High Throughput And Mechano-Active Platforms To Promote Cartilage Regeneration And Repair

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High Throughput And Mechano-Active Platforms To Promote Cartilage Regeneration And Repair

Abstract
Traumatic joint injuries initiate acute degenerative changes in articular cartilage that can lead to progressive loss of load-bearing function. As a result, patients often develop post-traumatic osteoarthritis (PTOA), a condition for which there currently exists no biologic interventions. To address this need, tissue engineering aims to mimic the structure and function of healthy, native counterparts. These constructs can be used to not only replace degenerated tissue, but also build in vitro, pre-clinical models of disease. Towards this latter goal, this thesis focuses on the design of a high throughput system to screen new therapeutics in a micro-engineered model of PTOA, and the development of a mechanically-responsive drug delivery system to augment tissue-engineered approaches for cartilage repair.

High throughput screening is a powerful tool for drug discovery that can be adapted to include 3D tissue constructs. To facilitate this process for cartilage repair, we built a high throughput mechanical injury platform to create an engineered cartilage model of PTOA. Compressive injury of functionally mature constructs increased cell death and proteoglycan loss, two hallmarks of injury observed in vivo. Comparison of this response to that of native cartilage explants, and evaluation of putative therapeutics, validated this model for subsequent use in small molecule screens. A primary screen of 118 compounds identified a number of ‘hits’ and relevant pathways that may modulate pathologic signaling post-injury.

To complement this process of therapeutic discovery, a stimuli-responsive delivery system was designed that used mechanical inputs as the ‘trigger’ mechanism for controlled release. The failure thresholds of these mechanically-activated microcapsules (MAMCs) were influenced by physical properties and composition, as well as matrix mechanical properties in 3D environments. TGF-beta released from the system upon mechano-activation stimulated stem cell chondrogenesis, demonstrating the potential of MAMCs to actively deliver therapeutics within demanding mechanical environments.

Taken together, this work advances our capacity to identify and deliver new compounds of clinical relevance to modulate disease progression following traumatic injury using state-of-the-art micro-engineered screening tools and a novel mechanically-activated delivery system. These platforms advance strategies for cartilage repair and regeneration in PTOA and provide new options for the treatment of this debilitating condition.

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HIGH THROUGHPUT AND MECHANICAL-ACTIVE PLATFORMS TO PROMOTE CARTILAGE REGENERATION AND REPAIR

Bhavana Mohanraj

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HIGH THROUGHPUT AND MECHANO-ACTIVE PLATFORMS TO PROMOTE CARTILAGE
REGENERATION AND REPAIR

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To Robert,

I am so glad we did this together.

Love, Chaos Bhavana
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Nearly 6 years ago, I joined the McKay labs, first as a research engineer and then as PhD student advised by both Rob and George. To this day, I still cannot believe how lucky I am to have the both of them as my advisors. They have been patient and supportive of my scientific pursuits, giving me the time to learn how to be a scientist and the ability to freely explore new ideas and interests. Not only did they instill in me (and I think all of their students) a love for research itself, but also the importance of a close-knit research environment. This sentiment was also echoed in the larger orthopaedic research community that Rob and George welcomed me into- one that is full of decades of familial academic history and friendship. As I finish my time at Penn, any success that I have had here is due in no small part to having Rob and George as my advisors. Their dedication to their students knows no bounds, and I am thankful to have had the opportunity to work with them during my PhD.

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ABSTRACT

HIGH THROUGHPUT AND MECHANO-ACTIVE PLATFORMS TO PROMOTE CARTILAGE REGENERATION AND REPAIR

Bhavana Mohanraj
Robert L. Mauck
George R. Dodge

Traumatic joint injuries initiate acute degenerative changes in articular cartilage that can lead to progressive loss of load-bearing function. As a result, patients often develop post-traumatic osteoarthritis (PTOA), a condition for which there currently exists no biologic interventions. To address this need, tissue engineering aims to mimic the structure and function of healthy, native counterparts. These constructs can be used to not only replace degenerated tissue, but also build in vitro, pre-clinical models of disease. Towards this latter goal, this thesis focuses on the design of a high throughput system to screen new therapeutics in a micro-engineered model of PTOA, and the development of a mechanically-responsive drug delivery system to augment tissue-engineered approaches for cartilage repair.

High throughput screening is a powerful tool for drug discovery that can be adapted to include 3D tissue constructs. To facilitate this process for cartilage repair, we built a high throughput mechanical injury platform to create an engineered cartilage model of PTOA. Compressive injury of functionally mature constructs increased cell death and proteoglycan loss, two hallmarks of injury observed in vivo. Comparison of this response
to that of native cartilage explants, and evaluation of putative therapeutics, validated this model for subsequent use in small molecule screens. A primary screen of 118 compounds identified a number of ‘hits’ and relevant pathways that may modulate pathologic signaling post-injury.

To complement this process of therapeutic discovery, a stimuli-responsive delivery system was designed that used mechanical inputs as the ‘trigger’ mechanism for controlled release. The failure thresholds of these mechanically-activated microcapsules (MAMCs) were influenced by physical properties and composition, as well as matrix mechanical properties in 3D environments. TGF-β released from the system upon mechano-activation stimulated stem cell chondrogenesis, demonstrating the potential of MAMCs to actively deliver therapeutics within demanding mechanical environments.

Taken together, this work advances our capacity to identify and deliver new compounds of clinical relevance to modulate disease progression following traumatic injury using state-of-the-art micro-engineered screening tools and a novel mechanically-activated delivery system. These platforms advance strategies for cartilage repair and regeneration in PTOA and provide new options for the treatment of this debilitating condition.
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Chapter 1. Introduction

The discovery and development of therapeutic strategies for early intervention in osteoarthritis (OA) remains a challenge and represents an unmet clinical need for patients who require joint replacements due to the lack of therapeutics and biological options. Articular cartilage functions as a load-bearing structure within the joint that uses mechanical feedback to maintain tissue homeostasis. While normal, physiologic loading is beneficial, injury or supra-physiologic loading can initiate acute degenerative processes that lead to progressive failure of the articular surface. This subset of joint disease, termed post-traumatic osteoarthritis (PTOA), is often the direct consequence of a traumatic event such as an ACL or meniscus tear. Although end-stage OA is well characterized clinically and experimentally, the initial signaling cascades post-injury that eventually culminate in cartilage degeneration, inflammation, and overall joint destruction are still poorly understood. This gap in knowledge presents an opportunity to discover therapeutic targets and advance treatment strategies for patients at different stages of disease.

Cartilage tissue engineering is one platform that can be used to tackle this problem. Over the past thirty years, tissue engineering has generally focused on the fabrication of constructs in vitro that can repair or replace the structure and function of diseased tissues in vivo. The progress made in this field has also enabled the development of pre-clinical models that can mimic physiologic features of disease to screen drug candidates and delivery systems for safety and efficacy. This dissertation focuses on the design of a high throughput platform for small molecule screening in an engineered cartilage model of PTOA and a mechanically-responsive drug delivery system to advance new strategies for cartilage repair.
To provide a framework for the work described herein, Chapter 2 reviews existing tissue-engineered models of musculoskeletal disease. Mechanical systems are described that both initiate measure degenerative responses in these micro-engineered constructs under disease conditions. *In vitro* observations are also compared to *in vivo* hallmarks of disease, and the effect of compounds known to modulate pathology *in vivo* are discussed relative to against clinical observations in these micro-engineered systems.

Towards the development of an *in vitro* model of PTOA, Chapter 3 describes the fabrication and maturation of cartilage tissue analogs (CTAs). Mechanical properties of CTAs are measured as a function of culture duration and benchmarked against those of native cartilage explants. Biochemical and histological assays are also used to evaluate structure-function relationships with regard to proteoglycan and collagen content in CTAs.

Chapter 4 reports on the scale-up of a mechanical testing platform as a step towards high throughput mechanical perturbation of engineered cartilage. The compressive mechanical properties of acellular biomaterials and engineered constructs are rapidly assessed in a 48-well format and validated against conventional single-sample testing methods. The system is able to measure constructs with equilibrium moduli between 25 and 300 kPa and identify changes in engineered cartilage properties to due exogenous stimuli.

This high throughput mechanical testing system is subsequently adapted for mechanical injury of functionally mature CTAs as described in Chapter 5. The ability of the CTA injury response to mimic the acute hallmarks of PTOA in native cartilage is first assessed as a function of strain and strain-rate. CTAs exhibited increased proteoglycan loss and cell
death in response to high strains (50 and 75%) within 48 hours of injury. High throughput application of compressive injury was consistent with single-sample results, and direct comparison to cartilage explants showed similar patterns of GAG loss and LDH release (an indirect soluble measure of cell death). To demonstrate the screening potential of this \textit{in vitro} model, putative therapeutic compounds known to modulate the injury response in explants and \textit{in vivo} are also evaluated. These molecules show similar levels of efficacy in attenuating matrix damage and/or loss of viability in our high throughput system as has previously been reported in the literature, validating the system.

Chapter 6 continues to build on the concept of a high throughput mechanical injury platform as an \textit{in vitro}, pre-clinical model for screening and identifying potential drug candidates. The commercial small molecule libraries chosen for these screens target pathways relevant to cell apoptosis and necrosis, as well as chondrocyte signaling and cartilage development. Out of the 118 compounds evaluated, 7 ‘high-performing hits’ were found that strongly reduced either cell death alone, or reduced both cell death and matrix loss. These ‘high-performing hits’ were subsequently the focus of secondary screens to evaluate donor-specific variability in small molecule efficacy.

While mechanical injury initiates specific, acute changes in cartilage, long-term cartilage degradation is likely mediated by the the inflammatory environment within an osteoarthritic joint. Chondrocytes and MSCs have previously been explored as potential cell sources for cartilage repair; however, the relative sensitivity of each cell type to pro-inflammatory stimuli is unknown. Understanding these differences in cellular behavior supports not only clinical translation of tissue engineered cartilage repair approaches, but also the development of \textit{in vitro} models to study agents that can inhibit cytokine-mediated catabolic
processes. Therefore, the focus of Chapter 7 is to evaluate the response chondrocyte- or MSC-derived engineered cartilage to IL-1β or TNF-α, two of the primary inflammatory cytokines found in OA. MSC-derived constructs exhibit a greater sensitivity to cytokine challenge as measured by a dose-dependent decrease in mechanical properties and matrix content. Differential regulation of nitric oxide production and MMP activity, catabolic mediators induced by IL-1β and TNF-α, was also observed between cell types. These results suggest that chondrocytes may have an inherent advantage in surviving within an inflammatory environment. In addition, screens to identify compounds of interest in vitro and in vivo should consider the role of cell source in interpreting results.

To complement this process of therapeutic discovery using a tissue-engineered model of PTOA, Chapter 8 reports on the design of a mechanically regulated drug delivery system. Mechanically-activated microcapsules (MAMCs) are hollow spheres containing an aqueous drug core and can be tuned to rupture and release under defined loading patterns. This chapter defines how physical properties, such as the shell thickness-to-diameter ratio, influence failure thresholds under direction compression in 2D. It also shows that when MAMCs are embedded in 3D hydrogels analogous to engineered tissues, matrix mechanical properties affected rupture under dynamic loading. The power of this system as a delivery vehicle was shown in the encapsulation and release of bioactive compounds (the chondrogenic growth factor TGF-β3) with mechanical activation. TGF-β3 released from MAMCs induced MSC chondrogenesis in an engineered cartilage construct, validating this drug delivery system for pre-clinical translation.
Finally, Chapter 9 summarizes the work presented here and discusses the potential limitations and implications of these approaches for treating the progressive stages of cartilage degeneration in OA. Future studies and strategies are also outlined that could improve our *in vitro* high throughput model of PTOA for screening applications and further demonstrate the potential of MAMCs to be used for cartilage repair.
Chapter 2. Micro-scale Tissue Engineered Models of Musculoskeletal Disease for High Throughput Screening

2.1 Introduction

The musculoskeletal system integrates several types of tissues (e.g. muscles, tendons, cartilage, bone) to support structural and load-bearing functions that are required for human motion. Injury, aging, and disease can cause degeneration of components of this system, resulting in debilitating pain and disability. As of 2012, 126.6 million adults within the US (1 in 2 adults) was diagnosed with a musculoskeletal condition; this is twice as many as any other condition (Watkins et al. 2016). Although this currently primarily affects working age adults, this population will continue to age, and by 2040, 1 in 5 adults over the age of 65 will be impacted. In addition, children and adolescents who experience trauma or sports injuries may have to contend with life-long chronic pain or instability. Musculoskeletal disorders correspondingly represent a growing percentage of national health expenditures. In 2011, treatment for musculoskeletal disorders (direct) and indirect costs (e.g. lost wages) represented 1.4% of US GDP or 213 billion dollars; however, as of 2015, this number had grown to 5.7% of US GDP or $874 billion dollars. While current clinical strategies use artificial materials (e.g. plastics, metals) in an attempt to restore function, and reduce pain and disability for patients with late stage disease (e.g. fully degenerated discs, osteoarthritis), these approaches have significant limitations (e.g. revision surgeries). Therefore, there has been a concentrated effort to develop disease modifying treatments for early intervention to offset degeneration in order to repair and regenerate tissues by mimicking native biological processes.
Tissue engineering is one such strategy that utilizes a combination of cell sources, biomaterials, chemical, and mechanical cues to fabricate constructs that mimic the cellular phenotype, as well as structure and function of native tissues. The first techniques developed in the early 1980’s focused on engineering skin substitutes to cover severe burn wounds (Green et al. 1979; Yannas et al. 1982), but quickly expanded to include musculoskeletal tissues including cartilage, bone, meniscus, tendon, and muscle. To date, however, few strategies have reached commercial success, including autologous chondrocyte implantation for cartilage repair (ACI, Carticel by Genzyme) and biomaterial-based delivery of BMPs to stimulate bone formation (Infuse by Medtronic, OP-1 implant by Stryker) (Mao et al. 2015). Although a number of engineered tissues are able to achieve native tissue properties in vitro, challenges remain in translating these strategies into clinically successful solutions due to the complexity of the in vivo environment. Despite these difficulties, advances in tissue engineering have supported the development of non-therapeutic applications, including micro-scale disease models.

Over a decade ago, Griffith and Naughton speculated that one of the greatest impacts of tissue engineering would be “the design of in vitro physiological models for the study of disease pathogenesis and the development of therapeutics” (Griffith et al. 2002). The ability to specifically control the design parameters and culture microenvironment of a 3D tissue in vitro make mimicking the cellular and tissue pathology of a given disease feasible. Preclinical models for drug discovery conventionally proceed from 2D cellular assays to in vivo animal models to evaluate the safety and efficacy of lead candidates (Breslin et al. 2013). However, 2D models often fail to recapitulate key aspects of 3D tissue features, including appropriate cell morphology, cell-cell and cell-ECM interactions, biophysical cues (e.g. matrix stiffness), and external stimuli (e.g. mechanical loading), which may also
play a role in cellular response to potential therapeutics (Astashkina et al. 2012; Breslin et al. 2013). In addition, while in vivo models confer the benefit of studying a whole system response to particular drug, screening hundreds or thousands of compounds in animal models is neither economically efficient nor ethically responsible (Mobasheri et al. 2013).

High throughput assays and reliable engineered disease models are required to conduct combinatorial screens of drug libraries and accurately predict in vivo responses in an in vitro system. Combinatorial studies often become increasingly complex as the number of factors (e.g. cell source, environmental cues, therapeutic compounds) increases, resulting in large sample sets that need to be rapidly evaluated (Figure 2-1). A micro-scaled approach would represent a novel and versatile tool for not only understanding mechanisms of disease at the cellular level, but also for identifying new biological targets and therapeutic compounds. In addition, as a high throughput format requires micro-scale tissues, cells could be isolated from patient biopsies to fabricate constructs and screen for drug combinations that have the greatest efficacy, realizing our goal of personalized medicine. The development of viability and functional assays to rapidly assess biomarkers relevant to the disease state (Kimlin et al. 2013) would further support this platform. This overview Chapter surveys the current state of the art for musculoskeletal disease models, with a specific focus on mechanical systems used to create or evaluate disease states in micro-scale engineered tissues and the potential utilization of these models in therapeutic discovery.
Figure 2-1. An example of an experimental tree for combinatorial screens that are possible using high throughput, *in vitro* micro-engineered disease models. In this scenario screening only two compounds to combat inflammation, a number of variables can be modulated, including dosage, timing of administration, and outcome measures to assess relevant disease biomarkers.

2.2. Skeletal Muscle Models

Clinical disorders of skeletal muscles generally fall into two main categories, those that are inherited (e.g. genetic abnormalities) or are acquired (e.g. due to inflammatory diseases, exposure to toxins, infection) (Muthusamy et al. 2010). These myopathies clinically present as various patterns of weakness and dysfunction, and are the result of
disruptions in structural integrity and abnormal cell metabolic activity. Experimentally, skeletal muscle is frequently characterized by phenotypic or morphological changes in myotubes or myofibrils, including expression of differentiation or hypertrophic markers (e.g. insulin-like growth factor I (IGF-1), myogenin (Sharples et al. 2012) and alterations in cell and histological features (e.g. myofiber size and fusion (Sharples et al. 2012; Lee et al. 2013)). Although these measures provide insight into the signaling pathways that regulate skeletal muscle remodeling, they are not necessarily reflective of muscle functional properties. Active and passive force generation represent the overall integration of these structural and signaling events and may better predict the in vivo physiological response to a potential therapeutic, as compared to characterization of cellular behavior (Vandenburgh 2010). In this section, bioengineered skeletal muscle models will be described wherein force generation serves as the primary outcome measure for determining how degenerative muscle diseases affect muscle function.

2.2.1. Micro-bioartificial Muscles

The MyoForce Analysis System developed by Vandenburgh et. al., is a high-content screening system capable of measuring active forces generated by micro-bioartificial muscles (mBAMs) in response to electrical stimulation (Vandenburgh et al. 2008) (Figure 2-2A). Proliferating primary mouse myoblasts were mixed with naturally-derived matrix products (fibrin (Vandenburgh et al. 2008; Vandenburgh et al. 2009), type I collagen (Vandenburgh et al. 2009), or collagen-Matrigel (Vandenburgh et al. 2008; Lee et al. 2013)) and seeded in wells containing elastomeric PDMS posts. The cell-seeded gels were observed to contract around the posts, facilitating alignment of the myoblasts. The addition of differentiation medium (low-serum conditions) following this initial contraction induced myoblast fusion to form aligned and striated muscle fibers. Under electrical
stimulation, fibers were able to rapidly generate isotonic tetanic forces, resulting in deflection of the PDMS posts. Post deflection was tracked using motion detection imaging; the magnitude of the active force generated by an mBAM (on the order of µN) was calculated based on the material properties and geometry of the posts.

Scale-up to a 96-well format, compatible with a robotic liquid handling system, demonstrated the feasibility of high throughput fabrication and culture of mBAMs, as well as the potential for conducting drug screens in a model of Duchenne muscular dystrophy (DMD) (Vandenburgh et al. 2009). DMD is a lethal disease caused by the absence of the functional dystrophin gene, which leads to progressive muscle breakdown and weakening (Matsumura et al. 1994). This genetic mutation occurs 1 in 3500 male births and onset of the disease is observed before the age of 13 (Muthusamy et al. 2010). Mechanical stress applied to muscle fibers lacking this cytoskeletal and structural support protein results in cell membrane damage and reduced force generation. In this in vitro model, myoblasts from a genetic murine homolog of DMD (mdx) were used to fabricate miniature mdx mBAMs to carry out combinatorial screens of therapeutic compounds that might strengthen muscles (Vandenburgh et al. 2009). Of the 31 compounds screened, those that markedly increased mdx mBAM force generation (by >30%) included anti-inflammatory glucocorticoids (e.g. prednisone and deflazacort which are currently used clinically), as well as IGF-1, creatine, and Trichostatin A. To determine if in vitro outcome measures were consistent with synergistic activity observed in vivo, several drug interactions were also evaluated. Treatment with creatine and glucocorticoids for 3 to 4 days doubled mdx mBAM maximum tetanic forces, consistent with improvements in muscle strength in the mdx mouse model (Figure 2-2B). Clinically observed negative interactions (i.e. lack of improvement in DMD patients) were also mimicked in the mdx in
vitro model, where the addition of pentoxifylline (an antifibrotic drug) reduced the tetanic forces generated by glucocorticoid or creatine treatment alone. Taken together, these results demonstrated that the functional properties of engineered skeletal muscle are consistent with and reflective of gross muscle changes observed in animal models, as well as patients, validating this system as a micro-scale musculoskeletal disease model.

Figure 2-2. Engineered muscle models can be used to induce and monitor disease conditions in vitro. (A) Micro-bioartificial muscle schematic of post deflection under electrical stimulation for measurements of active force generation; the 96-well mBAM format for high content screening is shown on the right (Adapted from Vandenburgh et al. 2008; Vandenburgh et al. 2009). (B) Treatment of mdx mBAMs with combined drug
treatment (Arginine and Deflazacor) improved tetanic force generation compared to either drug alone (Adapted from Vandenburgh et al. 2009). (C) Gross picture of constructs in the Culture Force System and fluorescence image of multi-nucleated myotubes that form during construct maturation (Adapted from Player et al. 2014). (D) A reduction in myotube area (atrophic response, MPD) was observed in a model of skeletal muscle aging, as compared to healthy, hypertrophic controls (CON) (Adapted from Sharples et al. 2012). (E) A model of static over-loading induced expression of hypertrophic markers, IGF-1 and MMP-9 (Adapted from Player et al. 2014).

Recently, mBAMs have also been utilized to investigate the molecular mechanisms by which tension reduction contributes to the development of disuse skeletal muscle atrophy (Lee et al. 2013). Mature mBAMs (able to generate a stable maximum isometric tetanic force) were subjected to a length reduction by shortening the post-to-post distance (25-50%), thereby reducing tension within the construct. Within 6 days, hallmarks of muscle atrophy were induced, including a reduction in active force generation, myofiber cross-sectional area, total protein synthesis rate, and non-collagenous protein content. However, these changes were considered modest compared to literature observations of atrophy in an in vivo rodent hindlimb unloading model. This difference in the magnitude of the degenerative response may be due to the absence of certain native muscle physiological factors (e.g. motor neuron innervation, endocrine effects, vascularity) in the mBAM microenvironment that contribute the skeletal muscle maturation (e.g. differential expression of contractile protein isoforms, higher level myofiber organization). Inclusion of these components in future iterations of the mBAM system will likely improve the accuracy of the pathological model in predicting in vivo responses, as well as support the discovery of new therapeutics that can inhibit or attenuate muscle wasting due

2.2.2. Culture Force System

The Culture Force System (CFS) was designed as a dual purpose system: (1) to measure passive force generation (Culture Force Monitor) and (2) to apply acute mechanical overload (Tensioning Culture Monitor) to engineered muscle (Sharples et al. 2012; Player et al. 2014) (Figure 2-2C). In an adaptation of the method described by Vandenburgh et. al., C2C12 murine skeletal muscle myoblasts were suspended in type I collagen and polymerized between polyethylene mesh flotation bars attached to stainless steel frames. Strain gauges attached to these frames measured the contraction of muscle constructs during differentiation and maturation of the tissue. In the first application of CFS (specifically as a Culture Force Monitor), an in vitro model was developed to investigate how aging contributes to skeletal muscle degeneration (sarcopenia) (Sharples et al. 2012). Expansion of myoblasts in monolayer culture to >50 population doubles has previously been shown to induce an ‘aged’ phenotype by mimicking the process of cellular expansion and self-renewal that occurs during a lifetime of endogenous muscle repair (Sharples et al. 2011). These expanded cells were subsequently seeded in collagen gels to form ‘MPD’ engineered muscle constructs. Within the first 24 hours of culture, a decrease in peak force generation was observed in MPD constructs as compared to non-expanded, parental control tissues. By day 14, MPD constructs were smaller and thinner (reduced myotube diameter and area) indicating an atrophied morphology as compared to control constructs (exhibited hypertrophic growth) (Figure 2-2D). These changes were taken as an indication of altered cell-matrix and cell-cell interactions, which regulate myoblast differentiation and fusion into myotubes. At early time points, gene expression showed a
reduction in MMP-9, myogenin, and IGF-1, as well as an increase in IGFBP-2 in MPD constructs compared to parental controls. This expression pattern supported the lack of differentiation capacity in engineered MPD muscles and mimicked the impaired regenerative capacity of aged muscles *in vivo*.

