and Smad3 by phosphorylation is necessary for chondrogenesis to occur and is required for articular chondrocyte homeostasis, and has been found to block terminal differentiation. The activation of Smad1, 5 and 8 has also been shown to be necessary for chondrogenesis during early stages of differentiation, however, in later stages it has been associated with hypertrophy as shown by enhanced runx2 expression and collagen X production. Other signaling mechanisms such as Notch, RhoA/Rock, and Wnt/β-catenin play a significant role in MSC differentiation, however, this thesis specifically focuses primarily on Smad signaling and its involvement in chondrogenesis and hypertrophy of MSCs.

Current limitations in cartilage tissue engineering

MSCs and PEG hydrogels hold promise to be an effective treatment for chondral defects, however, there are several other limitations that may hinder their success that have not yet been addressed. One major challenge with using hydrogels for cartilage tissue engineering is the mismatch in material properties. The physiological forces experienced by cartilage in a joint ranges between 1-2MPa, with peaks reaching 3-4 MPa during walking and as high as 7 MPa during running. The compressive modulus of non-degradable hydrogels used for cartilage tissue engineering range between 5-100 kPa and degradable hydrogels that would be used for cartilage repair are even softer. Using a soft hydrogel for cartilage repair in vivo may have limited success due to the inability of the hydrogel to be able to withstand such high forces. Not only does this reduce the efficacy of the hydrogels ability to regenerate cartilage tissue, the
mismatch in material properties can also lead to further degeneration of the cartilage tissue adjacent to the site of injury\textsuperscript{131,132}. Techniques such as 3D printing and hybrid scaffolds have the potential to eliminate this issue and will be investigated in this thesis.

**Thesis Approach**

MSCs are a promising cell source for the treatment of chondral defects due to their proliferation capabilities and chondrogenic differentiation potential. When combined with a tissue engineered scaffold, MSC chondrogenesis can be controlled and matrix deposition by the encapsulated cells can be encouraged. The ultimate goal of this thesis is to develop a degradable, biomimetic PEG hydrogel environment with mechanical stimulation that can support chondrogenesis of MSCs to be used as a treatment for chondral defect \textit{in vivo}. The extracellular cues effect on MSC differentiation and the corresponding cellular signaling mechanisms that control the differentiation will be investigated extensively. Multiple factors to take the biomimetic hydrogel developed \textit{in vitro} to an \textit{in vivo} treatment for chondral defects will be considered and evaluated.

Chapter 2 outlines the specific objectives that are addressed in this thesis. The first objective, explored in chapters 3-5, is to develop a degradable cartilage mimetic PEG hydrogel that enhances non-hypertrophic chondrogenesis of encapsulated MSCs by recapitulating the native cartilage environment. The incorporation of ECM analogs, mechanical stimulation, and an enzymatically degradable crosslinker were investigated. Objective 2, chapters 6 and 7, explore cellular signaling mechanisms that may be involved in non-hypertrophic condrogeinis of MSCs cultured in a cartilage mimetic hydrogel under unconfined dynamic compression. The effect of mechanical stimulation as well as soluble growth factors were investigated for bone marrow-derived MSCs as well as induced
pluripotent mesenchymal progenitor cells (IPS-MPs). The third and final objective is investigated in chapters 8-12. The goal of this objective was to investigate the effectiveness of the cartilage mimetic hydrogel as a treatment for chondral defects in vivo. An initial in vivo assessment of the hydrogel in a rabbit animal model led to modification of the cartilage mimetic hydrogel for future studies in a rat and horse model. Additionally, necessity for a hydrogel scaffold that has mechanical properties that match the surrounding cartilage tissue is investigated and 3D printed techniques are used to develop a hybrid hydrogel approach. Chapter 13 summarizes the overall conclusions of the work presented in this thesis and provides future recommendations to further improve hydrogels for cartilage tissue engineering.

References


Chapter 2
Objectives

Cartilage tissue lacks the regenerative capacity to repair damaged tissue, leaving the necessity for an alternative tissue engineering approach. A cell-based therapy to repair cartilage using bone marrow-derived mesenchymal stem cells (MSCs) is a promising treatment of cartilage defects. MSCs are advantageous over other cell types such as chondrocytes because they can be expanded to large numbers and maintain their multipotency and chondrogenic differentiation capacity\(^1\)\(^-\)\(^3\). Although promising, the ability of MSCs to regenerate hyaline cartilage \textit{in vivo} has been limited\(^9\). One challenge faced when using MSCs for cartilage tissue engineering is they readily undergo hypertrophy, which can ultimately result in endochondral ossification\(^4\). This hypertrophic phenotype is biologically and mechanically inferior to native cartilage, and can lead to mineralization and eventually be replaced by new bone when implanted \textit{in vivo}\(^4\). However, MSCs interaction with their environment can greatly influence their differentiation\(^5\)\(^-\)\(^7\). The cell-matrix interaction are advantageous to biomaterial design and can be used to develop an environment that can reduce hypertrophy while promoting chondrogenesis.

Poly(ethylene glycol) (PEG) hydrogels are an attractive platform for cartilage tissue engineering because they are hydrophilic, biointer and tunable. Specifically, the 3D scaffold material can be used as a vehicle to deliver MSCs, biochemical cues, and therapeutic agents to the defect site, be polymerized \textit{in situ}, and work as a microenvironment that supports a stable chondrogenic phenotype\(^8\)\(^-\)\(^10\). By using PEG-
based hydrogels, we are able to recapitulate the native cartilage environment by incorporating biomimetic moieties and cellular-mediated degradable crosslinks\textsuperscript{6,8,11–16}. The biomimetic cues can be incorporated and assessed to determine a hydrogel formulation that promotes chondrogenesis of the encapsulated MSCs, whereas, the degradable scaffolds are essential for macroscopic matrix deposition of the encapsulated MSCs.

The research presented in this thesis tests the overall hypothesis that a degradable PEG hydrogel that biochemically and mechanically recapitulates the native cartilage environment will promote chondrogenic differentiation of encapsulated MSCs and can be used as a treatment of focal chondral defects. Our approach is to create an injectable, photopolymerizable PEG hydrogel that can be tailored to allow for chondrogenic differentiation of encapsulated MSCs by the delivery of biochemical moieties, biomechanical cues, therapeutic agents, as well as cellular mediated degradation mechanisms. The ultimate goal of the project is to develop a hydrogel that can be used clinically and photopolymerized \textit{in situ} for a cartilage tissue engineering approach for the treatment of cartilage defects. The following research objectives were proposed:

2.1 Objective 1

The first objective of this thesis was to develop a degradable cartilage biomimetic PEG hydrogel by incorporating chemical and mechanical cues that enhance non-hypertrophic chondrogenesis of encapsulated MSCs. The interaction between MSCs and their environment plays an important role in their differentiation. This interaction raises the question as to what effect the unique environment of cartilage tissue has on MSCs. It was hypothesized that recapitulating the native environment would enhance
chondrogenesis of encapsulated MSCs. Biochemical moieties, mechanical stimulation, and cellular mediated degradation were investigated.

2.1.1 Objective 1a
The first sub-objective was to develop a cartilage biomimetic hydrogel with the ECM analogs, ChS and RGD, that enhance chondrogenesis of human MSCs (hMSCs). Native cartilage tissue is composed of chondrocytes embedded within a dense ECM consisting primarily of collagen II, aggrecan, and glycosaminoglycans (GAGs). The most abundant GAG is ChS which is negatively charged and responsible for the high extracellular osmolarity, high water content, and high load-bearing capabilities in tensile and compressive strength of cartilage tissue. The cell adhesion peptide RGD is found in many ECM proteins including fibronectin which is involved in early stages of chondrogenic differentiation. The effect of incorporating the ECM analogs in a PEG hydrogel at different concentrations on hMSC chondrogenesis was evaluated.

2.1.2 Objective 1b
The second sub-objective was to investigate the effect of unconfined dynamic mechanical loading on the differentiation of hMSCs in the cartilage biomimetic hydrogel. Results from Objective 1a showed that the presence of ECM analogs enhanced chondrogenesis, however the cartilage-like hydrogel environment did not provide cues to inhibit terminal differentiation and hypertrophy of the encapsulated MSCs. Mechanical stimulation is critical for cartilage homeostasis, however, studies have shown conflicting results on its effectiveness to promote chondrogenic differentiation of hMSCs. It was hypothesized that mechanical loading would improve non-hypertrophic chondrogenesis. Unconfined dynamic compression at different loading regimes was assessed for its effect
on chondrogenic differentiation of encapsulated hMSCs in a cartilage mimetic PEG hydrogel.

2.1.3 Objective 1c
Damage to joint tissue often extends from the articular cartilage to the subchondral bone, therefore, strategies that treat the entire osteochondral (bone-cartilage) interface are necessary. The third sub-objective was to design and develop a multilayered hydrogel environment that has the necessary biochemical and mechanical cues to control the differentiation of the encapsulated MSCs into their different tissue types: articular cartilage, calcified cartilage, and subchondral bone. This was done by creating a gradient of biochemical cues, specifically RGD and ChS, as well as a gradient of strain profiles throughout the hydrogel.

2.1.4 Objective 1d
The last sub-objective was to develop a chondrogenic-specific enzymatically degradable cartilage mimetic hydrogel to allow for ECM production and cellular mediated degradation of the synthetic hydrogel material. Objectives 1a and 1b developed a cartilage mimetic environment that promotes chondrogenesis of encapsulated MSCs, however, due to the non-degradable scaffold, large ECM molecules were prevented from diffusing and were restricted to matrix deposition in the pericellular space. As MSCs differentiate, they release matrix metalloproteinases (MMPs), specifically MMP7 to remodel the existing ECM, mobilize growth factors that enhance chondrogenesis, and facilitate collagen II production. The goal of this objective was to investigate the use of an MMP7-sensitive peptide crosslinker in a cartilage mimetic hydrogel and its effect on ECM production and matrix deposition of the encapsulated hMSCs.
2.2 Objective 2
The results from Objective 1 indicated that mechanical loading inhibits hypertrophy of hMSCs in a cartilage mimetic hydrogel in a strain rate dependent manner. These results are promising due to the inevitable terminal hypertrophic differentiation of chondrogenically differentiating MSCs. The chondrogenic maturation process is dependent on many factors such as transcription factors, growth factors, cell-cell and cell-matrix interaction and mechanotransduction. However, these interactions and the effects of mechanical loading are incredibly complex and multiple signaling pathways may be involved. The goal of Objective 2 was to investigate the signaling pathways underlying the biochemical and mechanical stimulation of encapsulated MSC differentiation.

2.2.1 Objective 2a.
The first sub-objective was to determine the affect mechanical loading has on Smad signaling of hMSCs encapsulated in the cartilage mimetic hydrogel. Smad signaling plays a critical role in chondrogenesis and hypertrophy of MSCs. The phosphorylation of Smad2/3 is vital for chondrogenesis and the production of collagen II, while Smad1/5/8 has been connected to collagen X expression and hypertrophy. It was hypothesized that dynamic loading inhibits hypertrophy via Smad signaling and that the inhibition is particular to the physiochemical cues exhibited by the cartilage mimetic hydrogel. The specific effects of the biochemical cues, chondroitin sulfate and RGD, were evaluated to decouple their individual roles on chondrogenesis. Additionally, mitogen activated protein kinase (MAPK) signaling pathways are also imperative for chondrogenesis to occur, and play a critical role in reacting to extracellular stimuli such as soluble growth factors, mechanical loading and osmolarity. As such, this signaling pathway was also investigated.
2.2.2 Objective 2b
The second sub-objective was to investigate the effect of growth factors and mechanical loading on MSC differentiation and Smad signaling. The results from Objective 2a showed that mechanical loading had an inhibitory effect on the pSmad1/5/8 signaling of bone marrow derived MSCs. The goal of this objective was to investigate the effect of mechanical loading on the chondrogenesis and Smad signaling of induced pluripotent mesenchymal progenitor cells (IPS-MPs). IPS-MPs are an attractive cell source for cartilage tissue engineering applications because they have unlimited proliferation potential independent of patient age and health. Additionally, the effect of soluble growth factors, TGFβ3 and BMP2, on IPS-MP differentiation was also evaluated. The phosphorylation of Smads are typically initiated through TGFβ and BMP receptors. It was hypothesized that mechanical loading and soluble growth factors will synergistically effect the differentiation of IPS-MPs through Smad signaling.

2.3 Objective 3
The third and final objective of this thesis was to investigate the cartilage biomimetic hydrogel as a treatment for chondral defects. Although there has been substantial progress in the field of biomaterials and tissue engineering over the past decade, an ideal treatment for chondral defects has yet to be developed. This is expected when translating biomaterials that are developed in vitro into a clinically relevant biomaterial that can be implanted in vivo and regenerate and repair cartilage tissue that matches both the biological and mechanical function of joint tissue. This objective investigates the use of the cartilage mimetic hydrogel developed in the previous objectives in animal models in vivo and ex vivo. Modifications were made to the cartilage mimetic hydrogel in order to deliver the necessary cues in vivo.
2.3.1 Objective 3a
The first sub-objective was to investigate the use of the cartilage biomimetic hydrogel with and without MSCs in a rabbit animal model. The cartilage mimetic hydrogel was injected and polymerized in situ chondral defects in adult New Zealand white rabbits. Three groups were tested: untreated empty defects, defects treated with hydrogel alone, and defects treated with hydrogel + MSCs. It was hypothesized that the cartilage mimetic hydrogel with and without MSCs would enhance cartilage repair when compared to the untreated empty defects.

2.3.2 Objective 3b
In the rabbit in vivo study in Objective 3a, cartilage regeneration was reduced in defects treated with hydrogel + MSCs. The contradictions between the in vitro and in vivo chondrogenesis of the encapsulated MSCs may be due to the lack of the growth factor TGFβ3 which was added to the media to promote chondrogenesis in vitro. The goal of the second sub-objective was to develop a cartilage biomimetic hydrogel with covalently tethered TGFβ3 to enhance chondrogenesis of encapsulated MSCs cultured with soluble growth factors. This TGFβ3-tethered cartilage mimetic hydrogel was evaluated for its ability to promote chondrogenesis of rat MSCs.

2.3.3 Objective 3c
Although the initial in vivo study in the rabbit animal model showed promising results, the rabbit animal model is not the best option for recapitulating the mechanics and biological function of human joints. Equine is a better animal model for cartilage degeneration and repair using biomaterials. In this sub-objective, the cartilage mimetic hydrogel with tethered TGF β3 was assessed for its ability to regenerate and repair cartilage tissue defects. Prior to moving in vivo, an extensive in vitro study was completed to investigate
equine MSC chondrogenesis from three different cell donors in a degradable cartilage mimetic hydrogel with tethered TGF β3 under free swelling and mechanical loading.

2.3.4 Objective 3d
Hydrogels used for cartilage tissue engineering and cell encapsulation are often soft in comparison to the native cartilage tissue. This mismatch in stiffness can lead to severe degeneration of the cartilage adjacent to the defect. The goal of the fourth sub-objective was to investigate the effect that hydrogel stiffness and physiological loading has on health of the surrounding cartilage tissue. Focal chondral defects were created in ex vivo porcine osteochondral explants and were either left empty or treated with a soft (~50 kPa) or stiff (~1MPa) acellular hydrogel and were cultured under static culture or subjected to physiological dynamic loading. The degeneration of the surrounding cartilage was evaluated for apoptosis, glycosaminoglycan retention, and degeneration of the ECM.

2.3.5 Objective 3e
The fifth and final sub-objective was to develop a stereolithography-based 3D printed hybrid hydrogel for cartilage defect repair. Following the previous sub-objective, the mechanical integrity of hydrogels used to treat cartilage defects is important when considering the effects the defect can have on the adjacent cartilage tissue. However, scaffold degradation is necessary for cartilaginous matrix deposition by the encapsulated cells. To combat this, a hybrid hydrogel that consists of a 3D printed, stable structural component that is infilled with a soft, cell-laden, degradable hydrogel component has the potential to maintain structural integrity of the construct while still allowing for cartilage tissue production. The development of the hybrid scaffold, as well as its application to be implanted in situ were investigated.
2.4 References


3.1 Abstract

Three dimensional hydrogels are a promising vehicle for delivery of adult human bone-marrow derived mesenchymal stem cells (hMSCs) for cartilage tissue engineering. One of the challenges with using this cell type is the default pathway is terminal differentiation, a hypertrophic phenotype and precursor to endochondral ossification. We hypothesized that a synthetic hydrogel consisting of extracellular matrix (ECM) analogs derived from cartilage when combined with dynamic loading provides physiochemical cues for achieving a stable chondrogenic phenotype. Hydrogels were formed from crosslinked poly(ethylene glycol) as the base chemistry and to which (meth)acrylate functionalized ECM analogs of RGD (cell adhesion peptide) and chondroitin sulfate (ChS, a negatively charged glycosaminoglycan) were introduced. Bone-marrow derived hMSCs from three donors were encapsulated in the hydrogels and cultured under free swelling conditions or under dynamic compressive loading with 2.5 ng/ml TGF-β3. hMSC differentiation was assessed by quantitative PCR and immunohistochemistry. Nine hydrogel formulations were initially screened containing 0, 0.1 or 1 mM RGD and 0, 1 or 2 wt% ChS. After 21 days, the 1% ChS and 0.1 mM RGD hydrogel had the highest collagen II gene expression, but this was accompanied by high collagen X gene expression. At the protein level, collagen II was detected in all formulations with ECM analogs, but minimally detectable in the hydrogel without ECM analogs. Collagen X
protein was present in all formulations. The 0.1 mM RGD and 1% ChS formulation was selected and subjected to five loading regimes: no loading, 5% strain 0.3 Hz (1.5%/s), 10% strain 0.3 Hz (3%/s), 5% strain 1 Hz (5%/s), and 10% strain 1 Hz (10%/s). After 21 days, ~70-90% of cells stained positive for collagen II protein regardless of the culture condition. On the contrary, only ~20-30% of cells stained positive for collagen X protein under 3 and 5%/s loading conditions, which was accompanied by minimal staining for RunX2. The other culture conditions had more cells staining positive for collagen X (40-60%) and was accompanied by positive staining for RunX2. In summary, a cartilage-like biomimetic hydrogel supports chondrogenesis of hMSCs, but dynamic loading only under select strain rates is able to inhibit hypertrophy.

3.2 Introduction

Bone marrow-derived mesenchymal stem cells (MSCs) are a promising autologous cell source for cartilage tissue engineering, offering several advantages over cartilage cells (i.e., chondrocytes). For example, MSCs can be expanded to achieve a large number of cells, which are needed for implantation, but without losing their multipotency and ability to differentiate down the chondrogenic lineage\(^1\). MSCs offer a one-step surgical procedure instead of two-steps, which is required in autologous chondrocyte implantation. Although promising, the ability of MSCs to regenerate hyaline cartilage \textit{in vivo} has been limited\(^2,3\). This finding is in part attributed to the fact that the default pathway in chondrogenically differentiating MSCs is terminal differentiation. This differentiation fate leads to a hypertrophic phenotype that is a precursor to endochondral ossification, where cartilage becomes hypertrophic, mineralizes, and eventually is replaced with new bone\(^4-6\). With the ability to deliver MSCs within a 3D scaffolding
material, such as a hydrogel, the opportunity arises to design 3D environments that support a stable chondrogenic phenotype. However, the cues that lead to a stable phenotype remain to be elucidated.

The interaction of MSCs with their environment is known to play an important role in differentiation\textsuperscript{7,8}. This raises the question as to whether the unique environment of cartilage is important for MSC chondrogenesis. In cartilage, chondrocytes are embedded within a dense extracellular matrix (ECM) comprised primarily of collagen type II and highly negatively charged aggrecan macromolecules. During physical activity, cartilage experiences large compressive strains that are transmitted through the tissue and converted into local physiochemical cues. These cues include deformation of cells, integrins acting as mechanoreceptors, and dynamic flow of ions and water that alters the local osmolarity and leads to fluid-induced shear stress\textsuperscript{9,10}. These effects, among others, have been shown to significantly affect ECM synthesis by chondrocytes\textsuperscript{11,12} and cartilage homeostasis; and therefore may also play a role in chondrogenesis of MSCs. Several studies have explored the individual effects of different cartilage-related ECM analogs and of dynamic loading on chondrogenesis of MSCs when encapsulated in hydrogels, but the results have been mixed.

The cell adhesion moiety RGD, which is found in many ECM proteins including fibronectin and collagens, has been investigated in MSC chondrogenesis. Fibronectin is involved in the early stages of chondrogenic differentiation, but is eventually down-regulated with maturation\textsuperscript{13}. The tethering of a cell adhesion peptide like RGD into a hydrogel provides a mechanism by which cells may interact with the hydrogel and this interaction has been shown to improve MSC viability within synthetic hydrogels. RGD,
however, has also been shown to inhibit MSC chondrogenesis\textsuperscript{14}, but at concentrations that are sufficiently high to affect the phenotype of fully differentiated chondrocytes\textsuperscript{15}. On the contrary, incorporating RGD either through degradable linkers\textsuperscript{16} or at low concentrations\textsuperscript{17} has been shown to support MSC chondrogenesis. These findings suggest that RGD supports MSC chondrogenesis, but depends on the contextual presentation of RGD.

Another ECM analog that has been investigated in MSC chondrogenesis is chondroitin sulfate (ChS), the main sulfated glycosaminoglycan found in aggrecan. Chondroitin sulfate can readily be functionalized enabling its incorporation into a hydrogel via covalent crosslinks\textsuperscript{18}. Similar to native cartilage, ChS introduces a high density of fixed negative charges into a hydrogel, which elevates the local osmolarity\textsuperscript{19}. Studies with fully differentiated chondrocytes have shown that osmolarities within the range of native cartilage support ECM synthesis, while lower or higher osmolarities, inhibit ECM synthesis\textsuperscript{20,21}. Similarly, high osmolarities have been shown to inhibit chondrogenesis of MSCs\textsuperscript{22–24}. Nonetheless, the incorporation of ChS into a synthetic hydrogel supports chondrogenesis by expression of cartilage-related genes and proteins in human MSCs\textsuperscript{25,26} and has been shown to downregulate hypertrophic genes in goat MSCs\textsuperscript{7}. However, concentration effects of ChS in a synthetic hydrogel have been shown to influence ECM synthesis of fully differentiated chondrocytes\textsuperscript{27} and therefore may also impact MSC chondrogenesis, but this effect has yet to be studied.

Dynamic mechanical compression applied to MSCs embedded in hydrogels has resulted in highly variable responses, enhancing chondrogenesis in some studies\textsuperscript{28,29}, while inhibiting chondrogenesis in other studies\textsuperscript{30,31}. A number of factors may contribute
to the variable responses in MSCs including hydrogel formulation, concentration of the
ECM analog, and loading parameters. For fully differentiated chondrocytes, the
combination of ECM analogs and dynamic compression has a synergistic and positive
effect on ECM synthesis, when compared to either cue alone, but the improved response
is dependent on the concentration of the ECM analog\textsuperscript{15,19,27}. Therefore, additional studies
are needed to better understand the impact of the physicochemical environment that
combines ECM analogs with dynamic compression on MSC chondrogenesis.

As cells in native cartilage experience multiple cues at any given time, a synthetic
hydrogel that captures multiple physicochemical cues may be provide a more biomimetic
environment for MSC chondrogenesis. Thus in this work, we hypothesized that a
biomimetic hydrogel containing RGD and chondroitin sulfate in combination with dynamic
mechanical compression enhances chondrogenesis of human MSCs (hMSCs), but in a
manner that depends on the magnitude of the cues. We employed a synthetic hydrogel
made from crosslinked poly(ethylene glycol), which serves as the base hydrogel to which
RGD and chondroitin sulfate are systemically incorporated. In this first part of this study,
RGD and chondroitin sulfate were incorporated into a PEG hydrogel over a concentration
range that was previously determined to be suitable for fully differentiated chondrocytes\textsuperscript{27}
and then screened for hMSC chondrogenesis and hypertrophy in the absence of loading.
In the second part of this study, we selected one combination of the ECM analogs and
investigated several different dynamic loading regimes that fall within the physiological
range for their impact on hMSC chondrogenesis and hypertrophy. Overall, findings from
this study support the hypothesis that the introduction of ECM analogs in the form of RGD
and chondroitin sulfate, improves hMSC chondrogenesis, but that dynamic loading under
certain loading regimes is necessary to preserve a stable chondrogenic phenotype and inhibit hypertrophy.

3.3 Materials and Methods

3.3.1 Macromer Synthesis

Poly(ethylene glycol) dimethacrylate (PEGDM) was synthesized via microwave methacrylation by reacting poly(ethylene glycol) (4600 g mol\(^{-1}\)) (Sigma) with methacrylic anhydride in the presence of hydroquinone (Sigma). The PEGDM product was recovered by dissolution in methylene chloride followed by precipitation in ethyl ether, filtration and drying. The degree of methacrylation was determined to be 94\% by \(^1\)HNMR (Varian VYR-500) by comparing the area under the peak for the vinyl groups (\(\delta=5.5-6.2\) ppm) to the area under the peak for the methyl groups in the PEG backbone (\(\delta=3.3-3.9\) ppm). Acryloyl-PEG-RGD was synthesized by reacting YRGDS (Genscript) in excess in a 1.1:1 molar ratio with acryloyl-PEG-N-hydroxysuccinimide (3400 g mol\(^{-1}\)) (Laysan Bio, Inc.) in 50 mM sodium bicarbonate. The degree of acylation was 94\% and the degree of conjugation of the peptide was 71\% determined by \(^1\)HNMR. Chondroitin sulfate methacrylate (ChSMA) was synthesized by dissolving chondroitin sulfate A (Sigma) at 25\% (w/v) in deionized water and reacted with excess methacrylic anhydride (1:8 molar ratio of chondroitin sulfate to methacrylic anhydride\(^{18,27}\). The product was sterile filtered and recovered by lyophilization. On average, 16\% of the disaccharide repeat units on each chondroitin sulfate polymer were substituted with a methacrylate as determined via \(^1\)HNMR (Bruker Ascend 400) by comparing the area of under the peak for the vinyl resonances (\(\delta=5.5-6.2\) ppm) to the area under the peak for the acetyl groups chondroitin sulfate sugar backbone (\(\delta=1.7-2.0\) ppm).
3.3.2 Acellular Hydrogel Formulation and Characterization

Hydrogels were fabricated via photopolymerization of macromers at concentrations of 8, 9 or 10 wt% PEGDM, 0, 0.1 or 1 mM Acryloyl-PEG-RGD, and 0, 1, or 2 wt% ChSMA with 0.5 wt% photoinitiator Igracure 2959 (I2959) (BASF) in phosphate buffered saline (PBS, pH 7.4) with 352 nm light at 5 mW cm$^{-2}$ for 10 minutes. Hydrogels were allowed to swell to equilibrium for 24 hours in PBS at 37°C prior to their characterization.

The tangent modulus under compression was determined using cylindrical hydrogels (5 mm in height x 5 mm in diameter) using a mechanical tester (MTS Synergie 100, 10N). The samples were strained at a constant strain rate of 0.5 mm min$^{-1}$ using nonporous platens to 15% strain and the resulting modulus was determined for the linear region of the stress-strain curve. A sample size of 10 was used.

The volumetric equilibrium swelling ratio (Q) was estimated from the experimentally determined mass equilibrium swelling ratio, which was determined by measuring swollen mass after 24 hours of free swelling in PBS relative to the dry polymer mass, and assuming a density of the polymer (1.07 g ml$^{-1}$) and density of the solvent (1 g ml$^{-1}$). A sample size of 10 was used.

The incorporation of RGD into the hydrogels was evaluated by qualitative and semi-quantitative fluorescence intensity using a fluorescently labelled acryloyl-PEG-RGD. In brief, cadaverine 488 was reacted with the acryloyl-PEG-RGD in the presence of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in 0.1 M sodium bicarbonate at a 1:1 molar ratio for 24 hours. The product was purified by dialysis and recovered by lyophilization. PEG hydrogels with fluorescently labeled RGD were created by initially
making a 100 mM stock solution of RGD consisting of 1 wt% acryloyl-PEG-RGD-fluorophore and the other 99 wt% acryloyl-PEG-RGD. This stock solution was diluted to the desired concentration of 1mM and 0.1 mM RGD, and the hydrogels were polymerized using a redox initiating system of ammonium persulfate (0.025 M APS) and tetramethylethylenediamine (0.0125 M TEMED) for 10 minutes. The hydrogels were placed in PBS for 24 hours after polymerization to remove unincorporated RGD. Images were acquired using the Molecular Imager VersaDoc system and intensity measurements were used to semi-quantify the amount of fluorescently labeled RGD incorporated into the hydrogels.

The incorporation of chondroitin sulfate into the PEG hydrogels was confirmed qualitatively using toluidine blue stain and quantitatively using the DMMB dimethylmethylene blue (DMMB) assay. Hydrogels were stained with a solution of toluidine blue, which stains for negatively charged glycosaminoglycans, for 24 hours in PBS (0.1% toluidine blue, 7% ethanol, 0.1% NaCl in PBS, pH<2.5) and imaged using light microscopy (Zeiss Pascal, Olympus DP70 100x magnification). DMMB assay was used to quantify the amount of ChSMA incorporated into the hydrogels by measuring the soluble fraction of ChSMA, i.e. the fraction not covalently attached into the hydrogel network, which had diffused out into the PBS bath after 24 hours of free swelling.

3.3.3 hMSC Donors and Expansion

Bone marrow derived adult hMSCs (passage 1) were purchased from Texas A&M Cell Distribution Center. Three donors were investigated for the free swelling studies: (1) Donor 1 is a 24 year old female, (2) Donor 2 is a 29 year old female, (3) Donor 3 is a 24
year old female. Three different donors were investigated for the loading studies: (4) Donor 4 is a 24 year old female, (5) Donor 5 is a 22 year old male, and (6) Donor 6 is a 24 year old female. The hMSCs were thawed and expanded in stem cell culture media (20% fetal bovine serum (FBS, Atlanta Biologicals), 50 U ml\(^{-1}\) penicillin, 50 mg ml\(^{-1}\) streptomycin, 10 ng ml\(^{-1}\) bFGF and 20 mg ml\(^{-1}\) gentamicin in low glucose Dulbecco's modified Eagle media (DMEM, Invitrogen)). The hMSCs were seeded at 3000 cells cm\(^{-2}\) and grown to 80% confluency in T225 flasks up to passage 2. The cells were grown under standard cell conditions of 37°C with 5% CO\(_2\).

### 3.3.4 hMSC Encapsulation and Culture in Hydrogels

hMSCs were encapsulated in hydrogels at a cell concentration of 10x10\(^6\) cells ml\(^{-1}\) of filter-sterilized (0.22 μm filter) macromer solution by photopolymerization as described above. The cell-laden constructs (5mm in diameter and 2mm in height) were cultured in chondrogenic differentiation medium composed of 1% ITS+ Premix, 100 nM dexamethasone, 2.5 ng ml\(^{-1}\) TGF-β3, 50 mg ml\(^{-1}\) l-ascorbic acid 2-phosphate, 50 U ml\(^{-1}\) penicillin, 50 mg ml\(^{-1}\) streptomycin, and 20 mg ml\(^{-1}\) gentamicin in high glucose Dulbecco’s modified Eagle media. The hMSC-laden hydrogels were cultured under standard cell conditions of 37°C with 5% CO\(_2\) under static conditions for 7 or 14 days.

### 3.3.5 Mechanical Loading

A custom-built bioreactor system\(^{32,33}\) was utilized to apply intermittent dynamic compressive strains to the hydrogels. The hydrogels were cultured under free swelling conditions for the first week and then subjected to an intermittent dynamic loading regime for up to an additional two weeks. Dynamic compression was applied in unconfined compression in a sinusoidal waveform for 1 hr followed by 23 hrs of rest. Four regimes
were investigated with strains and frequencies of: 5% peak-to-peak strain (2.5% amplitude strain) 1Hz, 5% peak-to-peak strain (2.5% amplitude strain) 0.3 Hz, 10% peak-to-peak strain (5% amplitude strain) 1Hz, and 10% peak-to-peak strain (5% amplitude strain) 0.3 Hz. Hydrogels were cultured individually in a 24-well plate with 2 ml per well of chondrogenic differentiation media, which was changed every other day for the duration of the study. Free swelling controls were removed at 7, 14, and 21 days and loaded hydrogels were removed at 14 and 21 days.

3.3.6 Quantitative PCR (qPCR)

RNA was extracted from constructs (n=3 technical replicates per donor) using MicroElute Total RNA Kit per manufacturer instruction (Omega). RNA was transcribed to cDNA with a High Capacity Reverse Transcription Kit (Applied Biosystems). Quantitative real-time polymerase chain reaction (qPCR) was performed with Fast SYBR Green Master Mix (Applied Biosystems) on a 7500 Fast Real-Time PCR Machine (Applied Biosystems). All genes of interest are relative to the housekeeping gene, L30. Relative expression levels were calculated from C\text{t} values. The genes of interest were collagen I (marker for undifferentiated hMSCs and osteogenesis), collagen II (marker for chondrogenesis), and collagen X (marker for hypertrophy) (Invitrogen) (Table 1). PCR efficiency was determined for each gene. Briefly, threshold C\text{t} values were determined from serial dilutions of cDNA and plotted. The slope between C\text{t} values of 15-25 was used to determine efficiency, resulting in the following efficiencies, 94% for L30, 98% for collagen I, 102% for collagen II, and 87% for collagen X. Normalized gene expression was determined using the actual efficiencies following methods described by Pfaffl\textsuperscript{34}.
3.3.7 Immunohistochemistry (IHC)

Hydrogels were fixed overnight in 4% paraformaldehyde and transferred to a 15% sucrose solution at 4°C for storage (n=4 technical replicates per donor). Constructs went through a series of dehydration steps and were embedded in paraffin. Sections (10 μm) were stained for the presence of aggrecan, collagen II, collagen X, and Runx2 by immunohistochemistry. For anti-collagen I, sections were pretreated with 1 mg ml⁻¹ pepsin. For anti-collagen II sections were pre-treated with 2000 U ml⁻¹ hyaluronidase. For anti-collagen X sections were pre-treated with 1 mg ml⁻¹ protease followed by 1 mg ml⁻¹ pepsin, followed by 0.25% trypsin in 1mM EDTA. For anti-aggrancan sections were pre-treated with antigen retrieval, followed by chondroitinase ABC (100 mU ml⁻¹) and keratinase I (1U ml⁻¹), and followed by hyaluronidase (2000 U ml⁻¹). Anti-RunX2 sections required no pretreatment. After permeabilization and blocking, samples were treated overnight at 4°C with primary antibody: 1:50 anti-collagen II (Abcam, ab3092), 1:5 anti-aggrancan (USBio, A1059-53F), 1:50 anti-collagen X (Abcam, ab3092), and 1:50 anti-Runx2 (Abcam, ab23981) in blocking solution. Constructs were treated for 2 hours with goat anti-mouse IgG or goat anti-rabbit IgG labeled Alexa Fluor 488 (1:200) and Alexa Fluor 546 (1:200) and counterstained with DAPI.

3.3.8 Enzyme Activity Analysis

A SensoLyte Plus 520 MMP-13 Assay Kit (Anaspec) was used to determine the concentration of active matrix metalloproteinase (MMP) in the media of the free swelling and loading conditions. Culture media from donor 5 and 6 was collected flash-frozen in liquid nitrogen from the loading and the free swelling cultures every other day from day 7 to day 21. The media was stored at -80°C until the assay was conducted. The media from
multiple time points were pooled together as such: day 11 (media collected from days 7-11), day 17 (media from days 13-17) day 21 (media from days 17-21). The assay was performed as described in the kit protocol containing recombinant human pro-MMP-13 as the standard and a fluorescent MMP-13 substrate, but without an activation step to measure only the endogenous active form of MMP-13 in the media. The fluorescent signal was measured (Ex/Em=490nm/520nm) using a FLUOstar Optima plate reader (BMG Labtech).

3.3.9 Statistical Analysis

Data are presented as mean with standard deviation. Mean and standard deviation for acellular hydrogel characterization (ChS incorporation, RGD relative fluorescence, compressive modulus, and swelling ratio) are from technical replicates and were analyzed using a one-way ANOVA with a post hoc Tukey’s test, $\alpha=0.05$. For all qPCR gene expression and immunohistochemistry data, the mean represents average of three biological replicates, where each donor is an average of the technical replicates ($n=3$ for gene expression, $n=4$ for IHC). Data were analyzed by a one-way ANOVA with a post hoc Tukey’s test, $\alpha=0.05$, to determine significant difference between conditions at specific time points.

3.4 Results

3.4.1 Effects of ECM Analogs Introduced in PEG-based Hydrogels

PEG hydrogels were fabricated by photopolymerization of (meth)acrylate functionalized monomers of PEGDM, monoacrylate-PEG-RGD, and ChSMA to form a crosslinked network composed of PEG and ChS with covalently tethered RGD (Fig. 1A).
Hydrogels were formed with compositional variations in RGD (0, 0.1 and 1 mM) and ChS (0, 1 and 2 wt%) (Fig. 1B). After hydrogel formation, the hydrogels were allowed to free swell to remove any unreacted ECM analog. For RGD, fluorescently labeled RGD was used to confirm its incorporation into the hydrogel (Fig. 1C). Photographs of the hydrogels show much greater fluorescence in the 1 mM RGD hydrogel formulation compared to the 0.1 mM RGD hydrogel formulation with no observable fluorescence in the PEG hydrogel with no RGD. The fluorescence intensity was semi-quantified and determined to be an order of magnitude greater in the 1 mM RGD hydrogel formulation when compared to the 0.1 mM RGD hydrogel. The incorporation of ChSMA was qualitatively confirmed using toluidine blue, which stains for sulfated glycosaminoglycans, and showed increased staining with increasing concentration of ChSMA (Fig. 1D). The total amount of ChS incorporated into the hydrogel was confirmed to be ~97% indicating that nearly all of the ChSMA monomer was incorporated into the hydrogel (Fig. 1D). The macroscopic properties, specifically compressive modulus (Fig. 1E) and the equilibrium volumetric swelling ratio (Fig. 1F), were also measured for the different hydrogel formulations. The compressive modulus was ~50 kPa and the volumetric swelling ratio was ~11. Both properties were statistically similar for all hydrogel formulations.
Figure 3.1. Formation and characterization of the PEG-based hydrogels. A) A schematic showing the monomers and the resulting network formed after photopolymerization. B) A schematic of the different hydrogel formulations investigated in this study. C) The incorporation of RGD was confirmed using fluorescently labeled RGD. Fluorescent images and the corresponding semi-quantification of fluorescence intensity (n=3) show increases in fluorescent intensity with the increasing RGD concentrations from 0.1 to 1 mM. D) The incorporation of ChS as confirmed by toluidine blue staining, which stains negatively charged molecules blue. Brightfield microscopy images of toluidine blue
stained hydrogels with 0, 1, or 2 wt\% ChS show increasing blue staining (scale bar=100\,μm). Note that PEG-only hydrogels exhibit some background staining. The amount of ChS incorporated into the hydrogel was also quantitatively assessed (n=3). E) The compressive modulus was measured for the different hydrogel formulations and show no significant differences (n=10, \( p=0.73 \)). F) The volumetric swelling ratio was measured for the different hydrogel formulations and show no significant differences (n=10, \( p=0.47 \)). All data are presented as mean with standard deviation parenthetically or as error bars.

3.4.2 Effect of ECM Analogs on hMSC Chondrogenic Differentiation in PEG Hydrogels

A total of nine hydrogel formulations were investigated to screen for RGD (0, 0.1 and 1 mM) and ChS (0, 1 and 2wt\%) concentrations that enhance hMSC chondrogenesis in the presence of low TGF-β3 concentration (2.5 ng/ml). hMSCs from three donors were investigated. After 14 days of culture in chondrogenic differentiation media, cell-laden hydrogels were assessed by qPCR and IHC for collagen II as a marker for chondrogenesis, collagen X as a marker for hypertrophy (Fig. 2) and collagen I, which is expressed by MSCs and is a marker of fibrocartilage (Supplementary Fig 1).
Figure 3.2. The effects of ECM analogs on hMSC chondrogenesis under free swelling culture. A) qPCR data obtained at day 14, normalized to hMSC-laden hydrogels 24 hours post encapsulation in differentiation media for each respective donor for A) collagen II and B) collagen X. The symbols (♦ (Donor 1), ■ (Donor 2), ● (Donor 3)) represent the mean of each donor (n=4 technical repeats). The line (▬) represents the average of all donors (n=3) and p values are relative to PEG only hydrogel. C) Immunohistochemistry staining for collagen II (green) and nuclei (blue) at day 14 of free swelling and for collagen X(green) and nuclei (blue). Scale bar= 20 µm.

Gene expression data represents the change in expression that occurred between initial encapsulation (i.e., 24 hours after encapsulation) and day 14. Collagen II mRNA levels were elevated (i.e., greater than one) across all hydrogel formulations by day 14. The formulation consisting of 0.1 mM RGD and 1 wt% ChS had the highest (~10x) (p=0.07) collagen II gene expression values over the PEG-only hydrogel. In addition, this formulation had higher (p=0.05-0.08) Collagen II expression when compared to the other
formulations with ECM analogs; although to a lesser extent ($p=0.4$) when compared to the formulation with 0.1 mM RGD and 2 wt% ChS. For collagen X, the formulation consisting of 0.1 mM RGD and 1 wt% ChS, which had the highest expression of collagen II, had higher (~4x) ($p=0.14$) expression of collagen X expression when compared to the PEG-only hydrogel. The mean expression was also higher when compared to the other formulations with ECM analogs, but the increased expression was less significant ($p=0.10-0.45$). Collagen I gene expression was reduced (i.e. less than one) across all hydrogel formulations by day 14 with no differences among the hydrogel formulations. At the protein level, there was very little if any collagen II detected in the PEG-only hydrogel. However, collagen II was present in all of the formulations with ECM analogs. Collagen X protein was present in all of the hydrogel formulations. Collagen I protein was not detectable in any of the hydrogel formulations.

3.4.3 hMSC Chondrogenesis under Dynamic Compressive Loading

Hydrogels were subjected to unconfined dynamic compression to investigate the effect of dynamic compressive loading on hMSC chondrogenesis. One hydrogel formulation (0.1 mM RGD: 1% ChS) was selected from the screening study and subjected to dynamic compressive strains for 1 hour per day at one of five different loading regimes with strain rates included in parentheses: (1) no loading, (2) 5% strain 0.3 Hz (1.5%/s), (3) 10% strain 0.3 Hz (3%/s), (4) 5% strain 1Hz (5%/s), and (5) 10% strain 1 Hz (10%/s). The hydrogels were initially cultured in a free swelling environment for one week, and then placed in bioreactors and subjected to dynamic compression for two weeks. Here gene expression was normalized to expression levels of the pre-encapsulated hMSCs for
each donor respectively to assess the overall differentiation of the hMSCs for each of the different culture conditions.

hMSC chondrogenesis was assessed after 14 days for collagen II (Fig. S2A,C) and more extensively after 21 days of culture for viability (Fig. S3), collagen II and aggregan (Fig. 3) and for collagen I (Fig S4). At 14 days all conditions had elevated \((p=0.07-0.19)\) collagen II gene expression levels when compared to pre-encapsulated hMSCs. However, there were no differences \((p=0.57)\) as a function of the culture condition. Collagen II protein was present in all conditions. At 21 days, cells were mostly viable with some dead cells present in the hydrogel (Fig. S3). There were no observable differences in viability under loading. Gene expression for collagen II (Fig. 3A) was variable across all loading conditions with no significant differences, but remained several orders of magnitude higher than prior to encapsulation. In all loading conditions, collagen II protein (Fig. 3B-C) was detected with an average of \(~70-90%\) of the cells staining positive. Although qualitatively, there appeared to be less staining for collagen II protein in the loading condition with a strain rate of \(1.5%/s\). Collagen I gene expression was approximately 2-3 orders of magnitude lower when compared to collagen II gene expression with no observable trends as a function of loading condition. Collagen I protein was not detectable in any of the hydrogels as a function of loading. The IHC analysis was extended to aggregan (Fig. 3D), which was highly expressed at the protein level with no observable differences among the culture conditions. These results suggest that collagen II and aggregan expressions are not substantially affected by dynamic compression across the loading regimes investigated.
Figure 3.3. The effects of dynamic compressive loading regime on hMSC chondrogenesis in a biomimetic PEG-based hydrogel containing 0.1 mM RGD and 1 wt% ChS. A) qPCR data for collagen type II obtained at day 21 and normalized to hMSC prior to encapsulation for each respective donor B) Immunohistochemistry staining for collagen II (green) and nuclei (blue) at day 21. C) Semi-quantitative analysis of the fraction of cell staining positive for collagen II at day 21. D) Immunohistochemistry staining for aggrecan (red) and nuclei (blue) at day 21. In B and C, the symbols (♦ (Donor 1), ■ (Donor 2), ● (Donor 3)) represent the mean of each donor (n=4 technical repeats). The line (▬) represents the average of all donors (n=3) in A and D.

3.3.4 hMSC Hypertrophy under Dynamic Compressive Loading

hMSC hypertrophy was assessed after 14 days by collagen X (Fig S2B,C) and more extensively after 21 days for collagen X, RunX2, and MMP13 (Fig. 4) under the same experimental conditions as described in the previous section. At 14 days all conditions had elevated \( p=0.06-0.21 \) collagen X gene expression levels when compared to pre-encapsulated hMSCs. However, there were no large differences \( p=0.39 \) as a function of the culture condition. Collagen X protein was not detected in two of the loading conditions with strain rates of 3%/s and 5%/s, but was detected in the free swelling condition and loading conditions at the extremes with strain rates of 1.5%/s and 10%/s. At 21 days, collagen X gene expression was reduced \( p=0.20-0.22 \) in the loading conditions with strain rates of 1.5-5%/s when compared to the free swelling (Fig. 4A). At the highest strain rate (10%/s) collagen X expression was similar to the free swelling condition. At the protein level, collagen X (Fig. 4B) was detected in the free swelling condition with ~60% of the cells expressing the protein. In the loading conditions at the extremes with strain rates of 1.5%/s and 10%/s, collagen X protein was also detected with 40-55% of the cells expressing the protein. On the contrary in the loading conditions with strain rates of 3 and 5 %/s, fewer cells expressed collagen X with only ~30% and 20%, respectively. Staining for RunX2 at the protein level (Fig. 4C) followed that of collagen X with prominent staining.
in the free swelling condition and in the extreme loading conditions with strain rates of 1.5
%/s and 10 %/s. However minimal staining was detected in the loading conditions with
strain rates of 3 and 5 %/s. MMP-13 activity was also assessed in the culture medium for
two of the three donors and data for day 21 is shown (Fig. 4D). Under the 5%/s strain
rate, the levels of MMP-13 activity were lower ($p=0.06$) when compared to the free
swelling condition, while all other loading conditions were more similar ($p=0.38-0.84$) to
the free swelling conditions. No differences were observed at the early time points of day
11 and day 17 (data not shown).
Figure 3.4. The effects of dynamic compressive loading regime on hMSC hypertrophy in a biomimetic PEG-based hydrogel containing 0.1 mM RGD and 1 wt% ChS. A) qPCR data for collagen type X obtained at day 21 and normalized to hMSC prior to encapsulation for each respective donor. B) Immunohistochemistry staining for collagen X (green) and nuclei (blue) at day 21 and semi-quantitative analysis of the fraction of cell staining positive for collagen X. C) The concentration of active MMP13 in accumulated media from day 21 From Donor 5 (■) and Donor 6 (●) D) Immunohistochemistry staining for RunX2 (green) and nuclei (blue) at day 21. In A, B and C, the symbols (♦ (Donor 4), ■ (Donor 5), ● (Donor 6)) represent the mean of each donor (n=4 technical repeats). The line (▬) represents the average of all donors (n=3).