Building on this platform, the Tensioning Culture Monitor was developed to apply acute mechanical overload to engineered muscle and the transcriptional signature associated with acute hypertrophy was evaluated as a function of loading parameters (Player et al. 2014). A ‘rapid static’ (immediate application of 10% strain held for 60 minutes) and ‘slow ramp’ (continuous stretch to reach 10% strain over 60 minutes) loading induced lactate release, indicating increased metabolic activity under overloading conditions. Measurement of gene expression focused on IGF-1 regulation, specifically IGF binding proteins and MMP-9. Together these factors control the bioavailability of IGF-1, which plays a role in potentiating the acute hypertrophic response. MMP-9 expression was significantly up-regulated and IGFBP-5 markedly down-regulated for both loading protocols, matching reported *in vivo* observations (*Figure 2-2E*). However, IGF-1 expression was only up-regulated following static stretch, suggesting that the cellular response to the rate of stretch may be differentially regulated and impact the acute adaptive response of skeletal muscle (*Figure 2-2E*). Genes related to inhibition of protein synthesis (myostatin) and activation of proteolytic pathways (MuRF-1 and MAFBx) were also considered as they play a role in regulating muscle mass. While *in vivo* models describe suppression of these genes, in engineered muscle, minimal down-regulation of myostatin was observed and no change was seen for MuRF-1 or MAFBx in the short duration of these experiments. Although challenges remain in recapitulating physiologic signaling events, a number of mechanisms involved in skeletal muscle atrophy and
hypertrophy were conserved in the CFS platform, supporting the continued development of engineered muscle as a preclinical model system.

2.3. Tendon Models

Repetitive or cyclic loading plays an important role in the development of tendinopathy, a common overuse injury in tendon, for which the etiology is not well understood. Tendinopathy is a blanket term for tendinitis (pain and inflammation in tendon), tendinosis (tendon degeneration), and tenosynovitis (inflammation of the synovial sheath); however, all conditions are clinically characterized by chronic tendon degeneration leading to pain and rupture (Hopkins et al. 2016). Athletes and workers represent the most at risk groups. Lower extremity tendinopathies are most common in athletes (e.g. patellar or Achilles’), due to the high intensity and frequency of physical activity. For workers who perform highly repetitive tasks, upper extremity tendinopathies are the most common, with the affected anatomical site dependent on the occupation (Hopkins et al. 2016). Experimental hallmarks of tendinopathy include expression catabolic mediators, as well as aberrant tenocyte differentiation and matrix remodeling. In particular, tendinotic tissues exhibit an increase in type III collagen and glycosaminoglycan (GAG) content as compared to healthy tendons which are primarily composed of type I collagen (Magnusson et al. 2010). Previous work has described the development of 2D in vitro and 3D ex vivo models to mimic specific pathologic features of tendon disease. In a scratch wound model of tenocytes isolated from normal, tendinopathic, or ruptured tendons, tenocytes from degenerated tissues expressed a higher collagen type III to type I ratio than healthy cells (Maffulli et al. 2000). An ex vivo explant model tested the hypothesis that increased GAG content was likely due to chondrogenic differentiation of tendon cells towards a
fibrocartilaginous phenotype (de Mos et al. 2009). Culture of explants under chondrogenic conditions (TGF-β) induced marked up-regulation of chondrogenic (SOX9, aggrecan, and collagen type II) and hypertrophic markers (RUNX2 and collagen type X), similar to human tendinotic tissues. These shifts in matrix composition may significantly affect the tensile load-bearing capacity of tendons (e.g. through a weakened collagen network structure), thus increasing the risk of rupture. To improve in vitro investigations of signaling pathways and molecules that regulate tendon injury and disease, this section will describe micro-scale models of mechanical stretch using tendon-derived cells in 3D environments.

2.3.1. Mechanical Stretch on Silicone Membranes

In vitro models of tendinopathy have frequently utilized isolated tenocytes or fibroblasts seeded on flexible silicone membranes or in microgrooves (as shown in Figure 2-3A) coated with adhesive proteins (Archambault et al. 2002; Yang et al. 2005; Zhang et al. 2009). These membranes were subjected to cyclic stretch at low (4%) strains to mechanically stimulate tissue growth and suppress inflammation, or at high (8%) strains to induce catabolic processes leading to injury (Agarwal et al. 2001; Agarwal et al. 2003). In particular, repetitive application of injurious strains was associated with arachidonic acid signaling and matrix metalloproteinase (MMP) activity. Mechanical stretch up-regulated gene expression of COX-2 (Archambault JOR 2002) in a strain-dependent manner, resulting in a down-stream increase in PGE-2 protein expression under injurious conditions (Yang et al. 2005) (Figure 2-3B). MMP-1 and -3 expression was also up-regulated with increasing strain magnitude (Figure 2-3B). Low strains, which have an anabolic effect, require matrix turnover as part of the process for tissue growth; however, an imbalance between protein synthesis and degradation rates at high strains can lead to overall protein loss and tissue atrophy. The effect of IL-1β, an inflammatory cytokine
associated with tendon injury, was also evaluated in the context of mechanical stretch of silicone membranes (Yang et al. 2005). While low strains were able to suppress expression (MMP-1, COX-2) and release (PGE$_2$) of catabolic mediators, high strains acted synergistically with IL-1β to further up-regulate these factors.

**Figure 2-3.** Tendon degeneration and de-differentiation *in vitro* is a function of the magnitude of applied strain. (A) Tendon stem cells seeded in microgrooved silicone surfaces exhibited an aligned morphology with uniaxial stretch (Adapted from Zhang et al. 2009). (B) MMP-1 and PGE$_2$ levels increased following cyclic stretch at high strain magnitudes (8%) in human patellar tendon cells (Adapted from Yang et al. 2005). (C-D) Application of high strain (injurious) regimens induced expression of abnormal non-tenocyte markers (e.g. LPL, SOX9, RUNX2) that were also up-regulated in an *in vivo* injury model of intensive treadmill running (ITR) (Adapted from Zhang et al. 2009; Zhang et al. 2013).
To further explore tendon differentiation potential in response to mechanical forces, Zhang et al. cultured primary tendon stem cells (TSCs) in silicone microgrooves and subjected them to cyclic tensile stretch at 4 or 8% strain for 12 hours (Zhang et al. 2009; Zhang et al. 2013). At both strain levels, TSC expression of collagen type I was significantly increased, indicating differentiation towards a tenocyte phenotype. However, aberrant differentiation of TSCs was observed under 8% strain, as evidenced by up-regulation of markers for chondrogenesis (SOX9, collagen type II), osteogenesis (RUNX2), and adipogenesis (PPARγ), compared to unloaded controls (Zhang et al. 2009) (Figure 2-3C). These phenotypic changes suggest that the GAGs, lipids, and calcified regions found in tendinopathic tissues may be the direct result of mechanical overload initiating several simultaneous cell differentiation programs. A follow-up study evaluating differentiation markers in an in vivo murine model of tendinopathy showed similar fold-increases in the expression of non-tenocyte genes following intense treadmill running (Zhang et al. 2013), as compared to TSCs in vitro (Figure 2-3D). One difference of note was the higher expression (~3-fold) of collagen type I and II under high strain conditions in vitro; however, overall, key hallmarks of altered progenitor cell fate due to an injurious mechanical environment were able to be captured in an engineered tissue system.

### 2.3.2. Microfabricated Tissue Gauges

While phenotypic changes are often characterized to identify mechanotransduction events associated with tendinopathy, tissue functional properties also play an important role in disease progression. Legant et al. recently developed a high throughput system to fabricate and evaluate the contractile properties of micro-tissues (Legant et al. 2009; Ramade et al. 2014). The μTUG array (micro-fabricated tissue gauges) passively
measures the interactions between cell contractile forces and matrix remodeling on micro-tissue force generation (on a nN scale, Figure 2-4 A-B). Similar to the mBAM system, cells (e.g. fibroblasts) are seeded in collagen matrix in PDMS wells containing raised cantilevers; cell compaction of the matrix around the posts causes cantilever deflection. Micro-tissue force generation increased with cell number, cantilever stiffness, and collagen concentration (Figure 2-4C). Changes in functional behavior also matched the observed increase in matrix production (measured by immunofluorescence of Tenascin C and fibronectin). This system has since been used to create arrays of cardiac microtissues to study the effects of electrical stimulation and drug compounds (Boudou et al. 2012). Since tendons undergo pathological matrix remodeling during degeneration, it may be possible to utilize this µTUG system to fabricate 3D micro-tendon-like tissues and create a disease model of tendinopathy with measurable force output. Micro-tendons could be treated with growth factors to induce differentiation (e.g. TGF-β), fabricated using combinations of collagen types and concentrations, or seeded with tenocytes and/or TSCs from healthy or diseased tendons. Control over cell source, matrix, and exogenous stimuli in this system would enable the development of a versatile, in vitro model of tendinopathy with the potential to track functional outcomes, in a high throughput manner, that have parallels in clinical observations.
Figure 2-4. Micro-fabricated tissue gauges (µTUGs) measure cell contractile forces and matrix remodeling in micro-patterned arrays of 3D constructs. (A) Large arrays of micro-tissues were fabricated simultaneously on a single substrate. (B) Tension generated within the construct reaches a plateau after cell contraction of the matrix (inset: fluorescence image of cytoskeletal and matrix components- actin, fibronectin, and tenascin C). (C) µTUGs fabricated on stiffer MEM cantilevers and seeded with a greater number of cells, exhibited greater tension generation within the construct. (Adapted from Legant et al. 2009).

2.4. Cartilage Models

Arthritis is the most common cause of disability among adults within the US and is projected to affect 67 million individuals (~25% of the adult population) by the year 2030 (Watkins et al. 2016). In 2011, the estimated annual cost of medical care for arthritis and
joint pain was 580 billion dollars; the total estimated cost of joint replacements alone was 66 billion dollars. In particular, osteoarthritis (OA) is the most common of all joint diseases, with the majority of affected individuals aged between 15 and 64 years (Economics 2007; Pereira et al. 2011). For a subset of this population, an acute instance of supraphysiologic loading (i.e. traumatic injury) was the initiating event that led to the development of cartilage lesions and full blown OA later in life. This sub-category of OA, termed post-traumatic osteoarthritis (PTOA), accounted for 12% of all OA cases (~6 million people) in the US as of 2006 (Brown et al. 2006). Traumatic joint injuries increase the risk of OA 5-fold, with 50 to 75% of patients with ACL tears or meniscal damage developing OA 10 to 15 years post-injury (Roos et al. 1995; Lohmander et al. 2004; Lohmander et al. 2007; Neuman et al. 2008). Clinically, OA is characterized by joint pain, as well as joint space narrowing and cartilage erosion (observed by MRI, (Kijowski et al. 2014)). Additional biological markers of PTOA observed experimentally (in vivo and in vitro explant models) include tissue swelling, cartilage fibrillation or erosion, cell death at or adjacent to the site of injury, increased expression of proteases and inflammatory cytokines, and overall depletion of proteoglycans, culminating in the loss of cartilage mechanical function (Kurz et al. 2001; Quinn et al. 2001; Borrelli et al. 2003; Patwari et al. 2003; DiMicco et al. 2004; Lee et al. 2005; Natoli et al. 2008; Hurtig et al. 2009; Boyce et al. 2013). While the native microenvironment plays an important role in the development of OA, animal and explant model systems are not amenable to scale-up for drug development due to considerations of cost, ethical issues, and tissue heterogeneity. To date, no pharmaceutical solution has been approved to stop the structural damage caused by OA. Yet as the number of joint replacements rise (expected ~10% annually), the need for disease modifying OA therapies for early intervention becomes apparent. Micro-scale engineered cartilage may therefore serve as an appropriate in vitro analog to study how inflammatory milieu and
mechanical injury contribute to progressive cartilage degeneration, and to evaluate the ability of putative therapeutics to inhibit this process.

2.4.1. Role of Inflammation in OA Pathogenesis

Classic inflammation is frequently associated with OA and contributes to the imbalance of catabolic and anabolic processes that lead to overall cartilage dysfunction. Cytokines (e.g. IL-1, TNF-α, IL-6 (Goldring 2000)) produced by a number of cell types within the joint (e.g. macrophages, synoviocytes, and chondrocytes (Benito 2005; Bondeson et al. 2006)) induce the production of catabolic mediators (e.g. nitric oxide, MMP, ADAMTS) that progressively change the quality and quantity of matrix components, including proteoglycans and type II collagen. Recently, engineered cartilage has been used as a platform to investigate the ability of specific cytokines to stimulate OA hallmarks and to test the inhibitory effects of candidate drugs (Willard et al. 2014). Using a high throughput platform, cartilage pellets were formed from mouse iPSCs and cultured for three weeks in the presence of chondrogenic factors (TGF-β3 and dexamethasone). Treatment of this engineered cartilage with IL-1α (1ng/mL) increased GAG, nitric oxide, PGE2, and MMP release to the medium, comparable to the response observed in treated native cartilage explants (Figure 2-5A). In a screen of candidate therapeutic agents known to inhibit inflammation (IL-4), COX-2 (NS398) and NF-kB (SC-514) signaling, and MMP activity (TIMP-3 and GM6001), only SC-514 was able to attenuate production of all catabolic mediators in response to IL-1α described above.

Although the above study demonstrated the scalability of an OA model and the ability of a single cytokine to mimic several features of cartilage pathology in vitro, the in vivo
environment contains several factors that modulate cell signaling all at once. To better recreate signaling events that occur in the inflamed joint environment \textit{in vivo}, Sun et al. cultured 3D silk-fibroin engineered cartilage with macrophage-conditioned medium (MCM) for 21 days (Sun et al. 2011). Chondrocyte response to MCM was compared to medium with exogenous addition of TNF-\(\alpha\) and IL-1\(\beta\) at the same concentration. Exogenous cytokines and MCM induced expression of MMP-1, -3, -13 and ADAMTS4, as well as suppression of GAG accumulation within the construct. Although the magnitude of these changes varied between the two conditions, the models mimicked many early hallmarks of OA observed \textit{in vivo}. However, only MCM was able to stimulate chondrocyte hypertrophy and apoptosis, suggesting exogenous cytokines alone may not be sufficient to capture key features (and potential targets) of OA cartilage.
Engineered cartilage models of OA can mimic features of the inflammatory and mechanical injury response. (A) Induced pluripotent stem cells (iPSCs) were used to fabricate cartilage pellets in a high throughput model of inflammation in OA. Increased GAG release and MMP activity observed in the engineered system was similar to the native cartilage response to IL-1α (1ng/mL). Evaluation of candidate drugs showed SC-514 (an inhibitor of NF-κB activity) was most effective at restoring matrix content (Adapted from Willard et al. 2014). (B) The ability of engineered cartilage to repair the surrounding matrix post-injury was assessed as a function of construct maturity. Chondrocytes in Immature (day 5) constructs were able to restore the structural integrity of the matrix by 5 weeks post-injury, while permanent loss of structural integrity was observed in mature constructs (day 35 pre-culture) (Adapted from Tan et al. 2010).
2.4.2. Compressive Injury Models using Engineered Cartilage

Mechanical injury, as it relates to PTOA, induces acute changes in cartilage (primarily cell death and proteoglycan loss). Over time, this initial traumatic event is potentiated by catabolic signaling that eventually leads to a loss in load-bearing capacity. To date, few \textit{in vitro} models have been developed to explore the mechanisms that cause mechanical injury to progress to cartilage degeneration. In recent work, Tan et al. described the intrinsic ability of chondrocytes to repair the surrounding matrix as a function of engineered cartilage maturity (Tan et al. 2010). Immature (day 5) or mature (day 35) constructs were subjected to compression-induced cracking (ramp to failure at 0.3\% strain/s), and subsequently cultured for up to 3 months (Figure 2-5B). Chondrocytes in immature constructs were able to infiltrate and repair discontinuous regions by 4 weeks post-injury, resulting in equivalent equilibrium and dynamic moduli as compared to un-injured constructs. In comparison, mature constructs did not recover with time in culture. A large fraction of cells remained non-viable and there was a permanent loss in mechanical integrity, similar to observations of injured explants \textit{in vitro}. This disparity in the injury response with construct ‘age’ may relate to the density of the surrounding matrix and cell-ECM interactions that influence strain transfer to embedded chondrocytes. A separate study also evaluated the contribution of chondrocyte phenotype to the injury response. Juvenile or adult chondrocytes were embedded in a poly(ethylene glycol) hydrogel and subjected to impact injury (Farnsworth et al. 2012). However, age-dependent differences were not clearly distinguishable, indicating that mechanical perturbation may be context dependent.

Although these systems represent a first step, further development of reliable, engineered cartilage injury platforms are needed to aid in the discovery of disease-modifying
therapies. Previously, the investigation of small molecules for the treatment of PTOA has focused on factors that target canonical early signaling events. These agents include those that block pathways related to cell death (e.g. pan-caspase inhibitors (D’Lima et al. 2001; Martin 2009)), repair cell membrane damage (e.g. amphiphilic surfactants (Phillips et al. 2004; Bajaj 2010)), reduce catabolic mediators (e.g. free radical scavengers (Martin 2009)), and attenuate or reverse proteoglycan loss (e.g. growth factors (Hurtig et al. 2009) and glucocorticoids (Lu et al. 2011)). However, since the mechanisms of PTOA have not yet been fully elucidated, there likely exist additional agents that modulate non-canonical or un-explored pathways with chondro-protective or chondro-regenerative effects (e.g. Kartogenin (Johnson et al. 2012)). A high throughput mechanical injury platform using engineered cartilage would address this need for drug discovery, and serve as a valuable, unbiased screening tool to identify new therapeutics for early intervention in PTOA.

This is approach is the major focus of this thesis. Characterization of an engineered cartilage model, design of a high throughput mechanical platform, and validation of the injury response in vitro, are all described as part of a concentrated effort to discover and deliver therapeutics that may attenuate the progression of degeneration initiated by traumatic mechanical injuries.

2.5. Conclusion

Tissue engineering is a promising strategy for the repair of diseased and degenerated musculoskeletal tissues, but difficulties still remain in translating this technology into clinically acceptable solutions. Despite this challenge, advances made in this field over the past thirty years have supported the development of in vitro pre-clinical models that more closely mimic the native tissue physiology. The current state of the art in tissue
engineered musculoskeletal disease models was described in this review, with a particular focus on micro-scaled engineered systems that can incorporate mechanical perturbations to reproduce key pathological features observed in vivo. Improvements in throughput, recapitulation of native tissue properties and injury responses in micro-scaled tissues, and most importantly, incorporation of mechanical stimuli in developing platforms will pave the way for the discovery of new therapeutics for the treatment of musculoskeletal disorders.
Chapter 3. Time-dependent Functional Maturation of Scaffold-Free Cartilage Tissue Analogs

3.1 Introduction

Articular cartilage is a dense connective tissue lining joint surfaces and is defined by the unique zonal architecture of extracellular matrix constituents and chondrocytes, as well as by its load bearing capacity during physiological activities. As a consequence of the avascular and aneural nature of this specialized tissue, cartilage has limited healing capacity following injury. Surgical methods to repair chondral defects include abrasion arthroplasty (Johnson 1986), subchondral drilling (Insall 1974), and osteochondral autografts (Hangody et al. 2004). Currently, only one cell-based regenerative approach is FDA approved; this technology is termed autologous chondrocyte implantation (ACI) (Brittberg et al. 1994). This approach utilizes patient cartilage biopsies that are digested to isolate chondrocytes. These cells are subsequently expanded and re-implanted at the site of cartilage damage to induce regeneration. However, these techniques have encountered limited success due to issues including fibrocartilage formation, chondrocyte de-differentiation, and lack of tissue integration and mechanical support (Furukawa 1980; Hunziker 2002). To address these limitations, tissue engineering strategies aim to repair cartilage by recapitulating the matrix architecture, cellular composition, and mechanical properties through the use of a various of design platforms. These systems combine scaffolds, cell sources, and environmental signaling cues to guide tissue regeneration and achieve native cartilage properties.

Research in this field is active, with numerous approaches to cartilage tissue engineering; these methods can be generally divided into two groups: scaffold-based vs. scaffold-free
constructs. Scaffold-based methods include the use of natural and synthetic hydrogels (Mauck et al. 2002; Erickson et al. 2009; McCall et al. 2012; Ingavle et al. 2013; Rahman et al. 2013) and fibrous meshes (Moutos et al. 2007; Kim et al. 2011), with or without cells (e.g. chondrocytes and stem cells) and other cues (e.g. growth factors), to grow engineered cartilage. Advantages of scaffold-based methods include the ability to temporarily re-differentiate passaged chondrocytes (Capito et al. 2006), to achieve near native mechanical properties (Moutos et al. 2007; Erickson et al. 2009; Erickson et al. 2012), to undergo bioreactor mechanical stimulation (Hu et al. 2006), and to entrap cells in a locally controlled environment. However, significant disadvantages of material-based approaches include phenotype instability, toxicity of degradation products and crosslinking methods, cell adhesion, and inappropriate biomaterial remodeling; each concern depends on the materials and methods utilized (Hu et al. 2006). Alternatively, scaffold-free methods utilize high cell density cultures in combination with low adhesion surfaces (Novotny et al. 2006; Ofek et al. 2008; Revell et al. 2008; Kim et al. 2011), bioreactors (Furukawa et al. 2003; Kelm et al. 2004), or centrifugation methods (Muraglia et al. 2003) to form aggregates (Anderer et al. 2002; Furukawa et al. 2003; Revell et al. 2008; Huey et al. 2011), pellets (Zhang et al. 2004; Bernstein et al. 2009) and micro-tissues (Kelm et al. 2004). These techniques can retain chondrocyte phenotype, facilitate cell-cell interactions and promote the elaboration a natural ECM (Anderer et al. 2002; Kelm et al. 2004; Bernstein et al. 2009). Enhancement of ECM characteristics (and thus improved cartilage-like characteristics) has also been achieved with the addition of real-time mechanical loading applied to these constructs (Elder et al. 2009; Kraft et al. 2011). In addition, the uniformity of scaffold-free fabrication enables compatibility with high throughput assay systems (Bhadriraju et al. 2002; Huang et al. 2008), which utilize small volumes and cell numbers.
We have previously developed a scaffold-free approach to cartilage tissue engineering that uses primary chondrocytes seeded in hydrogel-coated culture vessels that prevent cell adhesion and promote self-aggregation of the cell suspension, which subsequently forms into a cartilage-like biomass. In this model, chondrocytes are cultured at high density in tissue culture vessels coated with poly 2-hydroxyethyl methacrylate (polyHEMA); this hydrogel coating prevents cell attachment to the plastic substrate. Within 24 hours, chondrocytes coalesce to form a stable construct that remains in suspension and progressively increases in mass with time. We refer to these constructs as cartilage tissue analogs (CTA). Chondrocytes in CTAs possess appropriate phenotypic characteristics and deposit ECM that is similar to native cartilage (Estrada et al. 2001; Kim et al. 2011) and can be produced from several species, including neonatal porcine, bovine, equine, and human chondrocytes (Dodge et al. 1998; Richardson et al. 2000; Estrada et al. 2001). We have shown that CTAs in culture continue to produce collagen type II and do not produce collagen type I, which would be indicative of their differentiation to a fibroblastic phenotype (Novotny et al. 2006). However, the mechanical properties of CTAs have not yet been evaluated over an extended time course. One of the primary limiting factors in the clinical application of cartilage tissue engineering is the insufficient load-bearing capacity of the repair tissue (Hunziker 2002; Khoshgoftar et al. 2013). As such, this study evaluated the temporal development of CTA mechanical and biochemical properties, as well as the relationship between mechanics and matrix content in order to determine patterns of growth and maturation in this engineered cartilage model.
3.2. Methods

3.2.1. CTA Fabrication

Articular cartilage was harvested from juvenile bovine knees (N=5, age 2-6 months, Research 87, MA), minced, washed with PBS with 2X PSF (2% penicillin, streptomysin, fungizone) and digested in Type II Collagenase (298U/mg) in Basal Medium for up to 36 hours (1mg/mL in Dulbecco’s Modified Eagles Medium with 10% v/v Fetal Bovine Serum and 1% v/v PSF). Digested cartilage was filtered through 70µm strainers to separate cells from undigested matrix, diluted with 2X PBS-PSF, and centrifuged at 1750 rpm for 20 minutes at 12°C. The wash and centrifugation process was repeated 3X to pellet and isolate juvenile bovine chondrocytes.

Chondrocytes were seeded at 1x10^6 cells/well (200 µL/well) in poly 2-hydroxyethyl methacrylate (polyHEMA) hydrogel coated 96 well plates (Ultra-Low Adhesion 96 well plates, Corning) to form cartilage tissue analogs (CTA). As shown in Figure 3-1, chondrocytes initially form clusters at the bottom of the well that eventually condense into a uniform mass. The CTA constructs coalesce within 24-48 hours and continue to mature with time in culture; the chondrocytes produce cartilage specific extracellular matrix and increase in size over time. CTAs were cultured in Complete Medium (DMEM with 10% v/v FBS, 100 U/mL penicillin, 100µg/mL streptomysin, 2.5µg/mL fungizone, 1% v/v MEM Vitamin Solution (Gibco #11120), 25mM HEPES buffer, 50 µg/mL ascorbic acid) for up to 24 weeks and harvested throughout the culture period for mechanical, biochemical, and histological evaluation.
Figure 3-1. Chondrocytes aggregate in high density suspension culture to form CTAs. Chondrocytes seeded in 96 well plates begin to coalesce within 12 hours and form stable tissue analogs 24-48 hours post-seeding. CTAs 4 hours post-seeding show chondrocytes beginning to coalesce, form a mass and with increasing time, contract (125,000 chondrocytes/CTA), while higher seeding densities (1 million chondrocytes/CTA) result in more complete contraction and formation of a uniform construct. CTAs 4 and 42 weeks post seeding are cylindrical in shape and relatively uniform in size.

3.2.2. Adult Bovine Cartilage
To provide a suitable benchmark by which to compare CTAs, native cartilage plugs (Ø4mm) were harvested from the trochlear groove of fetal (second or third trimester, Animal Technologies, TX), juvenile (2-6 months, Research 87, MA), and adult (2-5 years) bovine knees (Animal Technologies, TX). Explanted cartilage plugs were trimmed to
remove the underlying subchondral bone, but retain the full thickness of articular cartilage. Adult explants were utilized for mechanical testing, while fetal, juvenile, and adult explants were assessed for cell-matrix distribution during histological analysis.

3.2.3. Mechanical and Biochemical Evaluation
CTAs and native cartilage explants were tested using a custom unconfined compression testing device (Mauck et al. 2006) to determine equilibrium (10% strain) and dynamic moduli (1% strain, 1Hz). Only CTAs cultured for more than four weeks were mechanically tested due to lack of mechanical integrity prior to this time point. After testing, samples were lyophilized, papain digested for 18 hours, and evaluated for glycosaminoglycan (DMMB) (Farndale et al. 1986), hydroxyproline (OHP) (Stegemann et al. 1967), and DNA (PicoGreen, Invitrogen) content.

3.2.4. Histology and Cell-to-Matrix Analysis
CTAs (N=3-5/age category with combined donors) and native cartilage explants (N=3/age/donor) were fixed in 4% paraformaldehyde, dehydrated in ethanol, and paraffin embedded for histological evaluation. Sections (8µm thick) were stained for proteoglycans (Alcian Blue 8GX, Sigma) and nuclei (Nuclear Fast Red, Master*TechTM), and a series of 20X images were taken of each CTA or cartilage plug (12 images/sample). The cell-matrix ratio for each image was quantified using ImageJ (V1.46, rsbweb.nih.gov/ij/). The following imaging processing steps were taken to calculate the cell area fraction of the total matrix. Each image was split into RGB channels, and the red channel selected in order to identify nuclear bodies for individual cells within matrix. Within the image, individual cell edges were found and contrast enhanced (0.4%); the image was then converted to a binary image. Cell area was determined by analyzing particles that fell
within a specified size range (determined by measuring the long axis of ~30 cells in an unprocessed image per sample, and calculating an idealized circular cell area). The sum of the area of all particles, or “cells”, was divided by the total matrix area of the image to generate the cell-matrix ratio. Fidelity of area measurements to unprocessed cells was manually verified; images which poorly represented cell area due to background noise or poor staining were excluded.

3.2.5. Statistical Analysis
Correlation of biochemical content and equilibrium modulus was calculated using linear regression (Pearson’s Coefficient) to determine the contribution of specific extracellular matrix components to the temporal development of mechanical properties. CTA cell-matrix ratios were grouped into multi-week categories and compared to that of fetal, juvenile, and adult bovine cartilage (one-way ANOVA, Tukey’s post-hoc; p<0.05). All mechanics, biochemical, and histological outcome measures are represented by mean ± standard deviation values.

3.3. Results
3.3.1. CTA Mechanical Properties
CTA mechanical properties increased in a uniform manner from four to twelve weeks across all donors. While the average equilibrium (E\text{\text{\text{\text{Y}}}) and dynamic (G*) modulus at 4 weeks was 8.7 ± 3.9 kPa and 352.6 ± 176.0 kPa, by 10 to 12 weeks E\text{\text{\text{\text{Y}} increased to 23.4 ± 7.0 kPa and G* to 576.3 ± 211.3 kPa, as shown in Figure 3-2. After 12 weeks of culture, mechanical properties began to diverge across donors. Equilibrium and dynamic moduli either continued to increase with culture duration or reached a plateau; past 16 weeks of culture, E\text{\text{\text{\text{Y averaged 110.9 ± 85.0 kPa and G* averaged 1215.9 ± 646.4 kPa; CTAs tested}}}}
at 24 weeks also fell within this range. These CTA properties approached those of adult cartilage, which had an average $E_y$ of $685.6 \pm 342.37$ kPa and $G^*$ of $8280.29 \pm 2757.11$ kPa.

![Figure 3-2. Mechanical properties of CTAs cultured over a period of 24 weeks. (A) Equilibrium modulus and (B) Dynamic modulus of CTAs increased with culture duration (N=4-5 samples/donor; each color represents a different bovine donor), and approached native adult bovine cartilage (N=6 donors) properties by 16 weeks.](image)

### 3.3.2. CTA Biochemical Content

Sulfated glycosaminoglycans (GAG) and collagen content of individual CTAs harvested throughout the 24-week culture period was plotted against the respective equilibrium modulus of each construct. GAG content of CTAs at early time points (between 4 to 8 weeks) averaged $2.92 \pm 0.7$ GAG%WW, and increased to $4.45 \pm 1.0$ GAG%WW for constructs 16 weeks or older. Collagen content also increased in a similar manner, averaging $0.66 \pm 0.19$ COL%WW at early time points and increasing to $1.65 \pm 0.32$ COL%WW by 16 weeks or older. When compared to native adult cartilage matrix content, GAG content was similar to that of matured CTAs, averaging $5.27 \pm 1.36$ GAG%WW;
however, collagen content in native tissue was approximately double, averaging 10.2 ± 1.61 COL%WW. The Pearson’s correlation coefficient for the relationship between biochemical content and mechanical properties was calculated to determine the contribution of a specific matrix element to mechanical integrity. GAG (Correlation Coefficient: 0.63, p<0.0001) and collagen (Correlation Coefficient: 0.64, p<0.0001) strongly correlated to the temporal increases in CTA equilibrium modulus (Figure 3-3A,C), while DNA content did not show any correlation (data not shown). Similarly strong, positive correlations were seen between these ECM components and the dynamic modulus (GAG-G* Correlation Coefficient: 0.51, p<0.0001; Collagen-G* Correlation Coefficient: 0.59, p<0.0001) (Figure 3-3B,D).

![Figure 3-3](image)

**Figure 3-3.** Correlation of GAG and collagen content to equilibrium moduli of CTAs. Correlation of GAG with (A) $E_Y$ and (D) $G^*$ showed significant contribution of proteoglycan
deposition to the development of CTA mechanical properties. Correlation of collagen with (B) $E_Y$ and (D) $G^*$ similarly showed that collagen content contributes to mechanical properties over 24 weeks of culture. Lightest color: youngest constructs. Darkest color: oldest constructs.

3.3.3. Cell-to-Matrix Ratio as a Function of Maturation

Paraffin-embedded CTAs and native cartilage were sectioned and stained with Alcian Blue and Nuclear Fast Red in order to analyze the ratio of cell area to matrix area. One week after establishment of CTAs, the cell-matrix ratio was $0.13 \pm 0.03$, indicating a high cellularity with a loosely woven matrix (Figure 3-4). With increasing culture duration, the cell-matrix ratio decreased to $0.08 \pm 0.01$ between 4-8 weeks, as matrix deposition increased ($p<0.05$). Between 10-24 weeks of culture, CTA cell-matrix ratio decreased further to $0.06 \pm 0.02$ ($p<0.05$). The majority of CTAs within this group had cell-matrix ratios not significantly different from each other. The cell-matrix ratio of CTAs cultured longer than 10 weeks was not different from that of juvenile cartilage ($0.05 \pm 0.02$). Juvenile and fetal cartilage ($0.05 \pm 0.01$) cell-matrix ratios were also not different from one another, but both were higher than adult cartilage values ($p<0.05$), which had a cell-matrix ratio of $0.04 \pm 0.08$. 
Figure 3-4. Quantification of cell-to-matrix ratios in Alcian blue (GAG) and Nuclear Fast Red (nuclei) stained histological sections (20X images). (Top) CTA cell-to-matrix ratios decrease with increasing culture duration as matrix deposition occurs. After long culture durations, CTA values approach that of native bovine cartilage (all groups p<0.05 except N.S.) (Bottom) Representative staining of CTAs and native cartilage.
3.4. Discussion

The importance of matching engineered cartilage properties to that of native cartilage cannot be overstated. While physiological mechanical forces are thought to have a positive influence on tissue repair, these forces can also induce long-term failure if the engineered construct does not closely mimic native cartilage characteristics or fails to integrate with the surrounding tissue. As such it is critical to bioengineer tissue equivalents that can survive and mature within the joint microenvironment. Enabling phenotypic correctness of the resident chondrocytes within constructs, as well as promoting the deposition of appropriate matrix constituents for the development of robust mechanical properties, will be necessary for creating functional engineered cartilage. To assess this functionality, the properties of engineered cartilage can be benchmarked against those of native cartilage. Achieving native tissue properties indicates the potential of the engineered tissue for application in model systems or in cartilage defect repair (Khoshgoftar et al. 2013). Here, we show that as CTAs mature over a period of 24 weeks, the constructs take on cartilage-like characteristics and maintain biochemical integrity throughout. As shown in previous studies using this culture model, chondrocytes undergo cell division over the first 3 weeks, but subsequently increase matrix synthesis, resulting in an increase in mass by as much as a 6-fold over the following 7 weeks. While this model is scaffold-free, over time, a natural scaffold is formed which provides the confinement necessary for the chondrocytes to elaborate an extracellular matrix (Estrada et al. 2001).

In the current study, chondrocytes within the CTA produced a robust extracellular matrix which correlated with increasing mechanical strength and decreasing cell-to-matrix ratios, leading to near native cartilage-like properties. Although scaffold-based methods are capable of supporting matrix production and can establish near native mechanical
properties more quickly, the use of foreign materials for cartilage repair is a clinically relevant concern. As such, scaffold-free, self-aggregating suspension cultures may present a viable alternative in which the lack of a foreign material might enhance integration potential and reduce the inflammatory response. Previous work has shown that scaffold-free constructs can form a functional extracellular matrix, which contributes to improved cartilage mechanical maturity (Ofek et al. 2008; Revell et al. 2008; Brenner et al. 2013). Aggregates of chondrocytes cultured in agarose coated wells for example, develop mechanical properties which increase with seeding density, with the aggregate modulus of these constructs ranging from ~70 kPa (2 million cells/construct) to ~300 kPa (55 million cells/construct) (Ofek et al. 2008; Revell et al. 2008). Additional methods of scaffold-free culture using ceramic carriers have reported a Young’s modulus of ~30 kPa after 2 weeks of static culture (4 million cells/carrier) (Hoenig et al. 2011), while intermittent dynamic stimulation of similarly cultured constructs increased the equilibrium modulus to ~80 kPa (Waldman et al. 2004). While this study reports that CTA mechanical properties improve with increased culture duration (and fall within a similar range to other scaffold-free engineered cartilage constructs), these values are still lower than that of native cartilage. Therefore, the use of combined strategies may be necessary to achieve the functional properties of native tissue. For example, dynamic loading systems (e.g. hydrostatic pressure (Kraft et al. 2011)) and/or environmental cues (e.g. growth factor additives (Waldman et al. 2004; Hoenig et al. 2011)), can enhance matrix production and further improve mechanical properties. However, if CTA mechanical properties exceed a certain threshold (i.e. the matrix composition is too dense), the ability of the engineered construct to integrate with native cartilage may be compromised (Brenner et al. 2013). As such, there may be a balance between preconditioned compositional / mechanical properties and integration potential in order for cartilage repair to be successful.
CTAs were observed to produce abundant matrix within the construct over the 24 week culture period. When compared to previous reports of scaffold-based engineered constructs, CTAs produce similar amounts of matrix as agarose constructs at eight weeks (20 million chondrocytes/mL, ~3.2 GAG%WW, ~2.1 COL%WW, (Erickson et al. 2009)), MeHA hydrogels at eight weeks (20 million MSCs/mL, ~3.5 GAG%WW, ~1.8 COL%WW, (Erickson et al. 2012)) and silk hydrogels at four weeks (20 million chondrocytes/mL, ~3.25 GAG%WW, ~2.75 COL%WW, (Chao et al. 2010)). In comparison, self-assembled aggregates in agarose wells cultured for four weeks show similar GAG content (2 million chondrocytes/construct, ~4 GAG%WW), although collagen content exceeded the values reported here (~25 COL%WW) (Revell et al. 2008). Scaffold-free methods using ceramic substrates have also produced constructs (2 million chondrocytes/construct) with similar amounts of both GAG and collagen (~2-3 GAG%WW and COL%WW) following intermittent dynamic loading during eight weeks of culture (Waldman et al. 2004). Temporal evaluation of CTAs also showed that both proteoglycan and collagen content strongly and positively correlated with increasing mechanical properties. This data meshes well with previous studies of engineered cartilage which have shown strong positive correlations between GAG density and compressive modulus in type II collagen scaffolds and methacrylated HA scaffolds (Pfeiffer et al. 2008; Erickson et al. 2009), as well as temporally matched increases in GAG content and aggregate modulus of chondrocyte aggregates over 8 weeks of culture (Ofek et al. 2008). Significant correlations of both GAG and collagen with aggregate modulus and hydraulic permeability have also been found for fetal, juvenile, and adult bovine articular cartilage (Williamson et al. 2001). Quantitation of cell-matrix ratios additionally showed a decreasing cell-to-matrix ratio as CTAs matured. Previous work by Jadin et al. has shown that cellularity of cartilage decreases with
increasing age, matching our results which showed that beyond 10 weeks of culture, CTA cell-to-matrix ratios decrease to approach that of adult native cartilage (Jadin et al. 2005). These studies support the idea that matrix deposition by chondrocytes in both tissue engineered cartilage and native cartilage is critical for the development and maintenance of mechanical properties.

3.5. Conclusion

In this study, the time-dependent functional maturation of cartilage tissue analogs (CTAs) was characterized and benchmarked against native tissue properties. Given our current understanding of the development of mechanical, biochemical, and phenotypic properties of CTAs, we have begun to explore applications of this engineered cartilage model for cartilage defect repair. Experiments are currently underway to explore the benefit of intermittent periods of hydrostatic loading (Novotny et al. 2006) on CTA characteristics, and whether any improvements due to loading would be reflective of in vivo maturation in animal models. In addition, due to the scalability of these constructs, we have started to investigate the use CTAs in high throughput screening studies of mechanical injury to investigate the progression of cartilage degeneration in the development of post-traumatic osteoarthritis (Mohanraj et al. 2013). With further development of the CTA model, this tissue engineered construct can serve both clinical and experimental applications in order to better understand mechanisms of cartilage repair and degeneration.

Chapter 4. A High Throughput Mechanical Screening Device for Cartilage Tissue Engineering

4.1 Introduction

Cartilage tissue engineering has made marked progress, with numerous studies developing methods for the production of mechanically functional cartilage, based on either native chondrocytes (Kelly et al. 2006; Novotny et al. 2006; Lima et al. 2007; Byers 2008; Bian et al. 2010; Cheng et al. 2011; Ng et al. 2011) or mesenchymal stem cells (MSCs) grown as three dimensional (3D) constructs (Mauck et al. 2006; Huang et al. 2010; Moutos et al. 2010; Thorpe et al. 2010; Erickson et al. 2012). However, the degrees of freedom present in any experimental design can make even the simplest of tissue engineering studies difficult to execute, where an investigator can vary materials (Mouw et al. 2005; Chung et al. 2009; Chung et al. 2009; Hwang et al. 2011), cell number (Mauck et al. 2003; Weinand et al. 2009), growth factor doses and combinations (Blunk et al. 2002; Gooch et al. 2002; Appel et al. 2009; Johnstone et al. 2013), and the mechanical loading environment (Ng et al. 2009; Thorpe et al. 2010). Moreover, complexity in experimental design leads to difficulties in capturing outcome parameters in a cost- and time-efficient manner. The need for increased throughput in assessing outcomes is not unique to tissue engineering. Indeed, high throughput screening (HTS) methods emerged very early in the pharmaceutical industry (Drews 2000), where such methods were essential for screening large chemical libraries to assess compound effects on biologic activities relevant to disease.
The underlying premise of HTS is that if a suitable assay can be developed that is 1) sufficiently sensitive to measure a relevant cellular response, 2) of a low cost per sample, 3) easy to automate, and 4) reproducible, then one can expedite drug discovery. While most HTS assays are performed in monolayer culture, recent studies have begun to implement assays in 3D constructs as well. For example, 3D multi-cellular spheroids have been used to screen for tumor suppressive agents (Kunz-Schughart 2004). A few studies have applied HTS principles towards applications in bone and cartilage biology and regeneration. For instance, HTS-based assays focused on MSC osteogenesis in monolayer (Brey et al. 2011) and chondrogenesis in micro-scaled pellet cultures (Huang et al. 2008) have been used to screen small molecule libraries in a 384-well format. The potential of such HTS approaches is perhaps best illustrated by a recent study, which employed an image-based HTS method that identified molecules that promoted the formation of chondrogenic MSC nodules, and was found to protect cartilage from degeneration in a small animal model of joint instability (Johnson et al. 2012).

While most HTS assays focus on molecular events, functional outcomes are equally important for musculoskeletal tissues (Vandenburgh 2010). This is particularly relevant for cartilage as the properties of the engineered tissue will dictate function in the load-bearing joint environment (Ateshian et al. 2005). Thus, it would be ideal if HTS approaches could be modified to include mechanical measures. However, traditional one-at-a-time assessments of mechanical properties can be prohibitively time consuming, where a typical stress relaxation test can take several hours per sample (Mauck et al. 2000; Soltz et al. 2000). In even relatively simple experimental designs (Erickson et al. 2012), involving just two different seeding densities, four different material formulations, one growth factor at a single dose (and a growth factor-free control), and five samples per
group, over 80 hours of testing is required at each time point. Given the continued development of novel materials and new factors influencing cartilage growth, and the requirement that each of these inputs be carefully evaluated in a combinatorial context, throughput in mechanical analysis has become a significant barrier to further progress. As such, the development of a high throughput mechanical screening (HTMS) system would represent a valuable tool to advance cartilage tissue engineering.

To address this need, several mechanical testing systems have been introduced that enable multi-sample evaluation. For instance, the Myoforce Analysis Device was developed to monitor bioartificial muscles to identify compounds that alter contractile strength (Vandenburgh 2010). The MATE system incorporated real-time measures of load during dynamic stimulation of engineered cartilage, using a six-sample actuating system (Lujan et al. 2011). Still more recently, a 12-sample tissue stimulator was developed that recorded load from each sample via individual force sensitive resistors (Salvetti et al. 2012). These devices illustrate how real-time and multi-sample mechanical analysis can be incorporated into tissue systems. While promising, throughput in these devices is restricted to a relatively small sample capacity, and expansion to higher throughput formats is a challenge for sensor technology (Lujan et al. 2011; Salvetti et al. 2012). Additional development is needed to make such devices compatible with HTS of chemical libraries.

We have developed a novel high throughput mechanical screening (HTMS) device that can assess mechanical properties of biomaterials and engineered cartilage in a 48-well format. Our system utilizes a custom force sensitive resistor (FSR) array to measure instantaneous and time-dependent mechanical response of up to 48 samples
simultaneously. The increased capacity of this device provides a platform to evaluate properties in complex, combinatorial studies for the screening and optimization of engineered cartilage. The objective of this study was to design, optimize, and validate this system for the screening of mechanical properties of various materials and engineered cartilage models in several experimental configurations.

4.2. Methods

4.2.1. HTMS Device Components

The HTMS device was designed to interface with mechanical testing systems utilized in most orthopaedic and bioengineering laboratories. A schematic is shown in Figure 4-1. The device housing consists of an aluminum base plate and two parallel side plates onto which linear bearings (Maintenance-Free Ball Bearing Carriages and Guide Rails, McMaster-Carr, GA) are affixed to align and maintain smooth vertical displacement of the platen. The sensor platen was integrated via two plates: a lower plate to which it is directly attached, and an upper plate fixed to the bottom plate. A custom force sensitive resistor (FSR) array was mounted via adhesive backing to this bottom plate (Custom 48 Matrix FSR Sensor Array, Sensitronics, WA). To control vertical displacement of the sensor platen, an Instron (Model 5848, Instron, MA) was connected via an adaptor to the top plate of the sensor platen (Figure 4-1A,D).

Opposing the sensor surface, a well plate assembly was designed to accommodate standard 48-well plates (BD Falcon, Multiwell Cell Culture Plate, #75875, NJ), as well as an indenter array and hole plate to align with each sensor on the FSR array (Figure 4-1B). The hole plate, which sits on top of the 48-well plate, was used to guide vertical and restrict horizontal movement of each indenter during loading. Indenters were composed
of PTFE (McMaster-Carr, GA) rods with a flat bottom surface that interacted with the sample during loading, and a beveled top for centered activation of the sensor (Figure 4-1C). Each indenter had a diameter of 9.5mm to fit within the 10mm diameter well of the 48-well plate to allow fluid displacement during unconfined compression testing. A small cotter pin (Dowel Pins, McMaster-Carr, GA) inserted at the top of the indenter allowed for simultaneous removal of all indenters after testing, without interfering with the motion of adjacent indenters in the array (Figure 4-1D).

Figure 4-1. Schematic of HTMS device. (A) The system includes an aluminum housing frame, sensor platen controlled via Instron displacement, and (B-C) well plate assembly designed for a standard 48-well culture dish with indenter platens. (D) Fully assembled HTMS device on Instron platform and complete 48 sample array of indenters in well plate assembly.
4.2.2. HTMS Device: Sensor, Components, and Software

The custom force sensor includes four layers: a force sensitive resistor (FSR) shorting layer, a spacer layer (0.0005”), a trace layer, and an adhesive layer (3M) (Figure 4-2B). The spacer layer between the FSR layer and trace layer creates a gap between the two conductive layers. Upon compression, the conductive layers come into contact, resulting in a decrease in resistance in the circuit. The sensor contains 48 “sensels” (force-sensitive locations) that match the well-plate layout and individually capture load during compression (Figure 4-2B). The sensor was connected via a voltage supply and resistor (10kΩ) to create a voltage divider to measure change in resistance. Each sensor on the trace layer has an individual conducting pin out, and all sensors have a common power supply pin (connected to a 5V source in the NI DAQ board, NI USB-6225 M Series DAQ, NI, TX). A ribbon (Nicomatic) connected the sensor to a custom wiring box (Figure 4-1D) with the circuit illustrated in Figure 4-2A. Each sensel voltage was captured as analog input to DAQ board and recorded using a custom LabVIEW program (LabVIEW 8.6, NI, TX), with data post-processing in MATLAB (MATLAB R2010a, Mathworks, MA).

4.2.3. HTMS Device: Sensor Calibration

The sensor was calibrated by inverting the sensor platen and applying displacement controlled compression to each sensel. Sensors were compressed to ~5N at a rate of 0.0004 mm/s via an indenter connected to the Instron. Force and voltage was recorded in LabView, and the resulting force-voltage data fit to an exponential curve (Figure 4-2C); this calibration was used for all subsequent testing. An example 3D representation of force output for silicone compression is shown in Figure 4-2D. The most sensitive (greatest force resolution) and nearly linear range of the sensor was 0-1V, corresponding to a load of ~1N. This sensitivity is determined by the thickness of the spacer layer; increasing the
thickness of the spacer layer decreases low-load sensitivity, but would allow for higher forces to be measured.

Figure 4-2. Sensor calibration and testing protocol. (A) Sensor circuit which measures changes in voltage due to compression and contact of sensor layers within (B) the custom
matrix array sensor. (C) Example force-voltage calibration curve for one position in the matrix array sensor. (D) 3D rendering of force signal from one step of HTMS testing. (E) Schematic illustration of multi-step stress relaxation and dynamic testing protocol. (F) Example time dependent response with three applied compression steps to agarose hydrogel (in blue) and silicone rubber (in black) samples in the HTMS device. Note that the sensor can capture the viscoelastic stress relaxation behavior of hydrogel sample.

4.2.4. HTMS Device Testing: Protocols and Data Analysis

To test the HTMS device, we first evaluated the properties of common elastic (silicone rubber) and viscoelastic (agarose) materials. To accommodate differences in sample height, we devised a step-wise stress relaxation testing profile consisting of multiple ramps of 10% compressive strain (relative to original average sample height) applied at 0.05% strain/sec with a 1000 second hold after each step. At each step, the relaxation phase was followed by a dynamic deformation phase (1% strain applied at 0.1 Hz), followed by a further 60 second hold (Figure 4-2E). Before starting each test, the device displacement was zeroed to the height of the tallest sample. The number of steps was determined by measuring the height of all samples, calculating average height and displacement per step, and determining how many steps would be required to ensure that multiple compression steps were applied to all samples. The sensor was able to capture the elastic and viscoelastic (relaxation behavior) of these hydrogels (Figure 4-2, D-F).