3.5 Discussion

Human mesenchymal stem cells are a promising cell source for cartilage tissue engineering given their ability to differentiate down the chondrogenic lineage. A successful tissue engineering approach using hMSCs requires that the scaffold (e.g., hydrogel) promote a stable chondrogenic phenotype with cartilage-specific ECM synthesis. The challenge is that the default pathway for hMSCs differentiating down the chondrogenic lineage is terminal differentiation, a precursor to endochondral ossification. However, the environment within healthy cartilage contains a host of physiochemical cues that maintain a stable chondrocyte phenotype throughout one’s life. Herein, we show that recapitulating some of the physiochemical cues from cartilage within a synthetic hydrogel enhances hMSC chondrogenesis and simultaneously maintains a stable chondrogenic phenotype by preventing terminal differentiation. A 3D hydrogel environment with carefully tuned biochemical and biomechanical cues offers a promising strategy to achieve hMSC-mediated cartilage regeneration.

In this study, we first screened two ECM analogs, a cell adhesion peptide (RGD) and a sulfated glycosaminoglycan (ChS), for their ability to promote hMSC chondrogenesis in a synthetic PEG hydrogel, but in the absence of mechanical loading.
Our results show that the presence of ECM analogs, regardless of type (RGD or ChS) or concentration, enhanced hMSC chondrogenesis. This observation was evident by positive staining for the protein, collagen II, a late marker for chondrogenesis. Collagen II was present in as early as two weeks of culture for all hydrogel formulations that contained ECM analogs, but was not detectable in the PEG-only hydrogels. Collagen II mRNA levels in the PEG-only hydrogel, however, were similar to many of the other formulations with ECM analogs. This result suggests that hMSCs are indeed undergoing chondrogenesis in all of the hydrogel formulations, but that the process may be slower in the absence of ECM analogs. This observation is supported by our previous work\textsuperscript{26}, which showed that when higher TGF-β3 concentrations were employed (i.e., 10 ng/ml instead of 2.5 ng/ml used in the current study), collagen II protein was detected in PEG-only hydrogels within two weeks of culture. We have also previously shown that when TGF-β3 was absent from the culture medium, ECM analogs of ChS or RGD were not sufficient to induce expression of collagen II protein in hMSCs over this same time frame\textsuperscript{26}. Since chondrogenesis is known to require TGF-β signaling\textsuperscript{39,40}, our findings suggest that the enhanced chondrogenesis, as observed by positive staining for collagen II protein, may be a result of a synergistic effect between the ECM analogs and TGF-β signalling.

In the case of RGD, it is well known that a cross-talk exists between growth factor receptors and integrins to control downstream signaling\textsuperscript{41,42}. In particular, TGF-β signalling has been shown to act synergistically with intracellular signalling pathways that depend on cell adhesion (e.g., Rho/Rock signalling)\textsuperscript{43}. Therefore, when hMSCs engage with the RGD tethers present in the PEG hydrogel, it is possible that integrin-mediated signalling may act synergistically with TGF-β3 to enhance chondrogenesis. Furthermore,
TGF-β binding to its receptor can lead to endogenous secretion of TGF-β, thus amplifying the signalling cascade\textsuperscript{44}. Interestingly, the latent form of TGF-β that is secreted by cells, can be activated by integrins that recognize RGD\textsuperscript{45-47}. We and others have reported that when cells are encapsulated in a PEG hydrogel with RGD, they up-regulate integrins that bind to RGD\textsuperscript{15,26}. While the exact mechanism remains to be elucidated, we hypothesize that the presence of RGD in the PEG hydrogel may enhance chondrogenesis through a cross-talk in the signalling pathways downstream of TGF-β signalling and/or by integrin-mediated activation of endogenously secreted TGF-β. Although further studies are needed to test this hypothesis.

In the case of chondroitin sulfate, its sulfate groups may help to sequester soluble TGF-β\textsubscript{3} and enhance its bioactivity. It is believed that the interaction between GAGs and growth factors prolongs their activity by increasing the half-life when bound to the matrix\textsuperscript{51}. Studies have shown that TGF-β\textsubscript{3} can bind to sulfated GAGs, reducing its release and improving its effectiveness in cartilage tissue engineering\textsuperscript{48}. In addition, the negatively charged chondroitin sulfate also attracts positive ions from the culture media leading to increased osmolarity within the hydrogel\textsuperscript{19}, which is similar to that of cartilage\textsuperscript{19,49,50}. It has been suggested that hyper-, but physiologically relevant, osmolarity can influence post-transcriptional regulation of Sox9 and therefore could lead to increased protein synthesis of collagen II\textsuperscript{51}. Furthermore, hyperosmolarity has been shown to enhance TGF-β activity in other cell types and was responsible for increased collagen synthesis\textsuperscript{52}. While the exact mechanism remains to be elucidated, we hypothesize that the presence of chondroitin sulfate enhances TGF-β\textsubscript{3} activity either through contextual presentation of the growth factor (i.e., binding of the growth factor) or through a hypertonic environment that acts
synergistically with TGF-β signalling. Although further studies are needed to test this hypothesis.

When the two ECM analogs were combined, the formulation containing 1wt% ChS and 0.1 mM RGD led to the highest collagen II mRNA levels across all three donors concomitant with collagen II protein expression in nearly all the cells. This observation suggests that while all formulations with ECM analogs induced hMSC chondrogenesis, there appears to be a synergistic effect between the ECM signals that depends on their magnitudes. Indeed, several studies have shown that there is an optimal osmolarity that enhances matrix synthesis by chondrocytes where supraphysical levels can be inhibitory to chondrocytes\textsuperscript{19,20} and to chondrogenically differentiating MSCs\textsuperscript{23}. High concentrations of RGD have been shown to support osteogenesis of hMSCs in 3D cultures through traction dependent processes\textsuperscript{53} and thus low RGD concentrations may favor chondrogenesis\textsuperscript{17}. The combination of RGD and ChS at the lower concentrations led to markedly improved chondrogenesis (as observed by the high mRNA levels for collagen II). We hypothesize that this effect occurs as a result of enhanced TGF-β signalling by the presence of both ECM analogs, where TGF-β induces Sox9-dependent transcriptional activity\textsuperscript{39} that ultimately controls collagen II expression\textsuperscript{39}. However, additional experiments are needed to better understand the observed synergistic effect between the RGD and ChS on chondrogenesis.

The hypertrophic protein, collagen X, was present in all formulations regardless of the presence of ECM analogs. Collagen X is the hallmark of terminal differentiation and the default pathway for chondrogenically differentiating MSCs. This result is consistent with our previous reports\textsuperscript{26} and reports by others, which use TGF-β to induce MSC
chondrogenesis\textsuperscript{4–6,36,54}. These findings show that despite the presence of ECM analogs that create a cartilage-like biomimetic hydrogel environment, the enhanced chondrogenesis was accompanied by a hypertrophic phenotype. However, these initial studies were performed in the absence of any mechanical stimulation.

It is well known that mechanical forces are critical to cartilage homeostasis\textsuperscript{55,56} and have been shown to regulate chondrogenesis of MSCs\textsuperscript{28,29}. We selected one formulation from the free swelling screening study, which showed robust chondrogenesis (i.e., 0.1 mM RGD and 1wt% ChS), and investigated the effects of dynamic compressive loading on MSC chondrogenesis and terminal differentiation. Here, a more in-depth analysis was performed. Interestingly, collagen II at the gene and protein levels did not appear to be affected by loading. This result is in contrast to our previous work, which showed inhibition of collagen II protein synthesis under dynamic compressive loading in PEG hydrogels with RGD\textsuperscript{31} and in PEG hydrogels with ChS\textsuperscript{26}. In our previous work, however, loading was applied at higher strains (i.e., 15%), where others have shown that relatively high fluid flow velocities can inhibit collagen II synthesis by chondrogenically differentiating hMSCs\textsuperscript{56}. Results from the current study, thus, suggest that lower strains (≤10%) and/or the presence of multiple ECM cues may be critical to maintaining chondrogenesis when hMSCs are encapsulated in PEG-based hydrogels and subjected to loading.

Most notably, dynamic mechanical loading affected terminal differentiation of the hMSCs and in a manner that depended on the loading rate. Interestingly, strain rates in the range of 3%/s (0.3 Hz 10%) to 5%/s (1 Hz 5%) inhibited collagen X protein expression across all three donors. There were notable differences between gene and protein expression levels for collagen X, which may in part be due to differences between
transcriptional and translational control as shown by others\textsuperscript{57}. The protein RunX2, a transcription factor involved in chondrocyte terminal differentiation and important to endochondral ossification\textsuperscript{58,59}, was also inhibited under similar strain rates as collagen X. On the contrary, RunX2 and collagen X proteins were detected in the hydrogels cultured in the absence of loading as well as cultured under the two extreme loading conditions, at 1.5\%/s and 10\%/s strain rates. Runx2 protein appeared to be co-localized with the nucleus in some cells, but also located in the cytoplasm. This observation is consistent with prior reports that show Runx2 shuttling between the nucleus and cytoplasm\textsuperscript{60,61}.

These results suggest that the hypertrophic phenotype in chondrogenically differentiating hMSCs may be highly mechanosensitive, which has been suggested by others\textsuperscript{62,63}. Our findings are consistent with others, which reported inhibition of hypertrophy, evident by reduced collagen X gene expression, by dynamic loading for hMSCs in a hydrogel\textsuperscript{64}. However, results from this study suggest that this effect may be strain rate dependent. Since collagen X is critical to endochondral ossification, mechanical loading may play a role in controlling this process. Indeed, studies have pointed to the mechanical environment as regulating the type of cartilage tissue that is formed during development and as well during homeostasis\textsuperscript{65}. Other studies have pointed to a role for non-collagenous extracellular matrix proteins (e.g., matrilin, which is involved in matrix assembly and detected early in chondrogenically differentiating MSCs\textsuperscript{54}) in regulating chondrocyte hypertrophy\textsuperscript{65} and which have been shown to be highly sensitive to dynamic loading\textsuperscript{66}. Furthermore, multiple physiochemical cues are generated in the hydrogel as a result of the combined presence of dynamic loading and ECM analogs. These cues include: (a) cell deformation and fluid flow induced by dynamic loading alone,
(b) integrins acting as mechanoreceptors to dynamic loading, and (c) dynamic changes in osmolarity that result from the movement of mobile cations in and out of the hydrogel during dynamic loading. In all cases, the magnitude of the cue will depend on the loading rate. While the exact mechanism remains to be elucidated, dynamic loading will dramatically change the environment surrounding the hMSCs. We hypothesize that a combination of cues are likely contributing to the regulation of the hypertrophic phenotype, while the chondrogenic phenotype appears to be less sensitive to the loading environment at least when TGFβ3 is supplemented to the media. Additional studies, however, are needed to identify the pathways that lead to the observed load-induced inhibition of hMSC hypertrophy.

MMP-13 is also known to be upregulated in hypertrophic chondrocytes along with collagen X and RunX2. In this study, MMP-13 activity was detected in the culture medium, and appeared to be reduced under the strain rate 5%/s that also resulted in minimal collagen X and RunX2 protein levels. MMP-13 is involved in the resorption of cartilage and the remodelling of new bone, and therefore its up-regulation in activity occurs later in this process. Indeed, others have reported that when hMSCs are cultured in pellets and in a hypertrophic-specific culture medium, MMP-13 mRNA was not detected until day 21. Here, MMP-13 was detectable, but differences were not apparent until late in our study. It is important to note that we measured active MMP-13 and therefore it is possible that MMP-13 protein may have been affected by other loading conditions, but that this was accompanied by the secretion of molecules, such as TIMPs, that regulate its activity.
There are several limitations of this study, which are worth noting. First, the number of human donors was limited (n=3), thus reducing the statistical power of the study. The large variability observed here, however, is consistent with other reports\textsuperscript{69–71}. As a result, our findings, particularly with respect to the gene expression data, are limited. However, we did observe notable differences in protein expression among different culture conditions that were consistent across all donors. While we did not quantify the total amount of protein deposited, the obvious presence or absence of a particular protein provided key insight into the effect of our culture condition on hMSC chondrogenesis. Second, our studies are limited to three weeks and therefore long-term studies are needed to determine whether the combined loading environment and biomimetic hydrogel can continue to maintain the chondrogenic phenotype while preventing terminal differentiation. Furthermore, terminal differentiation was evaluated in the presence of TGFβ3 by collagen type X, an early marker of hypertrophy. Additional studies are necessary to evaluate whether dynamic loading at the regimes identified here are sufficient to inhibit late stage hypertrophy in a more relevant hypertrophic medium\textsuperscript{68}. Third, our studies were also limited to non-degradable hydrogel. The use of a stable hydrogel was critical to ensure that the physiochemical cues, resulting from the hydrogel, were largely maintained throughout the culture period. However, non-degradable crosslinks prevent diffusion of large ECM molecules and thus limit their deposition to the pericellular space\textsuperscript{72}. While a pericellular matrix can shield cells from mechanical strain, the relatively short culture times and limited matrix deposition by the chondrogenically differentiated MSCs are not expected to affect strain transfer to the encapsulated cells. Previously we have shown that a relatively thick PCM is necessary to inhibit strain transfer
to cells. In addition, non-degradable hydrogels have also been shown to limit cell proliferation due to the restricted space; although cell proliferation was not measured in this study.

3.6 Conclusions

We describe a cartilage-biomimetic hydrogel that is composed of ECM analogs of a cell adhesion peptide based on RGD and a sulfated glycosaminoglycan based on ChS, which were incorporated, into a PEG hydrogel. This biomimetic hydrogel enhanced chondrogenesis of hMSCs over a range of ECM analog concentrations, but in all formulations and donors a hypertrophic phenotype was evident by the presence of collagen X. However when dynamic mechanical loading was introduced, chondrogenic differentiation did not appear to be affected, but hypertrophy was inhibited across all donors under moderate strain rates (i.e., 3%/s (10% 0.3 Hz) and 5%/s (5% 1 Hz). Our findings suggest that the combination of ECM analogs and dynamic mechanical loading contribute to the regulation of hypertrophy and help to promote a stable chondrogenic phenotype in MSCs.

3.7 Acknowledgments

Primary financial support for this work was provided by a NSF CAREER Award (#0847390). Additional support was provided by NIH (#1R01AR065441). The authors also acknowledge a NSF GRFP to EAA.

3.8 Supplementary Figure
Supplemental Figure 1. The effects of ECM analogs on collagen I expression in hMSCs undergoing chondrogenesis under free swelling culture conditions after 14 days of culture. A) Gene expression (by qPCR) for collagen I. The symbols (diamond (Donor 1), square (Donor 2), circle (Donor 3)) represent the mean for each donor (n=4 technical replicates). The line (▬) represents the mean for all donors (n=3) and p values are relative to PEG-only hydrogel. Normalized expression is defined as relative expression at day 14 normalized to relative expression at 24 hours after encapsulation. C) Immunohistochemistry staining for collagen I (green) (Cell nuclei (blue) were counterstained with Dapi. Scale bar = 20μm.
Supplemental Figure 2. The effects of dynamic compressive loading on hMSC chondrogenesis and hypertrophy in a biomimetic PEG-based hydrogel containing 0.1 mM RGD and 1 wt% ChS after 14 days of culture. Gene expression (by qPCR) for A) collagen II and B) collagen X. Normalized expression is defined as relative expression at day 21 normalized to relative expression for pre-encapsulated hMSCs. C) Immunohistochemistry staining for collagen II (green) (top row) and for collagen X (green) (bottom row). Cell nuclei (blue) were counterstained with Dapi. Scale bar = 20μm. In A and B, the symbols (diamond (Donor 1), square (Donor 2), circle (Donor 3)) represent the mean for each donor (n=4 technical replicates). The line (▬) represents the mean for all donors (n=3).
**Supplemental Figure 3.** The effects of mechanical loading on hMSC viability after 21 days of culture (7 days of free swelling followed by 14 days of dynamic loading). Representative images show live cells stained green (calcein-AM) and dead cells stained red (ethidium homodimer) at 21 days (100x magnification).
Supplemental Figure 4. The effects of dynamic loading on collagen I expression in hMSCs undergoing chondrogenesis in a biomimetic PEG-based hydrogel containing 0.1 mM RGD and 1 wt% ChS after 21 days of culture. A) Gene expression (by qPCR) for collagen I. Normalized expression is defined as relative expression at day 21 normalized to relative expression for pre-encapsulated hMSCs. C) Immunohistochemistry staining for collagen I (green). Cell nuclei (blue) were counterstained with Dapi. Scale bar = 20μm. In A and B, the symbols (diamond (Donor 1), square (Donor 2), circle (Donor 3)) represent the mean for each donor (n=4 technical replicates). The line (—) represents the mean for all donors (n=3).

Table S1. qPCR Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<td>L30 (housekeeping gene)</td>
<td>TTAGCGGCTGCTGTGGTT</td>
<td>TCCAGCGACTTTTTCGTCTTC</td>
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3.9 References


Chapter 4

Mechanical loading regulates human MSC differentiation in a multi-layered hydrogel for osteochondral tissue engineering

As appearing in Acta Biomaterialia 2015

4.1 Abstract

A bioinspired multi-layer hydrogel was developed for the encapsulation of human mesenchymal stem cells (hMSCs) as a platform for osteochondral tissue engineering. The spatial presentation of biochemical cues, via incorporation of extracellular matrix analogs, and mechanical cues, via both hydrogel crosslink density and externally applied mechanical loads, were characterized in each layer. A simple sequential photopolymerization method was employed to form stable poly(ethylene glycol)-based hydrogels with a soft cartilage-like layer of chondroitin sulfate and low RGD concentrations, a stiff bone-like layer with high RGD concentrations, and an intermediate interfacial layer. Under a compressive load, the variation in hydrogel stiffness within each layer produced high strains in the soft cartilage-like layer, low strains in the stiff bone-like layer, and moderate strains in the interfacial layer. When hMSC-laden hydrogels were cultured statically in osteochondral differentiation media, the local biochemical and matrix stiffness cues were not sufficient to spatially guide hMSC differentiation after 21 days. However dynamic mechanical stimulation led to differentially high expression of collagens with collagen II in the cartilage-like layer, collagen X in the interfacial layer and collagen I in the bone-like layer and mineral deposits localized to the bone layer. Overall, these
findings point to external mechanical stimulation as a potent regulator of hMSC differentiation toward osteochondral cellular phenotypes.

4.2 Introduction

Lesions to articular cartilage and the underlying subchondral bone in articulating joints can lead to joint pain, reduced joint function, and overtime osteoarthritis. This type of lesion is particularly problematic because it extends across two distinctly different tissues, highly compliant hyaline cartilage and stiff subchondral bone. These two tissues are connected by a thin interface that is defined by the tidemark flanking cartilage and the cement line flanking subchondral bone. This transitional region is critically important to the overall function of the joint as it enables efficient transfer of load between these two very different tissues, while minimizing stress concentration and reducing failure.

Tissue engineering is a promising strategy for regenerating osteochondral tissues, but the complexity of this type of tissue requires multi-layer scaffold designs and ultimately different cell types. Bi-layer scaffolds are the simplest approach, whereby a stiff layer, typically inorganic mineralized matrix (e.g., hydroxyapatite and β-tricalcium phosphate) representing the bone region is topped with a soft polymeric layer (e.g., poly(lactic-co-glycolic acid), collagen, agarose, etc.) representing the cartilage region. Other
approaches have employed the same scaffold chemistry in both layers and instead varied the physical properties (e.g., pore structure) and/or the biochemical cues (e.g., tissue-specific ECM-analogs, growth factors, or genes). A few studies have developed multi-layer scaffolds to capture the interfacial layer. These scaffold-based approaches have relied either on endogenous cells infiltrating into the scaffold or on exogenous cells delivered within the scaffold upon implantation. Several studies have used mesenchymal stem cells (MSCs) in their undifferentiated state within both layers, but achieving the appropriate differentiation has been limited. To overcome this shortcoming, MSCs pre-differentiated down the chondrogenic or osteogenic lineage have been used with some success. Recent studies have shown that using osteogenically differentiated MSCs in the bone-like layer with undifferentiated MSCs in the cartilage-like layer leads to improved chondrogenic differentiation in this layer.

To date, multi-layer scaffold designs for osteochondral tissue engineering have primarily focused on physical cues from scaffold architecture or biochemical cues to create layers that are conducive to tissue engineering cartilage or bone. One key component that has been largely missing is mechanical loading. It is well-known that mechanical cues are important in chondrogenesis and osteogenesis and that osteochondral tissues in articulating joints are continually subjected to mechanical forces. Therefore, a better understanding of how mechanical forces are translated through these complex multi-layer scaffolds and how they impact cells is needed. A few studies have utilized finite element modeling as a means to predict the local mechanical stresses and strains that are present in complex multi-layered osteochondral scaffolds. However, the impact on cells remains under-studied.
The overall objective for this study was to develop a multi-layered biomimetic hydrogel for osteochondral tissue engineering and to characterize the hydrogel within a mechanically relevant environment, specifically under dynamic compressive loading. Human MSCs (hMSCs) were investigated for their clinical relevance and potential in regenerating cartilage and bone. The hydrogel was designed from the same base chemistry, but with varying mechanical properties from soft to stiff and tissue-specific biomolecules within each layer. Specifically, a stable poly(ethylene glycol) hydrogel was chosen as the base chemistry because biological moieties are readily incorporated in a controlled manner while the mechanical properties can be independently tuned and maintained over the course of the experiment \(^{37-39}\). Two extracellular matrix (ECM) molecules, chondroitin sulfate and RGD, were chosen. Chondroitin sulfate is the main glycosaminoglycan in cartilage and creates a unique environment that is hyperosmotic, which for cartilage cells enhances tissue synthesis \(^{40}\) especially under dynamic compression\(^{41,42}\). RGD, a cell adhesion peptide, provides a mechanism for cells to sense substrate stiffness and may act as a mechanosensor to dynamic compression\(^{43}\). RGD has been shown to support chondrogenesis \(^{44,45}\) with low concentrations improving differentiation \(^{45}\). RGD has also been shown to support osteogenesis over a range of concentrations\(^{46,47}\).

The specific aims of this study were twofold. First, the aim was to develop and characterize a multi-layered hydrogel with controlled presentation of biochemical and mechanical cues for capturing the three main regions of osteochondral tissues: cartilage, bone, and the interface comprised of calcified cartilage. Specifically, a multi-layered hydrogel was designed based on (a) a compliant cartilage-like layer containing 1%
chondroitin sulfate\textsuperscript{41} and 0.1 mM RGD\textsuperscript{43}, (b) a stiffer bone-like layer consisting of 10 mM RGD, and (c) an interfacial region that combines the soft and stiff layers. The second aim was to employ undifferentiated hMSCs and investigate whether the local cues presented by a multi-layer hydrogel under the application of intermittent dynamic compression are sufficient to impact the fate of hMSCs when presented with a mixed osteochondral media.

4.3 Materials and Methods
4.3.1 Macromer Synthesis

Poly(ethylene glycol) dimethacrylate (PEGDM) macromer was synthesized via microwave methacrylation\textsuperscript{49}. Briefly, 4600 g mol\textsuperscript{-1} poly(ethylene glycol) (PEG) (Fluka, Sigma–Aldrich) was melted and reacted with methacrylic anhydride in the presence of hydroquinone (Sigma–Aldrich). The reaction mixture was dissolved in methylene chloride and purified by multiple precipitations with ethyl ether, filtration, and drying under vacuum. The degree of methacrylate substitution on each end of the PEG molecules was determined to be 93\% by \textsuperscript{1}HNMR (Varian VYR-500). Specifically, the area under the curve for the vinyl resonance peaks ($\delta = 5.7$ ppm, $\delta = 6.1$ ppm) was compared to the area under the curve for the methylene peaks associated with the PEG backbone ($\delta = 4.3$ ppm).

YRGDS (Genscript) was reacted in a 1:1.1 molar ratio with excess acryloyl-PEG-N-hydroxysuccinimide (3400 Da; Laysan Bio, Inc.) in 50 mM sodium bicarbonate buffer (pH 8.4) overnight at room temperature. The degree of attachment was determined to be 94\% using the spectroscopic Fluoraldehyde\textsuperscript{TM} o-Phthalaldehyde (Pierce) method of detection. In addition, fluorescently labeled YRGDS was synthesized as follows. In brief, acryloyl-PEG-RGD in 0.1 M sodium bicarbonate (pH 8.5) was reacted with Alexa Fluor 488 cadaverine in a 1:1 molar ratio using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
(EDC) as a coupling agent over 24 h while stirring. Acryloyl-PEG-RGD and acryloyl-PEG-fRGD were purified by dialysis against deionized water (dl-H₂O), recovered by lyophilization and stored at 4 °C.

Methacrylated chondroitin sulfate (ChSMA) was synthesized as previously described\textsuperscript{41–49}. Briefly, chondroitin sulfate A (Sigma), containing \(\sim 30\%\) chondroitin-6-sulfate and \(\sim 70\%\) chondroitin-4 sulfate was dissolved at 25\% (w/v) in dl-H₂O and reacted in a 1:8 ratio with methacrylic anhydride. The reaction temperature was held at 4 °C for 24 h and the reaction pH was maintained at 8. The reaction product was precipitated in chilled methanol and dialyzed against dl-H₂O. The purified product was recovered via lyophilization and the degree of methacrylation was determined to be 23\% via \(^1\)HNMR (Varian VYR-500), indicating that, on average, there were 23 methacrylate groups present on each ChSMA molecule. Specifically, the area under the curve for the vinyl resonance peaks (\(\delta = 5.5–6.2\) ppm) was compared to the area for the acetyl groups (\(\delta = 1.7–2.0\) ppm). Multiple methacrylate substitutions are possible due to the free hydroxyl groups present in each repeat unit of the ChS\textsuperscript{49}.

### 4.3.2 Acellular multi-layered hydrogel fabrication

Two macromer solutions were used to create the multi-layered hydrogels for fabrication characterization: 10\% (g/g) PEGDM, top, and 30\% (g/g) PEGDM, bottom. Each macromer solution was combined with 0.05\% (g/g) photoinitiator Irgacure 2959 (BASF). To visualize each layer in acellular hydrogels, 0.1\% (g/g) fluorescein-o-methacrylate (Sigma) was added to the 10\% PEGDM macromer solution, and 0.1\% (g/g) rhodamine methacrylate (Sigma) was added to the bulk 30\% PEGDM macromer solution. The macromer solutions were exposed to 365 nm light with an intensity of \(\sim 5\) mW cm\(^{-2}\) for a range of times, referred to as polymerization time (0–10 min: bottom,
plus 10 min: top). Cubic (5 × 5 × 5 mm) hydrogels were fabricated and imaged using confocal laser scanning microscopy. ImageJ software was used to characterize the resultant interface thicknesses. Multi-layered hydrogels were also fabricated with biochemical cues where the two macromer solutions were prepared: 10% (g/g) comprised of 90:10 (PEGDM:ChSMA) by weight with 0.1 mM RGD (top layer) or 30% (g/g) PEGDM with 10 mM RGD (bottom). Hydrogels were formed as described above. For hydrogels with the fluorescently labeled RGD, hydrogels were formed as described above and at room temperature, but with a redox initiating system consisting of ammonium persulfate (0.025M APS) and tetramethylethylenediamine (0.0125M TEMED). The concentration of the redox initiators was chosen to achieve similar polymerization behavior of the PEG hydrogels formed with the photoinitiator systems based on qualitative observations of the gel point and complete gelation. Specifically, the bottom layer was polymerized for 1 min and the top layer was polymerized for 10 min. In addition, only 1% of the RGD incorporated was the fluorescently labeled RGD.

4.3.3 Photorheometry
A rheometer (Ares 4400, TA Instruments) that was modified with a light source (365 nm, 5 mW cm⁻²) was used to characterize the gelation of the 30% (g/g) PEGDM solution. A macromer solution volume of 70 μL was placed between two 20 mm diameter plates with a separation gap of 150 μm. Measurements of the storage modulus (G’) and loss modulus (G’”) were taken during a dynamic time sweep at a frequency of 10 Hz and strain of 5% during photopolymerization. The light source was turned on after 60 s.

4.3.4 Mechanical testing
Single component hydrogels were formed from each macromer solution and polymerized as describe above for 10 min. Multi-layered hydrogels were also fabricated
using 1 min for the bottom layer plus 10 min for the top layer. Cylindrical hydrogels (5 mm in height × 5 mm in diameter) were fabricated. Hydrogels were allowed to free swell in PBS for 24 h at 37 °C. The tangent compressive modulus was determined in hydrated hydrogels under unconfined compression, using a mechanical tester (Bose LM1 Test Bench). The samples were strained at a constant strain rate of 0.3 mm s⁻¹ using nonporous platens. The resulting tangent modulus of the individual layers and the multi-layered hydrogels was determined for the linear region of the stress/strain curve (n = 5). It should be noted that because PEG hydrogels exhibit largely elastic behavior, the tangent modulus was assumed to be reasonably similar to the equilibrium modulus previously reported for similar hydrogel formulations⁵⁰.

4.3.5 Acellular composite biochemical analysis

The incorporation of chondroitin sulfate in the multi-layer hydrogels formed as described above was evaluated qualitatively by toluidine blue staining and quantitatively through biochemical assays for negatively charged glycosaminoglycans. Fully hydrated acellular multi-layered hydrogels were stained with a working solution of toluidine blue (stock: 1% toluidine blue in 70% ethanol, working solution: 10% stock solution in 1% NaCl, pH < 2.5). For quantitative assessment, cylindrical acellular tri-layered hydrogels were embedded in tissue mounting media and serially sectioned in the axial direction, starting at the top layer and continuing down the axis of the cylinder, using a cryosectioner (Leica). Sections (200 μm) were homogenized and then sequentially degraded in 1 M NaOH at 60 °C for 24 h, then neutralized followed by enzyme treatment in 2 ml of 6.6 × 10⁻³ U ml⁻¹ chondroitinase ABC (Sigma) at 37 °C for 24 h. Sections representing the top, interface, and bottom layers were pooled and analyzed for ChS content using the
DMMB dye assay\textsuperscript{51}. In addition, the soluble fraction of ChSMA, i.e., the fraction not incorporated into the hydrogel, was determined by assaying for the ChSMA (by DMMB) that diffused out of the hydrogel after the 24 h free swelling period. It was confirmed that 80\% of the ChSMA was incorporated into the hydrogel. The incorporation of RGD in the multi-layer hydrogels was evaluated qualitatively through fluorescent microscopy and semi-quantitatively by fluorescence intensity of a fluorescently labeled RGD (acryloyl-PEG-fRGD). Fluorescent images were acquired of the entire hydrogel and assembled to show the entire gel. In addition, a line was drawn down the center of the gel and the intensity from laser scanning confocal microscopy (Zeiss LSM 510) was measured as a function of the gel height at 400× magnification. Using ImageJ, the 16 color lookup table was applied to the 488 nm channel and the intensity was converted to a color spectrum scale from red (low) to violet (high). The intensity was semi-quantitatively measured using analysis of intensity plot profile in ImageJ and normalized to the intensity in the bottom layer.

4.3.6 Finite element modeling

The FEA software package ABAQUS (Simulia) was used to conduct simulations. Finite element discretization was carried out using 8-node axisymmetric hybrid elements (CAX8H) using a relatively fine mesh of 5000 elements. Because the model describes behavior in a relatively small stretch regime, Gaussian chain statistics are applicable\textsuperscript{52}. From experimental results, it was assumed the relative heights of the cylindrical hydrogel layers for the top, bottom, and interface layers were 2, 2, and 1 mm, respectively. Strain energy models were used to capture the stress–strain behavior for hyperelastic materials such as the hydrogels studied in this work. The neo-Hookean model was chosen because
it provided the best fit with experimental data and required only one fitting parameter. The strain energy ($U_{NH}$) for the neo-Hookean material model is given by:

$$U_{NH} = \frac{\mu}{2} (I_1 - 3)$$

Equation 1

where $\mu$ is the initial rubbery shear modulus and $I_1$ is the first strain invariant of the deviatoric, isochoric left Cauchy–Green strain tensor. The rubbery shear modulus used in the simulations for the top and bottom layer of the multi-layered hydrogel were estimated from the compressive modulus and assumed to be 16 and 115 kPa, respectively. The layers were assumed to be incompressible. The shear modulus for the interface was determined through curve fitting of the multi-layered experimental results and determined to be 33 kPa. The modeling results are reported as the axial logarithmic strain.

4.3.7 hMSC cell culture

Adult hMSCs from a single donor (24 year old female) were purchased from Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine and expanded in basal stem cell medium (20% fetal bovine serum (FBS, Atlanta Biologicals), 1 mg mL$^{-1}$ amphotericin B, 50 U mL$^{-1}$ penicillin, 50 mg mL$^{-1}$ streptomycin, and 20 mg mL$^{-1}$ gentamicin in low glucose Dulbecco’s modified Eagle medium (aMEM, Invitrogen) containing 1 g L$^{-1}$ glucose). The cells were grown under standard cell culture conditions (in a regulated incubator at 37 °C with 5% CO$_2$) and were plated at 3000 cells cm$^{-2}$ in T-275 tissue culture polystyrene flasks. Medium was changed twice weekly and cells were cultured in basal medium to 80% confluency until Passage 3.
4.3.8 Cell encapsulation

hMSCs were combined at a cell concentration of $10 \times 10^6$ cells mL$^{-1}$ with either a 10% (g/g) macromer (90:10 PEGDM:PEGDM/ChSMA) with 0.1 mM acryloyl-PEG-RGD macromer solution or a with a 30% (g/g) PEGDM solution containing 10 mM acryloyl-PEG-RGD and 0.05% (g/g) photoinitiator Irgacure 2959. The macromers and initiator were dissolved in defined osteochondral differentiation medium (OCDM). OCDM included 1 ml/100 ml media ITS + Premix (BD), 100 nM dexamethasone, 2.5 ng mL$^{-1}$ TGF-β3 (R&D Systems), 50 mg ml$^{-1}$ l-ascorbic acid 2-phosphate trisodium salt, 100 mg ml$^{-1}$ sodium pyruvate, 20 mM β-glycerophosphate, 1 mg mL$^{-1}$ amphotericin B, 50 U mL$^{-1}$ penicillin, 50 mg mL$^{-1}$ streptomycin, and 20 mg mL$^{-1}$ gentamicin in high glucose Dulbecco’s modified Eagle medium (DMEM, Invitrogen) containing 4.5 g L$^{-1}$ glucose, which contains the factors commonly present in both chondrogenic and osteogenic media $^{53}$. All monomer solutions were sterilized using a 0.2 μm sterile filter prior to combining with cells. A multi-layered hydrogel was produced as described above for the acellular hydrogel fabrication.

4.3.9 Cell straining

hMSC deformation was assessed for each of the two layers as well as the resulting interface. Cells were pretreated with Cell Tracker™ Green CMFDA (Invitrogen) per manufacturer and encapsulated as described above. Hydrogels with cubic dimensions of $5 \times 5 \times 5$ mm were required for the custom-built cell straining device, which is similar to that described by Knight et al. $^{54}$. Briefly, pre-swollen and cell-laden hydrogels were placed between two horizontal plates in the rig with one surface of the cube resting flat against a cover slip. In the horizontal direction, the hydrogel was subjected to a
compressive strain of 0, 7.5, or 15% strain and held while live cells were imaged in the hydrogel. Three samples were analyzed, and three areas within each layer (top, bottom, and interface of the multi-layered hydrogel) were visualized for \( n = \sim 30\)–45 cells/layer. Because the PEG hydrogels experience little stress relaxation\textsuperscript{54,55}, no changes in cell morphology were observed over the course of the experiment\textsuperscript{55}. Cellular deformation under strains less than 5% were not detectable due to the inherent variations in cellular diameter within the PEG hydrogels formed from PEGDM macromers\textsuperscript{55}.

4.3.10 Mechanical loading

A custom-built bioreactor system, as described elsewhere\textsuperscript{56,57} was utilized to apply intermittent dynamic compressive strains to hMSC-laden multi-layered hydrogels. In this experiment, cylindrical hydrogels (5 mm diameter \( \times \) 5 mm height) were used. The hydrogels were cultured under free swelling conditions for the first week and then subjected to an intermittent dynamic loading regime applied from 0% to 2.5% amplitude strain in a sinusoidal waveform at a frequency of 1 Hz (1 h on, 23 h off) in the second and third week. This amplitude strain was selected based on recent observations that 5% amplitude strains applied at 1 Hz stimulates chondrogenesis of hMSCs\textsuperscript{58}, while higher strains and lower frequencies have a negative effect on chondrogenesis\textsuperscript{53,59}. Hydrogels were cultured in individual wells with 2 ml per well of OCDM, which was changed daily for the duration of the study. Free swelling hydrogels \((n = 4)\) and loaded hydrogels \((n = 4)\) were removed at 14 and 21 days immediately following a complete 1 h loading cycle for analysis. Cell viability was assessed by the LIVE/DEAD® assay based on Calcein AM, which stains the cytosol of live cells, and ethidium homodimer, which enters cells with compromised membranes, and stains DNA.
4.3.11 (Immuno)histochemistry

Hydrogels were fixed overnight in 4% paraformaldehyde and transferred to a 15% sucrose solution for storage ($n = 4$). Constructs went through a series of dehydration steps and were embedded in paraffin. Sections (10 μm) were stained for the presence of collagens I, II, and X by immunohistochemistry or for mineral deposition by von Kossa following standard protocols. For immunohistochemistry, pretreatments included 1 mg ml$^{-1}$ pepsin for collagen I, 2000 U ml$^{-1}$ hyaluronidase for collagen II, and 1 mg ml$^{-1}$ protease followed by 1 mg ml$^{-1}$ pepsin for collagen X. After permeabilization and blocking, samples were treated overnight at 4 °C with primary antibody: 1:50 anti-collagen I (Abcam), 1:50 anti-collagen II (Abcam), and 1:50 anti-collagen X (Developmental Studies Hybridoma Bank) in blocking solution. Constructs were treated for 2 h with goat anti-mouse IgG labeled Alex Fluor 488 (1:200) and counterstained with DAPI. Sections were imaged by confocal microscopy at 400× magnification. Semi-quantitative analysis of each image was performed using ImageJ. Cells (identified by positive staining for nuclei) that stained positive for one of the proteins were manually selected across the entire image and the ImageJ cell counting add-on was used to determine the total number of positive cells per image. Total number of cells was determined by counting nuclei that were counterstained with DAPI in the corresponding image. Three to four images were analyzed per sample and four independent hydrogel samples were used per condition.

4.3.12 Statistical analysis

Data are reported as the mean with standard deviation in parenthesis in the text or as error bars in the text unless stated otherwise. Statistical differences between
experimental variables were assessed by statistical analysis using a One Way ANOVA with a Tukey HSD Post Hoc test. \( p \) values <0.05 were considered significant.

4.4 Results

4.4.1 Multi-layer hydrogel fabrication and characterization

Initially multi-layered hydrogels with spatially controlled crosslinked structures were investigated. Hydrogels were fabricated by a simple sequential photopolymerization of two macromolecular monomer (macromer) solutions of 10% (g/g) PEGDM and 30% (g/g) PEGDM to produce layered hydrogels whereby the base chemistry remained constant but the structure, via crosslinking density, varied. To create an interfacial region, the hydrogels were fabricated by partially polymerizing the bottom, more stiff layer (30% PEGDM) for various times followed by the addition of the second monomer solution (10% PEGDM) and a second polymerization step. This process yielded a middle layer as a result of mixing between the top and bottom layers and was defined as the interfacial layer. The general process is shown (Figure 1).

![Schematic of the process for fabricating multi-layer hydrogel through a sequential photopolymerization process. The bottom layer consisted of the 30% macromere solution. The top layer consisted of the 10% macromer solution.](image)

**Figure 4.1.** Schematic of the process for fabricating multi-layer hydrogel through a sequential photopolymerization process. The bottom layer consisted of the 30% macromere solution. The top layer consisted of the 10% macromer solution.
Photorheometry was performed to follow the gelation kinetics of the 30% PEGDM solution (Fig. 2A). The gelation point, referred to as the crossover point between the storage (G’) and loss (G”) modulus, occurred after 84(4) s of exposure to UV light. A steep rise in G’ occurred as the polymerization continued and by ∼500 s it stabilized indicating complete polymerization. G” increased only slightly at the gelation point and was ∼4 orders of magnitude lower than G’. Based on these observations, polymerization times for the bottom layer in Fig. 1 were selected to capture times associated with pre- (0 and 1 min), early (2.5 min), middle (5 min), late (7.5 min) and complete (10 min) gelation. Qualitatively, a continuous interfacial region formed with polymerization times corresponding to pre-gelation (Fig. 2B&E, C&F). For polymerization times that corresponded to after the onset of gelation, the formation of a more distinct boundary between the top and bottom layers became apparent (Fig. 2D&G, H&K) and was even more distinct with longer polymerization times (Fig. 2I&L, J&M). Semi-quantitative analysis of the interface revealed interface thicknesses that ranged from 2.7 to 0.5 mm or ∼45% to ∼10% of the total hydrogel height with increasing polymerization times for the bottom layer (Fig. 2N).
Figure 4.2. Characterization of multi-layered hydrogel. (A) Storage (G′) and loss (G″) modulus as a function of time during photopolymerization of 30% (g/g) PEGDM by photorheometry. The light (365 nm) was turned on at 0 s. The gel point, defined as the crossover of G′ and G″, is shown and occurred at 84(4) s. (B–M) Representative microscopy images of dually fluorescent multi-layered hydrogels (green, top; red, bottom) and the corresponding normalized fluorescence intensity plots. The hydrogels were fabricated using different polymerization times for the bottom layer (red): 0 min (B, E); 1 min (C, F); 2.5 min (D, G); 5 min (H, K); 7.5 min (I, L); and 10 min (J, M), with the top layer polymerization time held constant at 10 min. Original magnification is 25×. Scale bar = 1 mm. (N) Quantitative assessment of the interface thickness measured by the overlapping fluorescence intensities between the top and bottom layers as a function of polymerization time of the bottom layer.

One polymerization scheme was selected to investigate spatial control of both biochemical composition and mechanical properties in different layers. The formulation, bottom layer, polymerization time: 1 min, and top layer, polymerization time: 10 min (Fig. 2B), was chosen for subsequent experiments, because it yielded a measurable interface (~1 mm) that enabled its characterization and produced a mechanically robust interface to support repetitive mechanical loads. The local biochemical composition was varied by introducing different ECM analogs into each monomer solution. The bottom
layer was fabricated by co-polymerizing 30% PEGDM macromers with 10 mM RGD for the bone-like layer, while the top layer was fabricated by co-polymerizing 9% PEGDM with 1% ChSMA and 0.1 mM RGD for the cartilage-like layer. The interfacial layer then became a combination of RGD and chondroitin sulfate.

The axial spatial distribution of the chemical composition was probed for ChS and RGD (Fig. 3). Gross examination of the hydrogel stained with toluidine blue for glycosaminoglycans shows strong straining in the top layer with only background staining in the bottom layer (Fig. 3A). The amount of ChS was also determined in serial sections by measuring its concentration through a quantitative spectroscopy based assay. The highest concentration of ChS was measured in the top ~40% of the hydrogel, and it decreased rapidly beyond the interface layer with little to no ChS detected in the bottom ~20% of the hydrogel (Fig. 2B). The spatial distribution of RGD was assessed by imaging for a tethered fluorescently labeled RGD. Gross examination of the hydrogel shows strong fluorescence in the bottom layer along with an intermediate region of fluorescence and faint fluorescence in the top layer (Fig. 3C). The intensity was quantified confirming the highest concentration of RGD in the bottom layer, with less than 10% of that fluorescence intensity detected in the top layer (Fig. 3D). There was a steep decline in the intensity across the interfacial layer.
Figure 4.3. Characterization of biomimetic moieties in the multilayered hydrogel. (A) Representative image of a multi-layered hydrogel grossly stained with toluidine blue for glycosaminoglycans. There is a slight blue background staining by PEG in the bottom layer. Scale bar = 1 mm. (B) Biochemical analysis of ChS concentration ([ChS]) from pooled sections of a multi-layer hydrogel as a function of axial distance from top to bottom (*p < 0.05). (C) Representative image of a multi-layered hydrogel with fluorescently (green) labeled RGD. Scale bar = 1 mm. (D) Semi-quantitative fluorescence intensity measurements of a multi-layered hydrogel as a function of axial distance. Data are presented as mean with standard deviation as error bars for n = 3. Hydrogels were fabricated following the polymerization scheme of Fig. 2B, E with bottom layer, polymerization time of 1 min.

The mechanical properties of the multi-layered hydrogel were characterized by the unconfined compressive modulus (Table 1). Individual hydrogels were formed from 10% and 30% PEGDM resulting in an average compressive modulus of 48 and 345 kPa, respectively. When a multi-layered hydrogel was formed from these two formulations using the selected fabrication scheme (bottom layer 1 min, and top layer, polymerization time: 10 min) the resulting modulus was 90 kPa. The addition of 1% ChS did not
significantly alter the mechanical properties of the top layer, resulting in a compressive modulus of 53 ± 8 kPa.

Table 1. Unconfined compressive modulus for single and multi-layered hydrogels.