To calculate the compressive equilibrium modulus for each sample, voltage data was imported into a custom MATLAB program to extract strain and equilibrium load for each step. All sample and test information, including sample height, width, and step displacement magnitude was likewise imported into the program. The starting actuation
point for each sensor was determined using a pre-determined voltage threshold, which was then used to calculate applied strain for the sample for each step. A “first step” was selected for calculation of the compressive modulus based on an 8% strain threshold, i.e. for an individual sample the step was required to reach at least 8% strain for it to count as a first step. Since samples differed in height, this ‘first step’ was in some cases the first actuation of the sensor (i.e., for samples making good contact initially), and sometimes the second actuation of the sensor (i.e., for shorter samples that were not in contact, or not compressed by at least 8%, during the first actuation). Following capture of the transient and equilibrium response during this ‘first step’, all data (including step number, step strain, total strain, and equilibrium modulus) were exported to Excel for further analysis.

4.2.5. HTMS Validation: Multi-sample vs. Sample-by-sample Testing

To validate the HTMS device, we measured several materials with a range of compressive properties and compared the results to those derived from single sample-by-sample tests. Two types of hydrogels, agarose (Type VII, Sigma) and polyacrylamide (National Diagnostics), were cast between glass plates to create gels of uniform thickness and punched into cylindrical samples (H: 2.25mm, Ø 4mm). Agarose gels were cast at 4 or 10% (w/v) and polyacrylamide was cast at 15% (w/v). Construct dimensions were measured, and all samples were arranged in a 48-well plate for HTMS testing (n=10-11/material). HTMS testing consisted of a five-step stress relaxation, and for comparison, single sample-by-sample testing used a two-step stress relaxation test. Since HTMS results provide a modulus for compression ranging between 10 and 20% applied strain (depending on when the sensor actuated for a given sample), the equilibrium modulus at both strain levels were reported for the sample-by-sample testing. Materials screening
experiments were conducted at least three times, with one representative set of results shown here.

4.2.6. Fabrication and Screening of Engineered Cartilage

To evaluate engineered constructs using the HTMS device, mesenchymal stem cells (MSCs) were isolated from bone marrow from juvenile bovine femurs, and expanded (passage 2 or 3) as previously described (Huang 2010). Methacrylated hyaluronic acid (MeHA) was prepared as previously described (Burdick et al. 2005; Erickson et al. 2009).

To generate cell seeded constructs, MSCs were trypsinized, washed and centrifuged (300xg for 5 minutes), and encapsulated in 1% (w/v) MeHA at 20 or 60 million cells/mL (Erickson et al. 2012). The MeHA solution was cast between two glass plates and exposed to UV light for 10 minutes; gels were punched to form cylindrical constructs (H: 2.25 mm, Ø 4mm). Constructs were cultured in a chemically defined chondrogenic medium (CM+) as previously described (Mauck et al. 2006).

Two HTMS evaluations were performed with MSC-seeded MeHA constructs. In the first, we evaluated the concentration-dependent effect of a pro-inflammatory cytokine on the mechanical integrity of constructs. For this, constructs (20 million cells/mL; 1% MeHA) were cultured in CM+ for 12 weeks, at which point, constructs were cultured for a further 6 days in CM- medium (lacking TGF-β3), and treated with increasing concentrations of TNF-α (0, 1, 5, and 10ng/mL). Media was changed on day 3 and TNF-α replenished; all media was collected for further analysis of GAG loss using the DMMB assay (Farndale et al. 1986) and nitric oxide production via the Griess Assay (Promega). Mechanical properties were evaluated 6 days after exposure to TNF using the HTMS device (n=10-
12/group). To validate HTMS results, single sample unconfined compression (10% step only) was performed on a parallel set of samples (n=4/group) (Mauck et al. 2006). Construct GAG (DMMB assay) and DNA (PicoGreen, Invitrogen) content was also evaluated following mechanical testing.

In the second test, we evaluated the ability of the HTMS device to distinguish differences in properties arising from constructs seeded with MSCs at two densities (20 vs. 60 million cells/mL) in 1% w/v MeHA hydrogels. Mechanical properties of constructs were evaluated after 10 weeks of culture using the HTMS device (n=8-9/group) and compared to single sample-by-sample testing using the two-step protocol. Biochemical content of gels was also evaluated as described above.

4.2.6. Statistical Analysis

Mechanical properties of biomaterials and engineered cartilage constructs evaluated using the HTMS device were compared to single sample testing, and the effect of TNF-α on construct maturation was determined using one-way ANOVA with Tukey’s post-hoc test (p<0.05). To perform quality control (QC) and to assess validity of ‘hit’ criteria with mechanical screening, the strictly standardized mean difference (SSMD) was utilized (see below), with positive and negative controls from the log-transformed (for normalization) HTMS data set (Zhang 2007; Zhang 2011).
4.3. Results

4.3.1. HTMS of Materials and Engineered Cartilage

To evaluate the HTMS platform, three acellular hydrogel formulations, 4% and 10% agarose, and 15% polyacrylamide were simultaneously evaluated for compressive properties using the HTMS device, with results compared to sample-by-sample testing (Figure 4-3A). HTMS-derived equilibrium moduli for all materials closely approximated the results from single sample first and second step equilibrium moduli. Indeed, the means from HTMS testing were not different from sample-by-sample testing (HTMS vs. single samples: 4% agarose: 31.1 ± 24.0 kPa vs. 49.6 ± 18.7 kPa, 10% agarose: 173.6 ± 74.8 kPa vs. 194.2 ± 46.8 kPa, and 15% polyacrylamide: 140.9 ± 61.6 kPa vs. 109.9 ± 32.2 kPa; p>0.05). Engineered cartilage formed at 20 or 60 million cells/mL (20M or 60M) were also evaluated for mechanical properties. As shown in Figure 4-3B, the mean values from HTMS testing were not different from sample-by-sample testing, with 20M constructs having an equilibrium modulus of 105.6 ± 99.1 kPa vs. 128 ± 65.9 kPa and 60M constructs of 187.3 ± 65.7 kPa vs. 179.2 ± 62.1 kPa (p>0.05). These results show that the HTMS device can effectively determine and distinguish mechanical properties ranging from ~25 to ~300 kPa in engineered constructs.
Figure 4-3. HTMS evaluation of biomaterials and engineered constructs. (A) Compressive modulus of various biomaterials (single sample, N=4; HTMS, N=10-11) and (B) 1% MeHA hydrogels seeded with MSCs at two densities (20 million or 60 million cells/mL, single sample and HTMS, N=7-9). Comparison between single sample testing and HTMS-derived moduli show no significant differences in mean values (p>0.05).

4.3.2. HTMS of Engineered Cartilage Treated with Inflammatory Cytokines

Treatment of engineered constructs with TNF-α resulted in a dramatic loss of properties in a concentration-dependent manner. HTMS measurement of properties captured differences between treatment groups similar to that of the sample-by-sample testing (Figure 4-4A). Both testing methods showed that exposure to higher concentrations (5 and 10ng/mL) of TNF-α resulted in a decline in mechanical properties compared to control or 1ng/mL groups (p<0.05). Measurement of GAG in the media confirmed matrix breakdown, where sustained GAG release was observed during six days of treatment, with the higher TNF concentrations resulting in ~3-5 times more GAG release compared to control and 1ng/mL groups (Figure 4-4B). These results illustrate that the HTMS device can capture loss of properties in engineered cartilage as a consequence of graded exposure to TNF-α.
Figure 4-4. HTMS Screening of the Effect of Inflammatory Cytokines. (A) Treatment of MSC-seeded constructs with TNF-α results in a significant loss of mechanical properties in a concentration dependent manner that is readily captured using the HTMS device (single sample, N=4; HTMS, N=10-12). (B) Release of GAG to the media (SolGAG) correlates with the loss of mechanical integrity. p<0.05: * vs Control; # vs 1ng/mL.

4.3.3. Quality Control and ‘Hit’ Criteria for HTMS Screening

The value of a high throughput screening system lies not just in its ability to measure multiple samples at once, but specifically in its ability to distinguish between groups to identify ‘hits’, or factors that produce a response that is different from a given control. To further evaluate our HTMS system in terms of quality control (QC) and hit selection criteria, we used a statistical parameter developed by Zhang termed the strictly standardized mean difference (SSMD) (Zhang 2007; Zhang et al. 2007; Zhang 2011). SSMD measures the magnitude of difference between two populations, and can better account for non-normality, variability, skewness, and outliers within a population, compared to other statistical methods (such as the ‘z-factor’) (Zhang et al. 1999). Non-normality and
skewness are common features of HTS data, where ‘hits’ are few, and most conditions produce negative results (i.e., remain at the baseline level). To perform a quality control (QC) analysis, one first selects a condition that provides an acceptable baseline (negative control) level, and another condition that constitutes a known ‘hit’ (or positive control). For our biomaterial screen, we chose the 4% and 10% agarose groups as negative and positive control groups, respectively. For the TNF-α treatment data set, we chose free swelling controls as the baseline, and 10ng/mL as the positive control. Using log-transformed data, we then calculated the estimated SSMD from the ratio of median and median absolute deviation of both populations (Zhang 2011). From this analysis, SSMD was computed to be -2.16 for the biomaterial screen and -1.43 for TNF-α treatment. Comparing these values against published SSMD QC criteria (Zhang 2011), and assuming that our controls were of ‘moderate strength’, these SSMD values indicate that the HTMS screen is a “good to excellent” tool for identifying ‘hits’. Indeed, by randomly selecting three values (a reasonable number of replicates for HTS) from each group in the TNF-α study, and computing the SSMD value for each, the 1ng/mL TNF-α condition had an SSMD of -4.8, indicating a strong effect. In comparison, the values for 5ng/mL and 10ng/mL TNF-α treatments were -23.5 and -8.4, respectively, indicating that these two concentrations had extremely strong effects (|SSMD|>5) according to RNAi hit criteria (Zhang 2011). Candidate molecules with such strong effects would be identified as ‘hits’ based on empirically determined thresholds in a high throughput screen, and the molecules or conditions they represented would be further investigated in follow up screens.
4.3. Discussion

While mechanical properties are an essential outcome in any study of engineered cartilage, current sample-by-sample testing methods represent a significant bottleneck, constraining experimental designs. To address this limitation, we developed a novel HTMS device to enable the evaluation of up to 48 samples at one time. Our data show that this device can successfully determine the properties of various biomaterials and engineered constructs, in a rapid manner, while producing data that closely matches that of sample-by-sample testing. The current device was designed to measure mechanical properties of soft biomaterials and engineered cartilage (in the range of 25-300 kPa). However, given the flexibility of the sensor technology, stiffer materials or tissues (e.g. bone) could be evaluated by modifying the removable sensor to shift the load-sensitivity range for the desired application. With minor modifications (e.g. inclusion of an independent displacement control system), the device could likewise be adapted to apply long-term controlled mechanical stimulation to engineered tissues in culture, while measuring real-time evolution of properties. More importantly, this device reduces the burden of time spent in evaluation, where one can sequentially test several 48-well plates, thereby enabling a single user to derive properties from ~400 samples in a single 8-hour day. In contrast, single-sample testing would require at least 200 hours. The step protocol implemented here also takes into account differences in sample height, and allows the user to select a “first step” after completing the test, which is then used to calculate the equilibrium modulus. In contrast to previous HTS devices that used individual actuators (Lujan et al. 2011) or varying height plungers (Salvetti et al. 2012) to account for differences in sample heights, this protocol requires no additional parts and is easily tuned to apply a range strain magnitudes. Additional advantages of this system include individual force sensing capability, a semi-automated MATLAB program for data analysis, and the
ability to fabricate and assemble the device at a reasonable cost (~$6000 circa 2013).

While our data suggest that the HTMS device is a useful tool for the rapid evaluation of multiple materials and factors for cartilage tissue engineering, it is not without its limitations and opportunities for refinement. Namely, while the mean values from HTMS testing matched sample-by-sample testing, the standard deviations were larger. In developing screening tools, a balance must be achieved between accuracy, throughput, and cost. To address this, a number of statistical tests exist to evaluate whether a screening tool can ably distinguish ‘hits’ from conditions that fail to generate a response. One such test, the SSMD parameter, has been validated for high throughput RNAi assays, and its use is suggested for other HTS small-molecule screening assays (Zhang 2007). Using this method and data from HTMS testing, we validated the HTMS device as a “good-excellent” screening tool. One caveat to this statistical method, however, is the consideration of the strength of the controls, as this factor influences QC cutoff criteria. For some RNAi screens, hits by definition fall between an extremely strong positive control and negative reference, which defines the dynamic range (Zhang et al. 1999). In contrast, when screening properties in engineered materials, moderate or strong controls may be sufficient, as the effect size is unknown for assayed molecules, and their action might improve or reduce properties beyond controls. If we had considered our QC groups to be ‘extremely strong controls’, meaning that they represented a maximum possible compressive modulus, then the assay would only fall into the “inferior-good” range. This highlights the importance of understanding the expected differences in effect size in order to correctly set QC criteria as well as hit thresholds. Despite this limitation, using “moderate” controls, SSMD analysis identified the two higher doses of TNF-α as having extremely strong effects compared to controls, validating the HTMS device as a screening
tool. Moving forward, it will be necessary to empirically determine SSMD ‘hit’ thresholds based on the desired number of molecules or materials to be evaluated in more rigorous secondary (in vitro) and tertiary screens (in vivo) (Johnson et al. 2012).

While the current HTMS device is sufficient in its capacity to screen differences in material properties, it will be important in future iterations to not only increase throughput of the system, but also to improve the sensitivity and range of force detection and reduce measurement variability. To increase throughput capacity, we are currently scaling up to a 96-well format. This modification will double throughput, making screening of large chemical libraries more practical and enabling identification of compounds that promote cartilage growth and repair. To further adjust the range of load sensitivity, the thickness of the spacer layer in the FSR could be customized for a desired force range. Moreover, other sensor technologies could be incorporated into the design that may provide more stable calibrations and force readings (for example, via the inclusion of an integrated sensor with force interpolation). Additional applications of the HTMS system include controlled mechanical stimulation via the addition of an independent displacement control system (LVDT, stepper motor, and feedback control) to replace the Instron displacement control. This possibility opens a new platform whereby testing of multiple treatments can be carried out in a setting of real-time mechanical loading.

4.4. Conclusion

This HTMS device has the potential to dramatically alter the landscape of what is possible in the experimental design of studies directed towards cartilage tissue engineering, as well as injury and repair. Most sample-by-sample studies are predicated on a specific hypothesis, and as such use a defined set of experimental conditions to test that
hypothesis. Such an approach inherently limits the design space, and biases studies towards evaluation of a low number of conditions and interrogation of already well known mechanisms. In contrast, high throughput screening methods such as this allow discovery to drive hypothesis formation. A multitude of compounds can be tested rapidly, in a cost-efficient manner, and molecules previously unknown to have any relevance to the tissue or growth pattern of interest may unexpectedly produce a desired response. Using this HTMS device, our goal is to identify novel molecules and pathways that not only improve the functional properties of engineered cartilage, but also intervene to enhance cartilage repair in clinically relevant joint pathology.

Chapter 5. A High Throughput Model of Post-Traumatic Osteoarthritis using Engineered Cartilage Tissue Analogs

5.1 Introduction

The primary function of articular cartilage is as a load-bearing structure that supports and distributes the high stresses generated during normal physiological activities (Ateshian et al. 2005). While cartilage generally functions well over a lifetime of use, acute instances of supra-physiologic loading (e.g. an accident or other traumatic event), often result in tissue damage that initiates degenerative processes within the joint. Indeed, a subset of osteoarthritis (OA), termed post-traumatic osteoarthritis (PTOA) represents the significant fraction of patients who develop OA secondary to joint trauma. Based on the incidence of knee, hip, and ankle OA for patients with a history of joint injuries (Brown et al. 2006), it is estimated that up to ~6 million individuals are burdened with PTOA in the US alone. Cartilage pathology and PTOA incidence generally correlate with the intensity of the original injury. Patients with ligamentous or meniscal injuries are 10-fold more likely, and those with articular fractures are 20-fold more likely to develop knee OA compared to individuals without previous joint injuries (Roos et al. 1998; Gillquist et al. 1999). Despite a growing understanding of the mechanical thresholds that instigate PTOA, the molecular pathogenesis and mechanisms of disease progression are not yet well understood.

To that end, a number of in vitro, ex vivo, and in vivo models of cartilage injury have been developed to explore the temporal patterns of anabolic and catabolic events that culminate in cartilage degeneration. These models serve as useful platforms in which to explore variables that regulate the extent of damage, including the impact energy, peak
stress/strain, and stress/strain rate. Common markers of load induced injury include tissue swelling and fibrillation (Kurz et al. 2001), cell death at or near the injury site (Quinn et al. 2001; Natoli et al. 2008), and increased expression of proteases and inflammatory cytokines (Lee et al. 2005; Natoli et al. 2008). Biologic mediators of PTOA act collectively to decrease chondrocyte matrix biosynthesis (Kurz et al. 2001) and instigate a loss of proteoglycans and other matrix elements (Kurz et al. 2001; Quinn et al. 2001; Patwari et al. 2003; DiMicco et al. 2004). Together, these molecular and compositional changes culminate in a loss of tissue mechanical integrity (Kurz et al. 2001; Natoli et al. 2008).

The timeline of activation of these degenerative processes (and the controlling signaling mechanisms) is particularly important, as the different stages of response post-injury may represent opportunities for therapeutic intervention (Anderson et al. 2011). Indeed, previous studies have focused on small molecules targeting the early events, including mechanisms that lead to cell death, release of inflammatory mediators, and proteoglycan loss. Examples of such compounds include pan-caspase inhibitors (D'Lima et al. 2001; Martin 2009) to decrease cell death, amphiphilic surfactants (Phillips et al. 2004; Natoli et al. 2008; Bajaj 2010) to repair disrupted cell membranes, oxidative free radical scavengers (Martin 2009) to limit early inflammatory processes, as well as growth factors (Chubinskaya et al. 2007; Hurtig et al. 2009) and glucocorticoids (Lu et al. 2011) to increase anabolic response post-injury. In the above studies, these factors have shown varied success in reducing cell death and matrix degradation in in vitro and in vivo models of PTOA, indicating that these early pathologic changes are appropriate targets for therapeutic intervention.
To date, the selection of agents that might abrogate PTOA initiation has been based on their roles in canonical pathways involved in cell physiology and/or OA progression. Since the mechanisms of PTOA have not yet been fully elucidated, there may be other agents not previously known to play a role in PTOA that could have chondro-protective effects. In recent work, Sampson et al. showed that parathyroid hormone (clinically used to improve bone mass) administered to mice after meniscus destabilization surgery was chondro-protective (or regenerative) in that it limited hypertrophic changes after onset of instability (Sampson et al. 2011). Wang et al. also showed that the inflammatory complement system regulated cartilage degradation in mouse models of joint instability (Wang Q et al. 2011). These studies illustrate the significant role that such non-canonical pathways may play in mediating the degenerative response in situations of chronic overload; the acute injury response may similarly initiate heretofore unexplored signaling pathways.

High throughput (HT) screening enables the rapid evaluation of small molecule libraries for the discovery of novel compounds relevant to tissue development and healing without prior knowledge of the mechanism of action. Recently, Johnson et al. developed an image-based high throughput screening system to identify molecules that promoted chondrogenic differentiation of MSCs (Johnson et al. 2012). From the 1000s of molecules screened in that study, several “hits” were identified, with the small molecule kartogenin emerging as the most promising. Follow-up secondary in vitro assays (e.g. RT-PCR) and tertiary in vivo investigations (rodent joint instability models) illustrated that kartogenin also had a chondro-protective effect and acted by disrupting the binding of a specific transcription factor subunit to an actin associated protein. Given the non-intuitive mechanism of action, this study highlights the need for unbiased screening tools to guide molecular discovery specific to a particular disease process.
To enable such screens in the context of PTOA, we developed a high throughput in vitro mechanical injury platform that is compatible with drug screening. While in vitro models of injury using explants have been valuable in elucidating regional changes in cell viability and matrix loss, explants are not ideal for high throughput screening due to the large number of samples required and variation in the cellular and molecular stratifications found throughout the joint. Cartilage tissue engineering, which aims to mimic the biochemical and mechanical properties of native cartilage for joint repair, can generate cartilage-like analogs with which to study the pathogenesis of PTOA. Engineered cartilage can also be fabricated in a uniform manner and in large quantity, and as such are ideal for high throughput screening applications. In particular, we have studied a scaffold-less method to generate cartilage tissue analogs (CTAs) that closely mimic native cartilage both in terms of extracellular matrix composition and biomechanical properties (Estrada et al. 2001; Novotny et al. 2006; Mohanraj et al. 2013).

Here, we adapted our high throughput mechanical testing system (Mohanraj et al. 2014) to apply compressive injury to CTAs in a rapid and reproducible manner. The primary goals of this study were to determine the strain and rate dependent response of engineered cartilage to compressive injury, to evaluate the acute progression of degeneration, and to validate this response with respect to native articular cartilage explants treated similarly. Our findings validate the use of engineered cartilage as a surrogate for studying mechanisms of PTOA pathogenesis and introduce a new screening tool with which to identify novel compounds that can attenuate degeneration following cartilage injury.
5.2. Methods

5.2.1. Fabrication of Cartilage Tissue Analogs

Engineered cartilage tissue analogs (CTAs) were produced as described previously (Novotny et al. 2006; Mohanraj et al. 2013). Briefly, articular cartilage was harvested from juvenile bovine knees (2-6 months old, Research 87, MA), finely minced, and digested overnight (12-16 hours) in DMEM containing collagenase Type II (298U/mL Worthington, NJ). Tissue digests were filtered (70µm pore mesh), washed with PBS containing 200U/mL penicillin, 200µg/mL streptomycin, 5µg/mL Fungizone (PSF, Life Technologies, NY), and centrifuged at 1750 rpm for 15 minutes at 12°C (3X) until collected into a single suspension. Chondrocytes were resuspended at 5x10^6 cells/mL in complete medium (high glucose DMEM containing 10% FBS, 100U/mL penicillin, 100µg/mL streptomycin, 2.5µg/mL Fungizone, 1% MEM Vitamin Solution (Gibco), 25mM HEPES buffer, 50µg/mL ascorbic acid (Mohanraj et al. 2013)). This cell suspension was plated into ultra-low adhesion (polyHEMA coated) 96 well plates (Corning, NY) at 1x10^6 cells/well, where chondrocytes coalesced within 24-48 hours to form a CTA (Mohanraj et al. 2013). CTAs were cultured for a minimum of 14-16 weeks in complete medium prior to injury.

5.2.2. Injurious Compression of CTAs and Native Tissue Explants

To determine the level of injury necessary to induce pathological changes in CTAs that mimic changes in cartilage explants, four different injurious compression protocols were applied in a single-sample manner based on previously established injury parameters (Kurz et al. 2001; Quinn et al. 2001; Lee et al. 2005). CTAs were subjected to either 50 or 75% strain at one of two strain rates, 10% strain/sec or 50% strain/sec, followed by a hold period for a total ramp-hold compression time of 10 seconds. Constructs were then cultured for 5 days after injury, and both CTAs and media were harvested at 12, 24, and
120 hours post-injury for evaluation of biochemical content and presence of soluble catabolic markers as described below. Based on the outcomes of single-sample injury, high throughput injury was applied to constructs at 75% strain at 50% strain/sec. For this, a custom high throughput mechanical screening device (Mohanraj et al. 2014) was used. The device consisted of an aluminum housing with linear bearings to guide the vertical displacement of a loading platen which included a force-sensitive resistor (FSR) array for real-time monitoring of compressive forces during injury. In the current version of the device, 48 samples are housed in a standard 48-well plate and are compressed via PTFE indenters, with load recorded continuously during injury using a NI-DAQ board (National Instruments, USB-2665) and a custom Labview program (National Instruments, V8.6) with post-processing in MATLAB (Mathworks, R2012a). Injurious strain and strain rate were calculated based on the average height of constructs. After injury, constructs were cultured for up to 5 days with sample harvest at 24, 48, and 120 hours post-injury. As a positive control, constructs were treated with IL-1β (10ng/mL) for 5 days (Cook et al. 2001; Wehling et al. 2009; Ousema et al. 2012). To validate the injury response in CTAs, cartilage explants were injured in a similar manner. Full-thickness articular cartilage (chondral only) explants (4mm diameter) were harvested from the trochlear groove of juvenile bovine knees and trimmed to 3-4mm thickness, keeping the superficial layer intact. Cartilage cylinders were subjected to 75% strain at 50% strain/s using a single-sample injury protocol matching the high throughput injury of the CTAs. Cartilage cylinders were cultured for 5 days and evaluated as above in order to make comparisons between native and engineered cartilage response to injury.
5.2.3. Treatment with Putative Therapeutic PTOA Compounds

In a subset of studies, and immediately following injury at 75% strain applied at 50%/sec with the high throughput device, engineered cartilage was treated with one of the three following agents: (1) N-Acetyl-Cysteine (NAC, 2mm, Sigma, MO) a reactive oxygen species scavenger, (2) Z-VAD-FMK (ZVF, 100µM, Promega, WI) a pan-caspase inhibitor, or (3) Poloxamer 188 (P188, 8mg/mL, Corning, NY), an amphiphilic polymer capable of inserting into the cell membrane. Each compound was included in the culture medium for the initial 48 hours post-injury at levels previously reported to have beneficial effects in the context of cartilage injury (D'Lima et al. 2001; Phillips et al. 2004; Natoli et al. 2008; Martin 2009). Harvest time points and outcome measures were the same as described above.