<table>
<thead>
<tr>
<th>Compressive modulus (kPa)</th>
<th>Top layer</th>
<th>Interfacial layer</th>
<th>Bottom layer</th>
<th>Multi-layered hydrogel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top layer</td>
<td>48 (6)</td>
<td>~100</td>
<td>345 (35)</td>
<td>90 (22)</td>
</tr>
</tbody>
</table>

4.4.2 Hydrogel and cellular deformations under a compressive strain

A FE model was employed to predict the local strain profile produced within the multi-layered hydrogel when subjected to an unconfined apparent compressive strain. The model assumed three distinct regions each with a homogeneous modulus. The moduli for the top and bottom layers were taken from experimental measurements of the individual components of their respective layer (Table 1). The modulus of the interfacial layer was approximated through curve fitting the overall multi-layer hydrogel modulus data and estimated to be ~100 kPa (Table 1). Using this model, strain profiles were generated for two scenarios, the application of a low (i.e., 7.5%) and a high (i.e., 15%) unconfined compressive strain applied along the cylindrical axis. The strain profiles, presented in Fig. 4A, illustrate that the local strain decreases from top to bottom, with the majority of the applied strain being transferred to the top layer and to a lesser extent the interface with the stiffer bottom layer experiencing little deformation. When comparing the two apparent strains, it is evident that the smaller strain produces more uniform radial and
axial strain profiles. Under the higher strain, the hydrogel undergoes greater lateral expansion in the top and interfacial layers. The axial strains penetrate farther into the interfacial region at the periphery of the hydrogel while the center of the hydrogel resists lateral expansion. As a result, the center of the hydrogel experiences greater axial strains compared to the periphery.
**Figure 4.4. Hydrogel and cellular deformation under compressive strain.** (A) Results from finite elemental (FE) models describing the axial true strain for a multi-layered hydrogel subjected to a 7.5% apparent strain under unconfined compression and 15% apparent strain under unconfined compression. Negative values correspond to compressive strain. (B) Representative confocal microscopy images of a single hMSC (cytosol stained green) encapsulated in multi-layer hydrogels within the top layer, interfacial layer, and bottom layer. The hydrogel was subjected to no strain (0%), 7.5% compressive strain, or 15% compressive strain. Original magnification is 200×. Scale bar = 5 μm. (C) Deformation of hMSCs was quantitatively assessed by a diameter ratio when the multi-layer hydrogels were subjected to 0% (black), 7.5% (white), and 15% (gray) compressive strains. A diameter ratio of one indicates a perfectly round cell with no deformation and a diameter ratio of less than one indicates cell deformation. Data are presented as mean with standard error of the mean as error bars (n = ~90–135) and * indicates p < 0.05 within a layer, # indicates p < 0.05 relative to bottom layer, and & indicates p < 0.1 relative to interface. (D) Line plots for the experimentally determined diameter ratio (closed circles) and the strain predicted by FE model (open circles) for each layer in the multi-layered hydrogel as a function of the nominal strain. Hydrogels were fabricated following the polymerization scheme of Fig. 2B, E with bottom layer, polymerization time of 1 min.

hMSCs were encapsulated in the multi-layered hydrogels and subjected to an apparent compressive strain. Using a custom cell straining device in combination with confocal microscopy, changes in cell morphology were investigated as a measure of cell deformation (Fig. 4B&C). Representative images of cells in each of the three layers are shown in Fig. 4B for no strain (0%) and for low (i.e., 7.5%) and high (i.e., 15%) applied strains. Cell deformation was quantified from confocal microscopy images taken of each cell at full width half maximum diameter for each strain and reported as a diameter ratio \((x/y)\) (Fig. 4C). The x value is the cell diameter in the axis parallel to the applied strain and the y value is the cell diameter in the axis perpendicular to the applied strain. The application of a compressive strain to the hydrogel led to a general change in cell morphology from rounded to an ellipsoidal shape under strain in all layers. However, the degree to which the cells deformed depended on the magnitude of the strain and the location of the cell, i.e., the layer within the hydrogel. Qualitative analysis from confocal
microscopy images revealed that cells in the top least stiff layer of the hydrogel underwent the most dramatic morphological change with higher apparent strains, which was confirmed by the smallest diameter ratio. In contrast, changes in cell morphology in the bottom (stiffest) layer were not substantial under the lower strains but were observable under the higher strains with a significantly lower diameter ratio. Cells in the interfacial layer exhibited cell deformation, but to a higher degree than those in the stiffer bottom layer. For small strains (i.e., 2.5%), it was not possible to evaluate cell deformation due to the heterogeneity in cell morphology for cells encapsulated in the hydrogels.

Cell deformation results were compared to the FE model analysis by plotting diameter ratios alongside the average axial strain predicted from the FE model, where the average strain was determined from the central axis region of each layer. Experimental and simulation data display similar trends with increasing apparent strain (Fig. 4D). It is important to note that one limitation with directly comparing the trends predicted by the FE model to those in the cell straining device is that the shapes of the hydrogel are different, cylindrical (in the FE model) versus square (in the cell deformation studies). However to minimize edge effects, cells were imaged in the central region of the hydrogel and strains were also determined in the central region of the hydrogel.

4.4.3 hMSC differentiation in multi-layered hydrogels under free swelling and dynamic loading conditions

Undifferentiated hMSCs were encapsulated in multi-layered hydrogels with compositional and stiffness variations. The hydrogels were fabricated as described above following the polymerization scheme in Fig. 2B and the compositional make-up described in Fig. 3. The cell-laden multi-layered hydrogels were cultured in osteochondral media
containing both chondrogenic and osteogenic differentiation factors under free swelling conditions for the first week to initiate differentiation. The hydrogels were either continued in free swelling cultures or subjected to intermittent, unconfined, and dynamic compressive strains for the following two weeks. Viable cells were confirmed by a membrane integrity assay after the 21 day experiment (Fig. S1). Samples were assessed by immunohistochemistry for collagens type I, II, and X at days 14 and 21 and by semi-quantitative analysis of confocal microscopy images at day 21 for the fraction of positively stained cells for each collagen type and qualitatively for mineral deposition. Day 14 results are shown in supplemental figures (Fig. S2) and day 21 results are shown in Fig. 5.
Figure 4.5. hMSC differentiation in a multi-layered hydrogel. (A) Immunohistochemical images of protein expression by hMSCs encapsulated in multi-layered hydrogels and cultured under free swelling or dynamic loading conditions after 21 days. Green stains for protein, blue stains for nuclei (DAPI). Representative confocal microscopy images
from \( n = 4 \) hydrogels over several (\( \sim 3–5 \)) sections. Original magnification is 400×. Scale bar is 20 \( \mu \)m. (B) Semi-quantitative immunohistochemical analysis of the fraction of hMSCs staining positive for protein expression when encapsulated in multi-layered hydrogels. The top row describes the fraction of positively stained cells for each layer when cultured under free swelling after 21 days. The bottom row describes the fraction of positively stained cells for each layer when subjected to intermittent dynamic loading after 21 days. Data are from \( n = 4 \) hydrogels over several (\( \sim 3–5 \)) sections and are mean with standard deviation as error bars and \( ^* p < 0.05 \). (C) Histological images of von Kossa stained sections showing mineral deposits in black. Scale bar is 200 \( \mu \)m.

Under free swelling conditions at day 21, all three collagen types were detected in each layer. Collagen X was the most abundant protein detected, evidence by greater staining around the encapsulated cells. Approximately 60% of the encapsulated cells stained positive for collagen I and 60% for collagen X in all three layers. There was a similar fraction of cells (\( \sim 60\% \)) that stained positive for collagen II in the top and interfacial layers, but only 30% of the cells stained for collagen II in the bottom layer. After 21 days of dynamic compressive strains applied at 1 Hz in a sinusoidal waveform from 0% to 2.5% strain, there were no signs of mechanical failure. Collagen X deposition was dramatically reduced by loading in all layers with fewer cells staining positive for collagen X in the top and bottom layers. In the top layer under loading, nearly 90% of the cells stained positive for collagen II, but only \( \sim 30\% \) stained positive for collagen I and \( \sim 10\% \) for collagen X. In the bottom layer under loading, 80% of the cells stained positive for collagen I with only 2% staining positive for collagen II and \( \sim 30\% \) for collagen X. In the interfacial region under loading, \( \sim 50\% \) of the cells stained positive for collagen X and similarly 50% stained positive for collagen I, but only 30% for collagen II.

Under free swelling conditions, mineral deposition around the cells was evident throughout the multi-layer hydrogel. There appeared to be increased staining in the bottom layer. Under dynamic compressive strains, there was no observable staining for
mineral deposition in the top layer. However, there appeared to be a gradual increase in mineral deposition across the interface and in the bottom layer.

4.5 Discussion

A multi-layer PEG-based hydrogel was developed that combined spatially presented ECM analogs and varying hydrogel stiffness, such that under a mechanical load distinct biochemical and mechanical cues were generated within each layer to mimic aspects of cartilage, bone, and the interface. Most significantly, this work demonstrates that when undifferentiated hMSCs were encapsulated in the multi-layer hydrogel, dynamic mechanical loading was necessary to differentially direct differentiation within each layer. This study points toward the importance of dynamic mechanical cues for hMSC-driven osteochondral tissue engineering.

To create a hydrogel environment that captured two distinct tissue niches, a compliant cartilage-like layer and a stiffer bone-like layer, separated by an interfacial region, a simple sequential polymerization method was developed. The thickness of the interfacial layer was controlled by manipulating the polymerization time and hence the state of gelation for the bottom layer during fabrication. The type of interface formed varied from gradual, when the gelation point of the bottom layer had not yet been reached prior to applying the top layer, to distinct interfaces, after the onset of gelation had occurred in the bottom layer prior to applying the top layer. While we chose a relatively thick interfacial layer to facilitate characterization of the interface, this method can be used to produce a thin and more abrupt interface that is characteristic of the native osteochondral interface. The biochemical composition of the multi-layer hydrogels was also controlled through the amount of functionalized ECM analogs that were covalently incorporated into each layer.
during the polymerization process. This sequential polymerization method produced an interfacial layer that comprised an intermediate composition of ECM cues and hydrogel stiffness and in some ways, similar to calcified cartilage, which is comprised of a combination of cartilage and bone ECM molecules.

While the mechanical properties (i.e., compressive modulus) of the materials in the multi-layer hydrogels did not match the mechanical properties of native cartilage and bone, the differential strain levels were similar to native osteochondral tissues under physiological loads. Under large 15% apparent strains applied to the hydrogel, the local strains in the cartilage-like layer were ~30%, while in the bone-like layer were ~4%. Under the smaller 7.5% apparent strain, the local strains in the cartilage-like layer were ~15%, while in the bone-like layer were ~2%. In native osteochondral tissue, articular cartilage experiences strains typically of 2–9%\textsuperscript{60}, but as high as 30% can be generated\textsuperscript{61,62}. The underlying bone, however, experiences very little strain, typically less than 1%\textsuperscript{63}. While local strains in the interfacial region of the native osteochondral tissue are less well characterized, the Young’s modulus has been estimated to be ~10-fold lower than that of the subchondral bone\textsuperscript{64} and atomic force microscopy measurements confirm an intermediate modulus\textsuperscript{4,64} supporting the idea that the strains will be in between cartilage and bone. During healing, strains are often larger than those that exist normally in the native tissue where, for example in fracture healing, ~5% strains have been shown to lead to bone formation, while higher strains can preferentially lead to cartilage formation\textsuperscript{65,66}. While optimal strains remain to be determined for osteochondral tissue engineering, we confirm that the local modulus controls the local strains generated within
each layer and that the strains generated in the hydrogel, as determined through FE model analysis, are translated into cellular strain.

The ability to use undifferentiated human adult MSCs in a simple multi-layer hydrogel with controlled presentation of biochemical and mechanical cues was investigated. Differentiation was probed after 21 days through collagen protein expression since collagen type is the main defining phenotype for hyaline (i.e., collagen II) and calcified cartilage (i.e., collagen X) and is typically expressed in the later stages of chondrogenesis. Mineral deposition was also probed as a marker for osteogenesis. In all formulations, collagen expression was limited to intracellular or pericellular spaces, which is expected due to the early stages of differentiation and the use of stable hydrogels, which restricts ECM diffusion to the pericellular space. Interestingly under free swelling conditions, collagens I, II and X and mineral deposits were present in all three layers with the most abundant collagen being type X. Collagen X is one of the primary molecules upregulated during terminal differentiation of MSCs, which can lead to MSC hypertrophy and eventually to bone formation through endochondral ossification. The presence of mineral deposits supports the idea that the hMSCs are undergoing hypertrophy within the hydrogels regardless of the local cues. The culture medium contained both chondrogenic and osteogenic factors, which may have contributed to the large presence of collagen X and mineral deposits throughout the hydrogel. Studies have shown that TGF-β3, which is commonly used for chondrogenesis, can promote bone formation via endochondral ossification in the presence of other growth factors. Under free swelling culture conditions, the main difference in collagen expression was that fewer cells expressed collagen II in the bone-like layer. This finding
may in part be attributed to a more restricted diffusion of TGF-β3, the primary chondrogenic factor in the culture medium, into the bone-like layer due to its higher crosslink density. There were no significant differences in collagen protein expression between the cartilage and interfacial layers suggesting that the differences in the concentration of the ECM analogs and stiffness were not sufficient to alter differentiation.

On the contrary under dynamic loading, a more defined and directed differentiation was observed. Interestingly, loading led to a dramatic down-regulation in collagen X deposition regardless of the layer, suggesting that collagen X may be highly sensitive to dynamic mechanical loading. However, the role of loading on regulating collagen X has not yet been established. It is also possible that this response may involve cross-talk between cells from different layers that evolved over the culture period. In the top cartilage-like layer, the majority of the cells, ~90%, expressed collagen II, which is the defining marker for chondrogenic differentiation. The low expressions of collagen X, ~10%, and collagen I, ~30%, along with no observable mineral deposits support the idea that the cues generated in the top layer favored a more mature chondrogenetic phenotype. In the interfacial layer, there were 50% collagen X expressing cells along with 50% collagen I and 30% collagen II expressing cells and evidence of mineral deposits, which is more characteristic of a hypertrophic chondrogenic phenotype. In the bottom bone-like layer, the majority of the cells, ~80%, expressed collagen I with only 30% collagen X and 2% collagen II expressing cells, which along with the presence of mineral deposits is characteristic of osteogenic differentiation.

Findings from this study strongly point to the idea that mechanical cues arising from dynamic mechanical stimulation are stronger regulators of hMSC differentiation
within the multi-layer PEG hydrogel compared to the cues arising from the ECM analogs alone or from stiffness. The dynamic mechanical cues may arise from differences in cellular strain, differences due to the number of cellular adhesion ligands that are engaged, which may alter the signals received by the cells under dynamic loading, and/or from dynamic oscillations in osmolarity, which result from the fixed negative charges of chondroitin sulfate. In the former, the cellular strains based on the FE model are assumed to be ~5% in the top layer, 2.5% in the interfacial layer, and <1% in the bone-like layer, which are within the physiological ranges especially for cartilage. In the latter, oscillations in osmolarity will depend on the concentration of fixed charges and on the fluid flow induced by loading, both of which will differ between the cartilage and interfacial layers. In support of the latter, chondrocytes encapsulated in hydrogels and subjected to dynamic compressive loading were reported to be more responsive to dynamic oscillations in osmolarity resulting from a charged hydrogel than to the mechanical cues arising from a neutral hydrogel. It is important to note that the role of dynamic loading in the absence of biochemical cues on hMSC chondrogenesis was not evaluated, but studies with cartilage cells have shown improved response to dynamic loading when biochemical cues are present.

Interestingly, dynamic loading also had an effect on hMSCs in the bottom layer. While cellular strains are expected to be minimal, it is likely that the bottom layer experienced low levels of fluid flow. Studies have shown that very low levels of shear stress, correlating to that of interstitial flow in bone under loading, enhance hMSC osteogenesis. Furthermore, the round cellular morphology supported osteogenesis, which has been shown by others. It therefore possible that the combination of the
integrin-ligand interactions that sense the stiff hydrogel combined with interstitial flow may have contributed to the hMSC response under loading in the bottom layer. While the exact mechanisms remain to be elucidated, we hypothesize that a combination of extracellular signals from the dynamic loading and ECM analogs contributed to the observed differential response in hMSC differentiation with loading.

Overall, this study demonstrates that starting with undifferentiated hMSCs in a single differentiation medium cocktail, it is possible to direct hMSC differentiation within each layer. Other studies have shown the need to pre-differentiate cells in one layer prior to embedding in a bi-layer hydrogel to enable differential differentiation and ECM synthesis for osteochondral tissue engineering. Our findings suggest that the introduction of mechanical stimulation may be the critical link to enabling differential differentiation of MSCs without the need for pre-differentiation strategies. Furthermore, this simple sequential polymerization method can be easily translated to *in situ* delivery of MSCs within an osteochondral defect. There are, however, several limitations of this study, which are important to note. Assessment of differentiation was limited to short culture times of 21 days in non-degrading hydrogels. While this is sufficient to initiate differentiation, this platform and short culture times are not sufficient to support ECM secretion, deposition and the formation of a macroscopic tissue. Longer culture times and a degradable hydrogel will be necessary to confirm the effects of mechanical stimulation on not only hMSC differentiation, as investigated in this study, but on hMSC-mediated osteochondral tissue growth. Assessment of differentiation was limited to (immuno)histochemical analysis for collagen type and mineralization. While these are key
markers of differentiating hMSCs into osteochondral cellular phenotypes, further analysis is needed to fully characterize differentiation of the hMSCs.

4.6 Conclusions

This study describes the development and characterization of a multi-layered biomimetic hydrogel for osteochondral tissue engineering applications. Spatially controlling the local biochemical and mechanical properties gave rise to characteristically different levels of cell deformation under an applied apparent strain and to characteristically different collagen expressions and mineral deposition by hMSCs under free swelling and dynamic loading culture conditions. Interestingly, the incorporation of biomolecules and variations in substrate stiffness had minimal effect on the differentiation of hMSCs. However, the addition of mechanical stimulation was critical to spatially directing differentiation. Most exciting was that hMSC differentiation, based on collagen expression and mineral deposition, could be directed toward the desired differentiation fates for hyaline cartilage, calcified cartilage in the interface, and bone. Long-term studies, however, will require the incorporation of degradable crosslinks to facilitate macroscopic tissue elaboration and are needed to determine whether differentiation fate is maintained and maturation occurs. Nonetheless, this study points toward the importance of a dynamic mechanical environment in directing hMSC differentiation for osteochondral tissue engineering.
4.7 Supplementary Figures

**Figure 4.S1.** hMSC viability in a multi-layered hydrogel. Representative images of the live (green) and dead (red) cells in the top and bottom layers of the multi-layered hydrogels after 21 days of culture under free swelling and loading (scale bar = 100 µm).

**Figure 4.S2.** Collagen expression in the multi-layered hydrogel. Representative images of the collagen type I, II, and X expression in the top, interfacial, and bottom layers of the hydrogels under free swelling and loading conditions.
Figure 4.S2. Immunohistochemical images of protein expression by hMSCs encapsulated in multi-layered hydrogels and cultured under free swelling or dynamic loading conditions after 21 days. Green stains for protein, blue stains for nuclei (DAPI). Representative confocal microscopy images from $n = 4$ hydrogels over several (~3–5) sections. Original magnification is 400×. Scale bar is 20 μm.

4.8 Acknowledgments

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4.9 References


Chapter 5
An MMP7 sensitive biomimetic hydrogel for engineering human cartilage by MSCs

5.1 Abstract
Cartilage tissue engineering strategies that use in situ forming degradable hydrogels for mesenchymal stem cell (MSC) delivery are promising for treating chondral defects. Hydrogels that recapitulate aspects of the native tissue have the potential to encourage chondrogenesis, permit cellular mediated degradation, and facilitate tissue growth. This study investigated photoclickable poly(ethylene glycol) (PEG) hydrogels, which were tailored to mimic the cartilage microenvironment by incorporating extracellular matrix analogs, chondroitin sulfate and RGD, and crosslinks sensitive to matrix metalloproteinase 7 (MMP7). Human MSCs were encapsulated in the hydrogel, cultured up to nine weeks, and assessed by mRNA expression, protein production and biochemical analysis. Chondrogenic genes, SOX9, ACAN, and COL2A1, significantly increased with culture time, and the ratios of COL2A1:COL10A1 and SOX9:RUNX2 reached values of ~20-100 by week six. The encapsulated MSCs degraded the hydrogel, which was nearly undetectable by week nine. There was substantial deposition of aggrecan and collagen II, which correlated with degradation of the hydrogel. Minimal collagen X was detectable, but collagen I was prevalent. After week one, extracellular matrix elaboration was accompanied by a ~two-fold increase in compressive modulus with culture time. The MMP7-sensitive cartilage mimetic hydrogel supported MSC chondrogenesis and promoted macroscopic neo-cartilaginous matrix elaboration representative of fibrocartilage.
5.2 Introduction

Cartilage repair is a significant clinical challenge due to its limited self-healing capacity\(^1\),\(^2\). Autologous chondrocyte implantation (ACI) is a clinically available cell-based therapy for the treatment of articular chondral defects. In ACI, autologous chondrocytes are harvested from a non-loading bearing region of the joint, expanded and injected into the defect covered by a membrane\(^3\). ACI has shown some success\(^4\); however, long-term, randomized clinical trials indicate that ACI does not outperform microfracture\(^5\). Moreover, several limitations associated with ACI include donor site morbidity, limited number of cells that can be harvested, and de-differentiation of the chondrocytes during expansion\(^6\),\(^7\). An alternative cell source for ACI is mesenchymal stem cells (MSCs), which eliminate donor site morbidity, have a higher proliferation capacity, and undergo chondrogenesis\(^8\),\(^9\),\(^10\). Although promising, MSCs introduce several challenges\(^11\). Most notably, MSCs have an intrinsic differentiation profile to undergo hypertrophy during chondrogenic differentiation\(^12\),\(^13\),\(^14\), which is a precursor to endochondral ossification and osteoarthritis. In addition, robust cartilage regeneration by MSCs has not yet been demonstrated. Thus, a better understanding of the factors that control MSC chondrogenesis and cartilage regeneration is needed for their effective use in repairing focal chondral defects.

Delivering cells *in situ* within a three-dimensional (3D) matrix, such as a synthetic-based hydrogel, provides cells with structural support and as well creates the opportunity to introduce biochemical cues into the matrix to encourage differentiation. The extracellular matrix (ECM) of cartilage is comprised predominantly of collagen type II and aggrecan. Aggrecan is a proteoglycan made from sulfated glycosaminoglycans (GAGs)
(e.g., chondroitin sulfate) and is linked by hyaluronic acid to create large aggregan aggregates. On the contrary, hypertrophic cartilage is characterized by collagen type X and mineralization\(^{15}\). A number of studies have shown that incorporating different types of cartilage-derived ECM moieties into an otherwise synthetic hydrogel improves chondrogenesis of MSCs\(^{10},^{16},^{17}\). Importantly, several of these studies have shown that creating a cartilage mimetic environment within the hydrogel suppresses the hypertrophic phenotype of MSCs \(^{18}--^{20}\).

Hydrogels comprised of cartilage’s GAGs, specifically hyaluronic acid and/or chondroitin sulfate, have been extensively studied as a vehicle to encapsulate MSCs and support chondrogenesis. Hyaluronic acid hydrogels\(^{21}--^{26}\) and chondroitin sulfate hydrogel\(^{20}\) support chondrogenesis with the former showing improvements over synthetic hydrogels, such as crosslinked poly(ethylene glycol) (PEG)\(^{27}\). On the other hand, PEG hydrogels have served as a base chemistry to which cartilage ECM moieties are introduced. For example, the addition of covalently linked chondroitin sulfate into a PEG hydrogel led to enhanced chondrogenesis through cartilage-specific gene expression and matrix production when compared to PEG-only hydrogels\(^{20},^{28},^{29}\). Interestingly, chondrogenesis was improved and hypertrophy was suppressed when sulfate groups were introduced into the backbone of hyaluronic acid,\(^{30}\) suggesting an important role of sulfated GAGs.

Incorporating multiple ECM moieties into a hydrogel provides an opportunity to recreate the complexity of the native ECM. For example, when hyaluronic acid hydrogels were formed with either unmodified collagen type I, which introduces cell adhesion sites, or methacrylated chondroitin sulfate, cartilage matrix deposition was improved while
hypertrophic-induced mineralization was reduced when compared to hyaluronic acid-alone hydrogels.\(^{(18),(31)}\) The use of small peptides over full proteins, such as collagen, enables a facile method to controllably incorporate cell adhesion functionalities. For example, PEG hydrogels that combine tethered chondroitin sulfate with the cell adhesion peptide, RGD, supported chondrogenesis of MSCs under culture conditions that did not readily induce chondrogenesis in PEG-only hydrogels\(^{(29)}\). Alternatively, collagen mimetic hydrogels were created with peptides that bind cell-secreted GAGs,\(^{(32),(33)}\) showing enhanced chondrogenic differentiation when compared to hydrogels without peptides\(^{(32),(34)}\)–\(^{(36)}\). Collectively, these and other studies support the idea that biomimetic hydrogels, which introduce cartilage ECM moieties and create environments that are more reminiscent of cartilage, improve MSC chondrogenesis.

Hydrogel degradation is essential to forming a macroscopic engineered tissue and ultimately regenerating cartilage. When cells are encapsulated in a hydrogel, the mesh size of the hydrogel dictates diffusion of newly secreted ECM molecules\(^{(37)}\). One of the challenges is that the mesh size is smaller than that of most ECM molecules and notably that of collagen type II and the aggrecan aggregates\(^{(38)}\). As a result, the hydrogel must reach its reverse gelation point prior to the formation of a macroscopic tissue\(^{(38),(39)}\). Moreover, when using MSCs, chondrogenesis must occur prior to substantial degradation and then the rate of degradation must reasonably match ECM synthesis. Hyaluronic acid hydrogels have been designed with hydrolytically labile linkers enabling degradation by water and enzymes\(^{(40),(41)}\). The incorporation of hydrolytically labile linkers led to improved collagen II and chondroitin sulfate deposition over enzyme-only degradable hydrogels, but collagen II remained limited to the pericellular space\(^{(40),(41)}\). Crosslinks sensitive to cell
secreted matrix metalloproteinases (MMPs) have been introduced into synthetic-based hydrogels\(^{(42)}\). In particular, MMP7 has been investigated because it is upregulated during chondrogenesis\(^{(35),(43)}\). When MSCs were encapsulated in a MMP7-sensitive PEG hydrogel without any additional ECM analogs, macroscopic deposition of collagen II was evident, but the presence of hypertrophic proteins was not evaluated\(^{(43)}\). When a similar MMP7-sensitive crosslinker was incorporated into a collagen-based hydrogel that contained GAG binding peptides and the cell adhesion peptide RGD with MSCs, cartilaginous tissue deposition contained collagen II, but was limited to the pericellular space and was accompanied by collagen I and X, markers of fibrocartilage and hypertrophy, respectively\(^{(35),(36)}\). Although promising, macroscopic neocartilaginous tissue production by encapsulated MSCs has been limited and warrants further investigation.

The goal of this study was to develop an *in situ* forming enzyme-sensitive cartilage mimetic hydrogel for human MSC (hMSC) encapsulation and evaluate its potential for human cartilage tissue engineering. PEG hydrogels formed from the thiol:norbornene photoclick reaction were chosen as the base chemistry due to the mild photopolymerization conditions\(^{(44),(45)}\) and the ease with which thiolated ECM moieties and bis-cysteine crosslinks can be incorporated\(^{(46),(47)}\). ECM moieties of chondroitin sulfate and RGD were chosen, as they have shown enhanced chondrogenesis of MSCs\(^{(29)}\). A MMP7-sensitive peptide crosslinker was chosen given its promise in supporting macroscopic tissue deposition\(^{(43)}\). The hMSC-laden hydrogels were cultured for up to nine weeks and evaluated by mRNA expression, biochemical composition of cartilage ECM, hydrogel degradation, and mechanical properties. Overall, the results from this work indicate that a cartilage mimetic, MMP-7 sensitive PEG hydrogel formed from
a thiol:norbornene photoclick reaction supports chondrogenesis of encapsulated MSCs, promotes formation of a macroscopic neo-cartilage tissue, and suppresses hypertrophy.

5.3 Materials and Methods

5.3.1 Macromer Synthesis
An 8-arm PEG amine (10kDa) reactant was used to synthesize the ‘ene’ monomer, 8-arm PEG norbornene. The PEG amine was dissolved in dimethylformamide (DMF) and reacted with 8x molar excess of 5-norbornene-2-carboxylic acid in the presence of 4 molar excess n,n-diisopropylethylamine (DIEA) and 1-[Bis(dimethylamaino)methylene]-1H-1,2,3-triazolog[4,5-b]pyridinium 3-oxid hexafluorophosphat (HATU) overnight at room temperature under argon. The 8-arm PEG norbornene product was recovered by precipitation in ethyl ether, purified via dialysis for 2-3 days, filtered (0.2µm), and lyophilized. Conjugation of norbornene to each arm of the 8-arm PEG was determined to be ~100% via 1H NMR by comparing the area under the peak for the allylic hydrogen closest to the norbornene hydrocarbon group (δ=3.1-3.2ppm) to the peak of the PEG backbone methyl groups (δ=3.4-3.85ppm).

Thiolated chondroitin sulfate (ChS-SH) was synthesized as described by Shu et al. via a carbodiimide chemistry with thioacid dihydrazide[48]. ChS (Chondroitin sulfate A, Sigma Aldrich) was dissolved in water and reacted with 2x molar excess dithiobis(propanoic dihydrazide) (DTP) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) overnight at an adjusted pH of 4.75 using 1.0M HCl. To stop the reaction, the pH was raised to 7 with the addition of 1.0M NaOH. A 6.5 molar excess of dithiothreitol (DTT) was added and reacted overnight at a pH of 8.5 to reduce the thiol groups of the DTP. The thiolated chondroitin sulfate product (ChS-SH) was purified and
recovered by dialysis against 0.3mM HCl, centrifuged to remove any particulates, and the supernatant lyophilized. Conjugation of the thiol groups to the ChS was found to be ~15% (~7 thiol groups per molecule of ChS) via $^1$HNMR by comparing the area under the peaks for the methylene groups of DTP ($\delta=2.5$-2.6 and 2.6-2.8ppm) to the area under the peak of the methyl protons of the acetyl amine side chain of the chondroitin sulfate backbone ($\delta=1.8$-2.0 ppm).

5.3.2 Human MSC (hMSC) Culture

Human mesenchymal stem cells (26 year old female) were purchased from Texas A&M and expanded in MSC expansion media consisting of 20% fetal bovine serum (FBS, Atlanta Biologicals), 50 U ml$^{-1}$ penicillin, 50 mg ml$^{-1}$ streptomycin, 20 mg ml$^{-1}$ gentamicin, and 5 ng ml$^{-1}$ basic fibroblast growth factor (bFGF) (Invitrogen) in low glucose Dulbecco’s modified Eagle media (DMEM, Invitrogen). The hMSCs were expanded under standard cell conditions (37°C, 5% CO$_2$) to 80% confluency and passaged at 3000 cells cm$^{-2}$. Passage 3 was used.

5.3.3 Cell-laden Hydrogel Preparation

Cartilage biomimetic degradable hydrogels were fabricated via photopolymerization of 9wt% PEG-norbornene (8-arm, 10kDa), 1wt% ChS-SH, 0.1mM CRGDS (Genscript), and 2.5wt% MMP7 sensitive peptide (CRDPLE-LRADRC$^{(43)}$ (Genscript) in the presence of 0.05wt% photoinitiator Irgacure 2959 (I2959) in phosphate buffer saline (PBS) under 352nm light at 5 mW cm$^{-2}$ for 8 minutes. The hMSCs were encapsulated at 50 million cells ml$^{-1}$ of filter-sterilized (0.2 um filter) monomer precursor solution and photopolymerized.
Cell-laden hydrogels (5mm diameter x 2.5mm height) were placed in 24-well tissue culture plates in 2 milliliters of chondrogenic differentiation media (1% ITS+ Premix, 100 nM dexamethasone, 2.5 ng ml\(^{-1}\) TGF-\(\beta3\), 50 µg ml\(^{-1}\) l-ascorbic acid 2-phosphate, 50 U ml\(^{-1}\) penicillin, 50 mg ml\(^{-1}\) streptomycin, and 20 mg ml\(^{-1}\) gentamicin in high glucose Dulbecco's modified Eagle media) which was replaced every other day. The hMSC-laden hydrogels were cultured under standard cell conditions of 37°C with 5% CO\(_2\) up to 9 weeks.

5.3.4 Evaluation of mRNA by qPCR

Prior to encapsulation and at prescribed culture times, hMSC-laden hydrogels (n=3/time point) were removed from culture, homogenized (TissueLyzer II, Qiagen) at 30Hz for 10 minutes in RNA lysis buffer, and RNA was extracted using a MicroElute Total RNA Kit (Omega) per manufacturer instruction. RNA was transcribed to cDNA using a high capacity reverse transcription kit (Applied Biosystems) per manufacturer instruction. Quantitative PCR (qPCR) of chondrogenic genes, \textit{SOX9}, \textit{ACAN}, and \textit{COL2A1} and hypertrophic genes, \textit{RUNX2} and \textit{COL10A1}. Primers for each gene are given in Table 1 along with primary efficiency. The qPCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) and a 7500 Fast Real-time PCR machine (Applied Biosystems). Gene expression data was calculated from delta Ct values using true efficiencies, and relative to the house keeping gene \textit{L30}\textsuperscript{(49)}. Data were also normalized to the expression of the pre-encapsulated MSCs.

\textbf{Table 1.} Primer Sequences and Efficiency for qPCR Analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Efficiency</th>
</tr>
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<tbody>
<tr>
<td>\textit{L30}</td>
<td>5'-TTAGCGGCTGCTGTTGGTT-3'</td>
<td>5'-TCCAGCGACTTTTTGCCTTC-3'</td>
<td>94%</td>
</tr>
</tbody>
</table>
5.3.5 Histological and immunohistochemical analysis

At prescribed culture times, hMSC-laden hydrogels (n=3/time point) were fixed in 4% paraformaldehyde, dehydrated, and paraffin embedded following a protocol using gradual concentration of ethanol to Neoclear to paraffin. Paraffin embedded hydrogels were then sectioned to 10µm using a microtome. Sections were stained with Safranin-O/Fast Green to visualize sulfated glycosaminoglycans (sGAGs) using light microscopy (Ziess Pascal, Olympus DP70). Immunohistochemistry was performed as follows. Sections were pre-treated with appropriate enzyme treatments (hyaluronidase 200U/ml for aggrecan and collagen II, chondroitinase ABC (10mU) and keratinase I (4mU) for aggrecan, pepsin (280kU), protease (400U) and 0.25 %trypsin and EDTA for collagen X) for 1hr at 37 °C as well as antigen retrieval (collagen I, aggrecan). Sections were treated with primary antibodies against aggrecan (1:5), collagen type II (1:50), collagen type X (1:50) and collagen type I (1:50), followed by secondary antibodies with conjugated AlexaFluor 488 or 546 probes and counterstained with DAPI for nucleus detection. A laser scanning confocal microscope (Ziess LSM 5 Pascal) was used to acquire images at 400x magnification. Semiquantitative analysis of representative confocal images (n=4 images per hydrogel, n=3 hydrogels) was performed. Sections were stained simultaneously and the gain adjustment was set and maintained for all images to minimize variations in the
intensity of the stain between images. The total intensity of the positively stained protein or PEG was normalized to the number cells in each image.

5.3.6 Biochemical Analysis
At prescribed culture times, hMSC-laden hydrogel constructs (n=3/time point) were flash frozen in liquid nitrogen and stored at -80 °C. Hydrogels were lyophilized, homogenized (TissueLyzer II, Qiagen) at 30HZ for 10 minutes, and digested with papain for 16 hours at 60°C. DNA content in the hydrogel constructs was measured using Hoechst 33258 (n=3). Sulfated glycosaminoglycan (sGAG) content was assessed using dimethyl methylene blue (DMMB) assay (n=3)(50).

5.3.7 Hydrogel Characterization
The compressive modulus of the cell-laden hydrogels was evaluated at prescribed culture times (n=3/time point). Hydrogels were compressed to 15% strain at a strain rate of 0.1mm min\(^{-1}\) to obtain stress strain curves (MTS Synergie 100, 10N). The compressive modulus was determined by the slope tangential to the linear region of the stress-strain curves from 10 to 15% strain.

5.3.8 Statistical Analysis
Data are represented as the mean with standard deviation. A one-way analysis of variance (ANOVA) was performed with time as the factor followed by Tukey’s post-hoc analysis. P values are reported to indicate the level of significance with p<0.05 considered to be statistically significant.
5.4 Results

Figure 5.1. A schematic of the hydrogel precursors and the encapsulation of human mesenchymal stem cells (hMSCs) in a MMP7-sensitive cartilage mimetic hydrogel. Hydrogel precursors included 8-arm PEG functionalized with norbornene, MMP7-sensitive peptide flanked with cysteines on each end, thiolated chondroitin sulfate, and cysteine containing RGD sequence.

A photoclickable MMP7-sensitive cartilage mimetic PEG hydrogel was developed to encapsulate hMSCs (Figure 1). Chondrogenesis of hMSCs was evaluated by mRNA expression over the course of nine weeks in culture (Figure 2). Chondrogenic genes of SOX9, a transcription factor, and ACAN and COL2A1, the main ECM molecules in cartilage, were evaluated. Time was a factor for ACAN ($p<0.0001$) and COL2A1 ($p=0.0018$), but was not for SOX9 expression. ACAN levels were maintained from week one to three and then increased ($p<0.0001$) by 500-fold at week six. From week six to week nine, ACAN levels decreased ($p<0.0001$) by 30-fold. COL2A1 levels exhibited a similar trend to that of ACAN with a 10,000-fold increase ($p=0.0002$) from week three to six followed by a 200-fold decrease from week six to nine.
Figure 2. Gene expression of MSCs encapsulated in an MMP7 degradable hydrogel normalized to gene expression of pre-encapsulated MSCs. The chondrogenic genes SOX9, ACAN, and COL2A1, and the hypertrophic genes RUNX2 and COL10A1 were evaluated with culture time. Data are represented as the mean with error bars as standard deviation (n=3).

Hypertrophic markers were evaluated by RUNX2, a transcription factor, and COL10A1. Time was a factor for RUNX2 ($p=0.0045$) and COL10A1 ($p=0.043$). Both genes were significantly up-regulated at week one when compared to the pre-encapsulated MSCs. RUNX2 levels decreased from week one to three and by week nine
remained low ($p=0.005$) when compared to week one. COL10A1 levels increased ($p=0.04$) by ~100-fold at week six, but by week nine had returned to levels similar to that of week one. To further probe the phenotype of the differentiating MSCs, the ratios of COL2A1 to COL10A1 gene expression and the ratio of SOX9 to RUNX2 gene expression were evaluated. By week six, the COL2A1 to COL10A1 ratio increased ($p=0.03$) by ~100-fold when compared to week one or week three. SOX9 to RUNX2 ratio increased ($p=0.00003$) by ~100-fold from week one and increased ($p=0.0004$) by 10-fold from week three. By week nine, the ratio was lower ($p=0.0008$) than week six and not significantly different from week one.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Week 1</th>
<th>Week 3</th>
<th>Week 6</th>
<th>Week 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA per construct (µg)</td>
<td>2.79 (.33)</td>
<td>2.43 (.25)</td>
<td>2.62 (.29)</td>
<td>2.31 (.27)</td>
</tr>
</tbody>
</table>

Figure 5.3. Biochemical analysis of DNA and sGAGs in MMP7 degradable hydrogels A. Total DNA content per construct is shown as a function of culture time. B. sGAGs per construct are shown as a function of culture time. Data are represented as the mean with standard deviation shown parenthetically or as error bars (n=3). C. Representative microscopy images of histological assessment by Safranin O/Fast Green, which stains sulfated glycosaminoglycans (sGAGs) red, scale bar is 100µm.
The constructs were also evaluated by their DNA content and total sulfated GAG (sGAG) content as a function of culture time (Figure 3). For total DNA content per construct (Figure 3A), time was not a factor indicating that cell number remained constant in the hydrogels for the duration of the study. Time was a factor \((p=0.0005)\) in the amount of sGAGs per construct (Figure 3B). The sGAG content was relatively constant from week one to six, with a slight mean decrease \((p=0.06)\) by \(~20\%)\. By week nine, the sGAGs per construct were the lowest, decreasing \((p=0.005)\) by \(~40\%)\ from week one. The spatial distribution of sGAGs in the hydrogels was also evaluated (Figure 3C). At all time points, positive sGAG staining was present throughout the construct. However, it was not possible to differentiate between the ChS that was incorporated into the hyrogel and the newly synthesized sGAG.
The spatial distribution of cartilage-related proteins and the PEG polymer associated with the hydrogel were assessed by immunohistochemistry (Figure 4). The proteins included aggrecan and collagen II for hyaline cartilage, collagen X for hypertrophic cartilage, and collagen I for fibrocartilage. There was minimal aggrecan

Figure 5.4. Representative immunohistochemical images for aggrecan (red), collagen II (green), collagen I (green), collagen X (green), and PEG (green) as a function of culture time. Images were acquired by confocal microscopy. Nuclei are stained blue. Scale bars are 20 µm.
deposition detected at week 1, but its presence appeared to increase throughout the duration of the study and by week nine was present throughout the construct. There was some detectable staining for collagen II at week one. Similar to aggrecan, collagen II presence appeared to increase with culture time and was prevalent throughout the constructs by week nine. There was minimal collagen I detected at week one, but it also appeared to increase with culture time and was present throughout the construct by week nine. There was minimal collagen X detected at weeks one and three, but its presence became apparent by weeks six and nine. Its deposition, however, appeared to be localized pericellularly and not all of the cells stained positive. The spatial presence and disappearance of PEG was also evaluated with culture time. Positive staining for PEG was evident throughout the construct at week one, but its staining diminished over time with minimal staining by week nine.
Figure 5.5. Semi-quantitative analysis of immunohistochemical images of (A) collagen II intensity per nuclei and (B) PEG intensity per nuclei as a function of culture time. (C) A scatter plot of PEG intensity per nuclei plotted against collagen II intensity per nuclei. A linear correlation between PEG and collagen II intensity is shown with a linear Pearson correlation coefficient of -0.87. The data points above a value of four for collagen II intensity per nuclei were not included in the linear correlation.

The deposition of collagen II and correspondingly PEG disappearance was quantified from the immunohistochemistry images by measuring the intensity of positively stained collagen II and the intensity of positively stained PEG, each normalized to cell nuclei (Figure 5A and 5B). The mean intensity of collagen II increased from week one to three, although not significantly, and was maintained at week six. By week nine, collagen
II intensity was the highest ($p=0.001-0.032$). The opposite trend was observed for PEG intensity. From weeks one to three, there was a decrease in mean PEG intensity per nuclei, although not significant, which continued to further decrease ($p=0.01$) by week six and remained similarly low at week nine. Additionally, PEG intensity per nuclei was plotted against collagen II intensity per nuclei (Figure 5C). A linear relationship was observed in the data from week one to six resulting in a Pearson correlation coefficient of -0.87. Interestingly, collagen intensity increased although the PEG intensity was already at its lowest, suggesting that the cells may continue to build their surrounding ECM even after the hydrogel has degraded.

Figure 5.6. Compressive modulus measurements initially (day 1) and with culture time of the cell-laden MMP7 degradable hydrogels. Data represent mean with standard deviation as error bars (n=3).

The construct modulus under compression was measured as a function of culture time (Figure 6). The initial modulus, which was measured after one day, was 18 (2) kPa. The modulus dropped ($p<0.0001$) by ~70% after one week of culture. By week six, the
modulus increased ($p=0.047$) from week 1 to 10 (2) kPa and was maintained at week nine. Although the construct modulus increased from weeks one to nine, the final modulus was lower ($p=0.006$) than the initial modulus.

5.5 Discussion

In this study, we present a MMP7-sensitive cartilage mimetic hydrogel that supports MSC chondrogenesis and promotes neo-cartilaginous matrix production. The MMP7 crosslinker facilitated cell-mediated hydrogel degradation, which is necessary for macroscopic tissue elaboration. In accordance with hydrogel degradation, there was an increase in ECM deposition and a concomitant rise in compressive modulus. The neo-cartilage tissue that was formed by the encapsulated hMSCs was comprised of aggrecan and collagen type II, the main ECM molecules that make up cartilage, with minimal hypertrophy. However, the presence of collagen type I indicates fibrocartilage formation.

The encapsulated hMSCs readily degraded the MMP7-sensitive crosslinks within the hydrogel as indicated by the loss of PEG as a function of time. MSCs are known to secrete MMPs and as they differentiate, the types of MMPs released can change\(^{(51)}\). In particular, MMP7 is highly expressed during early stages of chondrogenic differentiation, but is not known to be secreted by fully differentiated chondrocytes\(^{(34),(43)}\). In this study, a significant drop in compressive modulus was observed during the first week of culture. Bahney et al\(^{(43)}\) reported that the MMP7 gene was undetectable in MSCs, but its expression spiked during the first week of chondrogenesis and then rose slowly and eventually leveled off by three weeks. Since the initial stage of chondrogenesis was not accompanied by significant ECM synthesis and deposition as shown by minimal staining for aggrecan and collagens, it is not surprising that the modulus drop was observed in the
first week. This observation is consistent with other studies, which have reported a decrease in compressive modulus due to the unmatched rate of hydrogel degradation to ECM production\(^{(43),(52),(53)}\). It is important to mention that other enzymes, including aggrecanase and MMPs 1, 2, and 13 have been reported to degrade this particular MMP7 sensitive peptide sequence\(^{(34),(54)–(56)}\). Thus, it is possible that the hydrogel, especially during the first week, may have been degraded by enzymes (e.g., MMP2) that are known to be secreted by MSCs\(^{(35),(43)}\).

Over the nine weeks of culture, the MMP7-sensitive cartilage mimetic hydrogel supported MSC chondrogenesis and importantly the formation of a macroscopic neo-cartilaginous tissue that was composed of collagen II and aggrecan. Neo-tissue growth was accompanied by an increase in the modulus. While other studies have encapsulated MSCs in MMP-sensitive hydrogels to investigate *in vitro* chondrogenesis and cartilage formation, the elaborated ECM is often limited with deposition primarily restricted to the pericellular space and little to no interconnectivity in ECM\(^{(35),(43),(57),(58)}\). A few studies, however, have also reported an interconnected ECM within regions of the hydrogel similar to that reported in this study, however, at higher concentrations of the chondrogenic growth factor TGFβ\(^{(43)}\). The neo-cartilaginous tissue that was formed in this study is attributed to a combination of the biochemical cues, chondroitin sulfate and RGD, and the degradable crosslinks\(^{(29),(59)}\). The incorporation of chondroitin sulfate can enhance ECM synthesis by the introduction of fixed negative charges, which similar to cartilage elevates the local osmolarity\(^{(60),(61)}\), and by binding and retaining chondrogenic growth factors (e.g., TGFβ) within the hydrogel\(^{(62)}\). RGD, which is found in fibronectin, is known to enhance chondrogenesis of MSCs during early stages of differentiation and when
incorporated at low concentrations or through degradable tethers enhances chondrogenesis\textsuperscript{(36),(63)--(65)}. It is worth noting that MMP7 may also contribute to ECM synthesis. MMP7 has been shown to support cartilage development by facilitating collagen II production through mobilization and release of bound growth factors (e.g., TGFβ) from the negative charged sGAGs\textsuperscript{(55),(66)--(69)}. In addition, sGAGs have been shown to bind MMP7 and promote its activation\textsuperscript{(55)} which can lead to localization of MMP7 activity in the pericellular region\textsuperscript{(55)}. While MMP activity is critical to hydrogel degradation, additional studies are needed to determine whether MMP7 contributes positively to ECM synthesis. Moreover, as noted above, other enzymes that are expressed in differentiated chondrocytes (e.g., ADAMTS4), but which are inactive during chondrogenesis may also have contributed to the continued degradation of the hydrogel long-term to help facilitate neo-tissue growth\textsuperscript{(70),(71)}. Taken together, our results show that MSC chondrogenesis and cartilaginous matrix elaboration is supported by the MMP7-sensitive cartilage mimetic hydrogel.

The MMP7-sensitive cartilage mimetic hydrogel was able to inhibit hypertrophy evident by high expression of cartilage genes (\textit{SOX9} and \textit{COL2A1}) relative to hypertrophic genes (\textit{RUNX2} and \textit{COL10A1}), which was accompanied by minimal staining for collagen X relative to aggrecan and collagen II. Other studies have reported reduced hypertrophy in MMP-sensitive hyaluronic acid hydrogels when compared to non-degradable hydrogels\textsuperscript{(59)} suggesting that the elaboration of a cartilage ECM due to hydrogel degradation may help to create a stable chondrogenic phenotype. However, the presence of aggrecan and collagen II was accompanied by collagen I indicating the formation of fibrocartilage. Our results are consistent with previous studies which have
reported similar findings in degradable hydrogels, where chondrogenically differentiating MSCs produce collagen I alongside collagen II\(^{(17)}\). Engineering fibrocartilage is important for regenerating certain cartilage tissues in the body, most notably the intervertebral disc. However, fibrocartilage is undesirable for treating cartilage defects in articulating joints. It is important to note that the hydrogels in this study were cultured in the absence of any mechanical stimulation, which has been suggested as an important factor in mediating fibrocartilage formation\(^{(72)}\). Our previous work has shown that mechanical stimulation can inhibit collagen I while maintaining aggrecan and collagen II in degradable hydrogels containing fully differentiated chondrocytes\(^{(44)}\). Thus, for articular cartilage tissue engineering, future work will need to investigate the effects of mechanical stimulation in the ability to control fibrocartilage development.

There are several limitations of this study. The activity of MMPs was not determined and thus we cannot confirm if MMP7 or other MMPs were critical to the positive outcome in ECM growth observed in this study. The compressive modulus of the neo-cartilaginous tissue was much lower than native cartilage tissue. Scaffold-less approaches based on condensed mesenchymal cell bodies have resulted in moduli similar to that of native cartilage tissue. Scaffold-less has benefits for clinical translation and compatibility with arthroscopic assisted surgery\(^{(73)}\). However, the ability to deliver cells in an injectable hydrogel has benefits for clinical translation and compatibility with arthroscopic assisted surgery\(^{(74),(75)}\). The presence of mechanical stimulation\(^{(76)}\) and confinement within a cartilage defect\(^{(77)}\) may enhance the mechanical properties of the engineered cartilage within this hydrogel. Additionally, the study was limited to one donor and future work will need to evaluate more donors. Moreover, the initial hydrogel properties (e.g., crosslinking), which influences the degradation kinetics, may require optimization for
different donors depending on the relative rates of MMP synthesis rates and ECM synthesis rates\textsuperscript{(38)}.

5.6 Conclusion
In this study, we developed a photoclickable cartilage mimetic PEG hydrogel with MMP7-sensitive crosslinks that supported hMSC chondrogenesis and promoted macroscopic formation of human neo-cartilaginous tissue comprised of aggrecan and collagen II. Human MSCs were capable of degrading the MMP7-sensitive peptide crosslinks resulting in a loss of PEG within the construct, which correlated closely with ECM growth and an increase in compressive modulus. Notably, the hydrogel inhibited hypertrophy, but led to collagen I indicating fibrocartilage. Overall, this hydrogel holds promise for cartilage tissue engineering using MSCs and warrants further investigation into improving the tissue mechanical properties and promoting articular cartilage.

5.7 Acknowledgments
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5.8 References


54. Wayne GJ, Deng S-J, Amour A, Borman S, Matico R, Carter HL, Murphy G. TIMP-3 inhibition of ADAMTS-4 (Aggrecanase-1) is modulated by interactions between


Chapter 6

Mechanical loading in a cartilage mimetic hydrogel inhibits hypertrophy of MSCs via Smad signaling

6.1 Abstract
Mesenchymal stem cells (MSCs) are promising for cartilage regeneration, however they readily undergo terminal differentiation, resulting in a hypertrophic phenotype. The interaction between MSCs and their surrounding environment can influence their differentiation and offers a mechanism to control hypertrophy. The aim of this study was to investigate the role of physiochemical cues resulting from ECM analogs and dynamic compressive loading on MSC chondrogenesis and the involvement of Smad and p38 MAPK intracellular signaling. The ECM analogs, chondroitin sulfate and RGD were incorporated into a photoclickable poly(ethylene glycol) hydrogel to which MSCs were encapsulated and cultured under free swelling and dynamic loading for three weeks. MSC chondrogenesis was evaluated by qPCR and immunohistochemistry. The combined ECM analogs and mechanical loading inhibited hypertrophy leading to a reduction in collagen X protein expression, but maintained chondrogenesis by collagen II protein expression. When Smad1/5/8 phosphorylation was blocked under free swelling by dorsomorphin, collagen X protein expression was significantly reduced similar to that observed under loading. The incorporation of ChS into the hydrogel was necessary to inhibit collagen X under mechanical loading, where RGD alone was not sufficient. The inhibitory effect of the ChS-containing hydrogels corresponded to enhanced gene expression of the osmotic responsive genes, NFAT5 and s100a4. Blocking p38 MAPK signaling by SB203580 resulted in collagen X protein expression. In summary, the physiochemical cues provided
by ChS and mechanical loading can inhibit hypertrophy of MSCs via Smad and p38 MAPK signaling.

6.2 Introduction

Human bone-marrow derived mesenchymal stem cells (MSCs) are a promising cell source for hyaline cartilage tissue engineering due to their high proliferative capacity and their chondrogenic differentiation potential\(^1,2\). The chondrogenic differentiation of MSCs leads to the upregulation of genes and proteins present in hyaline cartilage, including collagen II and aggrecan\(^2\). However, MSCs naturally undergo endochondral ossification \textit{in vivo} during development which involves condensation of MSCs, chondrogenic differentiation, followed by hypertrophy and mineralization\(^3\). During hypertrophy, the collagen II-rich extracellular matrix (ECM) is replaced with collagen X which can bind calcium and induce calcification, which is eventually turned over into bone\(^4\). Studies from our group and others have reported co-expression of collagen II and collagen X proteins in scaffolds with MSCs undergoing chondrogenesis indicating hypertrophic differentiation\(^5\)–\(^9\). Therefore, in order to successfully use MSCs as a cell-based therapy for the treatment of chondral defects in articular cartilage, strategies that inhibit hypertrophy must be established.

The interaction between MSCs and their surrounding environment has been shown to influence their differentiation\(^10\)–\(^12\). Specifically, the incorporation of extracellular matrix (ECM) analogs, cell adhesion peptides, and soluble growth factors can recapitulate many aspects of the native environment and provide a unique set of cues to control MSC differentiation\(^11\). Hyaline cartilage is a relatively simply tissue comprised predominantly of collagen II and aggrecan, a proteoglycan that links to hyaluronic acid and encompasses
the negatively charged glycosaminoglycan chondroitin sulfate. Hyaluronic acid (HA) has been incorporated into hydrogels to create a biomimetic environment that allows encapsulated cells to interact with their surrounding matrix. Studies examining the effect of HA-containing hydrogels on MSC differentiation have shown enhanced chondrogenic gene and protein expression. Another ECM analog, chondroitin sulfate (ChS), has also been investigated. ChS is responsible for the local negative charge of cartilage tissue, and can attract positive mobile ions from the surrounding fluid ultimately increasing the osmolarity. Incorporating ChS into cell-laden scaffolds has been shown to enhance cartilaginous matrix production and potentially suppress or delay the hypertrophic phenotype of MSCs. In addition, other ECM proteins, such as fibronectin, are critical during condensation in the initial stages of chondrogenesis and development. RGD, a cell adhesion peptide found in fibronectin, has been tethered into MSC-laden scaffolds and found to support chondrogenesis and cartilage ECM deposition in a concentration dependent manner. When combined with mechanical loading, a cartilage mimetic hydrogel environment containing both ChS and RGD has been found to maintain chondrogenesis, while inhibiting hypertrophy of encapsulated MSCs in a strain rate dependent manner. Collectively, these studies and more demonstrate that recapitulating physiochemical cues from native cartilage tissue enhances chondrogenesis of MSCs.

However, the mechanisms by which physiochemical cues affect chondrogenesis and importantly hypertrophy of MSCs, remains to be elucidated. The differentiation of MSCs to chondrocytes is precisely controlled by growth factors, transcription factors and cell-cell and cell-matrix interactions, all of which involve multiple signaling pathways.
One pathway of interest is the Smad pathway which plays a critical role in chondrogenesis of MSCs\textsuperscript{22–26}. Smad2/3 is activated by TGF-β, a growth factor commonly used for \textit{in vitro} chondrogenic differentiation of MSCs, whereas, the Smad1/5/8 route is commonly activated by bone morphogenetic proteins (BMPs) which are known to induce bone formation\textsuperscript{23}. The activation of Smad2/3 via phosphorylation is necessary for chondrogenesis and the production of collagen II and has been found to block hypertrophy. In contrast, the activation of Smad1/5/8 may be necessary during early stages of chondrogenesis, however, has been linked to the expression of the hypertrophic markers Runx2 and collagen X and endochondral ossification\textsuperscript{27}. When the activation of Smad1/5/8 is inhibited by a small molecule inhibitor, studies have shown a significant reduction in these hypertrophic markers\textsuperscript{27}.

In addition to Smad signaling, p38 mitogen-activated protein kinase (MAPK) has been found to be a positive regulator of MSC chondrogenesis\textsuperscript{28}. P38 MAPK signaling is critical in translating extracellular stimuli such as loading, osmolarity, and soluble growth factors into cellular responses such as proliferation and differentiation\textsuperscript{29}. Inhibiting p38 MAPK in MSCs has been found to downregulate the expression of the chondrogenic markers Sox9, collagen II and aggrecan, and enhance the expression of the hypertrophic marker Runx2\textsuperscript{28}. P38 MAPK may also be involved in crosstalk with Smad signaling, whereby inhibiting p38 MAPK leads to a decrease of Smad2/3, ultimately downregulating the expression of chondrogenic specific genes\textsuperscript{22}. Taken together, it is believed that the non-hypertrophic chondrogenic differentiation of MSCs may rely on a delicate balance between Smad2/3 and Smad1/5/8 signaling and their interaction with p38 MAPK.
In this study, we hypothesize that the inhibition of hypertrophy in chondrogenically differentiating MSCs is mediated by the presence of physiochemical cues arising from ECM analogs and loading, which signal by downregulating Smad1/5/8 signaling and upregulating p38 MAPK signaling. To test this hypothesis, this study had four goals. The first goal was to establish a simple and robust 3D hydrogel platform that mimics aspects of the cartilage ECM and its physiochemical cues when subjected to dynamic compressive loading. Towards this goal, we designed a poly (ethylene glycol) (PEG) hydrogel formed from a facile, rapid, and highly efficient cytocompatible photoclick thiol:ene reaction. This platform readily enabled the incorporation of thiol modified ECM analogs to create a cartilage biomimetic hydrogel containing chondroitin sulfate (ChS) and RGD. The second goal was to investigate the role of Smad signaling, specifically Smad 1/5/8, in mediating hypertrophy within the cartilage biomimetic hydrogel. Thirdly, the biochemical cues, ChS and RGD, were decoupled to identify their individual contributing roles in the physiochemical cues driving chondrogenesis and load-inhibited hypertrophy. Lastly, the p38 MAPK signaling pathway, which may act synergistically with Smad signaling, was investigated for its role in mediating load-inhibited hypertrophy within the cartilage mimetic hydrogel. Overall the findings from this study provide evidence for the role of physiochemical cues arising from the cartilage mimetic hydrogel, and notably the presence of chondroitin sulfate, coupled with dynamic compression in preventing MSC hypertrophy during chondrogenesis by inhibiting Smad 1/5/8 signaling through crosstalk with p38 MAPK signaling.

6.3 Materials and Methods
6.3.1 *Macromer Synthesis*

The monomer, 8-arm PEG functionalized with norbornene (PEG-NB), was synthesized as described previously (REF). Briefly, poly(ethylene glycol) (PEG) amine (8 arm, 10kDA MW) was dissolved in dimethyleformamide (DMF) and reacted with 5-norbornene-2-carboxylic acid at 4 molar excess with n,n-diisopropylethylamine (DIEA) and 1-[bis(dimethylamno)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) under argon, overnight, and at room temperature. The product was precipitated in cold diethyl ether and vacuum filtered, purified via dialysis, and recovered by lyophilization. The percent conjugation of norbornene to each arm of the 8-arm PEG was determined to be ~100% by $^1$HNMR by comparing the area under the peak for the allylic hydrogen closest to the norbornene hydrocarbon group ($\delta=3.1 - 3.2$ ppm) to the area under the peak representing the methyl groups in the PEG backbone ($\delta=3.4 - 3.85$ ppm).

Thiolated chondroitin sulfate (ChS-SH) was synthesized following methods described previously $^{32}$. Briefly, chondroitin sulfate (ChS) was dissolved in water, with excess dithiobi(propanoi dihydrazide) (DTP) (2 moles DTP: 1 mole ChS). The pH was adjusted to 4.75 by the addition of 1 M HCl. Excess 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) (2 moles EDCI: 1 mole ChS) was added to the ChS and DTP solution and reacted overnight. The reaction was stopped by raising the pH to 7 with the addition of 1 M NaOH. Excess of dithiothreitol (DTT) (6.5 moles DTT: 1 mole ChS) was added to the solution and the pH adjusted to 8.8 with 1 M NaOH. The reaction was carried out for 24 hours to reduce the thiol groups of the DTP. The final product (ChS-SH) was purified and recovered by dialysis against 0.3 mM HCl, centrifuged to remove any particulates...
and the supernatant was then lyophilized. Conjugation of thiol groups to ChS was determined to be 15% by $^1$HNMR by comparing the area under the peak for the two side chain methylenes of DTP ($\delta=2.5$-$2.6$ and $2.6$-$2.8$ ppm) to the area under the peak representing the methyl protons of the acetyl amine side chain ($\delta=1.8$-$2.0$ ppm), indicating that there are approximately 7 thiol groups per molecule of ChS.

### 6.3.2 Mesenchymal Stem Cell (MSC) Culture

Adult human mesenchymal stem cells (hMSCs) (passage 1, donor 1: 24 year old female, donor 2: 24 year old female, donor 3: 25 year old female) were purchased from Texas A&M Cell Distribution Center. The hMSCs were expanded in growth media (20% fetal bovine serum (FBS, Atlanta Biologicals), 50 U ml$^{-1}$ penicillin, 50 mg ml$^{-1}$ streptomycin, 20 mg ml$^{-1}$ gentamicin, and 5 ng ml$^{-1}$ fibroblast growth factor-basic (bFGF) (Invitrogen) in low glucose Dulbecco’s modified Eagle media (DMEM, Invitrogen). The hMSCs were seeded at 3000 cells cm$^{-2}$ and grown under standard culture conditions (37°C with 5% CO$_2$ in a humid environment) to 80% confluency and passaged. hMSCs at passage three were used in all studies.

### 6.3.3 Hydrogel Formation and MSC Encapsulation

Hydrogels were fabricated via photopolymerization from a precursor solution. For the cartilage mimetic PEG hydrogels containing ChS and RGD (RGD+ChS hydrogels), the solution was 9% (g/g) PEG-NB, 1% (g/g) ChS-SH, 0.1 mM CRGDS (Genscript), 2.14 (g/g) PEG dithiol (1kDa) (Sigma-Aldrich). For PEG hydrogels containing only RGD (RGD hydrogels), the solution was 9% (g/g) PEG-NB, 0.1 mM CRGDS, 2.67 wt% PEG dithiol (1kDa). For PEG-only hydrogels, the solution was 9% (g/g) PEG-NB, 2.67 wt% PEG
dithiol (1kDa). All precursor solutions were photopolymerization with 0.05% (g/g) photoinitiator Irgacure 2959 (I2959) (BASF) in phosphate buffered saline (PBS, pH 7.4) with 352 nm light at 5 mW cm\(^{-2}\) for 8 minutes. Acellular hydrogels were swelled to equilibrium in PBS for 24 hours prior to performing characterization.

For cell-laden hydrogels, hMSCs were encapsulated in hydrogels at a cell concentration of 10 million cells per ml of filter-sterilized (0.22 μm filter) precursor solution followed by photopolymerization as aforementioned. The hMSC-laden constructs (5mm in diameter and 2mm in height) were cultured in chondrogenic differentiation medium (1% ITS+ Premix, 100 nM dexamethasone, 2.5 ng ml\(^{-1}\) TGF-β3, 50 mg ml\(^{-1}\) l-ascorbic acid 2-phosphate, 50 U ml\(^{-1}\) penicillin, 50 mg ml\(^{-1}\) streptomycin, and 20 mg ml\(^{-1}\) gentamicin in high glucose Dulbecco’s modified Eagle media).

6.3.4 Hydrogel Characterization

Acellular hydrogels were characterized to confirm the incorporation of ChS-SH into the hydrogel using toluidine blue, which stains negatively charged glycosaminoglycans. PEG-only and PEG-ChS hydrogels were stained with toluidine blue solution (0.1% toluidine blue, 7% ethanol, 0.1% NaCl in PBS, pH<2.5) for 24 hours rinsed well for 48 hours in deionized H\(_2\)O (diH\(_2\)O) and imaged using light microscopy (Zeiss Pascal, Olympus DP70 100x magnification). It was confirmed that toluidine blue did not stain the peptide RGD (data not shown).

The volumetric swelling ratio (Q) was estimated from the experimentally determined mass equilibrium swelling ratio. PEG-ChS hydrogels were swollen in either diH\(_2\)O (n=10) or PBS (n=10) for 24 for hours, at which time the swollen mass was measured. After mass measurements were taken, the hydrogels swollen in diH\(_2\)O were placed in diH\(_2\)O for two
additional days, while hydrogels swollen in PBS were placed in diH$_2$O which was changed 3 times per day for 2 days to lower the concentration of salts to remove those in the hydrogel as well as those that are interaction with the negative charges of ChS. These hydrogels were then lyophilized, and the dry polymer mass was measured after 24 hours. The volumetric swelling ratio was determined from the mass swelling ratio assuming a density of the polymer (1.07 g ml$^{-1}$) and a density of solvent (1 g ml$^{-1}$).

6.3.5 Experimental Conditions

Three studies were performed. In all studies, the hMSC-laden hydrogels were cultured in standard culture conditions (37°C with 5% CO$_2$ in a humid environment) for one week under free swelling conditions. At which time, one set of hydrogels was continued under free swelling conditions while the remainder of the hydrogels was placed in a custom-built bioreactor$^{33,34}$ for an additional two weeks. In the bioreactor, the hydrogels were subjected to intermittent unconfined dynamic compressive strain applied daily for 1 hour at 5% peak-to-peak strain (2.5% amplitude strain) and 1Hz in a sinusoidal waveform followed by 23 hours of rest at a negligible tare strain of 0.5%. Hydrogels were cultured individually in a 24 well plate with two ml of chondrogenic medium per well, which was replaced every other day for the duration of the study. Study one used donor 1 in the PEG-ChS hydrogels and no additional treatment conditions were investigated. Study two used donor 2 in the PEG-ChS hydrogels and a second loading condition was investigated. The small molecule dorsomorphin (Biomol International), which inhibits the kinase domains of ALK 1,2, 3, and 6, was added at 10 µM$^{27}$ to the culture medium during the two weeks of dynamic loading (i.e., between day 7 and day 21). Dorsomorphin was added fresh to the culture medium at each media exchange. Study three used donor 3 in the
cartilage mimetic PEG hydrogels with RGD and ChS (RGD+ChS hydrogels) and in PEG hydrogels with RGD only (RGD hydrogels) with the small molecule SB203580, which inhibits p38 MAP Kinase. The inhibitor was added at 3 µM to the culture medium during day 7 and day 21 for all conditions (loading, free swelling, and hydrogel). SB203580 was added fresh to the culture medium at each media exchange.

6.3.6 Cell Viability

Cell viability within the cell-laden hydrogels used to investigate the pSmad1/5/8 inhibitor was assessed using Live/Dead™ (ThermoFisher). Briefly, the hydrogels were incubated with calcein-AM (1µM) for live cells (green) and ethidium homodimer-1 (2µM) for dead cells (red) in warm PBS for 20 minutes. Hydrogels were immediately imaged by confocal microscopy.

6.3.7 Gene Expression by qPCR

At prescribed time points, MSC-laden hydrogels (n=3) were removed from culture and homogenized (TissueLyzer II, Qiagen) at 30Hz for 10 minutes in RNA lysis buffer. RNA was extracted from the hydrogels using MicroElute Total RNA Kit per manufacturer (Omega). RNA was transcribed to cDNA using a high capacity reverse transcription kit per manufacturer (Applied Biosystems). Quantitative PCR (qPCR) was performed with Fast SYBR Green Master Mix (Applied Biosystems) and a 7500 Fast Real-time PCR Machine (Applied Biosystems). Gene expression data are reported relative to the housekeeping gene L30 and calculated from delta Ct values using the true efficiencies, however, for the first study, the relative gene expression is normalized to the gene expression of pre-encapsulated MSCs. PCR efficiency for each set of primers was
determined from serial dilutions of cDNA and the slope between Ct values of 15-25 following methods described in 35. The genes of interest included SOX9, RUNX2, ACAN, COL2A1, COL10A1, NFAT5, and S100A4 and their primer sequence and efficiency are given in Table 1.

Table 1. Primer Sequences and Efficiency for qPCR Analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>L30</td>
<td>5'-TTAGCGGCTGCTGTTGGT-3'</td>
<td>5'-TCCACGGACTTTCGTCTTC-3'</td>
<td>94%</td>
</tr>
<tr>
<td>SOX9</td>
<td>5'-TGACCTATCAAGCCATTACCA-3'</td>
<td>ATCATCCTCCACGGCTTGCTCTGAA-3'</td>
<td>95%</td>
</tr>
<tr>
<td>ACAN</td>
<td>5'-AGTATCATCGTCCAGAATCTAGCA-3'</td>
<td>5'-AATGCAGAGGTGTTTCCTCA-3'</td>
<td>88%</td>
</tr>
<tr>
<td>COL2A1</td>
<td>5'-CAACACTGCAACGCTCCAGAT-3'</td>
<td>TCTTGCAAGTGATGATTTCT-3'</td>
<td>102%</td>
</tr>
<tr>
<td>RUNX2</td>
<td>5'-TTGGGCTGTTGCTGTCAATTA-3'</td>
<td>5'-GAGTCTCGTCTGTCATGCA-3'</td>
<td>98%</td>
</tr>
<tr>
<td>COL10A1</td>
<td>5'-TTGGTCTGCTAGTATCTCTGACT-3'</td>
<td>5'-ACCTCTAGGCGCAAGGAC-3'</td>
<td>87%</td>
</tr>
<tr>
<td>NFAT5</td>
<td>5'-GGGTCAAACGACGAGATTGAGT-3'</td>
<td>5'-TTGTCGGTGTAAGCTGAA-3'</td>
<td>101%</td>
</tr>
<tr>
<td>S100A4</td>
<td>5'-GTCACCTTCCACAAGTATCG-3'</td>
<td>5'-TCATCTGGCTTTTTTCCAAG-3'</td>
<td>98%</td>
</tr>
</tbody>
</table>

6.3.8 Immunohistochemistry (IHC)

At prescribed time points, MSC-laden hydrogels (n=3) were removed from culture and processed for immunohistochemistry. MSC-laden hydrogels were fixed overnight at 4°C in 4% paraformaldehyde and transferred to 30% sucrose solution for storage at 4°C. Hydrogels were dehydrated following standard methods, embedded in paraffin, and sectioned (10 µm). For certain primary antibodies, sections were pre-treated with enzyme as follows: 2000 U ml⁻¹ hyaluronidase for anti-collagen II, 1 mg ml⁻¹ protease followed by 1 mg ml⁻¹ pepsin, and lastly 0.25% trypsin in 1mM EDTA for anti-collagen X, antigen retrieval followed by chondroitinase ABC (100 mU ml⁻¹) and keratinase I (1 U ml⁻¹) followed by hyaluronidase (2000 U ml⁻¹) for anti-aggrecan, and no enzyme pre-treatment was used for anti-Runx2. Anti-pSmad2/3 and anti-pSmad1/5/8 were pretreated with
Retrievagen A (BD Pharmingen) for antigen retrieval. Following permeabilization and blocking, sections were treated overnight at 4°C with the primary antibody: 1:50 anti-collagen II (US Biological, C7510-20F), 1:5 anti-aggrecan (US Biological, A1059-53F), 1:50 anti-collagen X (Abcam, ab49945), 1:50 anti-Runx2 (Abcam, ab23981), and 1:50 anti-pSmad2/3 (Santa Cruz Biotechnology, sc-11769) and anti-pSmad1/5/8 (Santa Cruz Biotechnology, sc-12353) in blocking solution. Sections were subsequently treated for 2 hours with goat anti-mouse IgG or goat anti-rabbit IgG labelled Alexa Fluor 488 (1:100) or Alexa Flour 546 (1:100) and the nuclei counterstained with DAPI.

Select conditions were further analyzed to quantify either the fraction of cells that stained positively for a protein or the relative intensity of staining for a protein. Representative confocal microscopy images (n=4 images per hydrogel, n=4 hydrogels per condition) were selected and processed using NIH ImageJ. For each image, the total number of nuclei was counted as an indication of cell number. For collagen II and collagen X proteins, the number of nuclei that were associated with positive staining for the protein was counted. Data are reported as a fraction of positively stained cells (i.e., positive cells/total cells) for each protein. For pSmad2/3 and pSmad1/5/8 proteins, the total intensity of the stain per image was determined. Data are reported as average intensity per nuclei.

6.3.9 Statistical Analysis

Data are presented as mean and standard deviation. Hydrogel characterization was analyzed with an unpaired t-test. The rest of the data met all of the requirements for an ANOVA and were analyzed using a one-way ANOVA as a function of time or hydrogel condition (given in the text) with a post hoc Tukey’s test, α=0.05 for compressive modulus
of acellular hydrogels and for gene expression and semi-quantitative IHC for cell-laden hydrogels.

### 6.4 Results

#### 6.4.1 Characterization of photoclickable cartilage-mimetic hydrogels

![Diagram showing the formation and characterization of a photoclickable cartilage-mimetic hydrogel.](image)

**Figure 6.1. The formation and characterization of a photoclickable cartilage-mimetic hydrogel**

A. A schematic of the monomers and resulting network structure formed after photopolymerization. B. The incorporation of ChS was confirmed by toluidine blue which stains negatively charged molecules blue. Brightfield microscopy images of PEG only and PEG-ChS hydrogels are shown (100x magnification). C. The volumetric swelling ratio (Q) of PEG-ChS hydrogels swollen in deionized H$_2$O (diH$_2$O) or phosphate buffered saline (PBS) for 24 hours. Data are reported as mean with standard deviation as error bar (n=10).

The photoclickable cartilage mimetic hydrogel was formed via photopolymerization of 8-arm PEG norbornene, PEG dithiol, thiolated chondroitin sulfate (ChS-SH) and CRGD (Figure1A). This formulation was designed to produce a stable hydrogel. Toluidine blue,
which stains negatively charged molecules, was used as an indicator for the presence of ChS. No staining was observed in the PEG-only hydrogel, whereas the PEG-ChS hydrogel stained blue confirming that ChS was incorporated into the hydrogel (Figure 1B). The presence of fixed negative charges that arise from the incorporated ChS was demonstrated by the volumetric swelling ratio measurements in two different solvents. In diH₂O, the PEG-ChS hydrogels exhibited a higher (p=0.05) volumetric swelling ratio than in PBS, where the presence of salts can shield the negative charges (Figure 1D).

6.4.2 Assessment of chondrogenesis of hMSCs in the photoclickable cartilage-mimetic hydrogel

Chondrogenesis of hMSCs encapsulated in the photoclickable cartilage-mimetic hydrogel (referred herein as RGD+ChS) and subjected to dynamic compressive loading was evaluated by mRNA expression for SOX9, ACAN and COL2A1 as markers for chondrogenesis and RUNX2 and COL10A1 as markers for hypertrophy and by IHC of collagen II and collagen X (Figure 2A and 2B).
Figure 6.2. Gene expression and immunohistochemical analysis in a cartilage mimetic hydrogel A. Gene expression of encapsulated hMSCs at day 21 of free swelling (solid) and loading (striped) conditions normalized to pre-encapsulated MSCs. The housekeeping gene was L30. B. Representative IHC images at day 21 of aggrecan (red), collagen II (green), and collagen X (green) and counterstained nuclei with DAPI (blue) in free swelling and loading conditions and quantitative IHC analysis of the fraction of positively stained cells for collagen II and collagen X at day 21 in free swelling (solid) and loading (striped). C. Representative IHC images of pSmad2/3 (red) and pSmad1/5/8 (red) and counterstained nuclei with DAPI (blue) at day 21 in free swelling and loading.
Under both free swelling and dynamic loading culture conditions, chondrogenesis of hMSCs in the cartilage mimetic hydrogel was confirmed by elevated SOX9, ACAN and COL2A1 levels relative to the hMSCs prior to encapsulation. Dynamic loading led to a reduction in COL2A1, but its expression remained high. Concomitantly, COL10A1 and RUNX2, which are markers of hypertrophy, were also elevated relative to hMSCs prior to encapsulation. Mean COL2A1 levels were higher than COL10A1 in both culture conditions, although to a lesser extent under loading. At the protein level, aggrecan and collagen II were present in both culture conditions. Collagen X protein was detected in the free swelling condition, but was minimally detectable under the loading condition. The percent of positively stained cells for collagen II was not different between free swelling and loading at ~60% under both culture conditions. However, for collagen X, there were ~60% positively stained cells under free swelling with significantly fewer ($p = 0.013$) at ~20% positively stained cells under dynamic loading. The presence of pSmad2/3 was detected by IHC in both culture conditions at day 21. However, pSmad1/5/8 was detected in the free swelling culture condition, but little to no positive staining observed in the loaded condition. These results demonstrate that dynamic loading does not alter hyaline cartilage ECM genes and proteins or pSmad 2/3, but downregulates hypertrophic markers at the protein level for collagen X and pSmad 1/5/8.
6.3.3 The Role of Smad 1/5/8 in chondrogenesis in the photoclickable cartilage-mimetic hydrogel

The role of Smad1/5/8 in mediating hypertrophy of hMSCs within the biomimetic hydrogels under free swelling was investigated using dorsomorphin, a small molecule inhibitor of the Smad 1/5/8 pathway. Dorsomorphin was introduced into the media of the free swelling cultures after one week of culture mirroring that of the hydrogels that were placed into the bioreactor and subjected to dynamic compressive loading.
Figure 6.3. Cell viability and Smad signaling immunohistochemistry under free swelling, loading, and with dorsomorphin. A. Cell viability showing live (green) and dead (red) hMSCs encapsulated in the cartilage mimetic hydrogel and cultured in free swelling, loading, and with the pSmad1/5/8 inhibitor, dorsomorphin, at day 21 (scale bar = 100 µm). B. Representative IHC images at day 14 and day 21 of pSmad2/3 and pSmad1/5/8 (red) and cell nuclei (blue) cultured in free swelling, loading and with inhibitor (scale bar = 20 µm). C. Semi-quantitative IHC of the pSmad2/3 and pSmad1/5/8 intensity per nuclei at day 14 (solid) and day 21 (striped) for the different culture conditions. Data are mean with standard deviation as error bars (n=4 hydrogels).
The presence of dorsomorphin in the culture medium did not affect the viability of the encapsulated hMSCs, where hMSCs remained viable at day 21 across all culture conditions (i.e. free swelling, dynamic compressive loading, and free swelling with dorsomorphin) (Figure 3A). Qualitative and quantitative IHC results for pSmad2/3 and pSmad1/5/8 are shown in Figure 3B and 3C, respectively. Dynamic loading did not affect the presence of pSmad2/3, which is consistent to that observed in Figure 2. The presence of pSmad1/5/8 was evident in the free swelling condition at days 14 and 21, but was lower (p<0.001) in the loading condition at both days 14 and 21. This finding is also consistent with that observed in Figure 2. Dorsomorphin inhibited (p< 0.001 and p < 0.001, respectively) pSmad1/5/8 at days 14 and 21, but did not affect pSmad 2/3. The presence of pSmad2/3 and pSmad1/5/8 was not different between the loaded condition and the inhibitor condition at days 14 and 21. These results overall confirm that in the cartilage-mimetic hydrogels, the addition of dorsomorphin inhibits pSmad1/5/8 under free swelling conditions similarly to the hydrogels that were cultured under loading.
Figure 6.4 Gene expression and immunohistochemistry of MSCs cultured under free swelling, loading and with dorsomorphin

A. Gene expression normalized to free swelling culture at day 14 (solid) and day 21 (striped) for cell-laden hydrogels cultured in loading (left) and with the pSmad1/5/8 inhibitor dorsomorphin (right). Data are represented as mean with standard deviation error bars (n=4) B. Representative IHC images at day 21 of collagen II (green) and collagen X (green) in the three different culturing conditions: free swelling, loading, and free swelling with Smad1/5/8 inhibitor dorsomorphin (scale bar = 20 µm) C. Quantitative IHC of the fraction of positively stained collagen II cells (left) and the fraction of positively stained collagen X cells (right) in the three culturing conditions at day 14 (solid) and day 21 (striped). Data are represented as mean with error bars as standard deviation (n=4) and * indicates significant differences from loading. D. Representative IHC images at day 21 of Runx2 (green) in the three different culturing conditions (scale bar = 20 µm)

The mRNA levels of the loaded condition and the free swelling condition with dorsomorphin were each normalized to free swelling culture conditions with no treatment. The hydrogels cultured with the dorsomorphin exhibited lower mRNA levels for the hypertrophic genes (RUNX2 (p=0.008), COL10A1 (p=0.01)) than the free swelling control and to a greater extent than that which was observed under loading (Figure 4A). However, dorsomorphin also reduced (p=0.01, p=0.006 and p=0.003, respectively) chondrogenic genes, SOX9, ACAN, and COL2A1, when compared to the hydrogels under loading (Figure 4A). At the protein level, collagen II was present across all three conditions with 95% of cells staining positively after 14 and 21 days (Figure 4B). Collagen X protein was present in the free swelling culture with ~75% of cells at day 14 and ~80% of cells at day 21 staining positive. Collagen X protein was reduced (p=0.02) under loading at days 14 and 21 where ~25% of cells stained positively for collagen X (Figure 4C). Dorsomorphin, however, led to a reduction (p=0.03) in collagen X at day 14, which was maintained at day 21 when compared to the free swelling hydrogels. These results demonstrate that dorsomorphin, which inhibits pSmad1/5/8, reduced COL2A1 and COL10A1 long-term. A reduction in collagen X protein expression was observed when cultured with...
dorsomorphin, and mirrored that of the load-induced inhibition in collagen X protein. Although \textit{COL2A1} was reduced with dorsomorphin at day 21, it did not appear to have an effect on collagen II protein production. Additionally, Runx2 protein expression was prevalent in the free swelling constructs at day 21, however, had little to no presence in the loaded or dorsormorphin cultured constructs (Figure 4D).
Figure 6.5. The effect of ChS+RGD and RGD hydrogels on MSCs cultured under free swelling and loading and without and with SB203580. A. Relative gene expression of MSCs encapsulated in PEG-ChS hydrogels (solid) and PEG-RGD hydrogels (striped) at day 7 (prior to loading) (n=3). B. Relative gene expression of the osmotic-responsive genes NFAT5 and s100a4 at day 21 of MSCs encapsulated in RGD+ChS hydrogels (solid) and PRGD hydrogels (striped) in free swelling and C loading culture without and with the p38 small molecule inhibitor SB203580. Data are represented as mean with error bars as standard deviation (n=3 where * denotes the comparison of free swelling to loading culture). D. Representative images of collagen II and collagen X in RGD+ChS and RGD hydrogels cultured under free swelling and loading conditions without and with the p38 inhibitor SB203580 (n=3, scale bar= 20 µm).

6.3.4 MSC Chondrogenesis in PEG-ChS and PEG-RGD hydrogels

To identify the role of the biochemical cues within the hydrogel on the load-inhibited hypertrophy of MSC differentiation, ChS and RGD (RGD+ChS) and RGD only (RGD) PEG hydrogels were investigated. mRNA levels were examined at day 7 (prior to mechanical loading) to investigate initial effects of ChS and RGD on MSC differentiation markers. COL2A1 was higher ($p = 0.008$) in the RGD+ChS hydrogels compared to the RGD hydrogels. NFAT5, an osmolarity-responsive binding protein that mediates the transcriptional activation of ion transporters, such as s100a4, a calcium binding protein, were also investigated. NFAT5 was upregulated ($p = 0.01$) in the RGD+ChS hydrogel compared to the RGD hydrogel after 7 days of free swelling culture.

From day 7 to day 21 the RGD+ChS and RGD hydrogels were cultured under dynamic compression and free swelling. There was no difference in NFAT5 between loading and free swelling culture at day 21 in the RGD+ChS hydrogels, however, S100A4 was significantly increased ($p=0.04$) with loading. The RGD hydrogels had an opposite effect, where NFAT5 levels were reduced ($p=0.002$) under loading. The S100A4 in the
RGD hydrogel cultured under mechanical loading was lower (p=0.014) than that in the RGD+ChS hydrogel. These results indicate that the load-induced inhibition of hypertrophy is dependent on ChS. The enhanced expression of the osmotic responsive genes, *NFAT5* and *s100a4*, in the ChS-containing hydrogels suggests that such ChS may be elevating the local osmolarity, ultimately inhibiting hypertrophy.

6.3.5 *The role of p38 MAPK signaling on MSC chondrogenesis in RGD+ChS and RGD hydrogels*

A subset of the RGD+ChS and RGD hydrogels were cultured in the presence of the p38 inhibitor SB203580 under free swelling and loading after an initial 7 days of culture. In the presence of SB203580, *S100A4* was reduced (p=0.04) in the RGD+ChS hydrogels cultured under free swelling and moderately reduced (p=0.09) under loading compared to untreated RGD+ChS hydrogel (Figure 5C). However, *NFAT5* was not affected by treatment with SB203580 under free swelling and loading in RGD+ChS hydrogels. SB203580 did not affect the expression of *NFAT5* and *S100A4* in the RGD hydrogels under loading when normalized to those not treated with SB203580. *NFAT5* and *S100A4* in RGD hydrogels cultured in free swelling were not affected by SB203580.

Collagen II and collagen X protein expression was assessed after 21 days of culture (Figure 5D). SB203580 did not appear to have an effect on collagen II protein expression, as collagen II was present in free swelling and loading in the RGD+ChS hydrogels. However, SB203580 reduced collagen II in the RGD hydrogels in both culturing conditions. Collagen X protein was prevalent in RGD hydrogels in free swelling and loading with SB203580. In the ChS hydrogels, collagen X was present in free swelling culture, similar to the previous two studies. Without the p38 inhibitor, collagen X
was reduced with mechanical loading in the RGD+ChS hydrogels, however, when cultured with SB203580 collagen X protein was present. These observations suggest that p38 MAPK signaling may be involved in the load-induced inhibition of hypertrophy in the RGD+ChS hydrogels, but may not have an effect on MSC differentiation in RGD hydrogels.

6.4 Discussion

In this study, we established a photoclickable PEG cartilage mimetic hydrogel by incorporating ChS and RGD. When combined with dynamic compression, this hydrogel environment provided the physiochemical cues to enhance chondrogenesis and inhibit hypertrophy of the encapsulated MSCs. Smad 1/5/8 signaling was confirmed to be involved in MSC hypertrophy under free swelling, which was consistent with the reduced Smad 1/5/8 signaling in the loaded constructs that showed inhibition of hypertrophy. The incorporation of ChS into the hydrogel was identified as being necessary in the load-induced inhibition of hypertrophy. MSCs encapsulated in the RGD+ChS hydrogels had increased expression of the osmotic responsive genes NFAT5 and S100A4, which were downregulated when p38 MAPK signaling was inhibited and ultimately led to hypertrophy. Taken together, findings from this study identified that ChS incorporated in a cartilage mimetic hydrogel combined with dynamic compression enhanced chondrogenesis and inhibited hypertrophy by inhibiting Smad 1/5/8 signaling and up-regulating p38 MAPK signaling.

Similar to the findings of others, the results from this study confirm the involvement of Smad1/5/8 signaling in the hypertrophy of MSCs. The inhibition of Smad1/5/8 signaling through the use of the small molecule inhibitor, dorsomorphin, resulted in a significant
reduction in collagen X protein expression. However, the mRNA expression of all genes in the MSCs cultured with dorsomorphin were significant reduced at day 21 when compared to free swelling and loading culture. One possible explanation for the reduced gene expression of the MSCs cultured with dorsomorphin could be due to early inhibition of Smad1/5/8 signaling. In this study, dorsomorphin was added to the culture at day 7 to coincide with the addition of mechanical loading, whereas previous studies added dorsomorphin at day 14. The addition of the inhibitor at the earlier time point in this study may have led to reduced chondrogenic gene expression, as early stages of differentiation require Smad1/5/8 signaling\textsuperscript{23}. In addition to adding the inhibitor prematurely, the concentration of dorsmorphin used was taken from literature and was not optimized for this specific study\textsuperscript{27}. Although these limitations may have reduced the mRNA expression of chondrogenic specific genes, it did not appear to have a significant effect on the protein expression of collagen II which were found to be similar in all culture conditions.

Smad1/5/8 signaling was down regulated in the loaded cartilage mimetic hydrogels, which also showed load-inhibited hypertrophy. This was confirmed by a reduction of pSmad1/5/8 and collagen X protein expression when compared to the free swelling constructs. mRNA expression was not reduced under loading as it was with the addition of the inhibitor, which we believe may be due to a more robust inhibition by the addition of dorsomorphin which effectively downregulated all chondrogenic genes. Simultaneously, mechanical loading in the cartilage mimetic hydrogels did not have an effect on Smad2/3 signaling and collagen II production, as both were present under free swelling and loading. Mechanical loading has been found to promote the phosphorylation of Smad2/3 while simultaneously enhancing cartilage formation and suppressing
chondrocyte hypertrophy. Although, enhanced expression of Smad2/3 was not found in this study, there was a significant reduction in pSmad1/5/8, which could result in a shift to Smad2/3 dominated signaling. Smad2/3 dominated signaling has been known to protect articular cartilage and block chondrocyte terminal differentiation, which is supported by our results.

The load-induced inhibition of hypertrophy is specific to ChS-containing hydrogels. The incorporation of ChS into the hydrogel introduces a fixed negative charge which can interact with mobile cations from the media to increase the local osmolarity within the hydrogel. This higher osmolarity more closely resembles that of physiological conditions in cartilage and has been found to promote neotissue production of chondrocytes and chondrogenically differentiating MSCs. Upon hyperosmotic culture conditions, the osmosensitive transcription factor NFAT5 is upregulated. NFAT5 activation induces the expression of osmoprotective genes such as the osmolyte transporter s100a4 in order to adapt to hyperosmolarity. Thus, an upregulation of the gene s100a4 is a direct indication of NFAT5 activity. In this study, MSCs encapsulated in ChS-containing hydrogels had higher NFAT5 and S100A4 expression compared to hydrogels with RGD after 7 days. The mRNA expression of the calcium binding protein, s100a4, is significantly increased in ChS-containing hydrogels under mechanical loading compared to free swelling culture at day 21. In contrast, there was a significant reduction in S100A4 in RGD hydrogels under mechanical loading. Dynamic changes in osmotic loading have previously been reported to enhance tissue production of chondrocytes. Therefore, it is hypothesized that the combination of ChS and mechanical loading is leading to dynamic changes in osmolarity and a subsequent increase in non-hypertrophic tissue production,
however, additional studies will be needed to investigate changes in osmolarity within the hydrogels.

The highly negatively charged ChS is known to interact with soluble growth factors, and therefore we cannot rule out the possibility that the ChS in the hydrogels may interact with the soluble TGFβ3 in the media and play a role in the enhanced non-hypertrophic chondrogenesis of the encapsulated MSCs. Smad2/3 is activated by the TGFβ superfamily through membrane-bound type I and type II serine/threonine kinase receptors. Alternatively, TGFβ3 can also bind to the BMP receptor and activate the phosphorylation of Smad1/5/8. Activated Smad2/3 signaling inhibits hypertrophic differentiation of chondrocytes and maintains normal articular cartilage, however, a shift in signaling dominance from activated Smad2/3 to Smad1/5/8 signaling is sufficient to induce articular cartilage maturation and hypertrophy. We believe that canonical Smad signaling through TGFβ may be contributing to our results, however, the significant effect of mechanical loading on Smad1/5/8 and collagen X suggests that the physiochemical cues are also playing a pivotal role.

In addition to Smad signaling, the physiochemical cues of the cartilage mimetic hydrogel under loading may affect chondrogenesis of MSCs through other signaling pathways. Results from this study found that with the addition of the p38 inhibitor, SB230580, mechanical loading no longer inhibited hypertrophy in the RGD+ChS hydrogels. On the mRNA level, there was no significant difference in the expression of NFAT5 and S100A4 due to the addition of SB203580, however, hypertrophy was confirmed in the RGD+ChS hydrogels under mechanical loading by the presence of collagen X. The addition of SB203580 in the RGD hydrogels had no observable effect on
mRNA or protein expression. P38 MAPK signaling is highly responsive to extracellular stimuli that may be emulated by the hydrogel environment such as osmotic stress \textsuperscript{28,55} and mechanical loading \textsuperscript{29,58}. P38 has been associated with NFAT5 expression and its downstream transcription of osmolality responsive genes, where cells treated with p38 inhibitor in hyperosmotic conditions no longer expressed NFAT5\textsuperscript{47}. Additionally, p38 MAPK has been associated with Smad2/3 signaling, whereby inhibiting p38 led to a significant reduction in pSmad2/3 and chondrogenic-specific genes. Inhibiting p38 in the RGD+ChS hydrogels under loading could potentially reduce the MSCs ability to respond to dynamic changes in osmolarity, ultimately affecting Smad signaling and hypertrophy.