5.2.4. Biochemical and Molecular Evaluation of Injury Response

Following injury, construct wet weight and dry weight (following lyophilization) were determined. Samples were then papain digested and glycosaminoglycan (GAG) content determined using the dimethylmethylene blue (DMMB) assay, with chondroitin-6-sulfate as a standard, as previously described (Farndale et al. 1986). DNA per construct was measured using the PicoGreen assay (Life Technologies, NY). Matrix content was measured per construct or normalized to DNA, and the swelling ratio was calculated as the ratio of wet to dry weight at the time of harvest. Medium was assayed at all harvest time points for GAG release and lactate dehydrogenase activity (LDH; CytoTox-ONE Homogeneous Membrane Integrity Assay, Promega, WI), which is released upon disruption of the cell membrane and is a measure of cell damage.
5.2.5. Histological Analysis of Injury Response

For viability analysis, constructs were stained using the Live/Dead staining kit (Live/Dead Viability/Cytotoxicity Kit, Life Technologies. NY) and imaged on a Nikon Eclipse TE2000-U (excitation wavelengths: Live- 420-495nm and Dead- 532-587nm) using 2X or 10X objectives. Additional samples were fixed in 4% paraformaldehyde (Affymetrix, CA), dehydrated, embedded in paraffin, and sectioned to 8µm thickness. Sections were stained for proteoglycan distribution with Alcian Blue (Rowley Biochemical Institute, MA) as previously described (Erickson et al. 2009).

5.2.6. Statistical Analysis

Effect of strain and strain-rate for single sample injury, differential effects of injury or IL-1β as a function of time, and comparisons to chondral explants were assessed by two-way ANOVA with Bonferroni’s post-hoc test (p<0.05). PTOA compound effects were compared to injury alone using a single sample t-test (p<0.05). All statistical analysis was conducted using the SYSTAT13 software (v.13.00.05, San Jose, CA).

5.3. Results

5.3.1. Effect of Strain Magnitude and Strain Rate on CTA Injury Response

CTAs were injured in a single sample manner, with compression to 50 or 75% strain at 10 or 50% strain/sec. Representative stress versus time profiles of injured CTAs showed that both strain and strain-rate significantly increased peak stress (with values reaching 1-2 MPa) (Figure 5-1). Interestingly, compression of CTAs up to 75% strain resulted in multiple peaks, likely indicative of construct fracture and re-compression during loading. Construct failure at high strains was confirmed by histological analysis. At 50% strain,
there was some internal fissuring of the construct and focal areas of GAG loss (Figure 5-2G). At the higher 75% strain level, there was obvious surface fibrillation, loss of construct shape, and widespread GAG depletion (Figure 5-2H). In comparison, control constructs maintained uniform GAG distribution throughout the construct (Figure 5-2F). Rapid application of 50% strain resulted in cell death throughout the intact construct thickness, while 75% strain primarily resulted in cell death in areas adjacent to fissures (Figure 5-2, C-E). GAG within the construct and released to the medium was measured 0-12, 0-24, and 24-120 hours post-injury. 75% strain applied at either strain rate reduced GAG/DNA in constructs for all time points post-injury (Figure 5-2A). Furthermore, injury at both 50 and 75% strain significantly increased GAG released to the medium compared to uninjured controls. Application of 75% strain resulted in an ~2-fold greater increase in GAG release compared to 50% strain applied at the same rate (Figure 5-2B). In these outcome measures, strain rate was not found to have differential effect on the injury response in CTAs.
Figure 5-1. Injurious compression protocol. (A,B) Engineering strain and stress profiles of CTAs for the four injury protocols tested: 50% or 75% strain applied at either 10 or 50% strain/sec for a total compression time (ramp and hold) of 10 sec. Profiles are representative of constructs injured in that group (N=24/group, all CTAs fabricated with pooled chondrocytes of a single animal). (C) Strain and strain-rate both significantly influence peak stress, with 75% strain applied at 50% strain/s yielding the highest peak stress.
Figure 5-2. Effect of strain and strain rate on matrix retention and loss following injury. (A) GAG content normalized to DNA content within CTAs (N=3/group, all CTAs fabricated with pooled chondrocytes of a single animal) showed consistent loss of matrix following injury applied to 75% strain at 10 and 50% strain/sec. (B) GAG released to the medium (N=3/group, all CTAs fabricated with pooled chondrocytes of a single animal) mirrored that of the construct, but with significant loss observed in a strain-dependent manner at both 50 and 75% strain, regardless of the strain-rate during injury. (C-E) Live/Dead staining (green: viable; red: non-viable) 24 hours post-injury for the highest strain rate illustrated that 50% strain resulted in focal regions of cell death with internal fissuring while control constructs contained viable cells throughout. 75% strain caused more extensive cell death superficially and adjacent to large and full-depth fissures (N=2/group). (D-F) Alcian blue staining for proteoglycans showed control constructs with
well distributed matrix, that 50% strain caused internal fissuring with local matrix loss (black arrows), and that 75% strain caused wide-spread matrix damage with fainter staining for proteoglycans throughout the construct (N=2/group). *p vs. control and #p vs. 50% strain for a respective time point; p<0.001 for * and # symbols alone.

5.3.2. High Throughput Injury of Engineered Cartilage
Based upon the outcomes of single sample injury of CTAs, 75% strain applied at 50% strain/sec was chosen for the high throughput application of compressive injury. Injury was carried out using the custom high throughput mechanical injury device, shown in Figure 5-3A. While CTAs had slight variations in height due to their free form assembly and growth during pre-culture, an applied target strain of 75% resulted in applied strains ranging from 59% to 99% in individual CTAs, with a mean strain of 78+/-10% for a full 48-well plate of constructs (Figure 5-3B). Simultaneous compression resulted in peak loads in CTAs comparable to single-sample compression; a 3D graphical representation of the real time peak voltages (peak load) is provided in Figure 5-3C, showing similarity across constructs. GAG released from high throughput constructs was 2 to 3-fold higher than uninjured controls for all time points (Figure 5-4A), a response similar to that evoked by the single sample injury. Interestingly, while IL-1 treatment initially caused GAG release at a similar magnitude as mechanical injury, by 120 hours of continuous treatment, GAG release was ~9-fold higher than uninjured controls and 4 to 5-fold greater than injured samples. Evaluation of LDH in the medium indicated that injury resulted in dramatic loss in viability over the short term (Figure 5-4B). Conversely, IL-1 treatment did not disrupt membrane integrity and so resulted in little LDH release relative to baseline levels. Tissue swelling was calculated as a measure of degeneration, as swelling is observed clinically in early stages of osteoarthritis. While injured constructs swelled significantly within 24
hours, control constructs did not change (Figure 5-4C). In contrast, treatment with IL-1 only affected swelling after 5 days due to continued matrix degradation.

Figure 5-3. HTMS device for applying compressive injuries to CTAs. (A) The HTMS injury device consisted of an aluminum frame with linear bearings to guide the vertical motion of the sensor loading platen. The sensor adhered to the underside of the loading platen comes into contact with a well plate assembly consisting of PTFE indenters aligned with a standard 48 well plate containing engineered constructs. Average sample height was measured prior to testing, with a target injurious compression of 75% strain at 50% strain/sec applied to constructs. (B) Example distribution of sample heights and applied strains for each construct (N=48/plate). Average strain was 78+/-10% strain for the population. (C) Peak voltage recordings in each well showed the uniformity of peak load responses in engineered cartilage during compressive injury.
Figure 5-4. Release of ECM and cellular enzymes and alterations in CTA properties following high throughput mechanical injury. (A) Injury of engineered cartilage significantly increased GAG release to the medium in a manner similar to that of IL-1 treatment alone for the first 48 hours; after 48 hours, IL-1 causes a 4 to 5-fold higher level of GAG release from the construct compared to injury alone (N=8 media collections per
sample combined into 4 aliquots, Sol-GAG is an average value per aliquot/group). (B) LDH release (a measure of cell viability) indicated that injury caused a large increase in chondrocyte membrane disruption in the first 24 hours after injury, while IL-1 resulted in little membrane damage with continuous exposure for 5 days (N=8 media collections per sample combined into 4 aliquots/group, LDH is an average value per aliquot). (C) The swelling ratio (calculated as the ratio of wet to dry weight) indicated that that injury caused gross tissue damage and swelling within 24 hours post-injury. In contrast, IL-1 treatment resulted in increased swelling only after 120 hours of treatment (N=4 samples/group; all CTAs fabricated with pooled chondrocytes of a single animal). *p vs. control and #p vs. IL-1 for a respective time point; p<0.001 for * and # symbols alone.

5.3.3. Validation of Engineered Cartilage as an Analog for Injury of Native Tissue

To validate the injury response of CTAs, explants were subjected to the same injury protocol and outcomes. A representative peak stress profile for a cartilage explant shows multiple peaks, similar to that observed with the engineered cartilage, with the first peak concurrent with gross tissue failure, followed by a second or third peak due to the further compression of the fragments (Figure 5-5A, inset). The first peak in stress occurred at ~16 MPa for cartilage explants, similar to previously reported values for explants subject to injurious compression at high strains or strain-rates (Torzilli et al. 1999; Kurz et al. 2001). GAG release from explants and CTAs 24 hours post-injury showed similar loss in matrix content per construct (Figure 5-5A). Similarly, LDH release showed comparable findings, with injured CTAs and explants both showing evidence of cell membrane damage compared to un-injured controls, though LDH release from explants was ~2-fold greater than from CTAs (Figure 5-5B).
Figure 5-5. Comparison of native and engineered cartilage injury response. Bovine cartilage explants were compressed to 75% strain at 50%/s. (A-inset) Representative peak stress profile of explants during injurious compression showing a first peak concurrent with gross tissue failure, followed by a second peak resulting from further compression of the fragments. Average first peak stress was 16.8+/−4.3 MPa (N=12, all explants harvested from a single animal). Soluble factors released to the medium in the first 24 hours showed that (A) GAG release was comparable between injured explants and CTAs and was significantly greater than un-injured samples (N=4 for explants, and N=8 media collections per sample combined into 4 aliquots/group). (B) In contrast, LDH release was approximately 2-fold greater in explants compared to CTAs following injury. (N=4 for explants, and N=8 media collections per sample combined into 4 aliquots/group). p<0.001 for * vs control within tissue type and # vs CTAs.

5.3.4. Response of Engineered Cartilage to Putative PTOA Therapeutic Compounds

As a secondary validation of this high throughput injury system, we subsequently screened several putative therapeutic PTOA compounds. In the initial 24 hours, ZVF treatment
significantly reduced LDH release by 20% compared to injury alone; however, NAC and P188 did not alter membrane disruption (Figure 5-6A). By 120 hours post-injury, however, both NAC and P188 decreased GAG loss (by 18 and 20% respectively) compared to injury alone, although neither agent restored GAG content to control levels (Figure 5-6B). Despite increasing initial cell viability after injury, ZVF treatment did not alter GAG content in injured samples.

Figure 5-6. Effect of putative PTOA therapeutics on matrix content and cell death after injury. (A) LDH release 24 hours post-injury was reduced by ~30% with ZVF
treatment while NAC and P188 had no effect compared to injury alone (N=8 media collections per sample combined into 4 aliquots/group). (B) GAG content in the construct 120 hours post-injury showed that NAC and P188 treatment resulted in retention of ~20% more GAG compared to injury alone (N=4/group). Dashed red line demarcates results from two separate experiments (CTAs for each experiment fabricated with pooled chondrocytes of a single animal) evaluating the effects of NAC, ZVF, and P188.

5.4. Discussion

Traumatic joint injury initiates a cascade of catabolic and anabolic processes, the imbalance of which often results in further cartilage degeneration. However, the pathways that underlie these irreversible changes remain poorly understood. As such, in order to conduct an efficient and unbiased evaluation of molecules that may modulate PTOA biologic processes, high throughput screening would be a valuable tool to evaluate compound libraries after injury. In this study, we utilized engineered cartilage analogs (CTAs) in conjunction with a high throughput mechanical injury device to develop a platform for studying PTOA pathology and to enable the discovery of potential therapeutics.

Primary markers of cartilage damage following traumatic injury include matrix disruptions, GAG loss from the matrix, and cell death. Using CTAs as an in vitro cartilage surrogate for studying PTOA, our objective was to define the thresholds for inducing such a response, to determine the uniformity of response using a high throughput device, and to benchmark the response against that of native cartilage. To define thresholds for injury, single sample compression was applied to constructs at strains (50% and 75%) and strain rates (10% and 50% strain/sec) previously explored (Kurz et al. 2001; Quinn et al. 2001;
Lee et al. 2005). In explants, while strains larger than 50% cause permanent deformation and surface fibrillation (stresses >15MPa), strains greater than 80% (stresses >20MPa) cause deep fibrillation and complete destruction of matrix integrity (Torzilli et al. 1999). In CTAs cultured for up to 16 weeks, whose properties approach that of native tissue (Mohanraj et al. 2013), application of 75% strain resulted in widespread fissuring, with a 2-fold increase in release of GAG to the medium compared to 50% strain. The greater release of GAG observed at this higher strain may be due to the increased surface area for diffusion. DiMicco et al. observed with injury of osteochondral explants an initially high rate of GAG release not blocked by MMP or biosynthesis inhibitors, indicating that this early release (≤4 days post-injury) likely consisted of diffusion of larger proteoglycan molecules out of the tissue rather than enzymatically-cleaved fragments, as is observed at later time points (DiMicco et al. 2004).

In our study, for both levels of injury, extensive cell death was observed adjacent to surface fissures and internal cavities. In cartilage explant studies, the extent and depth of cell death has been reported to be both strain and strain-rate dependent (Quinn et al. 2001; Morel et al. 2004; Green et al. 2006; Natoli et al. 2008). In addition, similar to our observations, extensive loss of cell viability occurs along fissure lines/regions both in whole joint (Tochigi et al. 2011) and osteochondral explant (Quinn et al. 2001; Stolberg-Stolberg et al. 2013) models with cell viability increasing with distance from the fissure line. However, with time, cell death expands to these non-fissure regions, which may suggest two mechanisms by which cell viability decreases with injury. Under high loading rates, cells may not be able to “recruit” sufficient membrane components in order to deform under compression, and in areas of cartilage fissuring, cells experience high strains and these cells rupture (Moo et al. 2013). The expansion of the region of cell death to non-
fissure regions with time may be the result of diffusible, soluble factors that induce apoptosis and contribute to the propagation of the injury response (Levin et al. 2001; Tochigi et al. 2011; Stolberg-Stolberg et al. 2013).

Upon determining that 75% strain at 50% strain/sec induced a degenerative response in CTAs, we next used our high throughput device to apply consistent compressive injury to up to 48 samples simultaneously. While there were small variations in applied strain due to differences in construct height, the response was sufficiently uniform so as to provoke a ~2-3 fold increase in GAG release compared to un-injured controls, comparable to the single sample response. Monitoring LDH release as a quantitative measure of cell death similarly confirmed that cell membrane damage is a repeatable effect of compressive injury in this engineered model of PTOA.

We subsequently validated the CTA response against that of native cartilage. Peak stresses during compressive injury of native tissue were ~20-fold higher than CTAs. This difference could explain the 2-fold greater increase in LDH release as compared to CTAs in the first 24 hours. Indeed, strain rate and peak stress dependent increases in LDH release have been observed in cartilage explants (Bush et al. 2005; Nishimuta et al. 2012). Additional factors which may also influence the extent of cell death include matrix composition and organization (Tan et al. 2010). In our study, GAG release was comparable between explants and CTAs, with a 4-fold increase in GAG release to the medium compared to un-injured controls within the first 24 hours. Previous work has similarly reported an ~2-fold increase in GAG release in the first 24 hours from explants subjected to injury at fracture levels (e.g. 50% strain at 100% strain/sec (Patwari et al. 2003; DiMicco et al. 2004) or high strain rates of 50% or 70% strain/sec (Quinn et al. 2004).
Given that the CTA can be injured at any point in its maturation, from the cell-rich, matrix-poor more fetal-like state, through to the mature, matrix-rich, cell poor adult-like state (Mohanraj et al. 2013), a range of studies may be performed to determine how injury and tissue maturation state interact.

Using our CTAs, we observed a consistent and marked effect of load-induced injury; however, in PTOA, degeneration is also potentiated by the presence of inflammatory cytokines, such as IL-1β, which may differentially govern the chondrocyte response in our in vitro system. While mechanical injury caused extensive cell membrane damage as measured by LDH release, IL-1 had a minimal effect. In contrast, while IL-1 and injury initially increased GAG release to similar levels, by 120 hours of exposure to IL-1, GAG release was 3-fold higher than injury alone. IL-1 is known to cause matrix degradation via increased NF-κB activation, expression of proteases (e.g. MMP and ADAMTS), and other pro-inflammatory molecules (e.g. NO and COX-2) (Lianxu et al. 2006; Montaseri et al. 2011). Such temporal patterns of matrix disruption may also explain why injury caused rapid construct swelling (mechanical disruption of the nascent collagen network), while this swelling response took longer with IL-1 mediated degradation (sustained enzymatic cleavage). These observations are consistent with reports of injury-induced increases in water content of explants in a strain dependent manner (Torzilli et al. 1999; Kurz et al. 2001), as well as of chondrocyte-seeded agarose hydrogels following a crush injury (Tan et al. 2010).

Finally to determine whether this CTA model is useful for screening new PTOA therapeutics in a high throughput manner, bioactive molecules previously reported to
reduce cell death and proteoglycan loss post-injury were evaluated. Application of these compounds to CTAs resulted in early protection against loss of viability (ZVF) and late protection against matrix loss (P188 and NAC) after injury. ZVF has been observed to increase cell viability by 15-20% with 48 hours of treatment following compression of explants (30% strain at 0.6s\(^{-1}\) (D'Lima et al. 2001) or impact at 7J/cm\(^2\) (Martin 2009)). Although NAC has likewise been shown to increase viability by ~30% following injury (Martin 2009), here we found no effect on reducing membrane damage. However, NAC was effective at reducing GAG loss, consistent with observations of a ~20% reduction in GAG loss from chondral explants following injury (7J/cm\(^2\) (Martin 2009)). While our findings also showed that P188 improved GAG retention, literature findings have been variable, with some studies noting a ~20% increase in cell viability (Phillips et al. 2004) with minimal changes in GAG loss (Natoli et al. 2008). One possible explanation is that P188 insertion into the membrane may prevent the expulsion of intracellular contents which in turn may preserve local ion concentration gradients (Yasuda et al. 2005) to allow living cells to function normally. It is important to note that although these compounds did have acute effects on matrix retention and cell viability, none were able to return constructs to control levels, highlighting the importance of continuing therapeutic discovery.

5.5. Conclusion

Taken together, these studies illustrate that injurious compression of CTAs replicates key markers of the injury response in native cartilage explants, validating this approach as a model system for studying the processes that govern cartilage degeneration in PTOA. While our current model focuses on mimicking articular fracture, this system can be adapted for applying insults that do not produce structural damage to mimic subtle injury scenarios and also for cyclic overloading injuries. In addition, our testing platform, along
with the ability to form large numbers of these cartilage analogs in a micro-scale format, sets the stage for high throughput screening of large chemical libraries to more rapidly identify therapeutics to attenuate progressive degenerative joint changes after injury.

Chapter 6. Small Molecule Library Screening for Modulators of Post-Traumatic Osteoarthritis

6.1 Introduction

High throughput screening (HTS) aims to use preclinical models to predict the efficacy and safety of drug candidates in vivo. In this early stage of drug development, it is critical to identify therapeutic candidates that meet (or fail to meet) the necessary targets for altered cell function or response and to prioritize those that are most promising. The use of appropriate preclinical models can reduce the number of drug failures that occur during clinical trials. Indeed, drug development attrition rates between 1964-2000 resulted in a success rate of less than 11% in bringing drugs to market in the US and Europe (Prentis et al. 1988; Kola et al. 2004; Astashkina et al. 2012). Conventionally, the ability of candidates to modulate a specific biologic target is assessed using an in vitro, 2D cell-based assay and lead candidates from these initial screens are directly assessed in vivo using a relevant animal model (Breslin et al. 2013). However, 2D-monolayer cultures may not recapitulate important aspects of the tissue microenvironment, including native cell morphology, cell-cell and cell-ECM interactions, and biophysical cues derived from the surrounding 3D matrix. These environmental factors also differentially regulate a number of cell behaviors as compared to 2D culture (e.g. adhesion (Cukierman et al. 2001), proliferation (Baker et al. 2015), and differentiation (Cosgrove et al. 2016)). These differences highlight the gap that exists between 2D monolayer cultures and animal models for understanding the cellular response to therapeutics in vivo. Therefore, given the time required for and expense of bringing a new drug to market (DiMasi et al. 2016), the adaptation of 3D in vitro cultures for screening applications presents an opportunity to
improve predictive efficacy of preclinical models. In this Chapter, we develop a system for use in musculoskeletal research, with a particular focus on post-traumatic osteoarthritis.

The primary focus of tissue engineering is to repair degenerated tissues through the use of constructs that mimic the structure, function, and cellular behaviors of its healthy, native tissue counterpart. Recently, however, engineered tissues have gained traction as 3D *in vitro* model systems to investigate mechanisms and microenvironments of disease, as well as screen therapeutic compounds (Griffith et al. 2006; Gibbons et al. 2013; Kimlin et al. 2013). While these systems may not capture the full complexity of the physiologic environment, key features of the tissue environment can be recapitulated *in vitro* to mimic hallmarks of the *in vivo* environment that may impact drug response (Rai et al. 2008; Hongisto et al. 2013). In addition, engineered tissues are fabricated as tightly controlled environments, enabling systematic and quantitative investigation of cellular responses to drug exposure in a reproducible context. Screening thousands of compounds in preclinical animal models is neither economically feasible nor ethically responsible (Astashkina et al. 2012). Therefore, scale-up of 3D *in vitro* models to HTS compatible platforms meets a critical need in the drug discovery process. A number of screening platforms for various tissue types are currently in development (Griffith et al. 2006; Gibbons et al. 2013; Kimlin et al. 2013), including engineered skeletal (Vandenburgh et al. 2008) and heart (Hansen et al. 2010; Boudou et al. 2012; Mathur et al. 2015) muscle models to measure the effect of therapeutics on contractile activity, as well as organoid-derived 3D cultures of kidney proximal tubules to screen drugs for nephrotoxicity (Astashkina et al. 2012). Our focus in this context, is the design of preclinical models for musculoskeletal conditions; more specifically, joint and cartilage pathologies for which the disease modifying therapies remain an unmet clinical need.
To date, several tissue engineering strategies have successfully matched functional and phenotypic characteristics of native cartilage using various cell sources, biomaterials, and environmental cues (Elisseeff et al. 2001; Wang et al. 2005; Novotny et al. 2006; Moutos et al. 2007; Ofek et al. 2008; Erickson et al. 2009; Johnstone et al. 2013; Sharma et al. 2013). While efforts to translate these native-like implants is ongoing (Gotterbarm et al. 2006; Holland et al. 2007; Mrugala et al. 2008; Wang et al. 2010; Kim et al. 2015; Fisher et al. 2016), there also exists the potential to use engineered cartilage for screening purposes to identify factors that can treat osteoarthritis (OA). OA is considered to be a disease that affects the entire joint, but the primary features include cartilage erosion, proteoglycan depletion within the tissue, and chondrocyte apoptosis leading to loss of load-bearing function (Little et al. 2013). A subset of OA, termed post-traumatic osteoarthritis (PTOA), is defined by the initial occurrence of a traumatic injury to the soft tissues within the joint (e.g. meniscus or ligament tear) that initiates progressive degenerative changes within cartilage. Towards the discovery of disease-modifying osteoarthritis drugs (DMOADs), genetically engineered mice have been used in preclinical models of PTOA (surgically-induced joint instability) to identify potential targets. Targets that have been validated across independent studies include ADAMTS5 (Glasson et al. 2005; Botter et al. 2009; Malfait et al. 2010), MMP13 (Little et al. 2009; Wang et al. 2013), and FGF2 (Chia et al. 2009). Although animal preclinical models provide strong support to further pharmaceutical investigations, *in vitro* drug screening platforms are needed to identify lead candidates that effectively modulate these targets.

To address this need for a 3D *in vitro* model of PTOA, our group recently developed a high throughput model of mechanical injury using engineered cartilage. A 48-well mechanical
testing system (Mohanraj et al. 2014) was adapted to apply compressive injury to cartilage tissue analogs (CTAs) (Mohanraj et al. 2014). Acute hallmarks of the injury response (cell death and matrix loss) were observed in vitro, mimicking the effects of injury applied to native cartilage tissue explants in vitro, as well as in destabilization models in vivo. A pilot screen of putative therapeutic compounds for early intervention in PTOA also showed similar efficacy in engineered cartilage compared to published results (Mohanraj et al. 2014). Here, we build upon this platform to conduct unbiased screens of small molecule libraries to identify novel candidates that have the capacity to modulate the acute injury response. HTS-compatible assays, measuring cell viability and matrix remodeling post-injury, were used to determine the effect size of screened compounds and to identify ‘hits’ for further analysis in secondary screens.

6.2. Methods

6.2.1. Cartilage Tissue Analog Fabrication for Screening Studies

Cartilage tissue analogs (CTAs) were fabricated as described previously (Novotny et al. 2006; Mohanraj et al. 2014) using primary chondrocytes seeded at high density in suspension culture to form aggregated masses (1 million cells per CTA in 96-well, poly(2-hydroxyethyl methacrylate)-coated plates). CTAs for screening experiments were cultured for at least 14 weeks to achieve functional maturity. For the initial library screen and secondary evaluation of individual small molecules, chondrocytes were harvested from the knees of a single juvenile bovine donor (2-6 months old, Research 87, MA). For the second library screen, CTAs were formed by pooling chondrocytes in equal number from bovine knees from different animals (N=7 knees from separate bovine donors). An aliquot of chondrocytes from each animal was also reserved to form donor-specific CTAs for validation of the behavior of pooled CTAs in a pre-screening study. All CTAs were cultured
in complete medium as described previously (Mohanraj et al. 2014) for the duration of construct growth and injury-screening experiments.

6.2.2. High Throughput Mechanical Injury for Compound Library Screening

A high throughput mechanical injury (HiTMI) system (Chapter 5) was used to apply compressive strains (50-75% strain at 50% strain/s) previously observed to induce key hallmarks of the acute injury response in engineered cartilage (Mohanraj et al. 2014). Prior to injury, the height of all CTAs was measured and CTAs were placed in a standard 48-well plate (Cat. No. 351178, BD Falcon, NJ). Injury strain and strain rate were calculated based on the average height of constructs in the well plate. The HiTMI device (as shown in Figure 5-3) consisted of an aluminum housing with linear bearings to guide the vertical displacement of a loading platen controlled by an Instron 5948. PTFE indenters were placed on CTAs in each well, the loading platen was brought to the average CTA height, and the displacement protocol applied. Immediately following injury, CTAs were treated with library compounds (all compounds: 10μM, N=1/compound; treatment applied <15 minutes post-injury). The commercial libraries chosen for these experiments targeted pathways relevant to the acute hallmarks of injury, cell death and matrix remodeling. The first library, containing 29 compounds, included small molecules relevant to apoptosis signaling pathways (e.g. capsases, TNF-α; Apoptosis Compound Library, L3300, SelleckChem, TX). The second library, containing 89 compounds, targeted pathways relevant to chondrocyte signaling and cartilage development (e.g. TGF-β, JAK; Stem Cell Signaling Library, L2100, SelleckChem, TX). A number of controls were also included to aid ‘hit’ identification and to define the range of the assay measures. Injured and un-injured CTAs cultured in complete medium alone, served as the baseline injury and naïve control,
respectively (N=2/plate). Injured CTAs were also treated with factors to induce known responses: TNF-α to induce GAG loss (10ng/mL), TGF-β3 to increase GAG production (10ng/mL), Triton-X to lyse all cells for maximal LDH release (0.18% w/v), and DMSO as a delivery vehicle control (0.1% v/v, matched to compound dilution) (N=1/plate). After 48 hours, medium and CTAs were collected and stored at -80°C until further analysis. The wet weight of each construct was measured at the time of harvest to normalize outcome measures (represented as %WW) to make comparisons across samples.

6.2.3. Screening Assays and ‘Hit’ Identification

CTAs for biochemical analysis were digested in proteinase K (1:200 in 10mM Tris-HCl) overnight. Glycosaminoglycan (GAG) content in the tissue and released to the medium was determined using the dimethylmethylene blue (DMMB) assay as previously described (Farndale). Lactate dehydrogenase (LDH) was measured as an indication of cell death (CytoTox-ONE Homogeneous Membrane Integrity Assay, Promega, WI); LDH is released into the medium following cell membrane disruption or damage. To identify ‘hits’ using data from these assays, dual flash-light plots were constructed to show the strictly standardized mean difference (SSMD, statistically-favored measure) vs. \( \log_2 \) fold change (biologically-favored measure) (Zhang et al. 2007; Zhang 2011) for each assay. SSMD indicates the magnitude of difference between two conditions and accounts for the non-normality, variability, skewness, and outliers within a population, compared to other statistical methods. These features are common in high throughput screening data where ‘hits’ are few, and the majority of compounds produce negative results (i.e. are similar to baseline). Assay values were log transformed, and the SSMD score was calculated as the ratio of two components: (1) the difference between the measured value for a compound
and the median of the negative reference (untreated, injured control) and (2) the median absolute deviation, adjusted for sample size (SSMD based on UMVUE, (Zhang 2011)). Gating criteria were applied, wherein compounds with an $-1 \leq \text{SSMD} \leq 1$ and $-1.5 \leq \text{Fold Change} \leq 1.5$, were identified as ‘hits’ (i.e. positive or negative regulators of the injury response). SSMD scores for ‘hits’ were also cross-referenced against an additional outcome measure (GAG release vs. LDH release) to determine if compounds were able to modulate multiple hallmarks of the injury response. ‘High-performing’ hits were subsequently evaluated in secondary screens in the HiTMI device using multiple ‘donor-specific’ CTAs (N=3-4 bovine donors, aged 6 months to 1 year) and biological replicates (N=3 CTAs per small molecule treatment).

### 6.2.4. Validation of Pooled CTA Response in an Inflammatory Environment

To determine if the response of pooled CTAs was comparable to that of the average response of donor-specific CTAs, constructs were subjected to inflammatory challenge. IL-1β is a pro-inflammatory cytokine found in the synovial fluid of osteoarthritic joints (McNulty et al. 2013) and is known to induce catabolic processes that cause matrix degradation and inhibit chondrocyte biosynthesis in engineered cartilage (Goldring 2000; Ousema et al. 2012). CTAs were pre-cultured for four weeks prior to exposure to IL-1β (10ng/mL) for 5 days. Medium collected during this period was analyzed for GAG (DMMB assay) and nitrite (Griess assay, Promega, WI) release. Nitrite is a breakdown product of nitric oxide and promotes cartilage degradation by inhibiting aggrecan synthesis, activating MMPs, and increasing chondrocyte susceptibility to apoptosis by other oxidants (Scher et al. 2007). The MTT assay was also used as a measure of cell metabolic activity in CTAs.
6.2.5. Statistical Analysis

Single sample t-tests (p<0.05) were used for analysis of pre-screening experiments comparing the effect of IL-1β to control for each donor-specific or pooled CTA. One-way ANOVA with Tukey’s post-hoc test (p<0.05) was used in secondary screens to determine differences between naïve control, injury alone, and treated injury groups. All statistical analysis was conducted using Graphpad Prism (v.5, CA) and SYSTAT13 software (v.13.00.05, CA).

6.3. Results

6.3.1. Identification of ‘Hits’ in a Screen Targeting Apoptosis-Related Pathways

Cell death immediately and acutely following compressive injury has been observed in both cartilage explant (Quinn et al. 2001; Morel et al. 2004; Green et al. 2006; Martin 2009; Rosenzweig et al. 2012) and engineered cartilage models (Tan et al. 2010; Mohanraj et al. 2014). While the parameters of mechanical loading may affect the location, depth, and expansion of the non-viable area, both necrosis and apoptosis are thought to be contributing mechanisms to the widespread loss of viability. Therefore, the objective of the first screen in the HiTMI platform was to evaluate a curated library of small molecules (29 compounds) that modulate signaling pathways related to apoptosis. A total of 9 ‘hits’ were identified that modulated the injury response across the three primary outcome measures. Compounds that modulated cell damage, and indirectly cell viability post-injury, were assessed by LDH release. Three molecules that attenuated LDH release were identified: Pomalidomide (an inhibitor of LPS-induced TNF-α release), Z-VAD-FMK (a pan-caspase inhibitor), and Necrostatin-1 (an inhibitor of TNF-α induced necroptosis) (Figure 6-1A). To
determine if compounds within this library also had off-target effects on remodeling as a secondary effect, GAG release and retention were also measured. Two compounds that attenuated GAG release were identified: ABT-199 (a Bcl-2 inhibitor) and JNJ-26854165 (a HDM2 ubiquitin ligase antagonist) (Figure 6-1B). In addition, one negative regulator of injury was identified: BV-6 (a cIAP and XIAP inhibitor) that increased GAG release, similar to exogenous TNF-α treatment. A separate group of molecules was also found that increased GAG content within the tissue; these included Apoptosis Activator 2 (induces caspase 3 activation), GDC-0152 (an IAP antagonist), and TW-37 (a non-peptide inhibitor of Bcl-2) (Figure 6-1C).
Figure 6-1. ‘Hits’ identified during screening of small molecules related to apoptosis signaling pathways. Black circles represent ‘hits’ that meet gating thresholds as compared to all other molecules (grey circles); controls are color-coded as described in the legend. (A) LDH release as an indirect measure of cell viability identified 3 ‘hits’ that attenuated cell death: ZVF, Pomalidomide, and Necrostatin 1. (B) GAG release identified two positive (JNJ-26854165 and ABT-199, and one negative (BV-6) regulator of the injury response. (C) GAG content in CTAs identified 3 ‘hits’ that improved retention post-injury: Apoptosis Activator 2, TW-37, and GDC-0152.
6.3.2. Scale-Up of the HiTMI Platform for Small Molecule Screens

To scale-up the throughput of screens using the HiTMI platform, CTAs needed to be fabricated in large quantity so as to match the size of large, small molecule libraries. To achieve this, chondrocytes were pooled from multiple bovine knees to form single batches of CTAs. Donor-specific batches of CTAs were also fabricated in parallel to enable comparisons of ‘pooled’ versus ‘donor-specific’ CTA responses to an inflammatory challenge, as a pre-screening test. We hypothesized that ‘pooled’ CTAs would exhibit the average response of ‘donor-specific’ CTAs following exposure to IL-1β (10ng/mL) for 5 days. As shown in Figure 6-2, in two separate studies, while nitrite and GAG release was variable across ‘donor-specific’ CTAs, the ‘pooled’ response was similar to the calculated average. MTT, a measure of metabolic activity, also showed a similar pattern (Figure 6-2C). These results indicate that a single ‘donor’ population of chondrocytes did not dominate the overall response and supported the use of a ‘pooled’ CTA fabrication method for larger screens.
Figure 6-2. Pre-screening comparison of ‘pooled’ vs. ‘donor-specific’ CTA response to an inflammatory challenge. (A) GAG and (B) Nitrite released to the medium following exposure to IL-1β for 5 days show that the pooled CTA response is similar to the average (green dashed line) response of individual CTAs. (C) MTT measurement of cell metabolic activity falls within the range of the donors tested. Individual bovine ‘donors’ are
represented in the figures as ‘B’ followed by a number (^p<0.05, †p<0.01, *p<0.001 vs. control).

In the second screen, a larger library of small molecules was chosen that broadly targeted signaling pathways related to chondrocyte signaling and cartilage development. A total of 89 unknown compounds were screened, along with controls on each 48-well plate (un-injured, injured un-treated, TNF-α, TGF-β, DMSO, and Triton-X). A total of 28 ‘hits’ were identified from LDH and/or GAG release outcome measures; 16 ‘hits’ exacerbated the injury response, while an additional 12 ‘hits’ attenuated cell damage or matrix loss (Figure 6-3). Those that reduced LDH release affected signaling related to JAK (Filgotinib, Tofacitinib Citrate, and WHI-P154), GSK-3 (1-Azakenpaullone), histamine receptor (Hesperetin), and Hedgehog/Smootherned (BMS-833923) signaling. Tofacitinib citrate (CP-690550, pan-JAK inhibitor) and BMS-833923 (smoothened antagonist) reduced LDH release to naïve, un-injured control levels. The 6 ‘hits’ that reduced GAG release included a number JAK pathway inhibitors (Filgotinib, Tofacitinib Citrate, Oclacitinib, and Pacritinib). LY2157299, a TGFβ receptor I inhibitor, and Wnt agonist 1, a cell permeable Wnt signaling pathway activator, were also identified factors that could reduce matrix loss post-injury.
Figure 6-3. ‘Hits’ identified during screening of small molecules related to chondrocyte signaling and cartilage development pathways. A total of 28 ‘hits’ were identified in this screen of 89 compounds. 16 ‘hits’ exacerbated the injury response, while 12 ‘hits’ attenuated markers of injury in terms of both (A) LDH and (B) GAG release outcome measures. (C) No compounds were identified that modulated GAG retention. Several positive regulators of the injury response affected JAK, as well as TGF-β, Hedgehog and Wnt signaling.
6.3.3. ‘Hits’ that Affect Multiple Injury Hallmarks and Secondary Screens

‘Hits’ derived from these two small molecule library screens were identified based on the effect size of a single outcome measure; however, these compounds likely regulate multiple cellular activities. To probe this, the SSMD score for GAG release and LDH release (those measures which contained the greatest number of ‘hits’) were plotted together (Figure 6-4). Only a few compounds exacerbated the injury response, including TW-37 and GDC-0152, which increased GAG retention in the first screen, but at the same time increased GAG and LDH release. Although Pacritinib and Wnt agonist 1 reduced GAG release, these compounds also decreased chondrocyte viability. Overall, most compounds either attenuated a single or both markers of injury. Pomalidomide and ZVF reduced only LDH release, indicating that their protective response was to maintain cell viability. Compounds that significantly reduced both cell damage and matrix loss included Necrostatin-1, Filgotinib, and WHI-p154. Finally, the two ‘hits’ that reduced LDH to uninjured, control levels (BMS-833923, Tofacitinib Citrate) also showed a marked reduction in GAG release. These 7 compounds were subsequently chosen for secondary in vitro screens to confirm the effects observed in the primary screen.
Figure 6-4. Candidate small molecules that modulate multiple hallmarks of the injury response. SSMD scores for GAG release and LDH release were plotted together to assess synergistic effects post-injury. While a few compounds exacerbated injury in at least one outcome measure, the majority of positive ‘hits’ attenuated either one or multiple measures of the injury response. In particular, ZVF and Pomalidomide particularly affected cell viability, while several JAK inhibitors (WHI-P154, Fligotinib, and Tofacitinib Citrate) and a Smoothened antagonist (BMS-833923) had the added effect of attenuating GAG loss.
‘Donor-specific’ CTAs (a total of four bovine donors) were used to independently evaluate the selected ‘high performing hits’. As shown in Figure 6-5, compounds had variable effect sizes in attenuating the injury response across donors, and some donors appeared to be more responsive than others to treatment (Figure 6-6). In terms of GAG release, WHI-P154 consistently had an effect across donors, wherein the level of matrix loss was between that of un-injured and injured CTAs (not significantly different from either). ZVF, Filgotinib, and BMS-833923 also similarly affected GAG release, but only for Donors 3 and 4. Measurement of LDH release showed greater variability, with only two out of the four donors responding to treatment (Figure 6-6). In particular, ZVF, Filgotinib, and BMS-833923 showed reduced levels of LDH release, approaching that of un-injured CTAs across both Donor 3 and Donor 4 CTAs.
Figure 6-5. Secondary screening of the 7 ‘high-performing hits’ was performed in several sets of ‘donor-specific’ CTAs. (A) Donor 1 and (B) Donor 2 showed attenuation of GAG release in response to WHI-P154 (not statistically different from un-injured or injured groups). No changes were seen with any treatment for LDH release. (C) Donor 3 showed a similar effect in terms of GAG and LDH release for ZVF, Filgotinib, and BMS-833923. WHI-P154 modulated only GAG release and the remaining compounds approached statistical significance compared to un-injured CTAs. (D) Donor 4 showed attenuated GAG release for all compounds, while LDH release was most affected by Pomalidomide, ZVF, Filgotinib, BMS-833923, and WHI-P154. (*p<0.05 vs. un-injured, ^p<0.05 vs. injury, NS p>0.05 vs. un-injured and injury).

Figure 6-6. Variability of ‘donor-specific’ CTA response to ‘high performing hits’ in a secondary screen. The average response of biological replicates for each donor (represented by a single point), in response to each compound is shown normalized to injury alone for GAG and LDH release. Donors 3 and 4 were the most responsive, with the greatest number of compounds attenuating the injury response, while WHI-P154 was the most consistent in terms of affecting matrix loss.
6.4. Discussion

Micro-engineered tissues have the potential to improve the predictive capability of preclinical screens as compared to 2D cellular assays. While the entirety of the *in vivo* milieu is difficult to recapitulate *in vitro*, 3D tissues can be used to assess how candidate drugs affect specific cellular behaviors already established to closely approximate the *in vivo* response. Furthermore, development of novel HTS assays and scale-up of biofabrication methods will support drug screening and discovery applications for engineered constructs. We previously described an *in vitro* model of compressive injury that induces the acute hallmarks of the injury response (cell death and proteoglycan loss) in engineered cartilage similar to native tissue (Mohanraj et al. 2014). In this work, we show the utility of our high throughput injury platform for conducting unbiased screens of small molecule libraries to identify ‘hits’ that modulate the injury response. Although the selected libraries were chosen for their relevance to signaling pathways associated with cartilage injury, the compounds were curated independently (SelleckChem). Of the 118 compounds screened, 20 ‘hits’ were identified that attenuated the injury response in at least one outcome measure. Within this group, 7 ‘high-performing hits’ were identified that strongly modulated either cell death alone, or matrix loss and cell death together. One of the two ‘hits’ that specifically improved viability was Z-VAD-FMK (ZVF), a pan-caspase inhibitor. This finding confirmed the protective effect of ZVF that has been observed in an *in vivo* ACL transection model of PTOA (D’Lima et al. 2006), as well as *in vitro* explant (D’Lima et al. 2001; Martin 2009) and engineered cartilage (Mohanraj et al. 2014) models. Although the remaining 6 ‘high-performing hits’ have not yet been explored in the context of early intervention for PTOA, the pathways targeted by these compounds are relevant to human disease and injury. Several JAK inhibitors were identified, including Tofacitinib
Citrate which inhibits cytokine mediated degradation (Jatiani et al. 2010) and is being investigated as a potential treatment for rheumatoid arthritis (Milici et al. 2008), and WHI-P154 which has been shown to reduce NO production in IFN-γ-stimulated macrophages (Sareila et al. 2006) and leptin-treated human OA cartilage (Vuolteenaho et al. 2009).

Secondary screens using multiple ‘donor-specific’ CTAs showed variable results across donors; however, ZVF, WHI-P154, and BMS-833923 trended towards attenuating GAG release and maintaining cell viability. Previous work has shown ‘hits’ in primary screens may have different effect sizes in secondary screens (Brey et al. 2011). It is also important to note that variability is inherently a part of the biological response in many systems. An investigation of donor to donor variability in cell sources commonly used for tissue engineering (chondrocytes and mesenchymal stem cells) showed heterogeneity not only between donors, but also within clones themselves (Cote et al. 2016). Moreover, the impact of drugs across patients is known to have widely varying efficacies (Bathon et al. 2000; Wolbink et al. 2006). These findings highlight the importance of understanding donor-based differences to potential therapeutics within pre-clinical disease models. Future experiments will focus on dosage, timing, and additional donors to tease out the effectiveness of these small molecules across a potential ‘patient’ population.

While we have demonstrated that engineered cartilage can be used to create a reproducible model of the acute changes in load-induced injury, long-term cartilage degeneration in PTOA is potentiated by the presence of inflammation. Pro-inflammatory cytokines found in the synovial fluid of osteoarthritic joints (e.g. IL-1β, TNF-α, IL-6, REF) stimulate catabolic processes (e.g. production of MMPs, inhibition of matrix biosynthesis)
that lead to progressive cartilage degradation (Goldring 2000). To screen for drug candidates that can reduce catabolic activity in this context, a number of studies have built engineered cartilage models of OA using inflammatory microenvironments. Rai et. al. described key temporal differences in the production of catabolic mediators (nitrite and PGE$_2$) by chondrocytes in 2D monolayer vs 3D culture (Rai et al. 2008). Willard et. al. developed a scalable platform (96-well) for engineering cartilage with a defined genetic background using iPSCs and demonstrated the effectiveness of known OA candidate drugs against an IL-1$\alpha$ challenge (Willard et al. 2014). Finally, towards mimicking the complexity of inflammation in vivo, Sun et. al. showed that 3D constructs treated with macrophage-conditioned medium (as compared to exogenous addition of cytokines) exhibited many of the early hallmarks of OA in vivo, including chondrocyte hypertrophy and apoptosis (Sun et al. 2011). Taken together, our work and others’ highlights the ability of engineered cartilage to mimic key aspects of disease progression. As multiple stages of OA progression become incorporated into a single model system, the predictive power of a 3D in vitro preclinical model is likely to improve, leading to efficient identification of lead drug candidates.

Using the current iteration of our high throughput mechanical injury system, a total of 118 compounds were screened and a number of promising ‘hits’ were identified. However, the strength of a high throughput system lies in size of the library (i.e. number of molecules) that can be rapidly screened using amenable outcome measures. As such, this study serves as a proof-of-concept for adapting our system to screen larger, small molecule libraries, including those that not specifically curated towards ‘known’ pathways. In support of this scale-up, assays for tissue engineering are being adapted for robotic liquid handling
systems. Automated cell seeding (Ibold et al. 2007), as well as biochemical measurements (Huang et al. 2008) and cellular activity assays (Brey et al. 2011) have been performed by robotic platforms in recent years. In particular, the discovery of the chondroprotective molecule Kartogenin (from 22,000 drug-like molecules, (Johnson et al. 2012)) serves as one of the best examples demonstrating the potential of engineered tissues in HTS screening applications.

6.5. Conclusion

This study demonstrated the ability of our high throughput mechanical injury model to screen small molecule libraries to identify drugs that attenuate the injury response through un-explored or non-canonical pathways. Future secondary screens will focus on constructing dose response curves, determining the duration of efficacy, and optimal timing of drug administration post-injury. From the results of these studies, should a lead candidate show a consistent effect in improving cartilage outcomes post-injury, tertiary screens will be conducted to evaluate drug safety and efficacy in an in vivo model of PTOA to determine the potential for clinical translation. The work presented here is a first step in the process of advancing an in vitro micro-engineered model of PTOA as a pre-clinical approach for discovery and development of new therapeutics for early intervention in OA.
Chapter 7. Chondrocytes and Mesenchymal Stem Cell Derived Engineered Cartilage Exhibits Differential Sensitivity to Pro-Inflammatory Cytokines

7.1 Introduction

Osteoarthritis (OA) is a progressive, degenerative joint disease characterized by articular cartilage fibrillation and erosion leading to loss of load-bearing function (Goldring 2000; Little et al. 2013). Tissue engineering aims to restore function via the combination of biomaterials, cells, and exogenous cues (e.g. growth factors, mechanical stimulation), in order to fabricate cartilage analogs in vitro for their eventual in vivo application. Decades of work have culminated in the ability to engineer cartilage tissue that recapitulates the native phenotype and structure-function properties (Elisseeff et al. 2001; Wang et al. 2005; Moutos et al. 2007; Erickson et al. 2009; Sharma et al. 2013). Despite this achievement, these tissues were realized under ‘optimal’ growth conditions, which are not representative of the implantation milieu. Indeed, inflammatory mediators in the OA joint environment (Goldring 2000) will challenge the survival and growth of these constructs when they are implanted in vivo. Interleukin-1 (IL-1α and IL-1β) and tumor necrosis factor-alpha (TNF-α) are the primary cytokines that induce matrix catabolism and are detected at elevated levels in cartilage, synovial fluid, and synovium of OA joints (Wood et al. 1983; Towle et al. 1997; Lotz 2001; McNulty et al. 2013). While TNF-α is considered an early marker of OA, IL-1β is present at both early and late stages of degeneration (Goldring 1999). In human OA cartilage, both cytokines co-localize with the expression and activity of matrix metalloproteinases (MMPs), which mediate degradation of the collagen network (Tetlow et al. 2001).
To investigate the mechanisms by which IL-1\(\beta\) and TNF-\(\alpha\) induce cartilage degradation, a number of in vitro explant and engineered cartilage models have been developed. Treatment of newborn and adult bovine explants with IL-1\(\beta\) and TNF-\(\alpha\) result in a dose-dependent increase in proteoglycan release, nitric oxide and MMP synthesis, and cell death, as well as a decrease in collagen content and tissue mechanical properties (Patwari et al. 2003; Wilson et al. 2007; Palmer et al. 2009; Torzilli et al. 2010). In engineered cartilage fabricated using articular chondrocytes, sensitivity to cytokine-mediate changes has been evaluated as a function of construct maturity and in a biomaterial system. Constructs allowed to mature prior to IL-1\(\beta\) (or IL-1\(\alpha\)) exposure were less susceptible to matrix degradation and showed attenuated loss of mechanical properties as compared to those exposed at earlier time points (Cook et al. 2001; Lima et al. 2008; Francioli et al. 2011). Kwon et al. showed that biomaterial selection can influence chondrocyte response to inflammation, where silk-based scaffolds were chondro-protective compared to PLA-based systems (Kwon et al. 2013). In addition, it has been noted that the ability of chondrocytes to robustly produce matrix and integrate with native cartilage is limited in an inflammatory environment (Djouad et al. 2009).