Hypertrophy of MSCs is inhibited when the encapsulated cells are provided with the necessary cues. Our study suggests that the combination of ChS and mechanical loading enhance chondrogenesis while inhibiting the hypertrophic phenotype. ChS adds a fixed negative charge into the hydrogel network which can lead to an increased concentration of ions, ultimately resulting in an increased osmolarity. When mechanical loading is combined with this hydrogel network, a complex system of mechanical stimulation as well as dynamic changes in osmolarity are introduced. P38 MAPK is involved in relaying these stimuli to the encapsulated MSCs, which ultimately reduces the expression of pSmad1/5/8 and inhibits hypertrophy. The cartilage mimetic hydrogel environment provides the necessary cues for non-hypertrophic chondrogenesis of the encapsulated MSCs and is a promising approach to develop a MSC-based therapy for chondral defects.

The findings from this study are promising, however, there were a few limitations. One cell donor was used for each experiment presented in this study. Although significant
effects of ECM analogs and mechanical loading on chondrogenic differentiation and cellular signaling were found, additional donors are necessary to extend this work to a broader population. Additionally, this study focused primarily on the effect of physiochemical cues on Smad and p38 MAPK signaling. We acknowledge that other signaling mechanisms are involved in MSC differentiation and may be detrimental for the MSC response to physiochemical cues, however, our results provide a strong argument for focusing on Smad and p38 MAPK signaling pathways within this study. Lastly, the duration of this study was limited to 21 days although longer studies are necessary to investigate long-term effects of ECM analogs and mechanical loading on MSC chondrogenesis. Degradable hydrogels are necessary for these longer term studies to allow for matrix elaboration that extends beyond the pericellular space for macroscopic tissue production.

6.6 Conclusions
In conclusion, our data strongly suggests that mechanical loading of MSC encapsulated in a cartilage mimetic PEG hydrogel blocks the phosphorylation of pSmad1/5/8, which prevents hypertrophy. The load-induced inhibition of hypertrophy is specific to the incorporation of ChS into the hydrogel, where RGD alone did not show similar results. ChS combined with mechanical loading could potentially lead to dynamic changes in osmolarity, as the encapsulated MSCs enhanced their expression of osmotic responsive genes. Additionally, p38 MAPK plays a vital role in responding to the physiochemical cues and inhibiting collagen X protein expression. By understanding the affect that external cues have on these signaling mechanisms, hydrogels for cartilage defect repair can be further improved to enhance and maintain stable chondrogenesis of MSCs.
6.7 References


Chapter 7

The effects of mechanical loading and growth factors on the chondrogenesis of induced pluripotent mesenchymal progenitor cells in a cartilage mimetic hydrogel

7.1 Abstract

Cell-based tissue engineering strategies have the potential to treat chondral defects and restore cartilage. Induced pluripotent stem cells (IPSCs) have emerged as a promising alternative cell source to chondrocytes and mesenchymal stem cells. However, the effect of biochemical and mechanical cues on the chondrogenesis of IPSCs is not well understood. The goal of this study was to evaluate chondrogenesis of IPSCs, which have been differentiated down the mesenchymal lineage to create induced pluripotent mesenchymal progenitor cells (IPS-MPs), then encapsulated in a cartilage mimetic hydrogel and exposed to free swelling or dynamic compressive loading culture with or without a growth factor cocktail containing TGFβ3 and/or BMP2. Chondrogenic differentiation was evaluated by gene expression (qPCR) and protein expression (immunohistochemistry) over three weeks. Dynamic loading promoted chondrogenesis of IPS-MPs encapsulated in the cartilage mimetic hydrogel in the absence of growth factors by enhancing collagen II protein production which coincided with increased pSmad2/3 expression, a signaling pathway necessary for chondrogenesis. In free swelling, TGFβ3, BMP2, and their combination enhanced chondrogenesis of IPS-MPs with similar collagen II expression, but this was accompanied by collagen X expression, a marker of hypertrophy, as well as pSmad1/5/8, a signaling pathway that is associated with hypertrophy. However, dynamic loading combined with TGFβ3 attenuated collagen X expression, via increased pSmad2/3 activation and reduced pSmad1/5/8. In summary,
local cues that combine dynamic loading and TGFβ3 provide IPS-MPs encapsulated in a cartilage mimetic hydrogel with key cues to promote chondrogenesis while attenuating hypertrophy.

7.2 Introduction

When damage occurs to articular cartilage, whether due to injury or disease, cartilage is unable to regenerate due to a lack of vascularity and low cellular density. If left untreated, such damage can lead to osteoarthritis, which has no cure. Current preventative treatments aim to repair damaged cartilage. One example of treatment is autologous chondrocyte implantation (ACI), where autologous chondrocytes are harvested from a non-loadbearing area of the joint, expanded ex vivo, and implanted into the defect. Although this treatment has shown improved patient-reported outcomes in young healthy patients, the repair tissue does not resemble hyaline cartilage and thus long-term function may be compromised. A new generation of ACI is matrix assisted chondrocyte implantation (MACI), which uses a natural scaffold, such as collagen to deliver the chondrocytes. Although this strategy shows promise over ACI, its effectiveness to treat chondral defects remains limited due to the need for a second surgery to harvest the chondrocytes, and as well as a limited number of cells harvested, and the potential for donor site morbidity.

An alternative cell source for cartilage tissue engineering is bone marrow derived mesenchymal stem cells (BM-MSCs). BM-MSCs can be harvested without invasive surgery, are expandable to a large number of cells, and can differentiate into multiple tissue types. However, BM-MSCs lose their multipotency and chondrogenic potential with increasing passage, thus the number of cells obtained is limited. Moreover, the chondrogenic potential of BM-MSCs decreases with age and age-related diseases.
Another shortcoming of BM-MSCs is their high propensity to undergo terminal differentiation during chondrogenesis, which is a precursor to endochondral ossification\textsuperscript{15,16}. When this occurs, the collagen II-rich extracellular matrix of the chondrogenically differentiated MSCs is replaced with collagen X, followed by calcification and mineralization, which can eventually lead to bone formation when implanted \textit{in vivo} \textsuperscript{15,17–19}. Thus, an alternative stem cell source for cartilage tissue engineering is needed.

Embryonic stem cells overcome some of the shortcomings with MSCs due to their unlimited proliferative capacity and their ability to differentiate into multiple cell types\textsuperscript{20}. They have also shown promise in cartilage tissue engineering\textsuperscript{21,22}. However, embryonic stem cells have several limitations including their potential to elicit an immune response due to their allogenic nature\textsuperscript{23} and the ethical controversy surrounding their use\textsuperscript{23}. The discovery of induced pluripotent stem cells (IPSCs) has paved the way towards a new cell source that avoids these concerns \textsuperscript{24,25}. IPSCs are generated from adult somatic cells, such as skin fibroblasts, which can be reprogrammed to an early state of differentiation similar to that of embryonic stem cells\textsuperscript{26}. Their pluripotency allows for a vast differentiation potential while their expansion capabilities allow for high yield, which is necessary for clinical applications\textsuperscript{27}. IPSCs can be isolated from older patients and maintain pluripotency similar to that of younger patients, which overcomes one of the limitations of using BM-MSCs\textsuperscript{28}. The reprogramming from skin fibroblasts from individuals older than 75 years of age has been reported and the resulting IPSCs have similar pluripotency and differentiation potential to IPSCs generated from somatic cells isolated from young patients\textsuperscript{29}. These characteristics make IPSCs a promising cell source for cartilage repair and regeneration.
There have been few reports using IPSCs with scaffolds for cartilage tissue engineering. IPSCs encapsulated in alginate hydrogels were reported to undergo chondrogenesis, however, this required coculture with chondrocytes. Polycaprolactone/gelatin electrospun scaffolds seeded with IPSCs promoted cartilaginous ECM production, however, when implanted in vivo, the formation of subchondral bone formation was reported, suggesting that the IPSCs supported endochondral ossification. None of these scaffolds to date provide IPSCs with local cues that mimic that of the native cartilage environment nor has the importance of the dynamic mechanical environment been investigated. Numerous studies have shown that the differentiation fate of MSCs is highly dependent on the scaffold environment, soluble growth factors and the presence of dynamic loading, where chondrogenesis can be enhanced given the right combination of cues. Thus, there is a need for additional assessment of scaffolds that promote chondrogenesis of IPSCs.

The goal of this study was to evaluate the effects of growth factors and dynamic compressive loading on chondrogenesis using IPCs as a cell source combined with our recently reported cartilage mimetic hydrogel. Our approach involves the direct induction of IPSCs into mesenchymal progenitor cells (IPS-MPs), which are then encapsulated in a photoclickable cartilage mimetic hydrogel. The hydrogel is based on the thiol-norbornene click chemistry using poly(ethylene glycol) (PEG) as the base to which extracellular matrix (ECM) analogs of chondroitin sulfate (ChS) and the adhesion peptide RGD are incorporated. We have previously reported that these two ECM analogs promote chondrogenesis of MSCs under dynamic compressive loading. Specifically, this study examined the effects of dynamic compressive loading and a growth factor cocktail.
containing TGFβ3, BMP2, or their combination on chondrogenesis of IPS-MPs in the
cartilage mimetic hydrogel. Chondrogenesis was evaluated through gene expression and
immunohistochemistry of chondrogenic and hypertrophic markers that included cartilage-
specific ECM molecules as well as TGFβ and Smad signaling. Findings from this study
demonstrate that creating an environment that mimics native cartilage enhances
chondrogenesis and cartilaginous matrix production of IPS-MPs while attenuating
hypertrophy.

7.3 Materials and Methods
7.3.1 Macromer synthesis
Poly(ethylene glycol) (PEG) norbornene (8 arm, 10kDa) was synthesized from
PEG amine as described previously. The 8-arm PEG amine (10kDa) molecule was
dissolved in dimethyleformamide (DMF) and reacted under argon with 5-norbornene-2-
carboxylic acid at 4 molar excess with n,n-diisopropylethylamine (DIEA) and 1-
[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid
hexafluorophosphate (HATU) overnight at room temperature. The 8-arm PEG
norbornene product was precipitated in cold diethyl ether, vacuum filtered, purified by
dialysis, and lyophilized to recover product. The conjugation of norbornene to each arm
of the PEG amine was found to be 100% as determined by comparing the area under the
peak for the allylic hydrogen closest to the norbornene hydrocarbon group (δ=3.1-
3.2ppm) to the area under the peak for the methyl groups of the PEG backbone (δ=3.4-
3.85 ppm) using 1H NMR.

Thiolated chondroitin sulfate (ChS-SH) was synthesized as described previously.
Briefly, ChS was dissolved in water and reacted with two molar excess
dithiobis(propanoic dihydrazide) (DTP). The pH was adjusted to 4.75 by the addition of
1.0M HCl. excess 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) (2 moles EDCI:1 mole ChS) was added to the ChS and DTB and reacted overnight at a maintained pH of 4.75. The reaction was stopped by the addition of 1.0M NaOH, raising the pH to 7. Excess dithiothreitol (DTT) (6.5 moles DTT: 1 mol ChS) was added to the solution and the pH was adjusted to 8.5 with the addition of 1.0M NaOH. The reaction was carried out for 24 hours to reduce the thiol groups of the DTP. The final product (ChS-SH) was purified and recovered by dialysis against 0.3 mM HCl, centrifuged to remove any particulates, and the supernatant was lyophilized. Conjugation of the thiol groups to the ChS was determined to be 15% by $^1$H NMR by comparing the area under the peaks for the two side chain methylenes of DTP ($\delta=2.5$-$2.6$ and $2.6$-$2.8$ ppm) to that of the methyl protons of the acetyl amine side chain ($\delta=1.8$-$2.0$ ppm). The 15% conjugation of thiols to ChS indicates that there are approximately 7 thiol groups per molecule of ChS.

7.3.2 Cell Culture

Induced pluripotent mesenchymal progenitor cells (IPS-MPs) were provided by Dr. Karin Payne at the University of Colorado Anschutz Medical Campus. The IPS-MPs were cultured in T175 flasks in mesenchymal expansion media (10% FBS, 1% nonessential amino acids, 1% penicillin, streptomycin, and 5 ng ml$^{-1}$ basic fibroblast growth factor (bFGF) in DMEM-GlutaMAX). IPS-MPs were expanded to 80% confluence and passaged up to passage 6.

7.3.3 Hydrogel Formation and IPS-MP Encapsulation

IPS-MPs were encapsulated in hydrogel at a cell concentration of 10 million cells per ml of filter sterilized (0.22 μm filter) precursor solution. The precursor solution was comprised of 9% (g/g) PEG-norbornene (8-arm, 10kDa), 1% (g/g) ChS-SH, and 0.1 mM CRGDS (Genscript) and 2.14 (g/g) PEG dithiol (1kDa) (Sigma-Aldrich). The cell-laden
precursor solution was photopolymerized with 0.5% (g/g) photoinitiator Irgacure 2959 (I2959, BASF) in phosphate buffered saline (PBS, pH 7.4) with 352 nm light at 5 mW cm\(^{-2}\) for 8 minutes. The IPS-MP-laden constructs (5mm in diameter and 2 mm in height) were cultured in chondrogenic differentiation medium (50 µg ml\(^{-1}\) l-ascorbic acid 2-phosphate, 40 µg ml\(^{-1}\) DL-proline, 1% ITS+ Premix, 1% penicillin/streptomycin in DMEM-Glutamax). Medium was supplemented with either no growth factor or with one of three growth factor conditions: 2.5 ng ml\(^{-1}\) TGF-β3 (TGF-β3), 25 ng ml\(^{-1}\) BMP2 (BMP2), or 2.5 ng ml\(^{-1}\) TGF-β3 plus 25 ng ml\(^{-1}\) BMP2 (TGF-β3+BMP2).

7.3.4 Cell-laden Hydrogel Culture
IPS-MP-laden hydrogels were cultured under standard cell conditions of 37°C with 5% CO\(_2\) under static, free swelling conditions for seven days. Hydrogels were cultured in individual wells of a 24 well plate with two milliliters of chondrogenic media per well. Medium was replaced every other day for the duration of the study. After one week, a subset of hydrogels were continued under free swelling culture conditions and the remaining hydrogels were placed in a custom-built bioreactor. The hydrogels were cultured for an additional two weeks. Hydrogels placed in the bioreactor were subjected to intermittent unconfined dynamic compressive strain at 5% peak to peak strain (2.5% amplitude strain) applied at 1 Hz in a sinusoidal waveform one hour daily and with 23 hours of insignificant tare strain (<0.1%).

7.3.5 Gene expression
At day 7 and day 21, IPS-MP-laden hydrogels (n=3) were removed from culture for RNA extraction. Briefly, hydrogels were homogenized (TissueLyzer II, Qiagen) at 30 Hz for 10 minutes in RNA lysis buffer. RNA was extracted from the hydrogels using MicroElute Total RNA Kit (Omega) per manufacturer instruction. RNA was transcribed to
cDNA using a high capacity reverse transcription kit (Applied Biosystems) per manufacturer instruction. Quantitative PCR (qPCR) was performed using Fast SYBR Green (Applied Biosystems) and a 7500 Fast Real-time PCR Machine (Applied Biosystems). Data for gene expression were calculated from delta Ct values and experimentally determined efficiencies. PCR efficiency for each set of primers were determined from the slope of Ct values of 15-25 from serial dilutions of cDNA following the methods described previously. Normalized gene expression is reported relative to the housekeeping gene L30 and normalized to day 7 gene expression of the same growth factor condition. The genes of interest included the chondrogenic markers of SOX9, ACAN, COL2A1, and the hypertrophic and osteogenic markers of RUNX2, COL10A1, and COL1. Primer sequences and efficiencies are given in Table 1.

### Table 1. qPCR Primer Sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Efficiency</th>
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</thead>
<tbody>
<tr>
<td>L30</td>
<td>5'-TTAGCGGCTGCTGTTGGTT-3'</td>
<td>5'-TCCAGCGACTTTTTGCCTTC-3'</td>
<td>94%</td>
</tr>
<tr>
<td>SOX9</td>
<td>5'-TGACCTATCCAAGCGCATTACCA-3'</td>
<td>5'-ATCATCCTCCACGCTTGCTGAA-3'</td>
<td>95%</td>
</tr>
<tr>
<td>ACAN</td>
<td>5'-AGTATCATCGTCCCAGAATCTAGCA-3'</td>
<td>5'-AATGCAGAGGTGTTTCCACTCA-3'</td>
<td>88%</td>
</tr>
<tr>
<td>COL2A1</td>
<td>5'-CAACACTGCCAAGTCCAGAT-3'</td>
<td>5'-TCTTGCAATGGTGGATGTTCTTCT-3'</td>
<td>102%</td>
</tr>
<tr>
<td>RUNX2</td>
<td>5'-TGGCTCTGTTGTCATTA-3'</td>
<td>5'-AGTCTTTCTGCGCTGCA-3'</td>
<td>98%</td>
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<tr>
<td>COL10A1</td>
<td>5'-TTTTGCTGCTATCCTTGTAACTC-3'</td>
<td>5'-ACCTCTAGGGCCAGAAGGAC-3'</td>
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<tr>
<td>COL1</td>
<td>5'-AGGAAGGCGCAAAAG-3'</td>
<td>5'-CAGTTACAAAGGAACAGAAGTCTCT-3</td>
<td>99%</td>
</tr>
</tbody>
</table>

#### 7.3.6 Immunohistochemistry (IHC)

At day 21, IPS-MP-laden hydrogels (n=3) were removed from culture and processed for immunohistochemistry. Hydrogels were fixed overnight in 4% paraformaldehyde at 4°C and transferred to a 30% sucrose solution at 4°C for storage. Constructs were subjected to a series of dehydration steps following standard methods, embedded in paraffin, and
sectioned at 10 μm. Sections were stained for collagen II, collagen X, pSmad2/3, pSmad1/5/8, and TGFβ I receptor (TGFβRI). Sections were pretreated depending on antibody as follows: 2000 U ml$^{-1}$ hyaluronidase for anti-collagen II, 1 mg ml$^{-1}$ protease followed by 1 mg ml$^{-1}$ pepsin, and lastly 0.25% trypsin in 1 mM EDTA for anti-collagen X, and antigen retrieval using Retrievagen (BD Biosciences) for pSmad2/3, pSmad1/5/8, and TGFβRI. Following permeabilization and blocking, sections were treated overnight at 4°C with the primary antibody: 1:50 anti-collagen II (US Biological, C7510-20F), 1:50 anti-collagen X (Abcam, ab49945), and 1:50 anti-pSmad2/3 (Santa Cruz Biotechnology, sc-11769), anti-pSmad1/5/8 (Santa Cruz Biotechnology, sc-12353) and anti-TGFβRI (Abcam, ab31013) in blocking solution. Sections were subsequently treated for 2 hours with goat anti-mouse IgG or goat anti-rabbit IgG labelled with either Alexa Fluor 488 (1:100) or Alexa Flour 546 (1:100) and nuclei counterstained with DAPI.

Stained sections were further analyzed to quantify the fraction of cells that stained positive for the protein of interest or the relative intensity of staining for the protein of interest. Representative confocal microscopy images (n=4 images per hydrogel, n=3 hydrogels per condition) were selected and processed using NIH ImageJ. For collagen II and collagen X proteins, the number of nuclei that were associated with positive staining for the protein were counted and relative to the total number of nuclei, and data are reported as a fraction of positively stained cells. For pSmad2/3, pSmad1/5/8, and TGFβR1, the total intensity of the protein stain per image was determined relative to the total number of nuclei, and data are reported as average intensity per nuclei. Different analysis for was used for Smads and TGFβR1 because basal level expression of these
signaling factors are expressed in MSCs, therefore all cells could potentially stain positively regardless of differentiation and signaling progress.

### 7.3.7 Statistical Analysis

Data are presented as mean and standard deviation. Quantitative PCR and IHC data were analyzed using a two-way analysis of variance (ANOVA, \( \alpha = 0.05 \)) with growth factor and culture condition (free swelling and loading) as factors followed by a post hoc Tukey’s test.

### 7.4 Results

![Figure 7.1. A schematic of hydrogel formation and experimental design](image)

A. A schematic of the monomers, cells and hydrogel formation. B. Experimental design of the IPS-MP-laden cartilage mimetic hydrogel with different growth factors, TGF\( \beta \)3 and BMP2, and
their combination and their culture conditions under free swelling or under free swelling for one week followed by two weeks under intermittent dynamic compressive loading.

The overall experimental design for this study is shown in Figure 1. Induced pluripotent mesenchymal progenitor cells (IPS-MPs) were encapsulated in a photoclickable cartilage mimetic hydrogels and cultured with different growth factor cocktails of TGFβ3, BMP2, or their combination. A set of cell-laden hydrogels were subjected to dynamic compressive loading while the others were cultured under free swelling (i.e., static) conditions. Chondrogenesis of IPS-MPs was evaluated by gene expression of the chondrogenic markers SOX9, ACAN, and COL2A1 and the hypertrophic markers COL1, RUNX2, and COL10A1. Protein expression was determined by immunohistochemistry of the collagen II, collagen X and the signaling pathways Smad2/3 and Smad1/5/8 and the TGFβ receptor (TGFβR1).
Figure 7.2. Gene expression data at day of 7 of various chondrogenic (Sox9, aggrecan, collagen II) and hypertrophic (collagen I, Runx2, collagen X) genes of MSCs cultured under free swelling without growth factors (black), or with TGFβ3 (white), BMP2 (dark grey), or TGFβ3+BMP2 (light grey). Data represented as mean with standard deviation as error bars (n=3).

The relative gene expression of IPS-MPs encapsulated in the cartilage mimetic hydrogel was investigated at day 7 to determine any initial effect of growth factor cocktail on chondrogenic differentiation. Specifically, the chondrogenic genes, SOX9, ACAN, and COL2A1, and the hypertrophic genes, COL1, RUNX2, and COL10A1 were evaluated.
There was no effect of TGFβ3 or BMP2 on SOX9, COL2A1 and ACAN compared to those cultured with no growth factors. However, culturing with TGFβ3+BMP2 reduced (p=0.009) SOX9 compared to no growth factors, and reduced (p=0.031) COL2A1 compared to TGFβ3-only cultured hydrogels. The hypertrophic marker, COL1, was reduced with BMP2 and TGFβ3+BMP2. RUNX2 expression was highest (p=0.0001 TGFβ3, p<0.0001 BMP2 and TGFβ3+BMP2) when cultured in the absence of growth factors. However, COL10A1 was enhanced when cultured with TGFβ3, where expression was higher (p=0.002) than no growth factors as well as higher (p=0.001) than BMP2.
Initially, the effects of mechanical loading in the absence of growth factors was evaluated. The gene expression of IPS-MPs encapsulated in the cartilage mimetic hydrogel was investigated in the absence of growth factors to determine the effects of the cartilage mimetic hydrogel environment and dynamic mechanical loading on IPS-MP chondrogenesis. Loading did not have an effect on chondrogenic or hypertrophic gene expression (Figure 3A). An immunohistochemical analysis of chondrogenesis was performed on day 21 for the chondrogenic marker collagen II, and the hypertrophic marker collagen X. At the protein level, there was a lack of collagen II in the free swelling condition (Figure 3B), where less than 10% of the cells stained positive for the protein (Figure 3C). However, under dynamic loading, the fraction of cells that stained positive for collagen II protein was significantly enhanced by ~8-fold in the absence of growth factors. Collagen X protein was detected in both the free swelling and loading conditions with approximately in all growth factor conditions under free swelling culture with approximately 60-80% of cells staining positively for the protein (Figure 3C).
Figure 7.4. Smad signaling of IPS-MPs under free swelling and loading in the absence of growth factors

A. Representative IHC images of pSmad2/3 (red), pSmad1/5/8 (red) and TGFβRI (green) and nuclei (blue) at day 21 of MSCs cultured without growth factors under free swelling or loading culture conditions. B. Quantitative IHC of the protein intensity per nuclei of the different proteins under free swelling (solid) and loading (striped). Scale bar is 20 µm. Data are mean with standard deviation as error bars (n=3).

The presence of pSmad2/3, pSmad1/5/8, and TGFβRI were evaluated to determine any observable differences in cellular signaling between the different culturing conditions. Smad2/3 signaling is critical for chondrogenesis to occur, whereas, Smad1/5/8 signaling has been linked to hypertrophy. Smad2/3 signaling is activated by TGFβ binding to its receptor TGFβRI which then forms a complex with TGFβRI and leads to the phosphorylation of Smad2/3. Quantitative analysis of protein intensity per nuclei were used for these proteins rather than fraction of positively stained cells due to the presence of basal levels of the phosphorylated proteins. pSmad2/3 and TGFβRI were detectible in the IPS-MPs cultured in the absence of growth factors under free swelling with approximately 60% of cells staining positively, while ~20% of cells stained positively for
pSmad1/5/8. Dynamic loading of the IPS-MPs increased \((p=0.01)\) the intensity of pSmad2/3 per cell. There was also an increase \((p=0.02)\) in TGFβRI intensity under dynamic loading. Dynamic loading appeared to have a significant effect on promoting chondrogenesis of IPS-MPs in the absence of growth factors.

The effect of growth factors in free swelling and mechanical loading culture on IPS-MP differentiation were investigated. Under free swelling culture, the effect of the different growth factors, TGFβ3, BMP2, and the combination of TGFβ3 + BMP2 were investigated. This was done to determine the effect of growth factors on the chondrogenesis of IPS-MPs in the absence of mechanical loading. There was no effect of growth factors on SOX9 or ACAN in free swelling culture (Figure 5A). However, \(COL2A1\) expression was enhanced \((p=0.03)\) when cultured with TGFβ3+BMP2. However, culturing with TGFβ3+BMP2 had significantly higher \((p=0.04)\) expression of \(RUNX2\). The effect of mechanical loading and growth factors were also investigated. Under mechanical loading, the different growth factor conditions did not have an effect on the chondrogenic markers SOX9, ACAN, and \(COL2A1\) or the hypertrophic markers \(COL1\), \(RUNX2\), and \(COL10A1\) (Figure 5A). Similar gene expression was found in the mechanical loading constructs, however, mechanical loading decreased \((p=0.02)\) \(COL2A1\) when cultured with TGFβ3+BMP2.
Figure 7.5. The chondrogenesis of IPS-MPs under free swelling and loading with TGFβ3, BMP2, or TGFβ3+BMP2 A. Normalized gene expression at day 21 under free swelling and loading condition and cultured with TGFβ3 (white), BMP2 (dark grey) and TGFβ3+BMP2 (light gray). Data was normalized to day 7 (prior to loading) of similar growth factor culturing conditions and are represented as mean with standard deviation (n=3). (* denotes difference from free swelling culture) B. Representative IHC images of
collagen II and collagen X (green) and nuclei (blue) at day 21 of MSCs cultured under free swelling (B) and loading (D) with TGFβ3, BMP2, and TGFβ3+BMP2 Quantitative IHC of the fraction of cells stained positively for collagen II (solid) and collagen X (striped) in free swelling (C) and loading (E) culture with the different growth factors. Scale bar is 20 µm. Data are mean with standard deviation as error bars (n=3).

Immunohistochemical analysis of chondrogenic and hypertrophic protein production were also evaluated (Figure 5B-E). The fraction of cells that stained positive for collagen II protein were not affected any of the culturing conditions, with approximately ~70% of cells staining positively for collagen II regardless of mechanical loading or growth factors. Under free swelling, collagen II protein detected in all growth factor conditions with approximately 80% of cells staining positively for the protein. Dynamic loading significantly reduced (p=0.001 (TGFβ3) and 0.006 (TGFβ3 + BMP2), respectively) the fraction of collagen X positively stained cells in those cultured with TGFβ3-only and with TGFβ3 + BMP2, where 20-30% of cells stained positively for the hypertrophic marker compared to ~90% under free swelling.
Figure 7.6. The Smad signaling of IPS-MPs under free swelling and loading with TGFβ3, BMP2, and TGFβ3+BMP2 A. Representative IHC images of pSmad2/3 (red), pSmad1/5/8 (red) and TGFβRI (green) and nuclei (blue) at day 21 of MSCs cultured with growth factors under free swelling (A) or loading (C) culture conditions. B. Quantitative IHC of the protein intensity per nuclei of the different proteins pSmad2/3 (solid), pSmad1/5/8 (striped) and TGFβRI (white) under free swelling (B) and loading (D). Scale bar is 20 µm. Data are mean with standard deviation as error bars (n=3).

The presence of pSmad2/3 was detected by immunohistochemistry in both free swelling and loading conditions at day 21. However, culturing under dynamic loading led to a significant increase (p=0.02) in staining intensity of pSmad2/3 with TGFβ3 only. This
staining intensity was higher (p=0.03 TGFβ3-only) than the pSmad2/3 intensity of those cultured with TGFβ3 + BMP2. Contrary to pSmad2/3 staining, pSmad1/5/8 staining was reduced, however, present, under free swelling in all culturing conditions. There was a reduction (p=0.06) in mean pSmad1/5/8 intensity under dynamic loading in those cultured with TGFβ3. In the presence of BMP-only, TGFβ3+BMP2, or without growth factors there was no significant effect of dynamic loading on pSmad1/5/8. These results demonstrate that dynamic loading alter pSmad signaling, but it is dependent on growth factors present.

Immunohistochemical analysis of the effect mechanical loading and TGFβ3 and/or BMP2 have on TGFβRI was examined. Under free swelling, the different growth factor conditions had similar protein levels of TGFβRI. However, similar to pSmad2/3 signaling, dynamic compressive loading significantly increased (p=0.005) protein levels of TGFβRI when cultured with TGFβ3-only under mechanical loading. Those cultured with BMP2 only or TGFβ3+BMP2 were not affected by loading.

7.5 Discussion
In this study, we investigated the effect of growth factors, TGFβ3 and BMP2, as well as the physiochemical cues provided by the cartilage mimetic hydrogel and dynamic loading on the differentiation of IPS-MPS. Our results suggest that mechanical loading enhances chondrogenesis in the absence of growth factors via enhanced Smad2/3 signaling. Under free swelling culture, all growth factor combinations promote collagen II protein, however, collagen X protein is also expressed. Mechanical loading maintained collagen II protein, while reducing collagen X when cultured in the presence of TGFβ3. This evaluation of culturing conditions on IPS-MP chondrogenesis indicates that
mechanical loading and growth factors play a significant role in promoting chondrogenesis and cartilaginous ECM production.

Dynamic compression of IPS-MPs encapsulated in the cartilage mimetic hydrogel promoted chondrogenesis in the absence of exogenous growth factors. This was confirmed by similar collagen II protein expression of IPS-MPs cultured under mechanical loading in the absence of growth factors to those cultured under free swelling and treated with TGFβ3, BMP, or TGFβ3+BMP2. Similar effects of mechanical loading of chondrogenesis have been shown in BM-MSCs\textsuperscript{[34–36]} however, to our knowledge, this is the first attempt at determining the effect of mechanical loading on IPS-MPs encapsulated in a mimetic hydrogel. In addition to collagen II production induced by loading, pSmad2/3 signaling was also enhanced. Smad signaling is critical to chondrogenesis, where the phosphorylation of Smad2/3 (pSmad2/3) is necessary for chondrogenesis to occur, and is activated by TGFβ binding to its receptor TGFβRI. The load-induced enhancement of Smad2/3 signaling shown in this study may be a result of increased endogenous secretion of TGFβ-1, -2, and -3. This has been found in BM-MSCs when cultured under mechanical loading in the absence of growth factors\textsuperscript{36}. Dynamic loading has also been found to enhance TGFβ receptors (TGFβRI and TGFβRII) in response to increased endogenous TGFβ secretion\textsuperscript{37}, ultimately increasing the activation of pSmad2/3\textsuperscript{38}. The results from this study suggest that mechanical loading enhances chondrogenesis of IPS-MPs through a similar mechanism, where dynamic loading may increase the endogenous secretion of TGFβ, results in increased TGFβRI and pSmad activation, ultimately promoting collagen II protein expression. However, it must be recognized that with collagen II protein expression, collagen X was also present in the constructs.
It is postulated that the effect of mechanical loading and growth factors on IPS-MP chondrogenesis shown in this study are specific to the cartilage mimetic hydrogel. The ECM analogs chondroitin sulfate and RGD both have been shown to play a pivotal role in the crosstalk between growth factors, growth factor receptors and integrins to control downstream signaling \(^{39–41}\). RGD allows for cell adhesion to the hydrogel network, and previous studies have shown cells encapsulated in a hydrogel with RGD leads to an upregulation of integrins that bind to RGD \(^{42,43}\). These integrins can also activate latent forms of TGFβ \(^{44–46}\), and when TGFβ binds to its receptor this can lead to endogenous secretion of TGFβ by the cells and result in a signaling cascade. The negatively charged sulfate molecules of chondroitin sulfate have been shown to interact with soluble TGFβ3, reducing its release, prolonging its half-life, and promoting chondrogenesis \(^{43,47}\). It has been reported that the latent form TGFβ can be stored in large amounts (300ng/g) in the extracellular matrix of articular cartilage \(^{48}\) and mechanical loading can act as an activator of latent TGFβ\(^{33}\). It is also possible that dynamic compression leads to the mechanical release of soluble TGFβ\(^{39}\) within our hydrogel constructs, ultimately enhances the chondrogenesis of IPS-MPs.

The growth factors TGFβ3 and BMP2 enhanced chondrogenesis of IPS-MPs in free swelling culture. These growth factors are known to modulate chondrogenesis\(^{33}\), and the results from this study suggest that the growth factors promoted chondrogenesis as shown by collagen II protein production. However, collagen X protein was present in all growth factor combinations when cultured under free swelling. This hypertrophic phenotype of chondrogenically differentiating mesenchymal cells is undesirable for cartilage tissue engineering\(^{49}\). Although the combination of growth factors and the
cartilage mimetic hydrogel are believed to play a role in promoting chondrogenesis of IPS-MPs, the cues presented are not enough to inhibit the hypertrophic phenotype. Our previous work has shown that the synergistic effect of ChS, RGD and dynamic compressive loading plays a more predominant role in inhibiting terminal differentiation while maintaining chondrogenesis of BM-MSCs\textsuperscript{35}. Therefore, it was hypothesized that dynamic loading with the correct combination of growth factors could potentially inhibit hypertrophy of IPS-MPs.

The combination of growth factors and dynamic loading reduced collagen X and inhibited hypertrophy, but only when cultured in the presence of TGFβ3. When cultured with BMP2 only, mechanical loading did not have an effect and collagen X remained present in the hydrogel. The activation of pSmad2/3 as well as TGFβRI also increased with mechanical loading when cultured with TGFβ3 only. Additionally, dynamic loading significant decreased pSmad1/5/8, but only when cultured with TGFβ3 alone. The inhibition of Smad1/5/8 signaling is known to inhibit hypertrophy of BM-MSCs\textsuperscript{50}. Our results suggest that mechanical loading and TGFβ3 results in pSmad2/3 dominated signaling, ultimately enhancing chondrogenesis while inhibiting the hypertrophic phenotype.

The proposed mechanism by which mechanical loading and TGFβ3 inhibit hypertrophy is through enhanced pSmad2/3 signaling as a result of endogenous TGFβ and exogenous TGFβ. It has been found that at low concentrations, TGFβ can bind to TGFβRI as well as BMP receptors and activate both pSmad2/3 and pSmad1/5/8 signaling cascades\textsuperscript{51,52}. This would ultimately result in collagen II and collagen X expression, and the hypertrophic phenotype. However, at high concentration of TGFβ, TGFβ preferentially
binds to TGFβRI and activates only pSmad2/3. pSmad2/3-dominated signaling has been found to inhibit hypertrophy. It is postulated that mechanical loading enhances the endogenous secretion of TGFβ, which combined with the exogenous TGFβ3 added to the media, increases the concentration of TGFβ in the loaded constructs to a greater extent than that in the free swelling cultures. This increase in TGFβ concentration preferentially activates pSmad2/3, resulting in a pSmad2/3 dominated signaling and inhibits pSmada1/5/8. This ultimately inhibits the hypertrophic phenotype.

There are several limitations of this study. A stable, non-degradable hydrogel was used in this study. This was important to maintain the local biochemical and mechanical cues within the hydrogel during the experiment. However, the ECM proteins deposited by the encapsulated cells is limited to the pericellular space. To assess the potential of this hydrogel platform for use with IPS-MPs for tissue engineering, future work will need to investigate the chondrogenesis of IPS-MPs in a degradable hydrogel. Lastly, the advantage of using IPS-MPs over BM-MSCs is that their chondrogenic differentiation capabilities are maintained in older or diseased patients. Future work will look to compare the chondrogenesis of IPS-MPs and BM-MSCs from the same patient in order to prove that IPS-MPs are a more attractive cell source for chondrogenesis.

7.6 Conclusions
A photoclickable PEG cartilage mimetic hydrogel cultured in the presence of TGFβ3 and mechanical stimulation was effective at promoting the chondrogenesis of IPS-MPS while limiting hypertrophy. Mechanical loading alone promoted chondrogenesis of IPS-MPs cultured in the absence of growth factors by enhancing Smad2/3 signaling. However, hypertrophy was only inhibited when mechanical loading was combined with
TGFβ3, ultimately enhancing Smad2/3 signaling while inhibiting pSmad1/5/8. Future efforts will examine the translatability of these findings into a degradable cartilage mimetic hydrogel where this hydrogel platform has clinical significance for its ability to be injected and photopolymerized into a chondral defect.

7.7 Acknowledgments
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7.8 References


Chapter 8
Cartilage repair with mesenchymal stem cells (MSCs) delivered in a cartilage mimetic PEG hydrogel in a rabbit animal model

8.1 Abstract

The treatment of symptomatic articular cartilage defects remain a clinical challenge. Tissue engineering provides a novel therapeutic tool that could provide regeneration of articular cartilage and improve clinical symptoms. The purpose of this study was 1) to determine the ability of the cartilage mimetic poly(ethylene glycol) (PEG) hydrogel to support chondrogenesis of rabbit mesenchymal stem cells (MSCs), 2) to assess cartilage repair of critical sized osteochondral defects by the cartilage mimetic hydrogel with and without MSCs in a rabbit animal model. Initially, rabbit bone marrow derived MSCs were cultured in the cartilage mimetic hydrogel in vitro to evaluate chondrogenesis through gene expression and immunohistochemistry. After 9 weeks of culture, encapsulated MSCs deposited their own extracellular matrix consisting of sulfated glycosaminoglycans (sGAGs) and collagen II. Validation of MSCs ability to degrade the synthetic hydrogel scaffold was confirmed by a reduction in immunohistochemistry staining for PEG and reduction in compressive modulus. A rabbit animal model was used to evaluate the MSC-laden hydrogel in vivo. Full thickness chondral defects were created on the trochlea of mature New Zealand rabbits, and treated with the cartilage mimetic hydrogel with and without MSCs. Repair tissue was evaluated at 6 months through macroscopic and histological evaluation of defect repair and showed cartilage repair in all groups. The hydrogel alone group had the best repair tissue per
O’Driscoll evaluation, however, there were no significant differences between the groups. Moreover, MSC-laden hydrogels did not enhance cartilage repair and showed similar repair as the untreated controls.

8.2 Introduction

Osteoarthritis (OA) is a serious clinical and economic burden for the orthopedic community and the public health system\(^1\). To date, surgical restoration techniques do not regenerate articular hyaline cartilage. Although patients’ symptoms improve, this improvement is temporary and there is a continued slow progression of disease resulting in osteoarthritis (OA). Tissue engineering approaches represent a promising therapeutic tool for the treatment of articular cartilage defects by providing a biomaterial scaffold as a vehicle to deliver chondrocytes or stem cells to the site of injury and allow for local production of new matrix\(^2\). Among these, injectable hydrogels have attracted great attention because of their performance characteristics. Hydrogels are three-dimensional networks that can be tuned for its biocompatibility, bioadhesiveness, and biodegradability\(^3\). In addition, hydrogels can be designed to be injected as an aqueous precursor solution and then polymerized \textit{in vivo} improving adhesion to the surrounding native tissue and allowing a near perfect fit\(^4\).

An enzymatically degradable cartilage mimetic biomaterial with incorporated chondroitin sulfate (ChS) and the adhesion peptide RGD has recently been developed for chondral defect repair\(^5\). ChS is the main glycosaminoglycan in cartilage and creates a unique environment that is hyperosmotic and promotes tissue synthesis\(^3, 7\), especially under dynamic compression\(^5, 8, \text{ and } 9\). RGD, a cell adhesion peptide found in fibronectin, has been shown to support MSC viability, chondrogenesis and cartilage ECM deposition.
When incorporated in the PEG hydrogel, RGD and ChS provide the MSCs with a biomimetic environment to enhance chondrogenic differentiation\textsuperscript{5,6}. This enzymatically degradable, injectable cartilage mimetic hydrogel can be injected directly into the defect site, formed \textit{in situ}, and provide the necessary biochemical cues to promote chondrogenesis and cartilage repair\textsuperscript{10}. The purpose of this study was: 1) to determine the ability of the cartilage mimetic hydrogel to support chondrogenesis of rabbit MSCs and 2) to evaluate cartilage repair of osteochondral defects by the cartilage mimetic hydrogel with and without MSCs in a rabbit animal model.

\section*{8.3 Methods}

\subsection*{8.3.1 Isolation of MSCs}

Rabbit bone marrow derived MSCs (MSCs) were isolated from the humerus of one (8-month-old) New Zealand white male rabbit. Following euthanasia of the rabbit, the forelimbs were dissected and the humerus were removed. The humerus were then placed in 70\% isopropyl alcohol for 5 seconds and then washed with 1X-phosphate buffer saline (PBS). The ends of each humerus were cut open by surgical bone cutters and flushed ~3 times with Dulbecco’s Modified Eagle Media (DMEM) into a 50ml conical tube. The bone marrow was then passed through a 70\(\mu\)m filter to remove bone and blood debris. The cells were then centrifuged at 200g at 4\(^\circ\)C for 5 minutes. The supernatant was removed and the cells were re-suspended in low-glucose media: Alpha Minimum Essential Medium (\(\alpha\)-MEM) containing 10\% Fetal Bovine Serum (FBS) and 1\% Penicillin, Streptomycin and Amphotericin A (PSA). The cells were then plated in T-75 flasks (cells from two humerus per flask) and kept in a 37\(^\circ\)C and 5\% CO\textsubscript{2} incubator. Media was changed every 3 days.
and supplemented with Fibroblastic Growth Factor (FGF) (4ng ml⁻¹) for maintenance and growth. When the cells reached 60% confluency, Accumax (San Diego, CA, USA) was used to passage them and then reseeded at 1 x 10³ cells cm⁻² up to passage 3.

8.3.2 Cell encapsulation

MSCs were encapsulated in the photopolymerizable, degradable, and biomimetic hydrogel consisting of 9% (w/v) PEG (8 arm, 10kDa) norbornene, 1.4% (w/v) of a matrix metalloproteinase 2 (MMP2) degradable peptide crosslinker CVPLSLYSGC, 1% (w/v) thiolated ChS, and 0.1mM CRGDS in sterile PBS at 50 million cells/ml of precursor solution via photo-polymerization with 352nm light at 5 mW cm⁻² for 8 minutes. The cell laden hydrogels were cultured in chondrogenic differentiation media for 9 weeks with media changed every other day. Samples were taken at weeks 3, 6, and 9 and analyzed for differentiation via qPCR and immunohistochemistry (IHC).

8.3.3 Mechanical Testing

Hydrogels were assessed for compressive modulus after 1 day (initial), and at weeks 3, 6, and 9 (n=3). Hydrogels were compressed to 15% strain at a strain rate of 0.1mm min⁻¹ to obtain stress strain curves (MTS Synergy 100, 10N). The compressive modulus was determined by the slope tangential to the linear region of the stress-strain curve between 10 and 15% strain.

8.3.4 Gene expression with qPCR

Gene expression was analyzed via qPCR for the chondrogenic markers: Sox9, aggregan and collagen II, and the hypertrophic markers Runx2 and collagen X. Hydrogels (n=3) were lysed with the TissueLyzer (Qiagen, MD, USA) and RNA was extracted using
a MicroElute Total RNA Kit per manufacturer instruction (Omega bio-tek, GA, USA). RNA was transcribed to cDNA following manufacturer procedure of a high capacity reverse transcription kit (Applied Biosystems, CA, USA), and RNA extraction was done following the procedure of RNA-isolation columns, and then converted to cDNA following kit instructions (Applied Biosystems, CA, USA). All gene expression data was calculated from delta Ct values and reported as relative to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and normalized to week 3 data.

Table 1. Primer Sequences and Efficiency for qPCR Analysis

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<th>Gene</th>
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<th>Reverse Sequence</th>
<th>Efficiency</th>
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<td>5’-GTAAGTCTCTCACGAGGGA-3’</td>
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<td>Collagen II</td>
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<td>Runx2</td>
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<td>5’-TAAGTAAGGTTGGCTGATAG-3’</td>
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<tr>
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<tr>
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<td>5’-GCTCCCTGAAGTCCCTGTGTC-3’</td>
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8.3.5 Immunohistochemistry and Histology Evaluation

Cell laden hydrogels (n=3) were fixed overnight in 4% paraformaldehyde in PBS at 4°C (n=3). After a series of dehydration steps, the constructs were embedded in paraffin. Hydrogel sections (10µm) were stained by immunohistochemistry (IHC) methods for the presence of PEG, collagen II and collagen X, and by histology methods with Safranin-O (Sigma-Aldrich, St. Louis, MO, USA) for sulfated glycosaminoglycans (GAGs). Enzyme pretreatments of 2000 U ml⁻¹ hyaluronidase for PEG and collagen II, and 1 mg ml⁻¹ protease followed by 1 mg ml⁻¹ pepsin for collagen X. Constructs permeabilization and blocking were treated with primary antibodies overnight at 4°C: 1:50 anti-PEG
(courtesy of Steve Roffler at Anti-PEG), 1:50 anti-collagen II (University of Iowa, Developmental Studies Hybridoma Bank), and 1:50 anti-collagen X (University of Iowa, Developmental Studies Hybridoma Bank) in 1% BSA blocking solution. Sections were then treated with secondary antibody for 2h with goat anti-mouse or anti-rabbit IgG labeled AlexaFluor 488 (1:100) and counterstained with 4′, 6-Diamidine-2′-phenylindole dihydrochloride (DAPI) for 10 minutes at room temperature.

8.3.6 Cell Harvest and Expansion

Allogenic MSCs were harvested from a New Zealand white rabbit (different from the in vitro study) as explained above and expanded in specific media (20% fetal bovine serum, 50 U ml⁻¹ penicillin, 50 mg ml⁻¹ streptomycin, 20 mg ml⁻¹ gentamicin, and 5 ng ml⁻¹ bFGF in low glucose DMEM under standard cell culture conditions) at 37° C with 5% CO₂ up to passage 3 and prepared for date of surgery.

8.3.7 Generation of Osteochondral Defect and Treatment

All surgical procedures were performed using a protocol approved by Institutional Animal Care and Use Committee. Under general anesthesia, a medial-parapatellar arthrotomy approach was performed, and the patella was luxated laterally in 10 skeletally mature (8 months old) New Zealand white rabbits. Then, a 3mm-width x 2mm-depth critically sized defect was bilaterally created, with a 3x2mm drill, in the central region of the femoral trochlear groove (20 knees), with previously reported technique (Figure 1)¹¹.
In each animal, one defect was randomly designated to be treated with hydrogel alone or hydrogel with MSCs. All contralateral knees were left untreated; hence each rabbit having its own matched control. Animals were allocated in three groups as follows: A (n=5) chondral defect treated with the cartilage mimetic hydrogel alone; B (n=5): chondral defect treated with hydrogel + BM-MSCs; and C (n=10, contralateral knees, 5 for each of the groups A and B): control defect with no treatment (Table 2). Prior to the application of the treatment, the defect was carefully dried with sterile filtered (0.2µm) CO\(_2\) to improve hydrogel adhesion to the lesion. Approximately 30-40 µl of precursor solution (3 x 10\(^6\) BM-MSCs per ml, for group B) was injected into the chondral defect until full and leveled with articular surface. The hydrogel was photopolymerization *in situ* using 405 nm light for ~40 seconds (Figure 2). Patella was then reduced and closure was performed using a monocryl through different layers. After surgery, animals were left to ambulate full weight bearing freely in kennels.

<table>
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<th>Rabbit N°</th>
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<th>Left Knee</th>
<th>Sex</th>
<th>N° of cells</th>
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<td>Hydrogel Alone</td>
<td>Control Defect</td>
<td>M</td>
<td>NA</td>
<td>40 µl</td>
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*Figure 8.1. Intraoperative drilling and chondral defect in the trochlea*
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<th>40 µl</th>
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<tr>
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<td>Control Defect</td>
<td>M</td>
<td>NA</td>
<td>40 µl</td>
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<tr>
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<td>Control Defect</td>
<td>Hydrogel Alone</td>
<td>M</td>
<td>NA</td>
<td>40 µl</td>
</tr>
<tr>
<td>5</td>
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<td>Control Defect</td>
<td>M</td>
<td>NA</td>
<td>40 µl</td>
</tr>
<tr>
<td>6</td>
<td>MSCs + Hydrogel</td>
<td>Control Defect</td>
<td>M</td>
<td>3X 10^6</td>
<td>40 µl</td>
</tr>
<tr>
<td>7</td>
<td>Control Defect</td>
<td>MSCs + Hydrogel</td>
<td>M</td>
<td>3X 10^6</td>
<td>40 µl</td>
</tr>
<tr>
<td>8</td>
<td>MSCs + Hydrogel</td>
<td>Control Defect</td>
<td>M</td>
<td>3X 10^6</td>
<td>40 µl</td>
</tr>
<tr>
<td>9</td>
<td>Control Defect</td>
<td>MSCs + Hydrogel</td>
<td>M</td>
<td>3X 10^6</td>
<td>40 µl</td>
</tr>
<tr>
<td>10</td>
<td>MSCs + Hydrogel</td>
<td>Control Defect</td>
<td>M</td>
<td>3X 10^6</td>
<td>40 µl</td>
</tr>
</tbody>
</table>

MSCs: mesenchymal stem cells, M: male; NA: non-applicable

**Figure 8.2. In situ polymerization of the hydrogel into the chondral defect.** A. In situ injection of hydrogel precursor under red light to prevent polymerization before inoculation. B. Photo-polymerization of the hydrogel in situ using visible 405nm light. C. Defect filled with polymerized hydrogel.

**8.3.8 Tissue Retrieval**
Six months after surgery, rabbits were humanely euthanized by an overdose of barbiturrate in accordance with the Guide for the Care and Use of Laboratory Animals, and the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals. The circular defect area was cut in half with a band saw (Exakt technologies Inc. OK, USA). The medial half of the repair tissue with surrounding native tissue was fixed in neutral-buffered 10% formalin for histology.

8.3.9 Macroscopic - Microscopic Evaluation and Scoring

Defects were macroscopically evaluated by 4 blinded experienced professionals in cartilage field. The International Cartilage Repair Society (ICRS) score was performed for each one of the defects and a mean was calculated for each one of the groups\textsuperscript{12}. (Grade 1: normal cartilage; grade 2: nearly normal, Grade 3: abnormal and grade 4: severely abnormal)

All histological sections were stained with H&E (Sigma-Aldrich, St. Louis, MO, USA) and Safranin-O (Sigma-Aldrich, St. Louis, MO, USA), followed by grading according to the ICRS II and the modified O’Driscoll scoring system\textsuperscript{13,14}. Grading was performed blinded by two orthopedic surgeons specialized on cartilage healing.

8.3.10 Statistical Analysis

\textit{In vivo} intergroup comparison was performed with a one-way Analysis of Variance (ANOVA) test. \textit{In vitro} data were analyzed by a one-way ANOVA as a function of time. Tukey’s posthoc correction was used to adjust for multiple group comparisons. Paired t-tests were run for intragroup comparisons: 1) compressive modulus and gene expression at different time points; 2) rabbits receiving hydrogel alone vs. their contralateral limb.
empty defect (control); 3) rabbits receiving hydrogel + MSCs vs. their respective controls; for ICRS, ICRS II, and modified O'Driscoll overall total scores and for comparison of each criteria. The tests were performed with SigmaPlot 11.0 statistical software (Systat Software, San Jose, CA, USA). Data are reported as mean with SD, and $P$ values are reported to indicate level of significance with $p \leq 0.05$ considered to be statistically significant.

8.4 Results

MSC chondrogenic differentiation was evaluated by qPCR for gene expression of Sox9, aggrecan, and collagen II as markers for chondrogenesis, and Runx2 and collagen X as markers for hypertrophy (Figure 3). Mean mRNA levels of the chondrogenic transcription factor, Sox9, were upregulated ($p=0.094$) from week 3 to week 9. Similarly, mean aggrecan mRNA levels were also elevated ($p=0.159$) throughout the study, indicating chondrogenic differentiation of encapsulated MSCs. Concomitantly, mean levels of the hypertrophic markers were also elevated over 9 weeks, with both Runx2 ($p=0.066$) and collagen X ($p=0.058$) increasing 10-fold. However, at week 6 the Sox9 expression was higher ($p=0.045$) than Runx2, and by week 9 collagen II mRNA levels were higher ($p=0.029$) than collagen X.
Figure 8.3. Relative gene expression data of chondrogenic and hypertrophic markers at week 3, 6 and 9 (n=3). Data is represented as the mean, and error bars are standard deviation. # and * represent significantly different expression between genes (# for Sox9 and Runx2, * for collagen II and collagen X) at a given time point (p<0.05).
At the protein level, the encapsulated MSCs expressed collagen II after 3 weeks, and by week 9 the ECM production of collagen II was prevalent throughout the hydrogel (Figure 4). Histological images of sGAGs stained by Safranin-O indicate the presence of sGAGs over 9 weeks. Positive staining at early time points was expected due to the incorporation of ChS in the hydrogel formulation, however, after 9 weeks the positive
sGAG stain was retained due to sGAGs synthesis despite hydrogel degradation. The IHC stain for PEG showed significant degradation of the polymer matrix in the hydrogel over the course of the 9-week study. By comparing the collagen II and PEG staining, it appeared that collagen II protein production correlated to the reduction in positive PEG staining, and that by week 9 there appeared to be more collagen II predominance over PEG.

![Graph showing compressive modulus over time](image)

*Figure 8.5. The bulk compressive modulus* initially (day 1) and at week 3, 6, and 9 (n=3)

The compressive modulus was evaluated to determine hydrogel degradation and matrix deposition (Figure 5). At day 1, the compressive modulus was determined to be ~65kPa, and decreased (p<0.0001) to ~30% after 3 weeks, ~15% after 6 weeks (p=0.029), where it was maintained up to 9 weeks. The results agree with the immunohistochemistry and histology images, indicating scaffold degradation coincides with deposition of matrix by the encapsulated MSCs.
**In vivo Results**

At time of euthanasia, all defects showed repaired tissue in the defect. Macroscopically, there was no synovial reaction or inflammation. Compared to control, study groups showed an inferior repaired tissue with nearly normal repaired tissue. Representative image and the average macroscopic ICRS scores for the groups are shown in Figure 6. The mean score for the control group was significantly higher (p=0.088) in the control group than those treated.
Figure 6. Macroscopic dissection of repaired cartilage lesions. A) Control B) Hydrogel alone and C) Hydrogel and MSCs. Arrow points to chondral defect. D) The mean evaluation score of the macroscopic tissue production with standard deviation. E) Plot comparing macroscopically the control to the corresponding contralateral treatment leg. Macroscopically, adding treatment, either hydrogel alone or hydrogel with MSCs reduced the amount of macroscopic normal tissue. (p=0.41). Each arrow shows the control compared to the treatment contralateral leg. The normal tissue was reduced when treatment was applied with abnormal tissue in some samples (these abnormal tissues were never evident in control group)
Repair quality and matrix deposition was evaluated histologically with safranin O and H&E (Figure 8). In the control group, fibrocartilage repair was observed in the defect site, with little to no positively stained sGAGs, suggesting the formation of fibrous neotissue. In the defects that were treated with hydrogel only, repaired tissue was evident and sGAGs were present in the defect site, indicating hyaline-like cartilage neotissue. However, there appeared to be a lack of integration between the hydrogel-formed neotissue and the defect walls. Defects treated with MSC-laden hydrogels were visually similar to the control with fibrocartilage tissue repair evident and a lack of sGAGs, as shown indicated by a lack of Safranin O staining.
No significant difference in treatment score was found between groups (Figure 9). Overall, the hydrogel alone had the highest average total score with O’Driscoll system (p= 0.11). When evaluated with ICRS II scoring system, the best result was seen in the control group, followed by hydrogel alone (p= 0.324). When comparing each criteria of the O’Driscoll score between treatment and contralateral control, the hydrogel alone had the most improvement. These included more Safranin O staining, better surface, better bonding to adjacent cartilage and reconstruction of the subchondral plate (p<0.05).
Figure 8.8. The mean scores for the different treatment conditions with standard deviation and a plot comparing the control value to the corresponding contralateral treatment leg. Treatment with hydrogel alone improved Safranin O staining (PG production) when compared to its contralateral leg. Contrary, adding MSCs did not improve the PG staining (p=0.49). Each arrow shows the control compared to its contralateral leg.

In addition to histological evaluation, collagen II protein expression was evaluated via immunohistochemistry. The best and worst regeneration of collagen II were chosen as representative images. Collagen II was found along the surface of the repaired tissue in the control group. Defects treated with hydrogel only showed a significant amount of collagen II in the defect site, however, the worst treated showed collagen II production similar to that of the untreated control. The MSC-laden hydrogel treatment showed a
range of outcomes from no collagen II, to a large presence of collagen II found in the defect site.

![Representative images of the best and worst immunohistochemically analysis of collagen II (green) at the chondral defect (indicated by arrows) and nuclei counterstained with DAPI (blue)](image)

*Figure 8.9. Representative images of the best and worst immunohistochemically analysis of collagen II (green) at the chondral defect (indicated by arrows) and nuclei counterstained with DAPI (blue)*

The integration of the hydrogel material to the surrounding defect tissue was also evaluated through immunohistochemistry of collagen II. At the site of the defect, those treated with hydrogel only had a distinguishable interface from where the neo-tissue formed in the hydrogel to the surrounding defect tissue. Although a disconnect appeared, the neotissue formed stained positively with collagen II. Defects that were treated with hydrogels and MSCs showed integration with the surrounding defect tissue, with little to no indication of a distinguishable tidemark between the two. However, in comparison to the hydrogel-only treated, these defects lacked collagen II at that interface.
Figure 8.10. Representative images of integration of the hydrogels to the surrounding tissue at the bottom of the defect (indicated by arrow) via collagen II (green) immunohistochemically analysis and nuclei counterstained with DAPI (blue)

8.5 Discussion

The goal of this study was to investigate the use of an MMP2 sensitive cartilage mimetic PEG hydrogel as a treatment for chondral defects in a rabbit animal model. Prior to in vivo applications, the chondrogenic differentiation of rabbit MSCs encapsulated in the degradable hydrogel were first evaluated in vitro. The encapsulated MSCs chondrogenically differentiated and were able to produce their own extracellular matrix and degrade the PEG hydrogel over the course of 9 weeks. In vivo the hydrogel was successfully injected and polymerized in situ focal chondral defects. Cartilage regeneration was found to be similar between all three conditions: hydrogel alone,
hydrogel with MSCs and the untreated control. However, histological evaluation show more sGAGs in the defect treated with the hydrogel alone than the other two conditions. Taken together, results suggest that the hydrogel alone may be promising for treating chondral defects.

Chondrogenesis of rabbit MSCs encapsulate in the cartilage mimetic hydrogel were investigated in vitro prior to surgery to ensure successful differentiation, degradation, and matrix production. mRNA levels of both chondrogenic and hypertrophic genes were increased over the course of the study. However, the mRNA levels of the chondrogenic genes Sox9 and collagen II were significantly higher than the hypertrophic genes Runx2 and collagen X. Over the duration of the study, the collagen II protein deposited by the encapsulated MSCs went from being deposited in the pericellular region at week 3, to an observable increase in deposition into the extracellular space, allowing for interconnectivity of the matrix produced by week 9. sGAGs, which are one of the first extracellular matrix molecules produced during chondrogenesis, were present at week 3 and were maintained from week 3 to week 9. These results are all indicative of successful chondrogenesis of the MSCs in the hydrogel network. However, by week 9 large holes in the construct can be seen in the histological images, which are most likely due to degradation of the hydrogel.

Degradation was further evaluated by measuring the compressive modulus. From the initial time point at day 1 to week 3 there was a significant drop in modulus. Although enzyme activity was not assessed in this study, we postulate that the decrease in modulus is due to degradation by cell secreted enzymes, such as MMP2. MMP2 is an enzyme that is involved in the remodeling of cartilage tissue and has been found to be secreted by
MSCs as well as chondrocytes, making it a promising candidate for cartilage defect repair. Additionally, rabbits are known to secrete MMP2 during healing and remodeling \(^{15-17}\). While there was a significant initial decrease in the modulus, the compressive modulus was maintained from week 3 to week 9. This is believed to be due to the matrix deposition by the encapsulated MSCs which is able to maintain the structure of the ECM and hydrogel construct while the hydrogel fully degrades. The analysis of PEG by immunohistochemistry further confirms this hypothesis, where by week 9 there is very little presence of PEG, however, the ECM and hydrogel construct still remains intact and the modulus is constant from week 3 to week 9. These results suggest that hydrogel degradation allows for macroscopic ECM synthesis, hence replacing the synthetic hydrogel with cartilage-like tissue.

The \textit{in situ} delivery of the photopolymerizable hydrogel for the treatment of focal chondral defect in a rabbit model was successfully achieved. Photopolymerizable PEG based hydrogels are advantageous to the treatment of chondral defects over other forms hydrogels and biomaterials used for this reason. Unlike materials that are made prior to surgery and are directly implanted into the defect as collagen-based scaffolds, the liquid precursor solution of the PEG hydrogel can be directly injected into the defect and fully fill and achieve the shape of the defect. This is advantageous for clinical practice of the hydrogel, as the chondral defects of patients will not all be the same size and shape as they are in an animal model. Additionally, unlike other hydrogels that require a UV light source to polymerize or cure, the hydrogels in this study are photopolymerizable by visible light (405nm). This eliminates the possibility of any additional damage that may be inflicted by the UV light on the cartilage tissue. Lastly, the PEG-based hydrogels are
attractive biomaterials because they have been found to be bioinert. This was evident in our study, where the hydrogel did not elicit an immune response. As our first in vivo study with this hydrogel material, the study confirmed that the photopolymerizable concept is successful for in situ delivery to chondral defects.

Results from this in vivo study follow suit of studies that proceed us, which have found that MSCs-based therapies do not enhance cartilage repair in a rabbit animal model\(^{18-22}\). Previous studies have reported reduced cartilage repair when treated with MSCs, and more evidence of hypertrophic cartilage and remodeling of the subchondral bone\(^{23}\). Additionally, the implantation of MSCs within scaffolds has been found to improve bone formation, but has had little to no success at regenerating cartilage\(^{22-24}\). This is especially common when defects extend into the subchondral bone\(^{22,23}\). It is possible that the encapsulated MSCs receive signals from surrounding tissue that leads to undesired tissue development and/or impedes their ability to differentiate to chondrocytes\(^{25}\). It is also possible that the tissue produced in the defect is as a result of infiltration by other cell types and may not be produced by the encapsulated MSCs. When the defect extends into the subchondral bone, an effect similar to microfracture can occur where cells and fluid from the underlying bone can migrate to the defect site, form a clot, and repair the defect with fibrocartilage. Due to the histological similarities between the MSC-treated and the untreated control, we hypothesize that the tissue produced is likely due to the migrating cells and is not a result of the encapsulated MSCs, however, further studies tracking cells may be needed to histologically probe this concept.

One strategy to improve the chondrogenesis of MSCs in vivo, is by providing a differentiation promoter like TGF-β or others such as Kartogenin and TD-198946, which
may promote chondrogenesis and collagen II synthesis. In the \textit{in vitro} study, the differentiation media contained dexamethasone and TGF-β3, which are known to improve chondrogenic differentiation and matrix deposition. However, it is possible that these growth factors were not delivered to the MSCs \textit{in vivo} at the same concentration or time. This could have negatively attributed to the lack of chondrogenic differentiation of the MSCs. Currently, we are developing a novel approach where we are incorporating TGF-β3 into the hydrogel scaffold in order to enhance differentiation of encapsulated MSCs and promote cartilage regeneration.

The chondral defects treated with the hydrogel alone improved cartilage repair, suggesting a promising scaffold for cartilage repair. The ChS incorporated in the hydrogel creates a unique environment within the hydrogel \textit{in vivo} by recapitulating some of the properties of native tissue such as increased water content and osmolarity, which has been shown to promote tissue synthesis. Previous studies have reported that ChS aids in tissue repair of chondral defects and has also been used as a bioadhesive to integrate to the surrounding native cartilage. The hydrogel did not appear to integrate well into the surrounding tissue in the histological evaluation of this study, however, this may have been due to processing techniques to prepare the tissue for histology. It is postulated that the sGAG production in the chondral defect is due to infiltration of cells from the underlying subchondral bone into the hydrogel network. It is possible that the hydrogel acts as a scaffold for those cells and enhances their production of ECM matrix.

The chondral defects that were left untreated (the control group) showed self-healing capabilities and intrinsic cartilage repair capacity, which has been previously reported in rabbit animal models. Nonetheless, this model has been widely used for
research on cartilage regeneration based on easy handling, low cost and care, and thus provides a useful model for feasibility testing of new materials prior to moving into larger and more complex animal models. The remarkable healing potential these animals have make rabbits a difficult model to evaluate the translational potential of cartilage repair treatments\textsuperscript{11}.

Some limitations to the present study are acknowledged. First, the high flexion degree of the rabbit’s knee creates a partial weight bearing joint, hence a suboptimal loading stimulus transmission for the hydrogel degradation and cartilage repair\textsuperscript{31}. Second, having a small sample size reduced the significance of the results due to high variability between the rabbits. Although high consistency was kept among the animal demographics, such as age, sex, and control defects in all samples, there was still a high degree of variability among the groups. Increasing the study population would provide a better understanding of the differences between the treatment groups, as there were notable differences when observed under light microscope, however they were not statistically significant.

8.6 Conclusion

In conclusion, the hydrogel alone supported cartilage repair, suggesting a cartilage mimetic environment that could potentially induce chondrogenesis of infiltrated cells. This treatment of hydrogel alone may be sufficient for cartilage repair, however, long-term evaluation and other animal models that better represent human cartilage would be necessary. MSC-treated defects did not exude similar results, and may be improved with the addition of the appropriate chondrogenic stimuli such as TGF-β or mechanical loading. To date, this is the first animal model reporting the proof of concept of
photopolymerization in situ of this particular MMP-sensitive cartilage mimetic hydrogel and evaluation of cartilage repair in a small animal model.

8.7 Acknowledgments
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8.8 References


Chapter 9

A TGF-β3-tethered MMP degradable PEG hydrogel enhances cartilage extracellular matrix production of encapsulated mesenchymal stem cells

9.1 Abstract
Mesenchymal stem cells (MSCs) are a promising cell therapy to treat chondral defect, however, chondrogenesis and cartilage regeneration in vivo has been limited. One potential solution is to combine MSCs with locally presented growth factors in a hydrogel scaffold in order to promote their chondrogenic differentiation when implanted in vivo. The objective of this study was to develop a cartilage mimetic hydrogel with the tethered growth factor, TGFβ3, to promote chondrogenesis of encapsulated MSCs and encourage cartilage extracellular matrix (ECM) production. Rat MSCs were encapsulated in a matrix metalloproteinase 2 (MMP2) degradable cartilage mimetic poly(ethylene glycol) (PEG) hydrogel containing the glycosaminoglycan (GAG), chondroitin sulfate (ChS), and the cell adhesion peptide, RGD. The chondrogenic growth factor, TGF-β3 was introduced either in the culture medium (soluble) or covalently linked (tethered) to the hydrogel network. After 9 weeks, both hydrogels systems showed significant degradation of the PEG hydrogel. Tethered TGF-β3 led to increased ECM deposition of sGAGs and collagen II by the encapsulated MSCs. Soluble TGF-β3 also led to an increase in collagen II protein deposition, however, sGAG retention decreased. Moreover, tethered TGF-β3 exhibited spatial distribution of collagen II similar to that of hyaline-like cartilage, soluble TGF-β3 produced tissue reminiscent of fibrocartilage, which was further confirmed by the presence of collagen I. In summary, tethering TGF-β3 into a cartilage mimetic, degradable hydrogel promotes chondrogenesis and cartilage-like ECM deposition by encapsulated MSCs and may be a promising scaffold for in vivo cartilage repair.
9.2 Introduction

Articular cartilage lacks the ability to self-heal when injured and requires treatment to maintain its function\textsuperscript{1, 2}. If left untreated, the damaged tissue will only worsen and become at risk of developing osteoarthritis. Osteoarthritis is characterized by loss of articular cartilage that causes pain, decreased mobility and is one of the major causes of disability in the United States\textsuperscript{2–5}. Tissue engineering is a promising treatment of articular cartilage lesions that could minimize the development of osteoarthritis. Many different approaches have been investigated\textsuperscript{6, 7}, which include the choice of material (natural and synthetic) \textsuperscript{1, 2, 7, 8}, cell type (chondrocytes, mesenchymal stem cells, etc.)\textsuperscript{9–11}, and therapeutic agents whether they be covalently tethered\textsuperscript{12, 13} or encapsulated\textsuperscript{14–16}. Although there have been a number of studies aimed at treating cartilage defects, regenerating hyaline cartilage remains challenging.

A promising cell source for cartilage tissue engineering are mesenchymal stem cells (MSCs). MSCs can be isolated from the patient, expanded to the large number of cells necessary for repair, and can differentiate into multiple tissue types. Many studies have investigated the use of MSCs for tissue engineering applications, and results suggest that when provided with the proper soluble and environmental cues, MSCs can differentiate along a number of mesenchymal lineages\textsuperscript{13, 17–20}. Environmental cues often come from recapitulating the native tissue. For instance, cartilage tissue is primarily made up of collagen II, hyaluronic acid, and aggrecan, which is comprised of the highly negative charged glycosaminoglycan (GAG) chondroitin sulfate (ChS). Studies have investigated incorporating one or multiple of these ECM analogs into hydrogels in order to create a biomimetic environment that allows for MSC chondrogenesis\textsuperscript{21–23}. Our previous studies have shown that incorporating ChS and the cell adhesion peptide RGD enhances
collagen II expression of encapsulated MSCs\textsuperscript{19}. Although promising, these environmental cues are often not enough to promote chondrogenesis on their own, and soluble cues such as growth factors are needed.

The chondrogenic differentiation of MSCs have been shown in many different matrix-based and matrix free systems when cultured with members of the transforming growth factor beta (TGF-β) family\textsuperscript{24}. In the presence of these growth factors, MSCs deposit cartilaginous matrix when cultured in pellets, hydrogels or fibrous scaffolds\textsuperscript{25–27}. These growth factors are commonly added in culture media during \textit{in vitro} experiments, however, delivering soluble growth factors \textit{in vivo} is challenging. For instance, the half-life of soluble growth factors \textit{in vivo} is limited\textsuperscript{28}. Growth factors can potentially react with other cell types in the area of the defect or be sequestered by the extracellular matrix (ECM)\textsuperscript{29}. Additionally, growth factors may not be present at concentrations similar to \textit{in vitro} culture\textsuperscript{30}. One mechanism used to deliver growth factors to encapsulated cells \textit{in vivo} is by incorporating them into microparticles and encapsulating those microparticles into the hydrogels\textsuperscript{15, 31}. Although results using this strategy are promising, there is high variability in protein loading and release kinetics, as well as complications with size distribution and scaffold preparation\textsuperscript{13}. In order to combat this, covalently tethering the growth factors to the hydrogel network allows for controlled concentrations and local presentation to the encapsulated cells.

Poly(ethylene glycol) (PEG) based hydrogels have been studied as a potential carrier of cells, biochemical cues and growth factors for cartilage tissue engineering\textsuperscript{32,33}. Notably, PEG hydrogels can be formed through cytocompatible methods enabling them to be injected directly into the defect site and formed \textit{in situ}, via a photoinitiated step-
growth polymerization between norbornene and thiol functionalized monomers. Biomimetic molecules, degradable crosslinkers, and growth factors can easily be tethered into the hydrogel through the thiol-reactive chemistry, making them an attractive platform for cartilage tissue engineering. Peptides that are sensitive to enzymatic degradation can be easily incorporated into PEG thiol-ene hydrogels through the addition of thiol-containing cysteines. Such peptides include those that are sensitive to matrix metalloproteinases (MMPs) which are secreted by cells during different stages of differentiation and maturation. These enzymatically sensitive crosslinkers allow for spatial and temporal control over hydrogel degradation. In addition to degradable crosslinks, growth factors such as TGF-β1 can be tethered into PEG thiol-ene hydrogels through thiol modification. The TGF-β1-tethered hydrogels have shown enhanced matrix production by encapsulated chondrocytes.

The goal of this study was to investigate the chondrogenic differentiation of encapsulated rat MSCs in an enzymatically degradable cartilage mimetic hydrogel with and without tethered TGF-β3. The overall hypothesis is that chondrogenesis and matrix elaboration in the tethered TGF-β3 hydrogels will be similar to hydrogels cultured in soluble TGF-β3. ChS and RGD were incorporated into the hydrogel network to recapitulate the biochemical cues of native cartilage tissue, which has previously been shown to enhance chondrogenesis of MSCs. Additionally, an MMP2 sensitive peptide crosslinker was used to allow for cell-mediated enzymatic degradation, as chondrocytes have been shown to secrete MMP2 during cartilage development and remodeling. Chondrogenesis was evaluated over the course of 9 weeks by examining the production of the chondrogenic markers, collagen II and sGAGs, as well as the degradation of the
PEG hydrogel. The tethered TGF-β3 hydrogel will be advantageous for cartilage repair in vivo because it provides the necessary cues without the need for additional soluble growth factor treatment.

9.3 Materials and Methods
9.3.1 Macromer Synthesis

Poly(ethylene glycol) (PEG) (8arm, 10kDa) norbornene was synthesized as previously described. Briefly, 8-arm PEG amine (10kDa) was dissolved in dimethylformamide (DMF) and reacted with 4 molar excess of 5-norbornene-2-carboxylic acid with N,N-diisopropylethylamine (DIEA) and 1-[Bis(dimethylamino0methylene]-1H-1,2,3-triazol[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) overnight at room temperature under argon purge. The PEG norbornene product was precipitated in cold diethyl ether, vacuum filtered, purified by dialysis and lyophilized to recover the product. The conjugation of norbornene to each arm of the PEG amine was determined via 1HNMR by comparing the area under the peak for the allylic hydrogel closest to the norbornene hydrocarbon group (δ=3.1-3.2ppm) to the area under the peak for the methyl groups of the PEG backbone (δ=3.4-3.85 ppm) and was found to be 100%.

Chondroitin sulfate was thiolated as previously described. Chondroitin sulfate (ChS) (Chondroitin sulfate A, Sigma Aldrich) was fully dissolved in water and reacted with two molar excess dithiobis(propanoic dihydrazide) (DTP) and the pH was adjusted to 4.7 with the addition of 1.0M HCl. Two molar excess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) was added to the ChS and DTP and reacted overnight and the pH was maintained at 4.75. The pH was raised to 7 with the addition of 1.0M NaOH to stop the reaction. The thiol groups of the DTP were reduced with the addition of 6.5 molar excess dithiothreitol (DTT) four 24 hours at a pH of 8.5. The final product, thiolated
chondroitin sulfate (ChS-SH) was purified and recovered by dialysis against 0.3mM HCl, centrifuged to remove any particulates, and the supernatant was lyophilized. Conjugation of thiol groups to ChS was found to be 15% (7 thiol groups per molecule of ChS) by $^1$HNMR by comparing the area under the peaks for the two side chain methylene groups of DTP ($\delta=2.5-2.6$ and $2.6-2.8$ppm) to the area under the peak representing the methyl protons of the acetyl amine side chain ($\delta=1.8-2.0$ ppm).

TGFβ3 (Peprotech) was thiolated using Pierce™ Traut’s Reagent (2-iminothiolane) (ThermoFisher). Briefly, Traut’s Reagent was reacted at a 4:1 molar ratio to TGFβ3 for 1 hour at room temperature. The thiolated TGFβ3 (TGF-β3-SH) was then pre-reacted with PEG norbornene to obtain a final concentration of 50nM via photopolymerization using 0.05wt% photoinitiator Irgacure 2959 (I2959) (BASF) for 30 seconds at 352 nm light at 5 mW cm$^{-2}$.

9.3.2 Rat MSC isolation and culture
Rat MSCs were isolated from Sprague-Dawley rats following the protocol provided by Jackson Laboratory. Briefly, rats were euthanized, the tibia/femur were harvested and the tibia was separated from the femur. All soft tissues were removed. The ends of the bones were snipped with scissors and placed in PBS. Warmed complete conditioned media (10% FBS, 1% penicillin/streptomycin, and 2 mM l-glutamine) was forced through the bone shaft to extract all red marrow, which was repeated until the solution was clear. The mixture was filtered (70µm filter) to remove any large debris, and the filtered cell suspension was plated on a vented T175 flask. Cells were cultured at standard conditions until they adhered and were confluent (4-7 days). The cells were then expanded up to passage 3 until 70-90% confluency.
9.3.3 Hydrogel Formation and MSC Encapsulation

Hydrogels were fabricated via photopolymerization from two precursor solution. A 9% (g/g) PEG norbornene solution was used for the control, whereas for the tethered-TGF-β3 hydrogels, 9% (g/g) norbornene with the attached 50nM TGFβ3-SH as previously described was used. The rest of the precursor solution consisted of 1% ChS-SH, 0.1mM CRGDS (Genscript), and 2.14% (g/g) of matrix metalloproteinase 2 (MMP2) degradable peptide flanked with cysteines CVPLS-LYSGC (Genscript). The precursor solution was filter-sterilized (0.22 µm filter) and photopolymerized with 0.05% (g/g) I2959 in phosphate buffered saline (PBS) with 352 nm light at 5 mW cm⁻² for 8 minutes. The MSC-laden hydrogels with tethered-TGF-β3 were cultured in chondrogenic differentiation media without TGF-β3 supplement (medium (1% ITS+ Premix, 100 nM dexamethasone, 50 mg ml⁻¹ l-ascorbic acid 2-phosphate, 50 U ml⁻¹ penicillin, 50 mg ml⁻¹ streptomycin, and 20 mg ml⁻¹ gentamicin in high glucose Dulbecco’s modified Eagle media), whereas the hydrogels without the tethered TGF-β3 were cultured in the same media with the addition of 1nM TGFβ3. MSC-laden hydrogels were cultured individually in a 24 well plate with two milliliters of chondrogenic media per well which was replaced every other day for the duration of the study. The cell-laden hydrogels were cultured under standard cell conditions of 37°C with 5% CO₂.
Figure 9.1. A schematic of the precursors and encapsulation of rat MSCs in an MMP degradable cartilage mimetic hydrogel via photopolymerization A) cultured with soluble TGF-β3 in the media and B) with a thiolated TGF-β3 covalently tethered into the hydrogel.

9.3.4 Cell viability
The viability of cells encapsulated in the hydrogels was examined using Live/Dead™ (ThermoFisher). Cell-laden hydrogels were removed from culture after 9 weeks, and incubated with calcien-AM (1µM) for live cells (green) and ethidium homodimer-1 (2µM) for dead cells (red) in warm PBS in a 24 well plate for 20 minutes. The hydrogels were then immediately imaged by confocal microscopy.
9.3.5 Immunohistochemistry (IHC) and Histology
At week 3, 6, and 9 rat MSC-laden hydrogels (n=3) were removed from culture and processed for immunohistochemistry. Initially, MSC-laden hydrogels were fixed overnight at 4°C in 4% paraformaldehyde and transferred to 30% sucrose until further processing. Hydrogels were preprocessed through a series a dehydration steps, and embedded in paraffin and sectioned (10µm). Sections were stained for the presence of collagen II and PEG. Sections stained for collagen II were pretreated with 2000 U ml⁻¹ hyaluronidase, followed by permeabilization and blocking, and treated overnight at 4°C with anti-collagen II (Abcam ab34712, 1:50). Sections stained for PEG were pretreated with Retrievagen (BD Biosciences) for antigen retrieval, and treated overnight at 4°C with anti-PEG primary antibody at (Anti-PEG 6.3, 1:50). Sections were subsequently treated for 2 hours with goat anti-mouse IgG and goat anti-rabbit IgG labelled AlexaFluor 488 (1:100) and counterstained with DAPI. Collagen II and PEG stained images were analyzed using confocal microscopy and NIH ImageJ. The total area occupied by positive staining for collagen II and for PEG was divided by the total number of nuclei to determine a percent area per cell (n=3 hydrogels, 4 images per hydrogel). Sections were also stained for sulfated glycosaminoglycans (sGAGs) by Safranin O and Fast Green and imaged at 100x by light microscopy (Zeiss Pascal, Olympus DP70). Representative images are shown (n=3 hydrogels, 4 images per hydrogel).

9.3.6 Mechanical Testing of Cell-laden Hydrogels
The compressive modulus was evaluated at week 1, 3, 6, and 9 (n=3) to investigate tissue production and hydrogel degradation. Using a mechanical tester (MTS Synergie 100, 10N) the tangent compressive modulus was determined by straining the hydrogels at a constant rate of 0.1 mm min⁻¹ to 15% strain and the compressive modulus was
determined from the linear region of the stress vs. strain curve between 10 and 15%. Hydrogels were then flash frozen in liquid nitrogen and used for biochemical analysis.

9.3.7 Biochemical Analysis
At 3, 6, and 9 weeks hydrogel constructs (n=3) cell-laden hydrogels were flash frozen in liquid nitrogen and stored at -80°C until processed. Hydrogels were lyophilized, the homogenized (TissueLyzer II, Qiagen) at 30Hz for 10 minutes, and digested by papain for 16 hours at 60°C. Cell-laden constructs were assessed for DNA content using Hoechst 33258. Glycosaminoglycan (GAG) content was assessed using the dimethyl methylene blue (DMMB) dye assay. GAG and collagen were normalized to the amount of DNA for each construct.

9.3.8 Statistical Analysis
Data are shown as the mean with standard deviation. Two way analysis of variance (ANOVA) was used to evaluate the statistical significant of data with a Tukey’s post-hoc to compare all combinations. P-values are provided in the plots and parenthetically in the text with p<0.05 considered to be statistically significant.

9.4 Results
Rat mesenchymal stem cells (MSCs) were encapsulated in a MMP2 sensitive cartilage mimetic poly(ethylene glycol) thiol-norbornene (PEG thiol-ene) hydrogel with either soluble or tethered TGF-β3. DNA content in the hydrogels was investigated to evaluate cell proliferation and cell loss whether by degradation or cell death (Figure 2A). From week 3 to week 6, there were no significant changes in DNA content in either of the hydrogel cultures. However, by week 9, there was a significant decrease (p=0.006) in DNA from week 3 in cell-laden hydrogels cultured with soluble TGF-β3. DNA content also decreased in the hydrogels with tethered-TGF-β3 by week 9, however, not to the same
extent \( (p=0.06) \). At the end of the experiment at nine weeks, encapsulated MSCs remained viable in the hydrogels with tethered or soluble TGF-\( \beta \)3 with few dead cells (Figure 2B). Cell viability appeared similar in the two TGF-\( \beta \)3 conditions.

**Figure 9.2. DNA content and cell viability** A) DNA content in the hydrogels with soluble TGF-\( \beta \)3 (solid) or tethered TGF-\( \beta \)3 (striped) throughout the culture period. Data is represented as the mean with error bars representing the standard deviation \((n=3)\). B) Representative images of live (green) and dead (red) MSCs encapsulated in the hydrogels after 9 weeks of culture \((n=3)\)

The deposition of chondroitin sulfate (ChS) in the hydrogels was also investigated (Figure 3A). In the hydrogels cultured with soluble TGF-\( \beta \)3, there was a decrease \((p=0.0001\) and \(p<0.0001\), respectively) in sGAG in the hydrogels at week 6 and week 9 from week 1. In contrast, in the hydrogels with tethered-TGF-\( \beta \)3, sGAG deposition at week 3 was maintained from throughout the remainder of the study. At week 9, the amount of ChS in the hydrogels with tethered TGF-\( \beta \)3 was higher \((p=0.026)\) than in
hydrogels cultured with soluble TGF-β3. This biochemical analysis of ChS deposition coincides with safranin O histology (Figure 3B).

Histological evaluation of sGAGs retained in the hydrogel was done via safranin O stain. Note, the incorporation of chondroitin sulfate in the hydrogel results in a basal level of positive stain. At 3 weeks of culture, sGAG deposition was evident in both TGF-β3 conditions. MSCs cultured in hydrogels with soluble TGF-β3 showed regions of positive stain that were adjacent to areas of little to no positive staining. MSCs cultured in hydrogels with tethered TGF-β3 showed a more uniform sGAG deposition throughout the hydrogel. By week 6, MSCs in hydrogels with soluble TGF-β3 showed a reduction in sGAG retention from week 3, and by week 9 there were even fewer regions of deposited sGAG in the hydrogel. In contrast, hydrogels with tethered TGF-β3 retained sGAGs from week 3, to week 6 and week 9 of culture, and exhibited observably more sGAGs than MSCs cultured with TGF-β3 in the media.

![Figure 9.3. sGAGs in the hydrogel constructs over time](image)

**Figure 9.3. sGAGs in the hydrogel constructs over time**

A. Chondroitin sulfate (ChS) content in the hydrogels with soluble TGF-β3 (solid) or tethered TGF-β3 (striped) throughout the culture period. Data is represented as the mean with standard bars representing the standard deviation (n=3). B. Representative images of safranin O stained sections counterstained with fast green.
Cartilaginous ECM deposition by encapsulated rat MSCS was analyzed and, as a result, indicated chondrogenic differentiation. After three weeks of culture, collagen II was present in both hydrogel cultures (Figure 3). Semi-quantitative analysis of collagen II stained sections indicated an increase ($p=0.024$) in collagen II area per cell for MSCs cultured in the hydrogels with soluble TGF-β3 from week 3 to week 6 (Figure 3). However, from week 6 to week 9 there was a significant decrease in collagen II area per cell when cultured with soluble TGF-β3. Collagen II was produced by MSCs encapsulated in the hydrogels with tethered TGF-β3 at week 3 and week 6. By week 9, there was an increase ($p=0.034$) in collagen II area per cell in the tethered-TGF-β3 hydrogel from week 3. Additionally, MSCs encapsulated in hydrogels with tethered TGF-β3 had significantly more ($p=0.039$) collagen II area per cell at week 9 than those cultured with soluble TGF-β3 in the media. The spatial deposition of the collagen II produced by MSCs encapsulated in hydrogels with tethered TGF-β3 also differed from those cultured with soluble TGF-β3 and resembled that of hyaline-like cartilage.

Sections were also stained for PEG to examine temporal degradation behavior of the hydrogel. PEG was present in both growth factor conditions after 3 weeks. By 6 weeks of culture, the percent area of PEG per cell was decreased in both hydrogels, although not significantly. After 9 weeks, there was a significant decrease ($p=0.006$ and $p=0.012$, respectively) in the percent area of PEG per cell from week 3 and week 6 in hydrogels cultured with soluble TGF-β3. In hydrogels with tethered TGF-β3, the percent area of PEG per cell significantly decreased ($p=0.032$) from week 3 to week 9.
Figure 9.3. Immunohistochemical analysis of collagen II and PEG A. Representative immunohistochemistry images of collagen II (left) and PEG (right) when cultured with tethered TGF-β3 and with soluble TGF-β3 in the media at week 3, 6, and 9. (scale bar = 20µm) B. Semi-quantitative analysis of immunohistochemistry images of the percent area of collagen II and C. PEG per cell at week 3, 6, and 9, and cultured with media (black) and tethered TGF-β3 (striped). Data shown as mean with error bars representing standard deviation.
In addition to collagen II, immunohistochemical analysis of collagen I was done to evaluate the formation of fibrocartilage (Figure 4). MSCs cultured with soluble TGF-β3 showed increased collagen I deposition from week 3 to week 9. By week 9 of culture, collagen I was prevalent and interconnected throughout the construct. Collagen I was also present in the tethered TGF-β3, however, the amount of collagen I was observably lower and appeared to localized to the pericellular space and not spread out throughout the construct.

![Image of immunohistochemical analysis of collagen I](image)

**Figure 4.** Immunohistochemical analysis of collagen I. Representative immunohistochemistry images of collagen I (green) when cultured with tethered TGF-β3 and soluble TGF-β3 at week 3, 6, and 9 (scale bar = 20µm).

The compressive modulus of the hydrogels was evaluated over the course of the study (Figure 4). After one week of culture, the modulus was similar in both hydrogel networks (~35-40 kPa). From 1 to 3 weeks, hydrogels cultured with soluble TGF-β3 decreased although not significantly and remained at a similar compressive modulus to
week 6. However by week 9 of culture, the compressive modulus had dropped \( (p=0.05) \) by \( \sim 2 \)-fold from week 1 to approximately 15 kPa. Hydrogels with tethered TGF-β3 maintained their compressive modulus from week 1 to week 6 (~35kPa). However by week 9, there was a decrease \( (p=0.006) \) in modulus to approximately 15kPa. By week nine, the compressive modulus was similar in both TGF-β3 conditions.

![Figure 9.4. Compressive modulus of the cell-laden hydrogels with soluble TGF-β3 (solid) or tethered TGF-β3 (striped) throughout the culture period. Data is represented as the mean with error bars representing the standard deviation (n=3).](image)

9.5 Discussion

Developing a tissue engineering approach to treat chondral defects is challenging. It requires a scaffold to which cells can be delivered to the defect site, polymerized \( \textit{in situ} \), and have the time and cues necessary to promote chondrogenesis and produce robust articular cartilage. Results from this study suggest that tethering TGF-β3 into a degradable cartilage mimetic hydrogel promotes chondrogenesis and encourages matrix deposition of encapsulated rat MSCs to a greater extent than hydrogels cultured with
soluble TGF-β3. By locally presenting the encapsulated MSCs with the tethered TGF-β3 in addition to the ECM analogs, ChS and RGD, we have shown quantitatively and qualitatively that MSCs chondrogenically differentiate and produce their own cartilage matrix while degrading the synthetic hydrogel scaffold \textit{in vitro}. The matrix produced by the cells encapsulated in the TGF-β3-tethered hydrogels was able to retain GAGs and deposit more collagen II when compared to the cell-laden hydrogels cultured in media containing soluble TGF-β3. Additionally, soluble TGF-β3 resulted in the formation of fibrocartilage, whereas the matrix deposited in the tethered TGF-β3 hydrogels was similar to that of hyaline-like cartilage. The data presented in this study suggests that the degradable, cartilage mimetic PEG thiol-ene hydrogel with tethered TGF-β3 is a potential tissue engineering strategy for \textit{in vivo} cartilage repair.

Tethering TGF-β3 to the cartilage mimetic hydrogel resulted in increased collagen II production and the retention of GAGs in the scaffold. Native articular cartilage is composed of a high concentration of sGAGs in the extracellular matrix such as chondroitin sulfate (ChS). ChS is responsible for the fixed negative charge which results in an increase in local osmolarity, water retention, and compressive properties of cartilage\textsuperscript{25,38–40}. We found that tethered-TGF-β3 hydrogels were able to retain ChS much better than hydrogels cultured with TGF-β3 in the media. The binding of TGF-β to its receptor is necessary for many cellular signaling mechanisms that are imperative to chondrogenesis such as Smad signaling\textsuperscript{41–44}. Negatively charged extracellular matrix molecules in native hyaline cartilage are known to interact and sequestering soluble growth factors from the surrounding fluid \textsuperscript{45,46}. The local presentation of the tethered TGF-β3 to the encapsulated cells may recapitulate the presentation of growth factors found in
native cartilage tissue and further enhance chondrogenesis and extracellular matrix elaboration.

The collagen II distribution was different in the two culturing systems. The collagen II produced in the tethered TGF-β3 hydrogels was rounded, similar to hyaline-like cartilage, whereas, the distribution in the hydrogels cultured with soluble TGF-β3 exhibited more of a sheet-like distribution of collagen II, reminiscent of fibrocartilage\textsuperscript{47–50}. The immunohistochemical analysis of collagen I further confirmed the deposition of fibrocartilage in the soluble TGF-β3 construct, where collagen I was prevalent and interconnected through the constructs cultured in soluble TGF-β3. Although collagen I was present in the TGF-β3 tethered constructs, the amount of collagen I was observably lower and appeared to be more localized to the pericellular space. These results suggest that tethered TGF-β3 may promote hyaline-like cartilaginous matrix production by encapsulated MSCs.

In additional to the changes observed in collagen II production, similar differences were seen in the degradation of PEG. In order for a degradable hydrogel to be beneficial \textit{in situ}, the rate of degradation should match tissue growth to maintain structural integrity. The PEG images in this study suggest that the tethered TGF-β3 the cells appear to be degrading the PEG locally around the deposited matrix. In contrast, hydrogels cultured in soluble TGF-β3 show large areas of degradation. Previously reports have extensively investigated enzymatically degradable hydrogels and the differences between local and bulk degradation as a function of reaction and diffusion kinetics of the enzymes\textsuperscript{35,51}. Although the mechanism by which these hydrogels degrade was not thoroughly investigated in this study, there was an observable difference in the degradation of the
hydrogel. This could be a function of many parameters including enzymes secreted, enzyme concentration, and ECM deposition. Additional studies will need to be done to investigate this further.