While chondrocytes can inherently synthesize cartilage matrix components, autologous cells are of limited supply for regenerative medicine applications, particularly in patients with advanced OA. Mesencymal stem cells (MSCs) are an alternative cell source that may be appropriate for cartilage repair. Bone marrow-derived MSCs can differentiate along chondrogenic, osteogenic, and adipogenic lineages, when cultured in the appropriate scaffold environment and with defined chemical stimuli (Pittenger 1999). A number of
studies have described MSC chondrogenesis in the presence of TGF-β to induce the production of proteoglycans and collagen type II and the gradual development of near-native functional properties (Huang et al. 2010; Moutos et al. 2010; Erickson et al. 2012; Kim et al. 2012; Bian et al. 2013). In addition, MSCs may also exhibit immunomodulatory behaviors (da Silva Meirelles et al. 2009; Bunnell et al. 2010). Despite the potential of this cell source, MSCs have been shown to produce less functional matrix compared to chondrocytes (Mauck et al. 2006; Farrell et al. 2012), to have a distinct molecule phenotype even after differentiation (Huang 2010), and to be more susceptible to nutrient deprivation-induced cell death (Farrell et al. 2015). Based on these findings, it is likely that MSC-based engineered cartilage will show a distinct response to cytokine challenge. Data supports that MSC-based engineered cartilage, similar to chondrocyte-based constructs, is affected by the presence of inflammatory cytokines. IL-1β and TNF-α inhibited MSC chondrogenesis in pellet and 3D constructs by down-regulating SOX9, COMP, collagen type II, and aggrecan expression, despite the continued presence of chondrogenic growth factors (Majumdar et al. 2001; Wehling et al. 2009). These results suggest that MSCs are sensitive to cytokines during and after chondrogenesis.

To determine how cell source impacts the response of engineered cartilage to inflammatory stimuli, this study directly compared the effect of IL-1β and TNF-α on chondrocyte- and MSC-based constructs. Cellular- and tissue-level response to cytokine exposure was evaluated by measuring release of catabolic mediators (nitric oxide, MMP activity) and changes in matrix composition and mechanical properties. Findings from this study may have implications of the clinical use of chondrocyte- or MSC-derived engineered cartilage in the inflammatory environment of a damaged or osteoarthritic joint.
7.2. Methods

7.2.1. Engineered Cartilage Fabrication and Culture with Inflammatory Cytokines

Articular cartilage was harvested from the trochlear groove and femoral condyles of juvenile bovine knees (aged 2 to 6 months; Research 87, MA). Chondrocytes were isolated as previously described (Mohanraj et al. 2014; Mohanraj et al. 2014). Briefly, cartilage was minced, digested in collagenase for 18 to 24 hours at 37°C (type 2 collagenase, 298 U/mg, Worthington Biochemical Corp, NJ). The cell suspension was filtered through a 70µm cell strainer and washed (2% penicillin/ streptomycin/ fungizone (PSF) in phosphate buffered saline, 1750 rpm for 15 minutes) to collect chondrocytes. Mesenchymal stem cells (MSCs) were isolated from bone marrow harvested from the tibia and femur of juvenile bovine knees and expanded in monolayer (P2 or P3) (Mauck et al. 2006). Each cell type was suspended in chemically defined medium (CM-, 40 million cells/mL) and combined with 4% w/v agarose (Type VII, in PBS) in equal volumes to form a cell-agarose solution at a final cell concentration of 20 million cells/mL in 2% w/v agarose. The cell slurry was cast between glass plates, gelled at room temperature for 10 minutes, and biopsy punched to form uniform cylindrical constructs (Ø: 4mm, H: 2.25mm). Constructs were pre-cultured for 21 days in chemically defined medium containing 10ng/mL TGF-β (CM+, DMEM, 1% PSF, 1% ITS+ premix, 40 µg/mL L-proline, 50 µg/mL ascorbic acid, 0.1 µM dexamethasone, 0.5% v/v bovine serum albumin, and 100µg/mL sodium pyruvate (Johnstone et al. 1998)). Following this pre-culture period, chondrocyte- and MSC-seeded constructs were transferred to CM- (medium lacking TGF-β) and exposed to either IL-1β or TNF-α for 6 days. To evaluate dose-response, cytokines were
added to the medium at increasing concentrations (1, 5, and 10 ng/mL). Medium and cytokines were refreshed on day 3, with medium harvested on days 3 and 6.

7.2.2. Assays to Measure Engineered Cartilage Response to Inflammatory Cytokines

Engineered cartilage mechanical properties were evaluated by unconfined compression testing (N=4 per condition) after 6 days of cytokine exposure, as previously described (Mauck et al. 2006). Constructs were subjected to a 2 gram creep load, followed by a stress relaxation protocol (10% compressive strain applied at 0.05 strain/s held for 1000s) and dynamic loading (1% strain, 1Hz, 10 cycles) to measure the equilibrium and dynamic modulus (Huang et al. 2009). For biochemical assays, constructs were subsequently lyophilized and papain digested overnight. Glycosaminoglycan (GAG) content was measured using the dimethylmethylene blue (DMMB) assay (Farndale et al. 1986) and collagen content via the OHP assay (Stegemann et al. 1967). GAG and OHP were normalized by construct wet weight. Medium (N=2 per condition, for 5 constructs cultured together) was analyzed for the GAG release, as well as established catabolic mediators, nitric oxide (NO, Griess assay, Promega (Henrotin 2003)) and MMP activity after APMA activation (Generic MMP 520 Fluorescence kit, Anaspec). At the terminal time point, constructs were also fixed (4% paraformaldehyde), ethanol dehydrated, and paraffin embedded (N=1 per condition). Constructs were sectioned across the cross-sectional face (8μm sections) and stained with Alcian Blue to qualitatively assess changes in proteoglycan content.
7.2.3. Statistical Analysis

The dose-dependent effects of IL-1β and TNF-α on mechanical properties and matrix content were analyzed using a one-way ANOVA with Tukey’s post-hoc test (p<0.05) for each cell type. GAG release, and NO and MMP production were evaluated using a two-way ANOVA with Tukey’s post-hoc test (p<0.05) for chondrocytes and MSCs individually, with time in culture (day 3 or day 6) and dose serving as independent variables. Differences in the magnitude of change between chondrocyte and MSC response, for each cytokine concentration, was also assessed using a two-way ANOVA with Bonferroni’s post-hoc test (p<0.05). Equilibrium modulus data was also fit to a single-phase exponential decay curve, to test the hypothesis that chondrocyte and MSC responses could be fit with the same parameters. Rejection of this hypothesis would indicate differences in the rate of decay in mechanical properties as a function of cytokine concentration between the two cell types. Results shown here are representative of two (chondrocytes) or three (MSCs) independent experiments.

7.3. Results

7.3.1. Effect of Cytokines on Mechanical Properties and Matrix Composition

Exposure to either IL-1β or TNF-α significantly reduced mechanical properties in a dose-dependent manner in chondrocyte- and MSC-derived constructs. Although exposure to IL-1β or TNF-α at 1ng/mL induced a moderate decrease in equilibrium (Figure 7-1, A-B) and dynamic (Figure 7-1, C-D) moduli, a greater reduction in properties was observed at both 5 and 10ng/mL (no differences were found between these two groups). MSCs were more sensitive than chondrocytes to cytokine exposure at a given dose (e.g. Eγ, 5ng/mL, IL-1β: -79% CH, -97% MSCs, TNF-α: - 71% CH, -99% MSCs vs. control), with nearly
complete loss of mechanical integrity at the highest concentrations of IL-1β and TNF-α assayed. A single-phase exponential decay curve fit to equilibrium modulus data showed, for both cytokines, a significant increase in the decay rate constant (K) for MSCs compared to chondrocytes (Figure 7-1, E-F). ‘Half-life’ was also calculated, or the cytokine concentration at which a 50% reduction in mechanical properties would be expected. For both IL-1β and TNF-α, chondrocytes required higher concentrations in order to elicit the same response in MSC-derived engineered cartilage (IL-1β, CH: 0.58 vs MSCs: 0.33 ng/mL and TNF-α, CH: 1.83 vs. MSCs 1.18 ng/mL).
Figure 7-1. Mechanical properties of MSC-derived engineered cartilage are more sensitive to cytokine challenge compared to chondrocyte-derived constructs. Construct equilibrium ($E_Y$) and dynamic ($G^*$) modulus show a dose-dependent effect for (A-C) IL-1β and (B-D) TNF-α. Cytokine challenge of MSC-derived cartilage shows a near complete loss of mechanical integrity at the highest cytokine concentrations (5 and...
10ng/mL). (E-F) Fit of an exponential decay curve indicated an accelerated rate of decline in mechanical properties (K) for MSC- compared to chondrocyte-derived constructs. p<0.05 for * vs. control, + vs. 1ng/mL and control, § vs. MSC-derived constructs at the same cytokine concentration.

GAG content in chondrocyte- and MSC-derived constructs was consistent with the dose-dependent effect of cytokines on mechanical properties (Figure 7-2, A-B). That is, MSC-derived constructs showed a greater magnitude of GAG loss compared to chondrocytes across all IL-1β or TNF-α concentrations (e.g. GAG%WW, 5ng/mL, IL-1β: -47% CH, -64% MSCs, TNF-α: - 37% CH, -74% MSCs vs. control). In contrast, collagen content was relatively stable with cytokine challenge (Figure 7-2, C-D). Chondrocyte-derived constructs exposed to either IL-1β or TNF-α consistently showed a modest decrease in collagen content at concentrations of 5 and 10ng/mL. No significant differences in collagen content were observed in MSC-derived constructs in response to either cytokine. Alcian Blue staining for proteoglycans reflected changes in GAG content and mechanical properties. In chondrocyte-derived constructs, less intense extracellular matrix staining was seen following exposure to IL-1β and TNF-α as compared to the control group (Figure 7-3, A-D, I-L). In all cases, pericellular matrix staining was present around chondrocytes regardless of the treatment condition. MSC-derived constructs, in comparison, showed reduced extracellular and pericellular matrix staining intensity with the greatest decrease seen at the highest concentrations (Figure 7-3, E-H, M-P).
Figure 7-2. Matrix loss from engineered constructs reflects changes in mechanical properties with IL-1β or TNF-α treatment. (A-B) GAG content was reduced in MSC-derived constructs to a greater extent than chondrocyte-derived constructs, and in a dose-dependent manner. Conversely, collagen content (C-D) was largely unaffected by cytokine exposure. Chondrocyte-derived constructs showed a moderate decrease in response to IL-1β or TNF-α exposure; no significant differences were found for MSCs. p<0.05 for * vs. control, + vs. 1ng/mL and control, $ vs. MSC-derived constructs at the same cytokine concentration.
Figure 7-3. Alcian Blue staining for proteoglycans in engineered cartilage treated with IL-1β or TNF-α. (A-D, I-L) In chondrocyte-derived constructs, a reduction in extracellular matrix staining intensity was observed. (E-H, M-P) In comparison, MSC-derived constructs showed progressive loss of extracellular matrix and pericellular matrix staining in a dose-dependent manner with cytokine treatment.

7.3.2. Cytokine-mediated Release of GAG, NO and MMPs

Catabolic mediators transiently increased in response to cytokine challenge, as measured by nitric oxide release to and MMP activity in the medium. Nitric oxide production (measured as nitrite, a byproduct of NO breakdown) was dose-dependent and was consistently greater on day 3 compared to day 6, and for chondrocyte- compared to MSC-
derived constructs at higher concentrations (Figure 7-4, A-B). In comparison, the pattern of MMP activity was both cytokine-specific and cell type-dependent. Although absolute MMP activity between chondrocyte- and MSC-derived constructs was similar, the activity levels relative to un-treated controls showed differences with IL-1β treatment. Chondrocyte-derived constructs showed an increase in relative activity compared to MSC-derived cartilage on both day 3 at 10ng/mL (CH: 27.6x CH, MSCs: 3.5x vs. control) and day 6 (CH: 26.2x, MSCs: 6x vs. control) at the highest concentrations (Figure 7-4C). However, only MSC-derived constructs were sensitive to IL-1β at the lowest concentration (1ng/mL) on day 6 and showed an increase in overall activity compared to day 3. A markedly different response was observed in constructs following exposure to TNF-α (Figure 7-4D). Although minimal MMP activity was detected on day 3, the response of chondrocyte-derived constructs was higher than that of MSCs. However, by day 6, activated MMP levels were significantly increased for MSC-derived constructs compared to control and chondrocytes for all TNF-α concentrations (e.g. 10ng/mL, CH: 0.6x, MSCs: 6.4x vs. control). To determine how these catabolic mediators effect matrix degradation, GAG release to the medium was also measured (Figure 7-4, E-F). In response to either cytokine, GAG release from chondrocyte-derived constructs was lower on day 3 compared to day 6 at concentrations of 5 and 10ng/mL. No differences were observed between time points for MSC-derived constructs, with GAG release maintained at this elevated level throughout the culture period. For IL-1β, matrix release was also higher for MSC-compared to chondrocyte-derived constructs on day 3 (e.g. 10ng/mL, CH: 1.9x, MSCs: 3.3x vs. control), but by day 6 the chondrocyte response was equivalent (e.g. 10ng/mL, CH: 5.5x, MSCs: 4x vs. control) (Figure 7-4E). Following exposure to TNF-α, a similar response difference between cell types was observed on day 3 (e.g. 10ng/mL, CH: 1.9x,
MSCs: 4.2x vs. control). However, by day 6 GAG release from chondrocyte-derived constructs matched that of MSCs (e.g. 10ng/mL, CH: 4x, MSCs: 5x vs. control; Figure 7-4F).
Figure 7-4. Differential release of catabolic mediators by chondrocyte- and MSC-derived engineered cartilage in response to cytokine challenge. (A-B) Nitric oxide production (nitrite) was higher for chondrocyte-derived constructs compared to MSCs, and on day 3 compared to day 6, for both cytokines and across all doses. (C-D) In response to IL-1β, MMP activity increased from day 3 to day 6, in a dose-dependent manner.
Although absolute measurement of activated MMPs was higher for MSC-derived constructs, the relative increase compared to control was larger for chondrocytes due to a lower baseline response. In contrast, TNF-α induced low levels of MMP activity on day 3 for both chondrocytes and MSCs; however, by day 6 MMP activity for MSC-derived constructs increased to match that observed for IL-1β. (E-F) GAG release to the media was consistently lower for chondrocyte-derived constructs than MSCs on day 3 for both cytokines. However, by day 6, minimal differences in matrix loss were observed between both cell types.

7.4. Discussion

The inflammatory environment of an osteoarthritic joint presents a significant challenge for the success of tissue engineered cartilage strategies. Here, we show that inflammatory cytokines, including IL-1β and TNF-α, induce degenerative changes in structure-function properties through the action of catabolic mediators (e.g. NO, MMPs, ADAMTS). These factors, in combination with cytokine-mediated inhibition of matrix biosynthesis, would be expected to prevent cell-based constructs from effectively restoring load-bearing capacity. Two of the primary cell sources utilized for cartilage tissue engineering are native chondrocytes and mesenchymal stem cells. Although both cell types have been successfully used to engineer cartilage in vitro (Elisseeff et al. 2001; Wang et al. 2005; Moutos et al. 2007; Erickson et al. 2009; Sharma et al. 2013), challenges remain in repairing cartilage within the in vivo joint environment (Gotterbarm et al. 2006; Holland et al. 2007; Mrugala et al. 2008; Wang et al. 2010; Kim et al. 2015; Fisher et al. 2016). To determine whether inflammation-induced degeneration is dependent on cell source in this context, we directly compared the response of chondrocyte- and MSC-derived constructs
to IL-1β and TNF-α in an in vitro model system. While both cell types showed a dose-dependent reduction in mechanical properties, MSC-derived constructs were more sensitive to inflammatory challenge, with complete loss of structural integrity measured at high cytokine concentrations. Consistent with these observations, construct GAG content and histological staining intensity were markedly reduced; however, collagen content was only moderately affected by cytokine exposure. Although collagen contributes to the equilibrium and dynamic mechanical properties (Charlebois et al. 2004; Park et al. 2008), the loss of GAG may play a larger role in engineered cartilage due to the immaturity of the collagen network (<1%WW vs. 15-20%WW in native tissue (Mow et al. 2005)). Our results complement previous studies describing matrix depletion in response to IL-1β and TNF-α in engineered tissues using chondrocytes (Cook et al. 2001; Lima et al. 2008; Francioli et al. 2011; Sun et al. 2011) and MSCs (Wehling et al. 2009; Ousema et al. 2012), as well as iPSCs (Willard et al. 2014). Similarites across cell types include 'maturation-dependent' effects of cytokines that have previously been characterized for chondrocytes constructs (Cook et al. 2001; Lima et al. 2008; Francioli et al. 2011), and more recently for MSCs. Ousema et al. showed that IL-α significantly impaired MSC chondrogenesis in woven PCL scaffolds when present in the media from the onset of culture (with TGF-β3) as compared to constructs pre-cultured for 2 weeks prior to cytokine treatment (Ousema et al. 2012). This inhibition of chondrogenic (and osteogenic) differentiation of MSCs (29, 30, 47) may be due to inhibition of Sox-9 transcriptional activity, which is critical for collagen and aggrecan synthesis (Murakami et al. 2000).

To determine if cell source affects the production of catabolic factors, nitric oxide and MMPs were measured in culture medium during exposure of constructs to IL-1β and TNF-
NO promotes cartilage degradation through inhibition of aggrecan and collagen synthesis, activation of MMPs, and increased susceptibility to other oxidants which cause apoptosis (Scher et al. 2007). Activated MMPs further contribute to destruction of the collagen network (Tetlow et al. 2001; Piecha et al. 2009), and together with NO may mediate the loss of construct functional properties and biochemical composition seen in this work. Higher NO concentrations were consistently found in the media of chondrocyte-derived constructs cultured with either IL-1β or TNF-α, as compared to MSCs at both time points (days 3 and 6). In comparison, MMP activity showed a differential response that depended on both the cytokine and cell type. Although the absolute measurements of MMP activity were higher for MSC-derived constructs than chondrocytes following IL-1β treatment, the baseline level of activity in naïve constructs was also higher. As a result, the relative increase in MMP activity (vs. control) was greater for chondrocyte-derived constructs than MSCs, though these findings suggest that MSC-derived constructs are actively degrading formed matrix at a higher rate in naïve (control) constructs. Despite the overall increase in the activity of catabolic mediators for chondrocyte-derived constructs, GAG released to the media was initially higher for MSC-derived constructs on day 3.

The exacerbated matrix loss from MSC-derived constructs supported the observed changes in mechanical and biochemical properties, and suggests that MSCs may exhibit greater sensitivity to IL-1β than chondrocytes. These results contrast with the response to TNF-α treatment, where only MSC-derived constructs showed a marked increase in activated MMPs on day 6 of culture. Although NO production was elevated for chondrocytes throughout, and MMP activity was only elevated at the later time point for
MSCs, GAG released to the media was still higher for MSC-derived constructs than chondrocytes on day 3. Similar to the effect of IL-1β, MSCs seem to be more sensitive to TNF-α than chondrocytes in engineered cartilage. Notably, the temporal patterns of NO and MMP production were different for each cytokine and cell type, highlighting the need for additional studies to investigate the regulation of relevant pro-inflammatory signaling pathways. The NF-κB pathway is one of particular interest, since IL-1β and TNF-α induce catabolic activity through associated signaling cascades (Hess et al. 2009; Wehling et al. 2009). This pathway is a primary target for therapeutic intervention in OA and studies have focused on the development of in vitro, engineered cartilage models to investigate the effect of NF-κB inhibition (e.g. small molecules (SC-514 (Willard et al. 2014)) and dominant negative expression of IkB (Wehling et al. 2009)). Recent work by Brunger et al. also demonstrated that genome engineering (CRISPR/Cas9) can be used to create stem cells that produce antagonists in response to IL-1 and TNF-α treatment in an auto-regulated manner to protect against cartilage degradation (Brunger et al. 2017). In this context, the choice of cell source for cartilage tissue engineering may not only influence the likelihood of repair in vivo, but also the response to candidate therapeutics for the treatment of OA.

7.5. Conclusion

Overall, this study demonstrated that cell source (native chondrocytes vs. MSCs) influences the response of engineered cartilage to pro-inflammatory cytokines. MSC-derived constructs were more responsive than chondrocytes to IL-1β and TNF-α, with greater loss of matrix and functional properties at lower doses of cytokine challenge. While elevated levels of NO and MMPs were generally observed for chondrocyte-derived
constructs, exacerbated construct degeneration was observed for MSCs, indicating an increased sensitivity of MSCs to catabolic mediators. Although MSCs and chondrocytes both have the capacity to produce matrix in engineered constructs, fundamental differences exist between the two cell types that impact their potential regenerative capacity upon implantation in vivo. Our findings illustrate these differences in the context of a cytokine challenge, and further support the notion that choice of cell source in tissue engineering approaches may influence the likelihood of successful repair within the inflammatory environment of OA.
Chapter 8. Mechanically-Activated Microcapsules for ‘On-Demand’ Drug Delivery in Dynamically Loaded Musculoskeletal Tissues

8.1 Introduction

Controlled drug delivery has several advantages over systemic delivery of therapeutics, including localized delivery to specific locations, maintenance of drug concentrations within the therapeutic range, and preservation of activity for long-term administration (Kost et al. 2012). More specifically, microcapsule systems encompass a subset of controlled drug delivery approaches that are characterized by size (diameter of 1-1000µm) and a core-shell morphology. The active agent encapsulated within a solid shell is protected against degradation and other environmental factors, and release can be controlled as a function of time or stimulus in order to program the release profile to match a desired profile (Singh et al. 2010; Datta et al. 2014). The use of a stimuli-responsive approach enables self-regulation, wherein physiological feedback actively controls release kinetics (Siegel 2014). These internal triggers include temperature (Windbergs et al. 2013) and pH (Abbaspourrad et al. 2013; Yang et al. 2014), as well as enzyme-substrate (Qi et al. 2009) reactions. When activated, such physicochemical mechanisms initiate capsule dissolution or disruption, resulting in the release of the encapsulated drug. The sensitivity of these triggers can be tuned for a particular physiologic or pathophysiologic state in order to initiate and maintain therapeutic release as is necessary.

To date however, few systems have taken advantage of the mechanical environment to initiate release. Recently, Korin et. al. designed microscale aggregates of nanoparticles which disaggregate under the abnormally high shear stresses found in narrowed blood
vessels to deliver tissue plasminogen activator to dissolve clots (Korin et al. 2012). Alternatively, mechanical stimuli have been combined with passive degradation mechanisms for drug release from microdepots within stretchable substrates (Di et al. 2015) or with thermal mechanisms for liquidization of a solid core to enable pressure-induced release in the GI tract (Wilde et al. 2014). Building upon the premise of mechanically regulated systems, we sought to design microcapsules with ‘on-demand’ release that could operate in the dynamic mechanical context of musculoskeletal tissues in order to stimulate tissue repair and regeneration.

Musculoskeletal tissues within the human body experience mechanical stimulation across multiple force magnitudes and length scales, from mechanotransduction at the cellular level (Engler et al. 2006) to the large and dynamic loads seen in articulating joints. These forces not only maintain tissue integrity (through modulation of cellular activity), but can also, at supraphysiologic levels, initiate degenerative processes that require surgical intervention to restore load-bearing function (Kijowski et al. 2014; Carter et al. 2015). Healing capacity is minimal in articular cartilage, which functions to bear and distribute loads in joints. Lesions in the articular surface can cause pain and disability, and may even initiate further degenerative processes (Lee et al. 2000). These mechanical demands, combined with the inflammatory milieu (Sward et al. 2012; McNulty et al. 2013) of an injured or osteoarthritic joint, present a challenging environment, and so repair of cartilage defects remains a clinical challenge. To date, tissue engineering strategies for cartilage repair have used a wide variety of cell sources (Chung et al. 2009; Erickson et al. 2009; Diekman et al. 2012; Craft et al. 2013), scaffold materials (Wang et al. 2005; Erickson et al. 2009; Moutos et al. 2010; Sharma et al. 2013), and growth factors (Johnstone et al. 1998; Elisseeff et al. 2001; Wang et al. 2009; Kim et al. 2012) to successfully grow tissues.
in vitro that mimic the matrix composition and mechanical properties of native cartilage. However, challenges remain in successfully repairing cartilage defects in vivo. In particular, growth factors, which are critical for engineered cartilage maturation in vitro, require local and controlled delivery in vivo in order to avoid the undesirable effects associated with supraphysiologic and systemic delivery (e.g. synovial fibrosis, endochondral ossification (van Beuningen et al. 2000; Bakker et al. 2001)).