In this study, TGF-β3 was covalently tethered into the hydrogel at a much higher concentration (50nM) than the TGF-β3 was provided to the media (1nM). This concentration was used because previous studies confirmed that tethered TGF-β at this concentration showed improved matrix production of encapsulated chondrocytes. Additionally, the concentration used for the soluble TGF-β3 has been found to promote chondrogenesis in previous studies that have investigated differentiation of MSCs in a similar cartilage mimetic PEG hydrogel. Preliminary results (not shown) using Smad2/3 reporter cells, have found that chemically-modifying TGF-β3 by adding thiol-reactive groups reduce the activity of the growth factor. The TGF-β3 in the media was replenished every other day for the duration of the study therefore maintaining its activity. Alternatively, the presence of the tethered TGF-β3 was dependent on the degradation of the hydrogel. Once the degradable hydrogel reaches its reverse gelation point, bulk degradation occurs and TGF-β3 is no longer present within the construct. Therefore, there may be a temporal response of the amount of TGF-β3 presented to the cells. Studies have shown TGF-β3 is necessary to initiate chondrogenesis and matrix deposition. However, long-term exposure to high concentrations of TGF-β3 can drive the differentiation into a hypertrophic phenotype. It is postulated that the tethered TGF-β3 promotes chondrogenesis during initial stages of differentiation, and as the matrix degrades, lower concentrations of TGF-β3 are present. The lower concentration of TGF-β3 may enhance GAG content and prevent hypertrophy, as shown by others.
9.6 Conclusions
We have confirmed that TGF-β3 can be modified with thiol groups, and conjugated into PEG thiol-ene hydrogels, resulting in increased sGAG retention, and collagen II. Additionally, tethered TGF-β3 promotes hyaline-like cartilage, whereas soluble TGF-β3 results in fibrocartilage. Furthermore, we found that the tethered TGF-β3 allowed for local degradation of the MMP-sensitive crosslinks, with little to no PEG left after 9 weeks of culture. Collectively, these results demonstrate that ability to deliver growth factors to MSCs encapsulated in 3D culture to promote and enhance matrix production. This hydrogel platform is a promising treatment for in vivo cartilage repair in future studies. We acknowledge that high concentrations of growth factors such as TGF-β3 can adversely affect other cell types\textsuperscript{30,52}, therefore, prior to in vivo application it is important to determine the minimum concentration necessary to promote chondrogenesis of encapsulated MSCs. The methodology used in this study can be translated to other hydrogel scaffolds that can be tailored to the differentiation of other cell types, such as bone, or multiple growth factors can be tethered in a spatially controlled manner for tissues such as the osteochondral interface.

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9.8 References


Chapter 10
The use of an enzymatically degradable cartilage mimetic PEG hydrogel with tethered TGFβ3 for horse MSC chondrogenesis: an in vitro and in vivo examination

10.1 Abstract

Animal models are a critical part of developing treatments for cartilage defects that will be used in clinical studies. Choosing the correct animal model to test tissue engineering strategies is imperative to their success. Horse animal models are promising for evaluating cartilage repair because they are similar in size, the endogenous cartilage lacks self-repair capabilities, and the joint is exposed to mechanical compression. The aim of this study was to investigate the chondrogenic potential of horse MSCs in an enzymatically degradable cartilage mimetic poly(ethylene glycol) (PEG) hydrogel with tethered TGFβ3 to treat chondral defects in the medial troclea. Prior to in vivo assessment, an initial study cultured in a free swelling environment showed significant proteoglycan and collagen matrix deposition with concomitant PEG hydrogel degradation. A second study was done to evaluate the effect of MSC differentiation when the cell-laden hydrogels were subjected to dynamic loading. Results suggest that mechanical loading does not affect cartilage extracellular matrix (ECM) deposition of horse MSCs, although there was variability of matrix production and degradation between the 3 cell donors. The successful chondrogenesis and cartilaginous matrix deposition by the encapsulated cells in vitro led to the investigation of the cartilage mimetic hydrogel with tethered TGFβ3 in vivo to treat chondral defects. If results of the in vivo equine animal model are successful,
the cartilage mimetic hydrogel has the potential to be used clinically as a treatment for chondral defects.

10.2 Introduction

Articular cartilage has poor intrinsic healing potential. Injuries to articular cartilage eventually degeneration and progress to osteoarthritis, the leading disability worldwide. Osteoarthritis is projected to affect 67 million Americans by 2030. There is also a financial burden associated with osteoarthritis, where estimated costs for treatment in the United States in 2001 were over 60 billion dollars. Therefore, there is clinical and financial need to develop new strategies to improve the repair and regeneration of articular cartilage.

Animal models are vital for developing effective treatments for cartilage defects. The rabbits (lapine) model has been widely used for research in cartilage regeneration. The ease in handling and relatively low costs for purchase and care allow for a sufficient number of replicates and make rabbits a promising animal model for cartilage tissue engineering. When compared to rodent animal models, rabbits are large enough to create chondral defects in the range of 3-4 mm in diameter to which implants can more easily be placed. This makes rabbits an attractive model for early evaluations of biomaterials, however there are many limitation in using rabbit models for cartilage repair. Rabbits have remarkable self-healing potential. Cartilage defects in humans show little to no repair when left untreated, however, full-thickness cartilage defects in rabbits have the ability to fully repair spontaneously. This is especially noticeable in osteochondral defects, those that extend down into the bone, which allow for the infiltration of recruitment of mesenchymal stem cells (MSCs) from the bone marrow. The MSCs can proliferate and differentiate into cartilage tissue without the participation of the
surrounding chondrocytes\textsuperscript{7}. Additionally, the biomechanics of rabbit joints are unlike that of humans. As jumpers, they have a high degree of knee flexion, and the trochlea groove is only used as a partial weight bearing surface\textsuperscript{11}. This is much different from humans and other large animals, and results in the relatively thin cartilage found in rabbit joints, where the mean cartilage thickness of the trochlear groove is approximately 0.44 +/- 0.08 mm\textsuperscript{12}. However, chondral defects in animal models often need to exceed this to allow for effective placement of implants, which can require depths of around 3mm. This ultimately requires the majority of defect to be in the subchondral bone, which can lead to concerns of self-repair as previously mentioned.

Our preliminary \textit{in vivo} study was done in a rabbit animal model in which a degradable, cartilage mimetic hydrogel was injected into osteochondral defects and polymerized \textit{in situ} with and without rabbit MSCs. Although our results were promising, the limitations explained herein were observed in our study. Due to the relatively thin cartilage defect, the defects extended well into the subchondral bone, which recruited bone marrow MSCs to the site of injury, even in those untreated. This allowed for spontaneous self-healing of the untreated defects that was similar, if not better than, those that were treated. Additionally, our \textit{in vitro} studies with encapsulated MSCs in the cartilage mimetic hydrogel have shown the importance of dynamic compression to maintain non-hypertrophic chondrogenesis\textsuperscript{13}, however, in the rabbit model there is a lack of compression on the joint articular cartilage. Therefore, a larger animal model is necessary.

The equine (horse) model offers several advantages over the rabbit model for cartilage repair studies. Horses suffer from similar cartilage problems to humans mainly
due to the racing industry, and as such, cartilage injury and repair are well understood in horses\(^\text{14}\). Additionally, equine cartilage shows low self-healing capabilities, similar to humans\(^\text{15,16}\). The equine model is highly beneficial for preclinical trials of biomaterial treatments as the cartilage thickness in horses (1.75 mm) most closely resembles that of humans (2.35 mm)\(^\text{17}\). The large joint dimensions also allow for the evaluation for larger defects (15-20 mm) which are similar to humans\(^\text{18-20}\). The articular cartilage in horse joints experiences compressive load as opposed to the rabbit mode, however, their weight and physiology results in loads that are greater than what are seen in humans\(^\text{21}\).

Cartilage repair techniques are well studied in horse models, and bone-marrow derived mesenchymal stem cells (MSCs) have shown mixed results for their ability to repair cartilage. Studies have found that equine MSCs have great chondrogenic potential \textit{in vitro}\(^\text{22,23}\), however, have limited chondrogenic potential \textit{in vivo}\(^\text{24,25}\). For some, MSC-treated defects have shown improved healing during early phases, however, found on differences in treatment long-term. Others have found that MSCs delivered to the defect site in biomaterials have exhibited similar, if not worse, cartilage repair than the biomaterial alone\(^\text{24}\). Biomaterials such as fibrinogen and hydrogels that have been used for cartilage defect repair often have not been tested and modified to improve MSC chondrogenesis \textit{in vitro} prior to \textit{in vivo} application.

The purpose of this study was to develop a cartilage mimetic hydrogel that promotes chondrogenesis of encapsulated equine MSCs. Specifically, a degradable cartilage mimetic hydrogel that was investigated in the preliminary rabbit animal model will be used with a slight modification. Herein, the cartilage mimetic hydrogel has been tethered with TGF-\(\beta\)3 to further enhance chondrogenesis. Prior to animal studies, an
extensive *in vitro* study was completed in which multiple MSC donors were evaluated for their chondrogenic potential\(^\text{13}\). Because horse joints experience compressive loading similar to human, we evaluated the chondrogenesis of the MSCs encapsulated in the cartilage mimetic hydrogel under free swelling and under unconfined dynamic compression. Following our *in vitro* investigation, the degradable cartilage mimetic hydrogel was tested for cartilage repair *in vivo* in horse chondral defect.

10.3 Materials and Methods

10.3.1 *Macromer Synthesis*

Poly(ethylene glycol) (PEG) (8arm, 10kDa) norbornene was synthesized from an 8-arm PEG amine (10kDa). Briefly, 8-arm PEG amine (10kDa) was dissolved in dimethylformamide (DMF) and reacted with 4 molar excess of 5-norbornene-2-carboxylic acid with N,N-diisopropylethylamine (DIEA) and 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) under argon at room temperature overnight. The PEG norbornene product was precipitated in cold diethyl ether, vacuum filtered, purified by dialysis and lyophilized to recover the product. The conjugation of norbornene to the PEG amine was determined via \(^1\)HNMR by comparing the area under the peak for the allylic hydrogel closest to the norbornene hydrocarbon group (\(\delta = 3.1-3.2 \text{ ppm}\)) to the area under the peak for the methyl groups of the PEG backbone (\(\delta = 3.4-3.85 \text{ ppm}\)) and was found to be 100%.

Chondroitin sulfate was thiolated as previously described\(^{26}\). Chondroitin sulfate (ChS) (Chondroitin sulfate A, Sigma Aldrich) was fully dissolved in water and reacted with two molar excess dithiobis(propanoic dihydrazide) (DTP) and the pH was adjusted to 4.7 with the addition of 1.0M HCl. Two molar excess of 1-ethyl-3-(3-dimethylaminopropyl)
carbodiimide (EDCI) was added to the ChS and DTP and reacted overnight and the pH was maintained at 4.75. The pH was raised to 7 with the addition of 1.0M NaOH to stop the reaction. The thiol groups of the DTP were reduced with the addition of 6.5 molar excess dithiothreitol (DTT) four 24 hours at a pH of 8.5. The final product, thiolated chondroitin sulfate (ChS-SH) was purified and recovered by dialysis against 0.3mM HCl, centrifuged to remove any particulates, and the supernatant was lyophilized. Conjugation of thiol groups to ChS was found to be 15% (7 thiol groups per molecule of ChS) by $^1$HNMR by comparing the area under the peaks for the two side chain methylene groups of DTP ($\delta$=2.5-2.6 and 2.6-2.8ppm) to the area under the peak representing the methyl protons of the acetyl amine side chain ($\delta$=1.8-2.0 ppm).

TGFβ3 (Peprotech) was thiolated using Pierce™ Traut’s Reagent (2-iminothiolane) (ThermoFisher). Briefly, Traut’s Reagent was reacted at a 4:1 molar ratio to TGFβ3 for 1 hour at room temperature. The thiolated TGFβ3 (TGF-β3-SH) was then pre-reacted with PEG norbornene to obtain a final concentration of 50nM via photopolymerization using 0.05wt% photoinitiator Igracure 2959 (I2959) (BASF) for 30 seconds at 352 nm light at 5 mW cm$^{-2}$.

10.3.2 Cell Culture
Horse mesenchymal stem cells were provided by Colorado State University and were all isolated from mares. For the free swelling study, one donor was used to study differentiation in the biomimetic degradable hydrogel. To determine the effects of mechanical loading on the encapsulated cells, a second study was done in which 3 donors were investigated.
10.3.3 Hydrogel Preparation
Degradable cartilage mimetic hydrogels were fabricated via photopolymerization of 9% (g/g) PEG norbornene (8-arm, 10kDa) with the attached 50 nM TGFβ3-SH, 1% ChS-SH, 0.1 mM CRGDS (Genscript), 2.14% of matrix metalloproteinase 2 (MMP2) degradable peptide flanked with cysteines and glycines (GCVPLS-LYSGCG) (Genscript) in phosphate buffer saline (PBS). The precursor solution was filter-sterilized (0.2 µm) and MSCs were encapsulated at 50 million cells ml\(^{-1}\) and photopolymerized with 0.05% (g/g) Irgacure 2959 (I2959, BASF) with 352nm light at 5 mW cm\(^{-2}\) for 8 minutes. Cell-laden hydrogels (3mm diameter x 3mm height) were placed in 24 well tissue culture plates in 2ml of chondrogenic differentiation media (1% ITS+ Premix, 100 nM dexamethasone, 2.5 ng ml\(^{-1}\) TGF-β3, 50 µg ml\(^{-1}\) l-ascorbic acid 2-phosphate, 50 U ml\(^{-1}\) penicillin, 50 mg ml\(^{-1}\) streptomycin, and 20 mg ml\(^{-1}\) gentamicin in high glucose Dulbecco’s modified Eagle media) (replaced every other day). For the first study, the cell-laden hydrogels were cultured under standard free swelling conditions at 37°C with 5% CO₂ for up to 9 weeks. For the loading study, MSCs from 3 donors were used and the cell laden hydrogels were cultured under free swelling for the initial 7 days of culture at which point a subset of hydrogels were mechanically loaded for the remainder of the 9 week period. All cell-laden hydrogels were cultured under standard cell conditions of 37°C with 5% CO₂.

10.3.4 Mechanical loading of cell-laden hydrogels
A custom-built bioreactor was used to apply intermittent unconfined dynamic compression to the hydrogels. The cell-laden hydrogels in the second study were cultured under free swelling (static) conditions for the first week and then subjected to dynamic compression in a sinusoidal waveform for 1 hour a day followed by 23 hours rest at 5% peak-to-peak strain (2.5% amplitude strain) and 1Hz for up to an additional 8 weeks. Hydrogels were
cultured individually in a 24 well plate with 2 ml per well of chondrogenic differentiation media which was replaced every other day for the duration of the study. Hydrogels were removed at week 3, 6, and 9 for analysis.

10.3.5 Hydrogel Characterization
Hydrogels were assessed for compressive modulus at week 3, 6, and 9 (n=3). Hydrogels were compressed to 15% strain at a strain rate of 0.1mm/min to obtain stress strain curves (MTS Synergie 100, 10N). The compressive modulus was determined by the slope tangential to the linear region of the stress-strain curves. Hydrogels were then flash frozen in liquid nitrogen and used for biochemical analysis.

10.3.6 Biochemical Analysis
At week 3, 6, and 9 hydrogel constructs (n=3) were flash frozen in liquid nitrogen and stored at -80°C. Hydrogels were lyophilized, then homogenized and digested with papain for 16 hours at 90°C. DNA content was measured using Hoechst 33258, and the constructs were analyzed for glycosaminoglycan content using the DMMB assay. Results were reported as the amount of the glycosaminoglycan chondroitin sulfate (ChS) per µg of DNA recovered from the gels.

10.3.7 Histological and immunohistochemical analysis
At week 3, 6, and 9, cell-laden hydrogels (n=3) were fixed in 4% paraformaldehyde, dehydrated, and paraffin embedded following a protocol using gradual concentration of ethanol to Neoclear to paraffin. Paraffin embedded hydrogels were then sectioned to 10um using a microtome. Sections were stained with Safranin-O/Fast Green to visualize sulfated glycosaminoglycans (sGAGs). Immunohistochemistry for the chondrogenic differentiation collagen II was done on the remaining sections. Prior to antibody treatment, sections were treated with the enzyme treatment hyaluronidase (200U/ml) for
1hr at 37°C. Sections were treated with primary antibodies against collagen type II (1:50) (University of Iowa Hybridoma Bank) followed by secondary antibodies with conjugated AlexFluor 488 and counterstained with DAPI for nucleus detection. A laser scanning confocal microscope (Zeiss LSM) was used to acquire images at 400x magnification for further semi-quantitative analysis using NIH ImageJ.

10.3.8 Statistical Analysis
Data are shown as the mean with standard deviation. A one-way analysis of variance (ANOVA) with a Tukey’s post-hoc was used to evaluate the statistically significant data as a function of time. P-values are provided in the plots and parenthetically in the text with p<0.05 considered to be statistically significant.

10.4 Results

10.4.1 Horse MSC differentiation in degradable biomimetic hydrogel

An initial study was conducted to determine the differentiation of horse bone marrow derived MSCs encapsulated in the MMP2 sensitive biomimetic hydrogel. Chondrogenesis was evaluated by histology, immunohistochemistry, and biochemical assays. After three weeks of culture, deposition of proteoglycans by the encapsulated MSCs was observed (Figure 1). From week 3 to week 6, there was an observable increase in sGAG production and deposition in the extracellular matrix. The distribution of extracellular matrix was similar to that of chondrocytes. In addition to sGAG production, there was a significant increase in collagen II protein expression shown in the representative immunohistochemistry images. At week 3, collagen II was detectable in the pericellular region surrounding the encapsulated MSCS. By week 9, collagen II was
prevalent throughout the extracellular matrix and interconnectivity of the protein was observed.

![Image of histological and immunohistochemical evaluation](image)

**Figure 10.1.** Histological and immunohistochemical evaluation of horse MSCs in a cartilage mimetic hydrogel with tethered TGFβ3. Representative images of sGAGs (safranin O, red) and collagen II (green) at 3, 6, and 9 weeks. Safranin O sections counterstained the nuclei with fast green (black) and collagen II sections counterstained the nuclei with DAPI (blue (scale bar=20um, n=3).

Biochemical evaluation of total DNA and sGAGs in the constructs was investigated (Figure 2). The total DNA in the constructs gradually increased from the initial time point (day 1) to week 9 where it was found to be significantly higher \( (p=0.039) \). The sGAGs retained in the constructs on a DNA basis were significantly increased \( (p=0.001) \) by week 3. From week 3 to week 9, the amount of sGAGs per DNA were maintained. These results agree with the representative histological images shown in Figure 1.
Figure 10.2. Biochemical analysis of DNA and ChS content in the hydrogel initially (24 hours post encapsulation) and at week 3, 6, and 9. Data are represented as the mean with error bars representing the standard deviation (n=3). ChS significantly increase from day 1 to week 3 an was maintained from week 3 to week 9.

The compressive modulus of the constructs increased over the course of the study and can be attributed to the extracellular matrix produced by the encapsulated MSCs shown in the previous figures as well as in photographs of the hydrogel constructs where macroscopic tissue production is evident (Figure 3). After 24 hours after encapsulation, the hydrogel constructs appeared to be much softer than right after the hydrogels were polymerized (data not shown), which may have been due to degradation of the MMP2 sensitive peptide crosslinks. This initial modulus (24 hours post encapsulation) was found to be 5.5 (0.5) kPa. After three weeks of culture, the modulus had increased (27 (5) kPa), and by week 6 there was a significant increase (p=0.027) to 40 (4) kPa. From week 6 to week 9, there was a significant ~3 fold increase (p=0.24) to 73 (21) kPa.
Figure 10.3. Macroscopic tissue production and compressive modulus data. Representative images of the MSC-laden hydrogels initially (24 hours after encapsulation) and after 9 weeks of culture. The compressive modulus of the cell laden hydrogels over the course of 9 weeks. Data is represented as the mean with error bars representing the standard deviation (n=3).

10.4.2 The effect of mechanical loading on MSC differentiation from multiple donors in a biomimetic hydrogel

The chondrogenesis of horse MSCs from three donors was investigate in the cartilage mimetic hydrogel under free swelling and under unconfined dynamic compression at 5% stain 1 Hz. Histological images of sGAGs show an increase in sGAG production throughout the study in all donors. Pericellular sGAGs were present at week 3 in donors 1 and 3, but similar to collagen II production, donor 2 exhibited limited sGAG production. However, by week 6 there was a significant increase in the number of cells with pericellular matrix production across all donors. At the end of the study (week 9), sGAGs were prevalent in both loading and free swelling conditions in observably similar amounts,
and tissue deposition appeared extend outward from the pericellular region to the extracellular matrix.

Figure 10.4. Histological images of Safranin O /Fast Green stained sulfated glycosaminoglycans (sGAGs) (red) of 3 donors cultured in free swelling and loading (unconfined dynamic compress 5% strain, 1Hz) for 9 weeks.

The collagen II IHC results verify the variation in chondrogenesis from donor to donor, with donor 1 and donor 3 producing more observable collagen II than donor 2 at week 3. However, by week 6, collagen II is present in the extracellular matrix for all donors in similar quantities between loading and free swelling. By 9 weeks, collagen II was prevalent across all donors and culturing conditions, however, donor 3 appeared to have more positive staining than donors 1 and 2. Interestingly, there does not appear to be an effect of loading on collagen II production.
Degradation of the MMP2 sensitive hydrogel under free swelling and loading of three different cell donors was determined by assessing the compressive modulus of the cell laden hydrogels. The degradation and ECM production appeared to be highly variable dependent on donor. For donor 1, there was a significant decrease in modulus under loading at week 3 compared to free swelling. This may be attributed to higher enzymatic activity or fluid flow induced transport of enzymes through the hydrogel resulting in greater degradation. However, in donor 2 and 3 there was no significant differences between the free swelling and loaded conditions. By week 6, there was a drop in modulus in the free swelling for donor 1, where the loaded construct modulus was significantly higher. A higher modulus was also found in the loaded constructs for donor 2. This may be due to the production of ECM by the encapsulated cells throughout the degraded hydrogel, ultimately increasing the compressive modulus.
Under free swelling, there was no significant changes in modulus between week 3 and week 6 for donors 2 and 3, and under loading donor 3 resulted in a significantly lower modulus compared to free swelling. Donor 3 saw a significantly higher compressive modulus at week 9 under mechanical loading compared to free swelling however, donors 1 and 2 under free swelling were either greater than the loading condition (donor 2) or there was no significant difference between them (donor 1). For all donors, there appears be an initial decrease followed by a slight increase in modulus throughout the 9 week study. The variable trends between donors can be attributed to differences in enzymatic activity and chondrogenic differentiation and ECM protein production of the encapsulated cells.

**Figure 10.5.** The compressive modulus and of the cell laden hydrogels in free swelling (solid) and loading (striped) for 3 donors. Data are represented as mean with error bars as standard deviation (n=3).

**10.4.3 Examining the MSC-laden cartilage mimetic hydrogel in vivo**

An examination of the degradable cartilage mimetic hydrogel *in vivo* was initiated in an equine animal model. Prior to the *in vivo* study, the procedure of creating the defects and treating them was performed in 3 cadaveric equine legs. Chondral defects (15 mm diameter) were created in the proximal and distal medial trochlea of the stifle joint. Acellular hydrogels were prepared and injected into the chondral defect and polymerized
in situ for 2 minutes. Flex and extension of the stifle was done to ensure that the hydrogel stayed in place. The hydrogel was able to fill the defect entirely, and the surface obtained had a similar smoothness to that of the surrounding cartilage.

Figure 10.6. Chondral defects treated in cadaveric equine knees A.) Two 15 mm defects were created in the distal and proximal aspect of the medial trochlea of the stifle. B) The hydrogel was injected and C) photopolymerized in situ at 405 nm for 2 minutes. E) Images show that hydrogels filled and polymerized in the defect and remained in place after the joint experienced flexion and extension.

The experimental design for the in vivo study is shown in Figure 7. Two defects were created in the medial trochlea in each stifle of 3 horses. In the left stifle, microfracture was performed in both defects. The defects were then treated with the hydrogel alone, hydrogel + autologous MSCs, or left empty. On the right stifle, the chondral defects were treated with hydrogel alone, and hydrogel + autologous MSCs. Images of the chondral defects and treatments at the time of surgery are shown in Figure 6.
Figure 10.7. Experimental design of the in vivo evaluation of a cartilage mimetic hydrogel in a horse animal model.

For those treated, the hydrogels were photopolymerized in situ in the chondral defects for 2 minutes using 405nm light. Autologous MSCs were encapsulated at 50 million cells/ml of hydrogel solution for those treated with hydrogel + MSCs (Figure 8). Synovial aspiration were taken at 7 and 14 days post-surgery. Results for cartilage treatment will be examined at 6 months post-surgery.
10.5 Discussion

Animal models are necessary to close the gap between cartilage tissue engineering designs developed in vitro to clinical practice in human patients. The animal models used for cartilage regeneration and repair each have their own limitations and benefits. Our previous in vivo study in a rabbit animal model showed marginal results when compared to our in vitro work, specifically in defects treated with MSCs. Rabbit animal models to investigate cartilage repair have their advantages in that they are relatively inexpensive and genetically similar, however, their joint mechanics are much different than humans, and their cartilage has the ability to self-heal. As such, we chose to investigate our hydrogel material in a horse animal model. Equine cartilage tissue is similar to humans in size, mechanical stimulation, and its inability to regenerate itself. The
goal of this study was to investigate the chondrogenic differentiation potential of horse MSCs in a cartilage mimetic hydrogel under free swelling and loading conditions.

Multiple donors of horse MSCs were used in this study to get a better understanding of their chondrogenic potential in cartilage mimetic hydrogels. Unlike New Zealand rabbits which are genotypically similar and bred for studies such as these, the horses used in this study add donor to donor variability. In the in vivo study, autologous MSCs were used in addition to the cartilage mimetic hydrogel in 2 of the 4 defects. However, the success of the MSC treated defects depends on the chondrogenic potential of donors MSCs, which has been shown to be highly variable in humans. In this study we found that all donors were chondrogenic and produced both sGAGs and collagen II over the course of 9 weeks. However, the degree of matrix produced by the encapsulated cells varied between the donors. The rate at which the encapsulated cells produced collagen II also varied between the donors, with collagen II present at week 3 with Donor 1 and 3, however, collagen II was not present until week 6 for Donor 2.

Our previous studies have shown that mechanical stimulation is necessary to produce non-hypertrophic chondrogenesis of encapsulated humans MSCs. The effect of mechanical loading on horse MSCs has yet to be fully investigated, however, strenuous mechanical loading of equine cartilage has been found to lead to deterioration and the development of lesions. The cartilage in horse joints experience high amounts of compression, therefore, we wanted to investigate the effect that mechanical loading has on encapsulated MSCs in the cartilage mimetic hydrogel prior to in vivo culture. The production of sGAGs and collagen II was produced in the loaded constructs similarly to free swelling, and did not appear to be adversely affected by mechanical loading.
In contrast to the rabbit animal model, TGFβ3 was tethered to the hydrogel scaffold to aid in MSC differentiation. One of the limitations of moving from in vitro to an in vivo environment is the lack of control of growth factor delivery to the encapsulated cells. Our previous studies using rat MSCs found that tethering TGFβ3 in the cartilage mimetic hydrogel showed better sGAG and collagen II matrix deposition than soluble TGFβ3. Similarly, the addition of TGFβ has been found to be imperative for successful chondrogenesis and ECM production of equine bone-marrow derived MSCs. Delivering soluble growth factors in vivo can be challenging; the half-life of soluble growth factors is limited, they can potentially react with other cell types in the area or be sequestered by the ECM and concentrations are hard to control and may not be similar to in vitro culture. In this study, TGFβ3 was tethered into the hydrogel at a higher concentration that TGFβ3 in the media, however, this concentration was chosen due to promising results of previous studies using tethered TGFβ. Growth factors such as TGFβ3 can adversely affect other cell types, therefore, synovial aspirations will be taken at day 7 and 14 post-surgery to determine any significant changes in concentration or cytokines in the fluid.

10.6 Conclusion

Horses are a promising model to test cartilage tissue engineering strategies to treat chondral defects because their cartilage, in size and biology, is similar to that of humans. This study confirmed that equine MSCs can chondrogenically differentiate and deposit cartilaginous matrix when cultured in a MMP2-sensitive cartilage mimetic hydrogel with tethered TGFβ3. Although there is donor to donor variability in the chondrogenic potential of the MSCs, after 9 weeks all deposited sGAGs and collagen II matrix in the extracellular space. Additionally, mechanical loading did not adversely affect chondrogenesis, as ECM
was similar in to two culturing conditions. We believe we have developed a suitable hydrogel environment to treat chondral defects in an equine animal model. If successful in vivo, we believe that our cartilage mimetic hydrogel has the potential for clinical use in humans.

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10.8 References


Chapter 11

A mechanically stiff hydrogel in focal chondral defects reduces degeneration of surrounding cartilage tissue in porcine osteochondral explants

11.1 Abstract

Hydrogels have gained increased popularity as an alternative treatment for chondral defects, however, hydrogels used for cell encapsulation are much softer than the surrounding tissue. The mismatch in mechanical properties can lead to degeneration of the surrounding cartilage tissue. The goal of this study was to evaluate the impact that treating the focal chondral defects with a poly(ethylene glycol) (PEG) hydrogel has on the cartilage adjacent to the injury when physiological loading is applied. Chondral defects were created in porcine osteochondral explants and filled with a soft hydrogel, with a modulus similar to that used for cell encapsulation, and a stiff hydrogel, with a modulus similar to cartilage. The degeneration of the adjacent cartilage was evaluated after 4 weeks of culture under free swelling and physiological load. Defects that were left empty showed significant degeneration of the adjacent cartilage as evident by a lack of sulfated glycosaminoglycans (sGAGs) along the defect site. Hydrogel-treated defects showed improved maintainance of the surrounding tissue with the soft hydrogel-filled showing reduced degeneration, and the stiff hydrogel-filled showing little to no degeneration. Additionally, mechanical loading appeared to further reduce degeneration of chondral defect that were treated with the stiff hydrogel and also reduced apoptosis of the adjacent chondrocytes. Taken together, this study suggests that a stiff hydrogel construct may be necessary to reduce further degeneration of chondral defects.
11.2 Introduction

Cartilage tissue lacks the ability to regenerate due to its low cellular density and avascular nature\(^1\). When damage occurs, whether caused by injury or disease, it can often lead to pain, loss of mobility, and further degeneration, ultimately resulting in osteoarthritis\(^1,2\). If left untreated, the progression of degeneration and the development of osteoarthritis not only affects the defect area but can lead to drastic changes to the cartilage tissue adjacent to the defect\(^3,4\). The degradation of the surrounding tissue is seen both biologically in extracellular matrix make-up and orientation as well as mechanically. Native cartilage tissue is a highly mechanically dynamic environment and when force is applied to cartilage defects, the tissue adjacent to the defect experience increased local strains and tissue deformation\(^5\textsuperscript{--}7\). These physiological loads can reach high enough strain levels that can induce cell death and matrix damage of the cartilage tissue surrounding the defect\(^8,9\). Therefore, there is a necessity to treat chondral defects not only to regenerate cartilage in the defect site, but also to maintain health of the surrounding tissue.

Currently, treatments such as autologous chondrocyte implantation (ACI) and microfracture are being used to treat chondral defects. ACI involves harvesting the patient’s cartilage from a non-load bearing site, isolating the chondrocytes and expanding the cells, and injecting the cells into the defect site\(^10\textsuperscript{--}12\). ACI has been most successful in young active patients under the age of 35, but repair of cartilage tissue has been limited and often results in fibrocartilage. In older patients, repair is limited due to the little regenerative capabilities of autologous chondrocytes \(^10,13,14\). Microfracture, a treatment method of creating small fractures to the underlying subchondral bone to induce the
recruitment of bone marrow and blood, has also been widely used to treat chondral defects\textsuperscript{15,16}. This treatment method has resulted in temporary improvement for some patients, however, the tissue produced is mechanically inferior and fibrous\textsuperscript{15,17,18}. These approved methods of treatment have primarily focused on treating the chondral defect, while neglecting any of the mechanical and biological affect they may have on the surrounding cartilage tissue. Taken together, there is still a necessity to develop new therapies to treat chondral defects and prevent the progression of osteoarthritis.

Hydrogels are a promising alternative treatment for cartilage defects. Photopolymerizable hydrogels are attractive because they can be injectable directly into the chondral defect, photopolymerized \textit{in situ} and work as a vehicle for \textit{in situ} cell delivery while maintaining the chondrocyte phenotype and supporting cartilage specific matrix production\textsuperscript{19–22}. Additionally, they are easily tailorable in chemical and mechanical properties\textsuperscript{23,24}. Hydrogels used for cell encapsulation have shown promising results in cell proliferation and tissue production\textsuperscript{25,26}. However, hydrogels used for cell encapsulation often have a compressive modulus that is orders of magnitude lower than that of the surrounding tissue. Utilizing a soft hydrogel may have limited success in repairing cartilage tissue by reducing its ability to withstand physiological forces which ranges between 1-2MPa\textsuperscript{27–29}, with peaks reaching 3-4 MPa during walking\textsuperscript{30} and as high as 7 MPa during running\textsuperscript{31}. This not only reduces the efficacy of the hydrogel for treating the chondral defect, but also puts the surrounding cartilage tissue as risk of further degeneration due to its lack of structural support.

The purpose of this study was to investigate the effects of treating a focal chondral defect with an injectable, photopolymerizable poly(ethylene glycol) (PEG) hydrogels on
the surrounding cartilage tissue. Specifically, hydrogels of two stiffness, soft (50kPa) and stiff (1MPa), were polymerized in situ in chondral defects of porcine osteochondral plug explants and cultured under dynamic compression. We hypothesize that untreated chondral defects that are left empty will lead to degeneration of the adjacent tissue. A mechanically stiff hydrogel, that resembles the modulus of native cartilage tissue (~1MPa) will maintain the surrounding tissue and protect it from degeneration better than a soft hydrogel. The cartilage tissue of the osteochondral plugs was examined after 4 weeks of culture through mechanical and biological characteristics.

11.3 Materials and Methods
11.3.1 Macromer Synthesis
Poly(ethylene glycol) dimethacrylate (PEGDM) was synthesized via microwave methacrylation as previously described 32. Briefly, poly(ethylene glycol) (4600 g mol\(^{-1}\)) (Sigma) was reacted with methacrylic anhydride in the presence of hydroquinone (Sigma). The product (PEGDM) was recovered by dissolving in methylene chloride and precipitated in ethyl ether, followed by filtration and drying. The degree of methacrylation was determined to be 94% by \(^1\)HNMR (Varian VYR-500) by comparing the area under the peak for the vinyl groups of the methacrylate (δ=5.5-6.2 ppm) to the area under the peak of the methyl groups of the PEG backbone (δ=3.3-3.9 ppm).

11.3.2 Acellular Hydrogel Formation and Characterization
Hydrogels were fabricated via photopolymerization of PEGDM at concentrations of 10 (g/g)% or 40 (g/g)% in phosphate buffered saline (PBS, pH 7.4) with 0.5% (g/g) photoinitiator Igracure 2959 (I2959) (BASF) with 352nm light at 5 mW cm\(^{-2}\) for 10 minutes to produce the soft and stiff hydrogels, respectively. Hydrogels were swelled in PBS for 24 hours to reach equilibrium prior to characterization. The tangent compressive modulus
was determined using cylindrical hydrogels (5 mm in height X 5 mm in diameter) using a mechanical tester (MTS Synergie 100, 10N). Hydrogels were strained at a constant rate of 0.5 mm min\(^{-1}\) to 15% strain and the compressive modulus was determined from the linear region of the stress strain curve between 10 and 15%.

11.3.3 In situ polymerization and culturing osteochondral explants

Porcine osteochondral explants (8.5mm diameter x 10 mm height) were taken from the trochlear groove of 3 month old female porcine from the population of Massachusetts General Hospital (MGH) miniature porcine (Transplantation Biology Research Center, Boston, MA) using a biopsy punch. Focal chondral defects (3 mm diameter) were created in the center of the osteochondral explants down to the bone (~2-3mm deep). After 24 hours, porcine chondral defects were filled.

Chondral defects were left empty, filled with the soft hydrogel (10% PEGDM), or filled with the stiff hydrogel (40% PEGDM). Prior to filling, the chondral defects of the osteochondral plugs were dried with 0.2µm filtered CO\(_2\) for 1 minute. Approximately 50µl of 0.2 µm sterile filtered hydrogel precursor described previously were injected into the chondral defects and the osteochondral plugs were placed under 352nm light for 10 minutes to polymerize the hydrogel in situ. Filled and empty plugs were cultured in chondrocyte media under free swelling for 4 weeks (empty n=4, soft n=4, stiff n=4) or free swelling conditions for 1 week followed by 3 weeks of unconfined dynamic compression in a custom bioreactor (empty n=4, soft n=4, stiff n=4) \(^{19,33}\). The plugs subjected to dynamic compression were held at a constant strain rate of 2.5% when not loaded. During the loading cycle the plugs were slowly compressed (0.1mm/min) to 20% strain and held at 20% strain for 15 minutes at which point the plugs were subjected to unconfined
dynamic compression at 2% peak to peak strain at 1Hz in a sinusoidal waveform for 1 hour \(^{34}\). After 1 hour of loading, the platens were slowly raised (0.1mm/min) to a tare strain of 2.5%. Osteochondral plugs were loaded 5 days a week (Monday-Friday). All subsets of osteochondral plugs were cultured individually in a custom-built metal 24-well plate, in which each row (6 wells) shared a pool of media with approximately \(~15\) milliliters of media. The media was changed Monday, Wednesday, and Friday.

11.3.4 **Histology and immunohistochemistry**

After 4 weeks, osteochondral plugs were removed from culture and the underlying bone was removed from the cartilage. The cartilage was then sectioned in half vertically and prepared for histology and immunohistochemistry. Explants for histology were fixed in 10% formalin for 2 days at room temperature, and transferred to 70% ethanol for storage, embedded in paraffin and processed for histology following standard protocols. Sections (20µm) were stained for sulfated glycosaminoglycans (sGAGs) by Safranin O and fast green and imaged at 100x by light microscopy (Zeiss Pascal, Olympus DP70). Semi-quantitative analysis of histological images was performed in which measurements of the width of generated tissue, defined by the lack of red stain, were taken using NIH ImageJ (n=10 measurements per image, 3 images per side of defect, 2 sides per sample, 4 samples per condition). Sections were also stained for mineralization by von Kossa. Briefly, sections were dewaxed and rehydrated and placed in coplin jars to which 1% silver nitrate was added and placed under UV for 1 hour followed by 5% sodium thiosulfate. Nuclei were counter stained with nuclear red and imaged at 100x by light microscopy (Zeiss Pascal, Olympus DP70).
Apoptotic cells along defect were examined using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. This assay is designed to detect apoptotic cells that undergo extensive DNA degradation. Antigen retrieval was done on sections using Borg Declocker (Biocare Medical) at 90° under vacuum for approximately 20 minutes until the solution turned faint purple. The slides were then transferred to staining centers and washed with PBS. TUNEL staining was done per manufacturer instructions (DeadEnd Florometric Tunel system, Promega), and slides mounted and counterstained with DAPI (Vetashield + DAPI, Vector Lab) and imaged immediately after staining. Confocal microscopy was used to image the apoptotic cells along the defect (100x magnification water). The percentage of apoptotic cells along the defect were determined using NIH ImageJ by counting the number of positively stained TUNEL cells and dividing it by the total number of cells (n=4 images per side, 2 sides per sample, 4 samples per condition).

Sections were also treated with a primary antibody against collagen 2 3/4C short (C1,2C) (1:100, IBEX 50-1035). Before primary antibody treatment, sections underwent an enzyme pretreatment with hyaluronidase (2000 U ml⁻¹), followed by permeabilization in TritonX-100 and blocking in BSA. Sections were probed with AlexFluor488 conjugated secondary antibodies (1:100) and the nuclei were counterstained with DAPI. Confocal microscopy was used to acquire images.

11.3.5 Statistical Analysis
Data are shown as the mean with standard deviation. Two way analysis of variance (ANOVA) was used to evaluate the statistical significant of data with a Tukey’s post-hoc
to compare all combinations. P-values are provided in the plots and parenthetically in the text with p<0.05 considered to be statistically significant.

11.4 Results
Focal chondral defects of porcine osteochondral plug explants were filled with a stable poly(ethylene glycol) (PEG) hydrogel of varying monomer concentrations and photopolymerized in situ to achieve soft (~50kPa) and stiff (~1MPa). The ability to fill the defects and polymerize in situ and the hydrogel stain in place was demonstrated (Figure 1A). The empty, soft-filled, and stiff-filled osteochondral plugs were then subjected to mechanical loading at 20% strain to recapitulate the strain of native cartilage joint tissue.
Figure 11.1. A schematic of the osteochondral explant experimental design A. A schematic of the chondral defects in the osteochondral plug explants being filled with hydrogel precursor solutions of a soft hydrogel and a stiff hydrogel and photopolymerized in situ. Images of the empty defects and filled defects indicate that the hydrogel remains in place. B. The experimental design of culturing the osteochondral plugs in free swelling and in loading and a schematic of the loading regime used. After one week of free swelling, a subset of plugs were subjected to unconfined dynamic compression. A static strain of 2.5% was applied to the plugs at all times except those during the 1 hour of loading, during which the plugs were strain to 20%, held there for 15 minutes, and then subjected to dynamic loading at a 2% peak to peak strain for one hour, then released by to 2.5% static strain.
After four weeks of culture, the cartilage tissue of the osteochondral plugs was examined. Apoptosis of the cells along the defect site was evaluated via TUNEL assay (Figure 2A). The percentage of dead cells was found to be highest in the stiff hydrogel-filled defect under free swelling, although it was not found to be significantly higher than the soft-filled and empty defect (Figure 3B). Mechanical loading significantly reduced the number of dead cells in the stiff-filled defects from 60% to 35%, while this affect was not found in the other conditions (Figure 2B). Although the stiff-filled defects had the highest percentage of dead cells, they also had more cells in the tissue adjacent to the defect than those that were filled with the soft hydrogel or left empty (Figure 2C).
Figure 11.2. Evaluation of apoptosis in cartilage explants. A. Representative images of TUNEL assay of the cartilage tissue adjacent to the defect to evaluate apoptotic cells (green) and nuclei (blue) (scale bar = 50 µm). B. Semi-quantitative analysis of the percentage of apoptotic cells along the defect of the various defect treatments and C. the total number of cells along the defect under free swelling (solid) and loading (striped). Data represented as mean with scale bars representing standard deviation (n=4).

Osteochondral plugs were stained with safranin O to stain for the negatively charged glycosaminoglycans in the cartilage tissue. Representative images of the cartilage tissue down to the subchondral bone show significantly different tissue health along the defect site in the different culturing conditions (Figure 3 A). The chondral defects
that were left empty and cultured under free swelling conditions had significantly more (p=0.0002) degenerated tissue (approximately 250µm) in the cartilage tissue adjacent to the defect than those that were filled with the stiff hydrogel (approximately 50 µm). Dynamic compression appeared to reduce the degeneration of the cartilage around the empty defects although not significantly. The width of degenerated tissue of the empty defects under mechanical loading was still significantly greater (p=0.004) than the plugs filled with the stiff hydrogel. The chondral defects filled with the soft hydrogel showed a reduction in the width of degenerated tissue compared to those left empty under both free swelling and loading with approximately 110-90 µm of degenerated tissue, although not significantly. Similar to the empty chondral defects, dynamic compressive loading reduced (p=0.014) the mean width of the degenerated tissue adjacent to the stiff hydrogel from 50 µm to 10 µm.
Figure 11.3. Analysis of GAGs in the cartilage tissue surrounding the defect A. Representative images of the entire cartilage tissue adjacent to the defect from the articular surface to the osteochondral interface. Images are stained for sulfated glycosaminoglycans (sGAGs) using safranin O after 4 weeks of culture in free swelling and loading. C. Semi-quantitative analysis of the width of degenerated tissue indicated by the lack of red stain from the histological images. Data are represented as the mean and error bars indicate standard deviation.
Figure 11.4. Analysis of degraded collagen II in the cartilage surrounding the defect. Representative images of C1,2C immunohistochemistry stain to evaluate degraded collagen II (green) and nuclei (blue) (scale bar = 20 µm).

Degradation of the cartilage was also evaluated by immunohistochemistry by staining for the short chains of degraded collagen II, (C1,C2) (Figure 3C). Representative images indicate that the degraded collagen II was prevalent when cultured under dynamic compression in the soft-filled and empty defects, however, was reduced in free swelling culture. There was little to no degraded collagen II present in the tissue surrounding the stiff-filled defect in either free swelling or loading culture.
Mineralization was also evaluated to determine the potential bone formation in the adjacent cartilage (Figure 5). Representative images of von Kossa stained sections show a lack of mineralization for all conditions.

11.5 Discussion

This study aimed to investigate the cartilage adjacent to the focal defect and the impact that loading and filling the defect has on that tissue. The degeneration of a focal chondral defect in porcine osteochondral plugs when left empty or filled with a hydrogel was investigated. Results show the importance of treating chondral defects with a material that has a similar compressive modulus to native cartilage tissue in order to maintain the tissue surrounding the defect. When left untreated, cartilage adjacent to the defect showed significant signs of degeneration, observed by the lack of sGAG retention along the edge of the defect. However, little to no degeneration was found when chondral
defects were filled with a stiff PEGDM hydrogel (~1MPa). Dynamic loading at physiological strains may help maintain cartilage homeostasis when stiff-filled, as evident by the reduction in apoptotic cells along the defect. In contrast, an increase in degraded collagen (C1,2C) was observed in the adjacent cartilage of defects left empty or treated with a soft hydrogel when cultured under loading compared to free swelling. Taken together, these results suggest that the progression of degeneration from a focal chondral defect can be minimized when treated with a hydrogel with a modulus similar to that of cartilage. Mechanical compression may play a vital role in the maintenance of healthy cartilage when defects are treated with a mechanically stiff hydrogel, however, may lead to increased degradation in defects that are left empty or treated with a soft hydrogel.

Cartilage focal defects that are left untreated do not self-heal after injury. Many studies have shown that overtime the articular cartilage at the edges of the defect become thinned, fold inwards, and are phenotypic of degenerated cartilage. Additionally, in accordance with our results, others have reported an acellular region directly adjacent to the defect. The degeneration of the cartilage tissue is suggested to be caused by a concentration of strain on the rim of the cartilage tissue and/or an increase in compliance due to damage to the matrix. Our study suggests that there is significant damage to the matrix due to the progression of degeneration from the defect site regardless of dynamic compression. This may be due to an increase in cytokines or matrix-degrading proteases in response to injury. The empty defect group cultured under dynamic compression showed a substantial bulge in the articular cartilage (Figure 3). This observation may be attributed to a lack of lateral constraint which permits the tissue to deform into the defect, as shown by others. By filling the defect with a mechanically stiff hydrogel that
resembles that of the native tissue, the integrity of the joint is reestablished and there is less of a discontinuity of biomechanics between the defect and the adjacent tissue. 42

Mechanical loading is imperative to the homeostasis of healthy cartilage tissue. While others have shown that mechanical loading further degenerates cartilage tissue, our findings suggest that mechanical loading can have a positive impact on preventing degeneration when the chondral defect is treated with a stiff hydrogel. As previously reported, loading of a chondral defect can lead to degeneration of the surrounding cartilage induced by altered strains in the tissue6,44. Introducing a mechanically stiff hydrogel into the chondral defect showed significant improvement of cartilage regeneration especially under mechanical loading. The stiff hydrogel may have reduced degeneration by incorporating a mechanically stable structure that resists any changes in strain through the tissue. Additionally, loading conditions which do not contain dynamic time-varying strains have been found to reduce synthesis and deposition of matrix molecules. Our results suggest that mechanical loading of the stiff-filled defects significantly decreased the fraction of the apoptotic cells compared to free swelling. Dynamic loading of acutely injured cartilage has been found to maintain chondrocyte viability by reducing apoptosis and subsequently increase the number of cells that survive the injury long-term45. Cartilage is avascular, therefore chondrocyte survival greatly relies on nutrient diffusion from the surrounding fluid in vivo, and dynamic loading may promote nutrient transport in vitro. Apoptosis maintains homeostasis by removing old stressed chondrocytes in order to increase the cell viability of new chondrocytes. The increase in apoptotic cells along the stiff-filled defect may be a response to maintaining the homeostasis of the tissue. Additionally, the number of cells adjacent to the empty defects
and the soft-filled defects were significantly lower than those adjacent to the stiff-filled defect. Studies have found that up to 50% of chondrocytes die by apoptosis within 6-96 hours after traumatic injury\textsuperscript{46,47}. Herein, the TUNEL assay was done after 4 weeks of culture. These results suggest that apoptotic cell death in response to the initial defect injury may have occurred up to 4 weeks prior to the time of analysis which may have reduced the detection of apoptotic cells by TUNEL.

The approach investigated in the study was not an attempt to repair or replace the defect with articular cartilage, but was rather a demonstration of maintaining the cartilage adjacent to the defect via \textit{in situ} polymerization of a stable, stiff hydrogel. Although hydrogels are a promising treatment to cartilage defects, one major drawback of using cell-encapsulating hydrogels is the significant reduction in mechanical properties to that of native cartilage tissue. Hydrogels used for cell encapsulation and tissue production often have a modulus in the range of 1s-10s of kPa due to the low crosslink density which is necessary to allow for diffusion of ECM proteins and allow for macroscopic tissue growth. Furthermore, a degradable hydrogel is necessary for cartilage repair, however, as the encapsulated cells begin to degrade the synthetic network, the structural support provided by the hydrogel and the modulus can drop dramatically. As shown here, a mismatch in the mechanical properties between the hydrogel and the surrounding native cartilage can increase the degeneration of the joint. However, treating chondral defects with even a soft hydrogel reduced the degeneration of the adjacent tissue. This may be due to other factors such as the high water content and hydrogel swelling introduced by the hydrogel that may play a role in maintaining the surrounding cartilage. Future work
in cartilage tissue engineering will need combine a mechanically stiff, stable structural component with a cell-laden, degradable hydrogel.

11.6 Conclusion
The treatment of chondral defects is necessary to maintain the surrounding cartilage tissue. If left untreated, there is significant progression of degeneration from the defect into the adjacent cartilage tissue as seen by a significant loss of sGAGs and degrade collagen. In order to combat the progression of degeneration and ultimately osteoarthritis, this study suggests treating the chondral defects with a hydrogel. Specifically, a hydrogel with a mechanical stiffness similar to that of the surrounding cartilage is able to minimize the degeneration of the adjacent cartilage to the greatest extent. Similarly, mechanical compression applied to stiff-treated defects maintained homeostasis of the cartilage by minimizing apoptosis. These results should be considered when designing a hydrogel treatment for chondral defects. Currently, a hydrogel construct that combines a mechanically stiff network with a soft hydrogel for cell encapsulation is being developed.

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11.8 References


Chapter 12

A stereolithography-based 3D printed hybrid scaffold for *in Situ* cartilage defect repair

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12.1 Abstract

Damage to articular cartilage can over time cause degeneration to the tissue surrounding the injury. To address this problem, scaffolds that prevent degeneration and promote neotissue growth are needed. We introduce a new hybrid scaffold that combines a stereolithography-based three-dimensional printed support structure with an injectable and photopolymerizable hydrogel for delivering cells to treat focal chondral defects. In this proof of concept study, we demonstrate the ability to a) infill the support structure with an injectable hydrogel precursor solution, b) incorporate cartilage cells during infilling using a degradable hydrogel that promotes neotissue deposition, and c) minimize damage to the surrounding cartilage when the hybrid scaffold is placed *in situ* in a focal chondral defect in an osteochondral plug that is cultured under mechanical loading. With the ability to independently control the properties of the structure and the injectable hydrogel, this hybrid scaffold approach holds promise for treating chondral defects.
12.2 Introduction

Focal defects that occur in articular cartilage as a result of injury pose a significant risk for developing osteoarthritis, a debilitating disease with no cure. Once damaged, articular cartilage has a limited capacity to self-repair. The lack of repair causes changes to the mechanical environment in the cartilage that surrounds the empty defect, which over time, can lead to degeneration and eventually progress to osteoarthritis. Unfortunately, the only effective solution to treat advanced osteoarthritis is joint arthroplasty. However, if the defect can be repaired while minimizing degeneration to the surrounding tissue, prevention of osteoarthritis may be possible. To this end, microfracture has been widely used to treat focal defects, where small fractures to the underlying subchondral bone induce bleeding and recruit bone marrow cells to facilitate repair. Although patient reported outcomes are temporarily improved, microfracture produces a mechanically inferior fibrous tissue that eventually leads to degeneration and is not suitable for large defects. Cellular therapies based on autologous chondrocyte implantation (ACI) were developed in an effort to improve cartilage repair. Results from ACI treated defects have shown some improvement in the short-term (up to one year), but long-term leads to similar repair when compared to microfracture. Taken together, there remains a great need to develop new therapies that are capable of repairing focal defects with functionally competent cartilage tissue and ultimately preventing progression to osteoarthritis.

One promising approach combines a hydrogel (i.e., matrix) with ACI to deliver chondrocytes into the defect within a three-dimensional (3D) matrix. In particular, hydrogels afford the opportunity to design the 3D environment with biochemical and mechanical cues to support the chondrogenic phenotype. Synthetic and degradable
hydrogels have been investigated for cartilage regeneration due to the ability to tune the chemistry, mechanical properties, and degradation behavior of the hydrogel and promote cartilage growth.\textsuperscript{23,24} Moreover, \textit{in situ} formation of the hydrogel in the defect enables the hydrogel to adhere to the surrounding tissue and ultimately facilitate integration while working as a vehicle for \textit{in situ} cell delivery to promote cartilage regeneration.\textsuperscript{25}

Designing a hydrogel that maintains the overall integrity of the construct while allowing for degradation and ECM synthesis of encapsulated cells remains challenging. Cell-laden hydrogels consist of a tight mesh of polymer crosslinks that surround the encapsulated cells. This mesh, which is on the nanometer length scale, restricts transport of the main cartilage ECM macromolecules (i.e., collagen type II and aggrecan) and thus prevents macroscopic neotissue growth.\textsuperscript{26} As a result, there is a temporary loss in the structural integrity as the hydrogel degrades, but before sufficient macroscopic tissue can be deposited.\textsuperscript{23,27,28} This loss in structural integrity may compromise the encapsulated cells and damage surrounding cartilage tissue when used \textit{in vivo} to treat focal defects, especially under physiological mechanical forces.

To overcome the shortcomings associated with using a single scaffold design for cell encapsulation, this proof of concept study sought to explore the potential of a new hybrid scaffold whereby a support structure is introduced into the cell-laden hydrogel (Figure 1). The overarching idea is to create a minimally invasive approach based on a hybrid scaffold containing a slow degrading temporary structure that provides support while an infilled fast degrading chondrocyte-laden hydrogel allows for neotissue formation. Collectively, the scaffold should also minimize damage to surrounding cartilage tissue. Stereolithography (SLA) 3D printing was chosen to fabricate the 3D support
structure due to its high resolution printing capabilities from photopolymerizable precursors and for its ability to create complex architectures in a layer-by-layer fashion without requiring a sacrificial material.29–33 A well-established poly(ethylene glycol) (PEG) hydrogel prepared from a step-growth photopolymerization reaction was chosen for the cell-laden hydrogel for its demonstrated promise in cell encapsulation34 and cartilage tissue engineering.35,36 Herein, the feasibility of our idea is demonstrated by the ability to infill the 3D support structure with a cell-compatible hydrogel and subsequently support cartilage cells and ECM deposition. Moreover, the in situ placement of the hybrid scaffold in an ex vivo model of a cartilage focal defect and the ability to protect the surrounding cartilage in a mechanically relevant loading environment are demonstrated.
12.3 Materials and Methods

12.3.1 Fabrication of the 3D support structure by SLA-based 3D printing

Solidworks® was used to generate a 3D model of the support structure made of an array of 250 µm diameter pillars evenly spaced to achieve a 25% volume fraction. A 250 µm thick lattice was then added at the top, middle and bottom to provide lateral
support. The dimensions of the structure were 2.06 x 2.06 x 2.00 mm (L x W x H). Autodesk’s Print Studio software was used to generate 10 µm thick 2D slices from the 3D model. The 2D digital image slices were uploaded to the Autodesk Ember projection SLA printer, which is optimized for printing 10 µm thick layers. In brief, the build head is lowered into a resin bath a distance of 10 µm from the printing window, whereby a 2D digital image is projected to polymerize the resin in regions exposed to light. The process is repeated layer-by-layer until the full 3D structure is complete. A separate single layer bulk material was printed following the same polymerization conditions and used for the contact angle experiments. The commercial resin for the Autodesk Ember projection SLA printer was used. The 3D printed support structure was removed, soaked in isopropanol for 15 min to remove monomer from the unexposed regions and then sterilized in 70% ethanol overnight followed by drying under sterile conditions.

12.3.2 Oxygen plasma treatment of the 3D printed support structures
Following aseptic protocols, the oxygen plasma chamber (Plasma Etch Inc., PE-25) was sterilized and stabilized by purging the chamber with oxygen plasma for 15 minutes. The sterile samples were transferred in sterile tissue culture plates, placed in the chamber, and then exposed to oxygen plasma for 1, 2 or 3 minutes. The treated materials (either the 3D printed support structure or the bulk printed material) were used immediately. The bulk printed materials were tested for hydrophilicity by photographing a water droplet on the surface.

12.3.3 Infilling of 3D printed support structures with a PEG hydrogel
A PEG hydrogel was formed from precursor solution of 10 (g/g)% 8-arm PEG (10kDa) norbornene, which was synthesized from 8-arm PEG (10kDa) amine following established protocols,35 PEG (1kDa) dithiol at a 1:1 thiol:ene ratio, and 0.05 (g/g)%
photoinitiator Irgacure 2959 (I2959) (BASF) in phosphate buffered saline (PBS). The hydrogel precursor solution was injected into the 3D printed support structure under vacuum and polymerized with 352 nm light at 5 mW cm\(^{-2}\) for 10 minutes. The hybrid scaffold was swollen to equilibrium for 24 hours in PBS prior to characterization. To image the hydrogel in the hybrid scaffold, 0.1 (v/v)\% Alexa-Fluor 546 maleimide (Thermo Fisher Scientific) was added to the precursor solution prior to polymerization. In a separate experiment, Alexa-Fluor 546 labeled microspheres (10µm) (FluoSpheres, Molecular Probes) at 1 million spheres per ml of precursor solution were infilled and photopolymerized. In both, the swollen hybrid scaffold was imaged by confocal microscopy (Zeiss LSM 5 Pascal). 3D printed constructs were sectioned using a razor blade to image infilling in the center of the 3D printed constructs.

12.3.4 Infilling a cell-laden hydrogel in 3D printed constructs

Bovine chondrocytes were isolated from the femoral-patellar groove of a skeletally immature (1-3 weeks old) calf (Arapahoe Meat Co., Lafayette, CO) following established protocols.\(^{35}\) Bovine chondrocytes were suspended in the 0.22 µm sterile filtered hydrogel precursor solution at 50 million cells/ml in a 10 (g/g)% 8-arm PEG (20kDa) norbornene, with a matrix metalloproteinase (MMP2) degradable peptide crosslinker (GCVPLSLYSGCG) at a 1:1 thiol:ene ratio. This solution was injected into the 3D printed support structures under vacuum for 30 seconds, and photopolymerized for 8 minutes at 352nm at 5 mW cm\(^{-2}\). The cell-laden hydrogels were cultured in chondrocyte medium (DMEM supplemented with 10% FBS, 0.2% Primocin, 10 mM HEPES, 0.1 M non-essential amino acids, 50 µg ml\(^{-1}\) L-ascorbic acid, 4mM l-glutamine, 0.4µM l-proline) for up to 14 days (n=3). Cell-laden hydrogels were fixed in 4\% paraformaldehyde for 24 hours at 4°C and were processed for histology following standard protocols. Sections (40 µm)
were pretreated with 200 U ml\(^{-1}\) hyaluronidase for 1 hour at 37°C, followed by permeabilization (0.25% Triton X, 1% BSA in PBS) and blocking (1% BSA), treatment with an anti-collagen II primary antibody (1:50) (abcam ab3092) overnight at 4°C, treatment with Alexa-Fluor 488 conjugated secondary antibodies, and treatment with DAPI. Images were taken using confocal microscopy (Ziess LSM 5 Pascal).

12.3.5 Ex vivo focal defect treatment of osteochondral plugs

Osteochondral plugs (8mm diameter, 10 mm deep) were explanted from the trochlear groove of porcine knees following aseptic protocols. Focal chondral defects (3mm diameter, ~2mm deep) were created in the center of the osteochondral plug. The inside of the chondral defect was dried using sterile filtered (0.2µm syringe filter) CO\(_2\) for 1 minute. The 3D printed support structure was placed into the chondral defect site, and a 0.22 µm sterile filtered hydrogel precursor solution (10wt% 8-arm PEG norbornene (10kDa), PEG dithiol (1kDa) at 1:1 thiol:ene ratio, 0.05wt% I2959) was injected into the 3D printed support structure, subjected to vacuum for 2 minutes and polymerized (352 nm, 5mW cm\(^{-2}\) for 8 minutes). A separate set of osteochondral plugs were left empty. Filled and empty plugs were cultured in chondrocyte media under either a) free swelling for 4 weeks (filled n=4, empty n=4) or b) free swelling conditions for 1 week followed by 3 weeks of unconfined dynamic compression in a custom bioreactor (filled n=4, empty n=4).\(^{54,55}\) In the latter, specimens were held at a constant strain of 2.5% throughout the culture, but for one hour per day were subjected to a 10% offset strain onto which a 2% peak to peak strain at 1Hz in a sinusoidal waveform was applied. Each plug was cultured individually in a 24-well plate with 2ml per well of chondrocyte media which was replaced every other day. After four weeks, the cartilage layer was removed from the bone layer, fixed in 10% formalin for 2 days at room temperature, transferred to 70% ethanol for
storage and then were processed for histology following standard protocols. Sections (20µm) were stained for sGAGs by Safranin O and fast green and imaged by light microscopy. Semi-quantitative analysis of histological images was performed in which measurements of the width of degenerated tissue defined by a lack of red stain were taken (n=10 measurements per image, 5 images per sample, 4 samples per condition).

12.4 Results and Discussion
The 3D support structure was designed to fulfill several criteria. First, the structure needed to be stable over the course of the experiments to maintain structural integrity. Second, the structure needed to be infilled with the aqueous cell-compatible hydrogel precursor solution and therefore needed to be hydrophilic. And lastly, the structure needed to maintain integrity while having sufficient void space to infill with cells and allow for neotissue deposition. From these criteria, a 3D support structure was designed with an array of evenly spaced 250 µm diameter pillars which were connected together using a lattice structure at the base, middle, and top. The continuous pillar design provided structural support to resist compressive forces, while the lattice provided lateral support to resist transverse movement. The 3D support structure was also designed to occupy ~25% of the total volume, such that ~75% of the 'open' space is available for the cell-laden hydrogel. A computational rendering of the 3D support structure is shown in Figure 2A. From this rendering, a series of 2D digital images were created and used to generate light projections for the SLA process using a commercially available Autodesk Ember Printer which prints a proprietary and stable photopolymerizable resin (Autodesk, PR-48) at a resolution reported to be 50 µm. Top view and side view images of the 3D printed structure are shown in Figure 2B, which confirm successful printing of the 3D design.
Due to the hydrophobicity of the commercial resin that formed the printed structure, oxygen plasma treatment was investigated. A bulk printed material was made from the same resin and exposed to oxygen plasma. Increasing the time of treatment led to a decrease in the water contact angle and thus an increase in hydrophilicity (Figure 2C). By three minutes of treatment, the water droplet nearly wet the surface resulting in a contact angle of 10 degrees indicating increased hydrophilicity. This condition was used for all subsequent experiments.

Figure 12.2 The SLA 3D printed structure. A. Solidworks® 3D drawings from the top and side of the 3D support structure with 250 µm diameter pillars and top, middle, and bottom lattice structures and occupying 25% volume fraction. B. Photographs of the SLA 3D printed structure from the top and side (scale bar is 1 mm). C. Photographs of water droplets on a bulk specimen made from the same material as the 3D support structure treated with oxygen plasma treatment for 0, 1, 2 and 3 minutes. A decrease in the water contact angle can be visualized with increasing oxygen plasma treatment. D. Contact angle measurements after oxygen plasma treatment. Data are represented as mean with standard deviation reported parenthetically for n=3.
The ability to infill the 3D printed support structure with a hydrogel was investigated (Figure 3). A precursor solution of 8-arm norbornene functionalized PEG monomer (10kDa), a PEG dithiol crosslinker (1kDa), and fluorescently labeled PEG monothiol (1kDa) in the presence of a maleimide-conjugated fluorophore was infilled into the 3D printed construct and photopolymerized. Successful infilling of the PEG hydrogel was demonstrated by confocal microscopy (Figure 3A). A top view of the 3D printed structure shows the infilled hydrogel surrounding the pillars of the support structure. To demonstrate distribution of the cells in the PEG hydrogel infill, fluorescently-labeled microspheres (10 µm diameter) of similar size to cells were suspended in the hydrogel precursor solution, injected into the support structure and then exposed to light (Figure 3B). Representative confocal microscopy images confirm a relatively uniform distribution of the microspheres within the void spaces of the support structure.
Figure 12.3. The infilling of the 3D printed structure to create a hybrid scaffold A. A schematic of the photopolymerizable PEG precursor solutions for the infill and the fabrication of the hybrid scaffold by injecting the precursors and photopolymerizing. B. A schematic of the infilling of the hybrid scaffold with a fluorescently-labeled, PEG hydrogel. Representative confocal microscopy images shows successful infilling of the hydrogel (red) around the 3D printed support structure (black) (scale bar = 100 µm). C. A schematic of the infilling of the hybrid scaffold with fluorescently-labeled microspheres that are suspended in the infill solution and then subsequently photopolymerized to encapsulate them in the PEG hydrogel in the hybrid scaffold. Representative confocal microscopy images show the distribution of microspheres (red) through the top of the lattice (left) and a side view through the pillars (right) (scale bar= 100 µm). D. A schematic of the infilling of a chondrocyte-laden hybrid scaffold to which bovine chondrocytes were suspended in the infill solution and then subsequently photopolymerized to encapsulate them in the PEG hydrogel. Here a MMP2-sensitive PEG hydrogel was used. Cell nuclei (blue) shows chondrocytes are successfully infilled around the pillars (indicated by dotted line) of the hybrid scaffold at 7 and 14 days (scale bar = 100 µm). High magnification images of regions of high cell density where extracellular matrix neotissue is forming as shown by
staining for collagen type II (green) and cell nuclei (blue) at 7 and 14 days (scale bar = 20 µm).

With the long-term goal being to use the hybrid scaffold to treat focal chondral defects, the hybrid scaffold was investigated for its ability to support chondrocytes and cartilage-specific ECM synthesis. Freshly isolated chondrocytes were suspended in a hydrogel precursor solution, injected into the support structure, exposed to light to entrap the cells within the hybrid scaffold and then cultured for two weeks (Figure 3C). In these studies, a degradable PEG hydrogel was used where the crosslinker was a matrix metalloproteinase 2 (MMP2) sensitive peptide crosslinker. This particular crosslinker was chosen because chondrocytes have been shown to secrete MMP2 during cartilage development and remodeling. Confocal microscopy images confirm that chondrocytes were entrapped in the hydrogel regions surrounding the support structure (Figure 3C). Chondrocyte phenotype and ECM synthesis was confirmed by staining for collagen type II, which is one of the main cartilage-specific ECM proteins found in cartilage (Figure 3C). These findings demonstrate the feasibility of delivering chondrocytes in a degradable hydrogel into a 3D printed support structure and their ability to produce cartilage-specific ECM.

The hybrid scaffold was tested in an \textit{ex vivo} focal chondral defect to demonstrate the ability to fill the defect \textit{in situ} and to investigate the surrounding cartilage once filled in a dynamic mechanical environment. A chondral defect was prepared in osteochondral plugs explanted from the trochlear groove of adult porcine knees (Figure 4). The 3D printed support structure was treated with oxygen plasma, physically placed into the focal chondral defect, infilled with a hydrogel precursor solution, and photopolymerized \textit{in situ}.
(Figure 4A). To assess the ability of the *in situ* hybrid scaffold to protect the surrounding tissue from further damage, a stable version of the hydrogel was employed to minimize confounding factors that could arise due to changes in the scaffold properties. To emulate the *in vivo* environment, the osteochondral plugs (filled and empty) were subjected to physiologically relevant dynamic compressive strains for one hour per day for three weeks in custom built bioreactors (Figure 4A). The loading profile consisted of an 10% offset strain followed by a 2% peak to peak dynamic loading strain, which has been previously shown to maintain cartilage explants *ex vivo* (Figure 4B).40 Photographs show an unfilled defect and a defect filled with the hybrid scaffold immediately after filling (Figure 4C). The osteochondral plugs were cultured for four weeks under free swelling or dynamic loading conditions and then removed, visualized, and then processed for histology.

All hybrid scaffolds filling the defects visually remained in place in both loading and free swelling culture conditions after four weeks. The surrounding tissue was characterized by staining for sulfated glycosaminoglycans (sGAGs), which are the main GAGs found in aggregcan, the most abundant proteoglycan in cartilage, and have been shown to be the first ECM molecule that is lost during early stages of cartilage degeneration (Figure 4D).41 Our results reveal that chondral defects left untreated displayed depletion of sGAGs in the regions adjacent to the defect (~140 µm from edge of defect), indicative of degeneration (Figure 4E). However, chondral defects that were treated with the hybrid scaffold showed significantly higher retention of sGAGs in the regions adjacent to the defect, regardless of the loading environment (Figure 4D and 4E). These results demonstrate that infilling of the defect with the hybrid scaffold prevents degeneration of cartilage adjacent to a defect regardless of the presence of loading.
Although the exact mechanism is not known, the physical confinement that results from \textit{in situ} polymerization of the infilled hydrogel may prevent tissue swelling along the defect boundary and protect the tissue.\textsuperscript{42} Additional studies, however, are needed to elucidate the mechanisms involved.
Herein, we demonstrate the applicability of combining stereolithography with injectable hydrogels to create a new hybrid scaffold for treating focal defects in cartilage. 3D printing strategies have been investigated for cartilage tissue engineering with the most common method being extrusion-based printing. Although promising, the resolution of the bioprinted material is restricted by nozzle size, polymer viscosity, and printing speed, limiting the choice of material and the resolution of the printed structure. Stereolithography, on the other hand, achieves higher resolution and can be applied to essentially any photopolymerizable monomer solution, thus, enabling structures to be printed at relevant lengthscales and properties that mimic native tissues (e.g., zonal regions within cartilage). A few studies have investigated stereolithography in cartilage tissue engineering to create 3D porous scaffolds, but have only investigated seeding chondrocytes directly onto the scaffold. Also, little is known on the applicability of the 3D printed structures in chondral defects with physiologically relevant mechanical loading. In this work, we introduced a novel approach that leverages the
advantages of stereolithography to create complex 3D architectures and simultaneously the advantages of injectable synthetic-based hydrogels for chondrocyte delivery and for ease of placement and in situ polymerization into chondral defects.

There are several limitations of this study. The resolution and mechanical properties of the 3D printed support structure were limited to the commercial resin, which is not designed to degrade, and the SLA 3D printing process. Moreover, under dynamic loading the in situ filled OC defect creates a complex mechanical environment due to several factors such as the confinement of the scaffold by the adjacent cartilage and the bond between the infilled hydrogel and the adjacent cartilage. To address these limitations, future work will use a custom-built SLA 3D printer, which has a resolution of 4 μm and which can be used with a wide range of polymer formulations to tune the degradation (e.g., with enzymatically and/or hydrolytically cleavable sites) and mechanical properties of the support structure. Other advanced 3D printing technologies have also been developed with enhanced resolution capabilities. Future work will also implement computational methods to characterize the complex mechanical properties of the hybrid scaffold within the defect. Another limitation of this study was the mechanical loading environment in the explant osteochondral model, which was limited to compressive strains. In the in vivo environment, understanding the effect of shear strains combined with compressive strains will be important.

12.5 Conclusions
In summary, treating focal defects in articular cartilage is a significant challenge clinically. Many current approaches still rely on a single scaffold, which limits the functions that the scaffold can perform \textit{in vivo}.\textsuperscript{53} Thus, there is a need in tissue engineering to develop more complex 3D scaffolds that have structural integrity and clinically relevant biological functions for supporting tissue regeneration and maintaining the tissue surrounding the implanted scaffold.\textsuperscript{32} We present in this proof of concept study, a hybrid scaffold that combines \textit{in situ} delivery of chondrocytes within a hydrogel, but which is supported through an embedded 3D printed structure. Our approach enables independent design of the support structure and the hydrogel such that in the future the hydrogel can be designed to degrade rapidly while the support structure can be designed to degrade slowly. Importantly, this study demonstrates that a hybrid scaffold maintains the health of the surrounding tissue. Long-term, this approach can be adapted using minimally invasive methods to repair focal chondral defects where imaging modalities, such as MRI, could be used to size the 3D printed support structure prior to surgery. Overall, this feasibility study presents a new hybrid scaffold approach as a potential therapy to treat focal defects and prevent cartilage degeneration long-term and warrants further research.

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12.7 References


Chapter 13

Conclusions and Recommendations for Future Directions

13.1 Conclusions

The initial goal of this thesis was to develop a PEG-based hydrogel system that enhanced the chondrogenesis of MSCs, encouraged matrix deposition, and allowed for scaffold degradation. The interaction of MSCs with their surrounding environment is known to play a critical role in their differentiation\(^1,^2\). This raised the question as to whether the unique environment of cartilage tissue was necessary to promote chondrogenesis of MSCs. Chapter 3 aimed to develop a PEG-based hydrogel environment that enhances the chondrogenesis of encapsulated MSCs. To investigate this, chondroitin sulfate (ChS), a negatively charged glycosaminoglycan as well as the adhesion peptide RGD were incorporated at varying concentrations within a PEG-based hydrogel. The ChS was added to resemble the fixed negative charge of native cartilage tissue \(^3\) and RGD was added to allow the encapsulated MSCs to interact with hydrogel environment \(^4\). Additionally, dynamic compression, which has resulted in variable responses both enhancing\(^5,^6\) and inhibiting\(^7,^8\) chondrogenesis, was applied to the hydrogel at various loading regimes. The results from this study found that the presence of the ECM analogs enhanced MSC chondrogenesis regardless of concentration, however, the hypertrophic marker collagen X was present in all hydrogel formulations. The formulation of 1% ChS (g/g) and 0.1 mM RGD led to the highest collagen II gene and protein expression, therefore, the 1% ChS 0.1mM RGD was selected to investigate the effects of dynamic compressive loading on MSC chondrogenesis. Results found that dynamic loading affected MSC differentiation in a strain rate dependent manner. Strain rates in the range
of 3%/s (10% strain 0.3 Hz) to 5%/s (5% strain 1Hz) inhibited collagen X. These results suggest that the hypertrophic phenotype of chondrogenically differentiating MSCs may be highly mechanosensitive\(^9\), and by applying the necessary biochemical cues with mechanical stimulation the hypertrophic phenotype can be inhibited.

In Chapter 4, previous results that suggested that physiochemical cues can greatly affect the differentiation of MSCs was applied to a multilayered, osteochondral hydrogel. Damage to joints often extends to the subchondral bone, however, many of the current tissue engineering strategies focus specifically on only repairing the cartilage which leads to insufficient treatment as well as a lack of anchoring to the bone\(^{10}\). Here, a high concentration of RGD (10mM), which is found in bone-specific proteins, was added to the stiffer bottom “bone-like” layer\(^{11}\). A soft “cartilage-like” layer, which was the hydrogel formulation from Chapter 3 (1% ChS, 0.1mM RGD) was added on top, and the diffusion of the top layer into the bottom created the “interface layer” which had a concentration of ChS and RGD and compressive modulus that was in-between the other two layers. Additionally, the hydrogel constructs were subjected to 2.5% strain to apply 5% strain to the cartilage-like layers, while having limited compression onto the bone-like layer\(^{12}\). Results from this study found that mechanical stimulation was key in controlling the differentiation of encapsulated MSCs into the specified regions of the hydrogel.

In addition to biochemical cues and mechanical stimulation, scaffold degradation is necessary to encourage macroscopic tissue deposition and to be considered a suitable material for cartilage defect repair\(^{13–15}\). Multiple methods of degradation exist, however, cellular mediated degradation is promising for cartilage tissue engineering\(^{16,17}\). As MSCs differentiate they secrete enzyme such as matrix metalloproteinase 7 (MMP7) that are
responsible for remodeling their extracellular matrix. The expression of MMP7 by chondrogenically differentiating MSCs has been found to be parallel to chondrogenesis and ECM tissue production\textsuperscript{16,18}. In Chapter 5, MMP7 sensitive hydrogels were investigated. An MMP7 sensitive peptide was used as the crosslinker of cartilage mimetic PEG thiol-ene hydrogel with the incorporated ChS and RGD. Results from this study found that the encapsulated MSCs were able to degrade the synthetic hydrogel and produce their own extracellular matrix. After an initial drop in compressive modulus due to the degradation of the PEG, the tissue produced by the encapsulated cells led to a significant increase in the compressive modulus of the construct. This study developed a cellular mediated degradable cartilage mimetic hydrogel that allows for macroscopic human cartilage tissue production and has the potential to be used as a treatment for chondral defects.

The cellular mechanisms involved in MSC differentiation are incredibly complex\textsuperscript{19}, and their response to stimuli in their surrounding environment is relatively unknown. The previous work in Chapters 3 and 4 show that MSC differentiation is highly sensitive to mechanical stimulation, however, the mechanism by which mechanical stimulation controls the differentiation remains to be elucidated. One mechanism of interest is the Smad signaling pathway, which is known to be crucial for chondrogenesis to occur\textsuperscript{20}, where the phosphorylation of Smad2/3 is vital for cartilage tissue development, whereas the phosphorylation of Smad1/5/8 is associated to hypertrophy\textsuperscript{21}. Initially, the inhibition of pSmad1/5/8 by a small molecule inhibitor, dorsomorphin, was found to inhibit collagen X production of the encapsulated MSCs. To a similar affect, dynamic loading was found to reduced pSmad1/5/8 signaling, and also inhibited collagen X production. The load-
induced inhibition of hypertrophy was specific to ChS-containing hydrogels, where RGD alone did not provide the physiochemical cues necessary to inhibit collagen X production. It is hypothesized that this is due to the fixed negative charge associated with the incorporation of ChS into the hydrogel, which allows for dynamic changes in osmolarity similar to that of cartilage tissue. The cellular response to extracellular stimuli such as osmolarity are also regulated by mitogen activated protein kinase (MAPK) signaling mechanisms such as p38. When p38 was inhibited in the MSCs encapsulated in the cartilage mimetic hydrogel, there was no longer an inhibitory effect on hypertrophy due to mechanical stimulation. These results suggest that dynamic loading of a ChS-containing cartilage mimetic hydrogel inhibits pSmad1/5/8 signaling and activates p38 MAPK to ultimately inhibit hypertrophy.

In Chapter 7 the effect of soluble growth factors and dynamic loading on the differentiation of induced pluripotent mesenchymal progenitor cells (IPS-MPs) were investigated. Currently, most cell-based therapies for treating cartilage tissue defects use chondrocytes or bone-marrow derived MSCs. Although these have been found to be promising, there are limitations associated with each cell source. An alternative cell source are induced pluripotent stem cells (IPSCs), which are reprogrammed somatic tissue such as skin fibroblasts, however, little research on cartilage regeneration of IPSCs has been done. This study found that the chondrogenesis of IPSCs is promoted by mechanical loading in the absence of growth factors, which coincided with an increase in pSmad2/3 as well as the TGFβ receptor (TGFβR1). In free swelling culture, TGFβ3 and BMP2 and their combination enhanced chondrogenesis to a similar degree, however, collagen X was also present. Similar to BM-MSCs in the previous chapters, mechanical
loading inhibited hypertrophy of the IPS-MPs but only when cultured in the presence of TGFβ3. Additionally, when cultured with TGFβ3 only, mechanical stimulation enhanced pSmad2/3 signaling and TGFβRI, while reducing pSmad1/5/8 signaling. These results suggest mechanical loading alone of IPS-MPs encapsulated in the cartilage mimetic hydrogel can promote chondrogenesis. Additionally, similar to BM-MSCs in the previous chapter, the chondrogenesis of IPSCs is dependent on Smad signaling. Mechanical loading in combination with TGFβ3 enhances pSmad2/3 dominated signaling, and non-hypertrophy differentiation of IPS-MPs.

After all of the hydrogel design and development, the biodegradable, cartilage mimetic PEG hydrogel's ability to treat chondral defects was tested in an in vivo rabbit animal model in Chapter 8. Prior to the in vivo study, rabbit MSC chondrogenesis was evaluated in the MMP2-sensitive cartilage mimetic hydrogel in vitro. Results showed collagen II and aggrecan production by the encapsulated rabbit MSCs after 9 weeks of culture, showing promise for in vivo applications. For the in vivo study, osteochondral defects were created and treated with the cartilage mimetic hydrogel with and without rabbit MSCs. After 6 months, results showed that the cartilage mimetic hydrogel alone (without MSCs) had the best cartilage repair and regeneration, whereas those treated with MSCs showed similar repair to the untreated defects. These results were not surprising as MSCs have found to be unsuccessful at cartilage regeneration in vivo. Many factors such as lack of mechanical stimulation and growth factors may have contributed to the poor repair by the MSCs.

In response to the inadequate results by the rabbit study, the cartilage mimetic hydrogel was modified with tethered TGFβ3 to encourage chondrogenesis of MSC in vivo.
in future studies\textsuperscript{36}. Specifically, Chapter 9 investigated chondrogenesis of rat MSCs in a degradable cartilage mimetic hydrogel with tethered TGFβ3. This study was done to determine a hydrogel formulation that can be used to treat growth plate defects in a rat animal model. Results found that after 9 weeks of culture, MSCs encapsulated in the hydrogels with tethered TGFβ3 showed increased collagen II and sGAG deposition than those cultured with soluble TGFβ3 in the media. The tethered TGFβ3 also resulted in hyaline-like cartilage tissue, whereas, the soluble TGFβ3 appeared to produce more of a fibrocartilage as confirmed by the presence of collagen I. Additionally, the rat MSCs were able to degrade the hydrogel environment and increased the compressive modulus due to the deposited ECM matrix. This study developed a hydrogel system that has the potential to improve \textit{in vivo} cartilage repair.

As such, the degradable cartilage mimetic hydrogel with the tethered TGFβ3 was investigated \textit{in vivo} in a horse animal model in Chapter 10. In addition to the lack of growth factors in the rabbit \textit{in vivo} study, other limitations by using a rabbit animal model may have contributed to our results. Rabbit models used for cartilage repair have shown variable results due to their inherent ability to self-repair damaged tissue, as well as their lack of compression to their articular joints\textsuperscript{35,37}. A horse model is a better representation of cartilage repair in humans, specifically, defects are similar in size, lack the ability to regenerate, and the joint experiences compressive loading. Prior to \textit{in vivo} examination, the horse MSCs were cultured in the hydrogel and evaluated for their chondrogenic capacity. The horse MSCs produced macroscopic tissue composed of collagen II and aggrecan after 9 weeks. Mechanical loading did not negatively affect ECM deposition. Variability among donors was evident, however, all donors showed enhanced matrix
produced by 9 weeks. The *in vivo* study is currently ongoing, but from the *in vitro* results, it is hypothesized the hydrogel will enhance cartilage repair.

One major limitation of using hydrogel for cartilage repair is that they are significantly softer than the native cartilage surrounding the defect. This mismatch in properties can lead to changes in strain profiles through the adjacent cartilage, and ultimately result in further degeneration. In Chapter 11, the effect of hydrogel stiffness on the surrounding cartilage in a physiologically loading environment was investigated. Chondral defects of *ex vivo* porcine osteochondral plugs were treated with a soft or stiff hydrogel or left empty. The empty defects resulted in significant degeneration of the surrounding cartilage. The stiff treated defects resulted in little to no degeneration adjacent to the defect, while the soft-filled defects showed some degeneration but not as much as the untreated defects. This study suggests that a stiff hydrogel construct with mechanical integrity is necessary to preserve the healthy cartilage surrounding the defect.

Although a stiff, mechanically stable scaffold might be necessary to maintain the surrounding cartilage tissue, a highly crosslinked, nondegradable hydrogel is not suitable for chondrogenesis and cartilage defect repair. Therefore, in Chapter 12 the development of a hybrid scaffold that has a stiff, stable support structure that is infilled with a soft, degradable cell-laden hydrogel was investigated. Stereolithography 3D printing was used to develop the support structure, which was then infilled with the cartilage mimetic hydrogel. Chondrocytes were successfully infilled in the hybrid hydrogel construct and produced ECM matrix around the 3D printed structure. The 3D printed structural support was implanted in cartilage defects in *ex vivo* porcine osteochondral plugs as a proof of concept. After 4 weeks of culture under physiological loading conditions, the implant
remained in place and was able to maintain the surrounding cartilage better than the untreated defects. This work shows the applicability of a 3D printed hydrogel construct for chondral defect repair.

The findings of this thesis provide a hydrogel platform for MSC chondrogenesis and cartilage repair that has the potential to be applied clinically. Throughout this work we have 1.) identified a cellular mediated degradable cartilage mimetic hydrogel environment with the necessary physiochemical cues to enhance chondrogenesis, 2.) we have investigated the cellular signaling mechanisms involved in MSC differentiation and their response to extracellular stimuli and 3.) we have applied this hydrogel to in vivo cartilage defect repair and have made modifications to the hydrogel construct to translate from in vitro development to in vivo regeneration.

13.2 Recommendations for Future Directions
This thesis demonstrates that a hydrogel environment can be tuned to control cell differentiation, cellular signaling, as well as the health of the surrounding tissue. The PEG-based hydrogels offer a platform to incorporate and tether different chemical cues, therapeutic agents and degradation mechanisms to further enhance tissue development. The cartilage mimetic hydrogel developed in this thesis was used with a wide range of species and a plethora of different applications. As such, multiple collaborators were involved which include veterinarians and orthopedic surgeons. While the hydrogel showed promising results in many of the applications, the main feedback that I received from the clinicians was that for in vivo application the simpler the material is, the better. At the same time, the feedback received from researchers and chemical engineers was that additional complexity can be added to the hydrogel to further control the cells
behavior. As such, my recommendations for future directions have been split between complex hydrogels for MSC differentiation that can be developed in vitro and “simple” materials for cartilage defect repair that are more translatable in vivo.

13.2.1 Complex hydrogels to better control MSC differentiation
13.2.1.1 Incorporation of small molecule inhibitors or drugs for spatial and temporal control

The work presented in this thesis showed the cellular mechanisms involved in controlling chondrogenesis and terminal differentiation of MSCs. Smad signaling is only one of many signaling pathways that may be involved. Although mechanical loading was found to inhibit pSmad1/5/8 and hypertrophy, the loading regime applied was very specific and does not necessarily recapitulate physiological conditions. However, the results showed that inhibiting pSmad1/5/8 inhibited hypertrophy of chondrogenically differentiation MSCs. One method to recreate the similar response found with mechanical loading is by incorporating small molecule inhibitors that can directly and locally inhibit certain cellular signaling pathways to control the differentiation. This may be promising, as precise control over the mechanical loading may be difficult, especially in an animal model.

The small molecule inhibitors could be tethered to the hydrogel network through a degradable crosslinker such as a poly(lactic acid) monomer or an MMP sensitive peptide. Similar work has been done for drug delivery. This would allow for temporal control of the release of the small molecule inhibitor, as pSmad1/5/8 is necessary for early stages of chondrogenesis. Multiple inhibitors or enhancers could be tethered into the same hydrogel by different degradable means to control the release and further control the MSC
differentiation. This could also be applied to multilayered osteochondral hydrogels to control the differentiation spatially as well as temporally.

13.2.1.2 CRISPR/Cas9 genetic modification of IPSCs and MSCs

Results from this thesis suggested that IPSCs are a promising cell source for chondrogenic differentiation. One major advantage of using IPSCs for cartilage tissue engineering is that they reportedly do not readily undergo hypertrophy as seen by BM-MSCs\(^{31}\). However, developing a homogenous culture of chondrogenic progenitor cells from IPSCs has been found to be challenging\(^{30,32}\). The use of clustered regularly interspaced short palindromic repeats (CRIPR) –based gene editing via the Cas9 protein (CRISPR/Cas9) has been used to engineer multiple cell types that control their expression of certain genes and proteins. It has also been used to create a novel cell line of IPSCs that express GFP relative to collagen II protein expression.\(^{40}\)

In conjunction with being able to provide a chondrogenically differentiation population of IPSCs, CRISPR/Cas9 has the potential to be used to repress the expression of genes as well, which has been shown in MSCs to reduce the immune response of diseased tissue\(^{41}\). As such, CRISPR/Cas9 could be used to suppress hypertrophic genes during MSC differentiation. Our results showed that inhibition of pSMAD1/5/8 leads to inhibition of hypertrophy. Thus, the expression of Smad1/5/8, or a regulatory signaling mediator, could be potentially repressed to inhibit hypertrophy of MSCs.

13.2.3 “Simple” materials for cartilage defect repair

13.2.3.1 Patient specific acellular hydrogels for cartilage repair

The work presented in this thesis focused on the development of a hydrogel that promotes and enhances the chondrogenesis of MSCs encapsulated within. However, results from our initial in vivo study showed that MSCs do not enhance cartilage repair,
and the biomimetic cartilage hydrogel alone showed the best results. Given the limitations of cell-based approaches for chondral defects, an acellular hydrogel that promotes the activity of endogenous cell sources could be developed to induce the chondrogenesis of cells infiltrating the hydrogel matrix\(^{42}\). Additionally, an acellular hydrogel would be advantageous to the clinical application of using hydrogel for cartilage defect repair.

Currently, studies have investigated the use of acellular materials for microfracture repair of cartilage defects. Materials are designed to attract cells through chemotaxis and provide a structurally stable environment for cells to initiate repair, however, the formed tissue is fibrocartilage\(^{42}\). Additionally, acellular materials that incorporate biological components such as serum and hyaluronic acid, which reportedly may participate in the homing of bone marrow cells have been investigated\(^{43}\). Photopolymerizable hydrogels have shown promising results in regenerating tissue that matches native cartilage tissue\(^{44}\). The currently existing materials show promise, however, patient variability might reduce the effectiveness of the constructs due to differences in endogenous cell repair.

One possible solution to the patient variability, is to provide patient-specific acellular scaffolds that encourage cartilage repair. Specifically, the enzymes secreted by the cells and the surrounding tissue can vary from donor to donor. To alleviate the hydrogel from degrading too quickly or too slowly, the degradation mechanisms can be tuned specifically for the patient, by the addition of different degradable peptides and the crosslinking density of the degradable peptides. Although this would require a lot of work on determining the specific enzyme activity of the patients’ cells, it could potentially enhance cartilage repair.
13.2.3.2 3D printed constructs for MSC recruitment and osteochondral repair

Our results have shown that mechanical stability is necessary for tissue repair. Although biomaterials have been used to treat chondral defects, often the results long-term show limited success. This may be due to the lack of mechanical stability to fully repair the cartilage tissue while maintaining the health of the cartilage adjacent. One proposed strategy to treat chondral defects is through 3D printing of stiff structures. Specifically, this could be applied to the osteochondral interface to allow for mechanical anchoring into the subchondral bone. The acellular hydrogel described above could be applied to this 3D printed construct, in which different biochemical cues that could potentially enhance the differentiation of the infiltrated cells could be spatially controlled and tethered to the 3D printed construct. This would allow the clinician to implant the 3D printed construct directly into the chondral defect, infill with a degradable hydrogel, and photopolymerize without an additional complexities of cells or multiple precursor solutions.

13.3 Long-term Project Goals

The long-term goal of this project is to provide a hydrogel environment that enhances chondrogenesis of MSCs for cartilage defect repair. Due to the complexities of controlling the differentiation and matrix production of MSCs, modifications of the hydrogel have been made throughout the thesis. Additional modifications such as tethering other growth factors, optimization of growth factor concentrations, and multimodal degradation mechanisms have the potential to be incorporated and further enhance chondrogenesis. In collaboration with Dr. Robert McLeod and Dr. Virginia Ferguson, the development of a 3D printed construct that recapitulates the osteochondral interface biologically and mechanically is currently being developed to be used as a
treatment for osteochondral defects. The osteochondral interface is incredibly complex and varies in cell type, mechanical properties, and soluble factors, therefore, engineering strategies to recapitulate this tissue are not trivial. The ultimate goal is to develop a construct that can be 3D printed directly in the osteochondral defect depending on the dimensions of the patient’s defect, infilled with the different hydrogel layers, and photopolymerized to allow for spatial control of the differentiation of the MSCs, as well as mechanical stability through the 3D printed structure. While this may be a few years down the road, significant progress has already been made in the 3D printing and hydrogel systems, and further progress will continue to improve the constructs, with the hope of being implemented to repair osteochondral defects clinically.

13.3 References


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Chapter 9


Chapter 11


Chapter 12


Chapter 13