Advances in biomaterials design for cartilage repair have allowed for greater control over biofactor release in vivo in terms of spatial, temporal, and multi-factor delivery (Gotterbarm et al. 2006; Holland et al. 2007; Mrugala et al. 2008; Fan et al. 2010; Wang et al. 2010; Kim et al. 2015; Lam et al. 2015; Fisher et al. 2016). While these delivery systems have improved defect fill, matrix deposition, and mechanical properties compared to biofactor-free formulations, achieving native-like cartilage properties remains elusive. Moreover, approaches delivering multiple factors (e.g. TGF-β and IGF-1) have often failed to elicit optimal in vivo responses, despite evidence of staged delivery in in vitro (Elisseeff et al. 2001; Anseth et al. 2002; Holland et al. 2007). These limitations may in part be due to the nature of release in these systems, where biofactors emerge from implanted materials based on passive-release mechanisms. Stimuli-responsive approaches, wherein release is based on features of the repair environment, may be required to enable more active and precise control over the timing and long-term maintenance of delivery to improve in vivo outcomes.

To address this need, we developed mechanically-activated microcapsules (MAMCs) as a stimuli-responsive drug delivery system in which the mechanical loading environment is
harnessed to elicit a therapeutic response. This bioengineering approach is inspired by self-healing polymer systems used in material science applications, where microcapsules embedded in a polymer matrix rupture upon physical damage releasing a catalyst for polymerization and repair of the surrounding material (White et al. 2001). We generated uniform populations of biocompatible and biodegradable microcapsules (Lee et al. 2012; Tu et al. 2012) using a custom microfluidic device, and established the tunability of their mechano-activation based on the physical attributes of the microcapsule and its composition. From this parameter space, we selected a cohort of MAMCs and evaluated their response in 3D matrices that mimicked regenerating cartilage tissue, and showed that the physical properties of the microenvironment modulated MAMC mechano-activation under dynamic loading. Finally, we demonstrated the feasibility of encapsulating biologically active compounds within MAMCs, and showed that mechanically mediated release of TGF-β3, a growth factor important in the maturation of engineered cartilage, could promote tissue formation. These data establish a novel means by which to tune biofactor release based on the physiologic dynamic mechanical loading environment of musculoskeletal tissues.

8.2. Methods

8.2.1. Polymer Microcapsule Fabrication

Microcapsules were fabricated using a glass capillary microfluidic device as previously described (Lee et al. 2012; Tu et al. 2012) to form a monodisperse Water/Oil/Water (W/O/W) double emulsion from three fluid phases (Figure 8-1 A,B). The inner aqueous phase was maintained at pH 7.4 and, for all experiments, included a fluorescent compound to visualize microcapsule integrity. The middle oil phase consisted of poly(D,L-lactide-co-glycolid) acid (PLGA) dissolved in chloroform or dichloromethane with the
addition of Nile Red (100µg/mL, Sigma, N3013) to fluorescently visualize the shell wall. PLGA of various molar ratios of lactic:glycolic acid were evaluated: ester-terminated PLGA 50:50 (MW 38,000-54000, ester-terminated, Sigma 739944), PLGA 75:25 (0.55-0.75 dL/g, MW 76,000-119,900, ester-terminated, Lactel B6007-1), PLGA 85:15 (0.55-0.75 dL/g, MW 76,000-119,900, ester-terminated, Lactel B6006-1). The outer aqueous phase contained 2% wt poly(vinyl alcohol) (PVA). All double emulsions were collected in 0.1-1% w/v bovine serum albumin (BSA, Sigma A7906) in phosphate buffered saline (PBS, P4417), with the pH tuned to the PLGA composition (PLGA 50:50 ≥ pH 7, PLGA 85:15 and 75:25 > pH12) to maintain stability during microcapsule condensation (Figure 8-1C). Shell thickness and outer diameter of the microcapsules was tuned by controlling the sheath flow rates (inner and middle flow rates, Q) and PLGA concentration (% w/v) in the middle phase. Average estimated shell thickness was derived from conservation of mass as previously described (Tu et al. 2012) where the inner and outer radii of the double emulsion was quantified from images taken during fabrication using an upright optical microscope. The average outer diameter of fully condensed microcapsules was measured from the maximum intensity projection of a confocal z-stack image (Nikon A1R+ confocal microscope, 20X magnification, NIS Elements AR software). Shell thickness to outer diameter (t/D) ratio was calculated as a metric to assess effect of microcapsule geometry on mechano-activation. MAMC concentration (as defined by the number of microcapsules/µL, n=3-4 aliquots per fabrication batch) was also measured by confocal microscopy within the first three days (‘Day 0’) following microcapsule condensation prior to conducting mechano-activation experiments.
Figure 8-1. Fabrication of mechanically-activated microcapsules in a glass capillary microfluidic device. (A) Schematic and (B) image of water/oil/water (W/O/W) double emulsion generation. (C) Schematic showing the time evolution of microcapsules.

8.2.2. 2D Mechano-Activation

A single layer of microcapsules (~500 MAMCs) was seeded between two glass coverslips and uniaxially compressed at a controlled strain rate ($\dot{\varepsilon} = 0.5/s$) to defined loads (0.1 to 5N) (based on parallel plate compression testing methods previously described (Fery et al. 2007)). Unloaded (0N) microcapsules seeded between coverslips served as negative controls. Microcapsules in these experiments contained a model drug, fluorescein isothiocyanate-dextran (0.01 to 0.05% w/v, FITC-dextran, 2MDa, Sigma FD2000S) with 1% w/v BSA in PBS. Following compression, microcapsules were collected into PBS for overnight incubation at 37C, 5% CO$_2$ to allow for complete release of encapsulated contents. Microcapsules were imaged on a confocal microscope (4X magnification, microcapsule mid-plane) before and after load application. Images were analyzed to
quantify the number of intact microcapsules based on a threshold of >50% of ‘Day 0’ fluorescence signal intensity (FITC signal), as well as the total number of microcapsules (Nile Red signal). The fraction of empty microcapsules (% empty) was then calculated at each load level. Representative confocal z-stack images (20X magnification) were also obtained for volume reconstructions. In select experiments, scanning electron micrographs (SEM, FEI Quanta 600 ESEM) were obtained to visualize the failure morphology of microcapsules. Mechano-activation was assessed on Day 1 for microcapsule geometry experiments and at weekly time points for up to 10 weeks for polymer degradation experiments, where microcapsules were incubated at 37°C and 5% CO₂.

8.2.3. 3D Mechano-Activation

Microcapsules were embedded in poly(ethylene glycol) diacrylate (PEGDA, MW 508 kDa) and cast between two glass plates to create a uniform hydrogel sheet. The polymer matrix was cross-linked using a free-radical initiation method as previously described (Temenoff et al. 2002; Saxena et al. 2016) using ammonium persulfate (APS, 1mg/mL, BioRad 1610700) and tetramethylethylenediamine (TEMED, 0.4% v/v, BioRad 1610801). A methacrylated dye (9-anthracenylmethyl methacrylate, Sigma 578207, 0.1mg/mL) was also included in some cases to visualize the hydrogel matrix in which the microcapsules were embedded. Cylindrical constructs (Ø: 4mm, H: 2.25mm) were cored via biopsy punch from the hydrogel sheet. Unconfined compression testing of blank hydrogels across a range of PEGDA concentrations (5-20% w/v) was used to determine the equilibrium modulus of the encapsulating matrix. For static-loading experiments, microcapsule-laden hydrogel constructs (0.1% v/v) were evaluated using a custom confocal-mounted...
compression device (Farrell et al. 2012). MAMC deformation was tracked over 20% compressive strain applied to the hydrogel surface, with strain steps of 5% followed by compression until hydrogel failure. At each strain step, confocal z-stack images were collected (10X magnification, depth: 300 µm), processed to acquire maximum intensity projections of the microcapsule shell, and thresholded to determine the bounding box lengths (x- and y-axis) for each identified microcapsule object in the binary image. Microcapsules in contact with one another or void of internal fluorescent contents were excluded from analysis. Deformation was quantified according to the MAMC strain in the direction of loading (E₁₁, x-direction) and perpendicular to (E₂₂, y-direction) loading. MAMC strain was computed as the difference in bounding box length between each strain step and the initial, un-loaded (ε=0) state, normalized to the initial, un-loaded state. For dynamic loading experiments, constructs were cyclically loaded in unconfined compression (Instron Electropuls) under physiologic conditions (PBS, 37°C). Free-swelling constructs maintained under the same conditions served as negative controls. Constructs were compressed to a 2% tare strain, followed by 20% cyclic strain applied at 5Hz for up to 1.5 hours (shorter time intervals were used for temporal experiments). After loading, all constructs were incubated at 37C overnight and imaged the following day. Maximum projection images of confocal z-stacks of constructs collected prior to dynamic loading (10X, depth: 300µm) were compared to post-dynamic loading images of the same microcapsule population in order to compute the number of ruptured microcapsules (% dynamic empty).
8.2.4. Biofactor Activity

Microcapsules were fabricated containing activated Transforming Growth Factor-β3 (TGF-β3, R&D 243-B3/CF, 10µg/mL) with BSA (1mg/mL) and AlexaFluor 488-BSA (100µg/mL, Life Technologies, A13100) in PBS. Microcapsules containing only AlexaFluor 488-BSA (protein control) or the TGF-β3 activation solution (vehicle control, 1mg/mL BSA, 4mM HCl) served as negative controls. All solutions were filtered for sterility prior to fabrication for use in cell culture experiments. TGF-β3 activity was assessed in the supernatant of ruptured and intact microcapsules via ELISA (R&D Systems, DY243). Microcapsules were ruptured using a TissueLyser LT system (Qiagen) in PBS. Both intact and ruptured microcapsules were diluted with an additional volume of PBS and centrifuged to separate the shell pellet from the supernatant. In a separate set of studies, microcapsules were stored at 4°C, with activity regularly assessed over three weeks to determine the shelf life of the growth factor. Functional activity of TGF-β3 released from microcapsules was measured using an engineered cartilage model previously established in the literature (Mauck et al. 2006). Briefly, mesenchymal stem cells were isolated from femoral and tibial bone marrow from juvenile bovine knees (Research 87, Bolyston, MA), cultured in basal medium during passaging (high glucose DMEM, 10% FBS, 1% penicillin/streptomycin/fungizone). MSCs (passage 2, 20 million cells/mL) were embedded in agarose (2% w/v, Type VII, Sigma) to generate cylindrical constructs (Ø: 4mm, H: 2.25mm). Microcapsules were ruptured as described above and diluted in chemically defined medium (CM). For this, the number of microcapsules was tuned such that 100% release would equate to a media concentration of 10ng/mL of TGF-β3 (Johnstone et al. 1998). Supernatant from intact TGF-β3 microcapsules, as well as from ruptured and intact ‘vehicle’ and ‘protein’ microcapsules served as delivery controls. CM with or without the
addition of exogenous TGF-β3 (10ng/mL) served as positive and negative aqueous controls, respectively. Cell-seeded constructs were cultured under the above media conditions for a period of 7 days. All media was prepared at the time of construct fabrication, with a separate aliquot frozen at -20°C for a media change on Day 4. Chondrogenesis was assessed by sulfated glycosaminoglycan (GAG) content (DMMB assay (Farndale et al. 1986)) and matrix deposition by histological evaluation (Alcian Blue, Nikon Eclipse Ni).

8.2.5. Statistical Analysis
To determine mechano-sensitivity in 2D environments, linear regression was applied to ‘% empty’ vs. load plots and the slopes compared between microcapsule batches (t/D ratios). For all following analysis, Bonferroni’s post-hoc test was used to make pairwise comparisons. In experiments evaluating the effect of polymer degradation kinetics on mechano-activation, one-way ANOVA was used to compare ‘% empty’ values across time points at 0.5N load. For static compression of 3D hydrogels, two-way ANOVA was used to compare microcapsule E_11 strain between 50 and 500 kPa hydrogels at each strain step. In dynamic loading experiments, one-way ANOVA was used to compare ‘dynamic % empty’ values across all hydrogel matrix stiffness values and free-swelling controls hydrogels. For TGF-β ELISA and GAG measurements, two-way ANOVA was used to compare intact vs. released conditions within a MAMC subset, and to determine dosage differences in TGF-MAMCs. One-way ANOVA was also used to compare all BSA, vehicle, TGF intact, and TGF released groups to CM- for GAG quantification. Finally, TGF activity monitored over time was analyzed using a one-way ANOVA to compare activity at each time point to Day 0 levels, for a given storage temperature.
8.3. Results

8.3.1. Microcapsule Physical Properties Determine Mechano-activation Thresholds

To determine how physical properties determine the thresholds for mechanical activation of MAMCs, two fabrication parameters were tuned to control shell thickness and the outer diameter of the microcapsule: (1) PLGA concentration and (2) fluid phase flow rates. Decreasing the PLGA concentration in the middle phase reduced the thickness of the shell wall while maintaining the same outer diameter (Figure 8-2 A,B, 1.8 and 2.7% w/v). The combined effect of a lower polymer concentration (0.9% w/v) and reduced flow rates of the inner and middle phases resulted in a thinner shell wall as well as a smaller outer diameter (Figure 8-2 A,B, 0.9% w/v). Thus, the resulting thickness to diameter (t/D) was not different between the two lower PLGA concentration MAMCs (0.9 and 1.8% w/v, t/D~0.006). Conversely, the highest PLGA concentration MAMCs had a higher t/D ratio (t/D=0.0096, 2.7% w/v). To determine how the mechano-activation was determined by these physical properties (shell thickness, outer diameter, and t/D ratio), a single layer of MAMCs was subjected to uniaxial compression. Mechano-activation on Day 1 was depended on the t/D ratio, with MAMCs having a lower t/D ratio (t/D~0.006) showing high sensitivity to increasing load, despite differences in outer diameter (Figure 8-2 C,D). In contrast, a higher t/D ratio (t/D=0.0096) rendered MAMCs insensitive to load within the same range.
Figure 8-2. MAMC physical attributes regulate mechano-activation. (A) Polymer concentration and fluid flow rates control MAMC shell thickness (t) and diameter (D) (N=7-8 double emulsion images/batch for shell thickness calculations). (B) Confocal mid-slices of MAMCs with different t/D ratios containing dextran (green) with labeled shells (red). (C-D) MAMCs rupture with increasing load, releasing fluorescent dextran from the aqueous core, dependent on the t/D ratio (*p<0.01 comparison of slopes, N=3 replicates/load/level/MAMC batch, mean ± SD). White bar = 100μm.

One consideration in the mechano-activation attributes of these MAMCs relates to the choice of polymer. Since PLGA is biodegradable, the rate of polymer degradation is likely to influence shell structural integrity and therefore, mechanoactivation as a function of time. To measure this, mechanical activation was assessed as a function of time for MAMCs stored in physiologic conditions (37°C, PBS), with comparisons made between a
fast-degrading MAMC (PLGA 50:50) and a slower degrading MAMC formulation (PLGA 85:15). Differences in the degradation profile of each PLGA type was confirmed by tracking the empty fraction of MAMCs over time under no load conditions (Figure 8-3). MAMCs that rapidly degraded (PLGA 50:50) showed a marked increase in sensitivity to applied load at Day 7 compared to Day 1 (Figure 8-4 B,C). The failure morphology of these MAMCs was confirmed by 3D volume reconstructions of confocal z-stack and SEM (Figure 8-4A), which showed a midline rupture in the shell wall and loss of internal fluorescent contents. By Day 14, degradation of the shell of PLGA 50:50 MAMCs resulted in 80% of the microcapsule population observed to be empty under zero load. Upon application of increasing loads, microcapsule shells fractured into fragments with 100% rupture observed. In comparison, for the slower degrading MAMC population (PLGA 85:15), the mechanical release profile remained stable over the first 3 weeks, and showed a slightly suppressed mechano-activation response at 4, 6, and 10 weeks of incubation (Figure 8-4 D,E).

Figure 8-3. Comparison of PLGA 50:50 and 85:15 MAMC degradation profiles under physiologic conditions. PLGA 50:50 MAMCs show an accelerated degradation rate (as determined by empty microcapsules under zero load) as compared to PLGA 85:15.
MAMCs with incubation at 37°C in PBS over 10 weeks (N=3 replicates/time point, mean ± SD).

Figure 8-4. Polymer degradation regulates MAMC mechano-activation profile. (A) Reconstruction of the volume (confocal) and morphology (SEM) shows the rupture mechanism in 3D as compared to intact microcapsules on day 7 (PLGA 50:50). (B,C) Fast-degrading microcapsules (PLGA 50:50) show a marked change in response between Day 1 (D1) and 7 (D7), with all microcapsules degrading by Day 14 (D14) and undergoing complete rupture. (D,E) Slow-degrading microcapsules (PLGA 85:15) exhibit a stable or slightly suppressed mechano-activation response compared to day 1 over 42 days of incubation under physiologic conditions. Comparison at 0.5N loading *p<0.01, *p<0.001,
and \#p<0.05 vs D1 (N=3 replicates/load level/time point with the exception of PLGA 85:15 D1 5N, D42 0.5N, and D70 0.25N where N=2 replicates/load level, mean ± SD).

8.3.2. Microcapsule Mechano-activation in 3D is Determined by Matrix Mechanics

To begin the translation of these mechano-activated microcapsules for release in a 3D setting, we next evaluated their release characteristics in engineered tissues under physiologic loading conditions. We hypothesized that microcapsule deformation and release in these 3D environments would depend on the stiffness of the surrounding matrix and its ability to transmit bulk deformation to the microcapsules. To track MAMC deformation as a function of matrix stiffness, MAMCs were embedded in PEGDA hydrogels with equilibrium moduli mimicking the mechanical properties of maturing engineered cartilage (Fisher Biomaterials 2014). Under static compression, MAMCs encapsulated within a soft, immature matrix (\(E_Y = 50\) kPa) deformed only minimally with increasing applied hydrogel strain (0 to 20% strain, 5% step strain increments, Figure 8-5A). Conversely, when situated in a stiff representative of a mature construct (\(E_Y = 500\) kPa), MAMC strain increased in both the direction of (\(E_{11}\)) and perpendicular (\(E_{22}\)) to that of applied hydrogel strain (Figure 8-5A), and upon hydrogel fracture, MAMCs remained permanently deformed. To further determine how loading cycles (in the context of changing matrix stiffness) influenced MAMC rupture and release, dynamic compressive loading was applied to MAMC-seeded hydrogel constructs. The fraction of microcapsules ruptured due to dynamic loading was quantified as a function of matrix stiffness by comparing pre- and post-loading confocal z-stacks of the same MAMC populations. We observed a graded microcapsule rupture response over a range of hydrogel equilibrium moduli (~25 to 150 kPa), with release dependent on the stiffness of the encapsulating matrix, with greater rupture in stiffer hydrogels (Figure 8-5, B). Loss of internal fluorescent
contents following dynamic loading in stiffer constructs is also visualized in representative 3D volume reconstructions (Figure 8-5 C,D). Together, these results highlight the role of matrix mechanical properties in regulating MAMC release in dynamic 3D environments.

Figure 8-5. MAMC mechano-activation in 3D. (A) Schematic of microcapsule stepwise compression in PEGDA hydrogels. Quantification of MAMC strain (E$_{11}$ and E$_{22}$) in hydrogels of two different stiffnesses demonstrates the effect of encapsulating hydrogel mechanical properties on microcapsule deformation, with representation images at each strain step shown (50 kPa hydrogels: N=41 microcapsules and 500 kPa hydrogels: N=30 microcapsules, *p<0.001 and +p<0.01 vs. 50 kPa, mean ± SEM). (B) Dynamic loading of hydrogels shows a graded increase in microcapsule rupture as the stiffness of the
encapsulating hydrogel increases (2% tare strain, 20% cyclic strain, 5Hz, 1.5 hours, FS = free swelling response range, N=3 to 6 hydrogels for dynamic loading, N=8 hydrogels for FS pooled across stiffness, *p<0.001 vs. FS, mean ± SD). Representative confocal images of MAMCs (C) free-swelling hydrogel conditions or (D) dynamically loaded hydrogels (matrix stiffness shown in the top left corner) show ruptured microcapsules mostly devoid of internal fluorescent contents (Alexa488-BSA, green).

8.3.3. Biofactors Encapsulated Within MAMCs Retain Biologic Activity

The clinical translation of MAMCs depends on the ability to encapsulate within microcapsules, biologically active compounds that can modulate or accelerate tissue repair upon mechanical-mediated release. In the context of cartilage tissue engineering for defect repair, TGF-β3 is known to stimulate the production of matrix constituents (e.g. proteoglycans, type II collagen) that contribute to the development of functional properties (Erickson et al. 2012). To determine if TGF-β3 remains active through fabrication and after release from MAMCs, the supernatant from intact and ruptured MAMCs was measured by ELISA (Figure 8-6A and 8-7). Microcapsules containing only the carrier protein (fluorescently-labelled bovine serum albumin, BSA) or the vehicle solution (BSA and HCl) were also tested as controls. No activity was measured in control MAMCs (intact or ruptured), and minimal TGF-β3 activity was measured in the supernatant of intact TGF-β3-containing MAMCs (Figure 8-6A). Conversely, TGF-β3 was measured in the supernatant of ruptured TGF-β3-containing MAMCs in a concentration-dependent manner based on the number of microcapsules ruptured (Figure 8-6A). To confirm that this released TGF-β3 from MAMCs could stimulate a biologic response, MSC chondrogenesis was assessed in agarose hydrogels cultured in the supernatant from intact and ruptured
MAMCs (BSA, Vehicle, and TGF-β3). Results were compared to constructs cultured with and without the exogenous addition of TGF-β3 as positive (CM+) and negative (CM-) controls, respectively. Quantification of sulfated GAGs produced within the engineered construct showed that TGF-β3 released from ruptured MAMCs stimulated matrix synthesis at the same level as exogenous addition of TGF-β3 (CM+; Figure 8-6B). While minimal TGF-β3 activity was measured in the supernatant of intact TGF-β3-MAMCs, the presence of the growth factor in the media was sufficient to induce GAG production in constructs, but at a lower concentration as compared to ruptured TGF-β3-MAMCs. Control MAMCs (BSA and Vehicle) did not stimulate a chondrogenic response, similar to base media lacking the growth factor (CM-). GAG accumulation within engineered constructs was confirmed by histological analysis using Alcian Blue, where expansion of pericellular staining and increased intensity of extracellular matrix staining was observed in CM+ and ruptured TGF-β3-MAMC conditions compared to CM- media (Figure 8-6C).
Figure 8-6. Biofactors released from MAMCs retain biological activity. (A) TGF-β3 released from MAMCs measured by ELISA shows a concentration-dependent response as determined by the number of microcapsules ruptured. BSA and Vehicle controls show no activity (N=3 replicates/group, *p<0.001 and +p<0.01 vs. intact MAMCs or indicated by line comparison, mean ± SD). MSCs seeded in agarose hydrogels undergo chondrogenic differentiation in response to released TGF-β3 released from MAMCs as measured by (B) GAG accumulation in the construct (N=4 replicates/group, *p<0.05, vs CM- or indicated by line comparison, mean ± SD) and (C) histological evaluation using Alcian Blue staining (N=2 replicates/group).
Figure 8.7. Bioactivity of TGF-β3 within MAMCs is sensitive to the storage temperature (4°C or 37°C in PBS). ELISA measurements over 22 days show a more rapid decline in TGF-b3 activity when MAMCs are stored at 37°C (within 4 days), as compared to storage at 4°C (within 8 days, N=3 replicates/time point/storage temperature; *p<0.001, +p<0.01, #p<0.05 vs. ruptured or intact D0 measurements, mean ± SD).

8.4. Discussion
To advance repair in dynamically loaded musculoskeletal tissues, we developed a novel class of mechanically-activated microcapsules (MAMCs) that enable autonomic healing through the release of growth promoting factors in response to the mechanical environment. MAMCs take advantage of this physiologic loading to initiate release when microcapsule failure thresholds are met. We demonstrated that these failure thresholds are governed by the physical attributes (t/D ratio), polymer degradation rate, and ratio of MAMC to matrix mechanical properties. Additional factors, such as the microcapsule-matrix adhesion properties (Wu et al. 2008) can also be tuned to influence MAMC rupture behavior. Interfacial adhesion may likewise be modulated by changing physical...
characteristics (e.g. surface roughness of the shell wall (Blaiszik et al. 2009)) or the nature of the interaction (e.g. electrostatic interactions (Raichur et al. 2006)). These and other modifications can be used to generate a suite of mechano-responsive microcapsules, whose release is governed by physical inputs.

Figure 8-8. MAMC repair of cartilage defects. MAMC physical properties can be tuned for programmed and extended release in maturing repair tissue exposed to physiologic dynamic loading. Biofactors released from microcapsules allow for matrix deposition in the repair tissue, resulting in a stiffer environment, which can then engage and initiate release of additional MAMC populations.

This novel delivery system will enable complex regulation of the healing environment. For instance, a population of MAMCs is not limited to a one-time release under a single loading event but rather, by combining cohorts of microcapsules with different rupture thresholds,
a distribution of release events can be programmed to enable simultaneous or sequential release to initiate and maintain tissue repair (Figure 8-8). This would allow for tuning and selection of a particular drug release sequence in a temporal fashion to regulate the variety of biological responses needed for repair. Growth factors can initiate a number of anabolic signaling cascades; however, the delivery of other factors including anti-inflammatory compounds, steroids, and small molecules (Gorth 2012; Kang et al. 2014; Bajpayee et al. 2016) may also be enable progression of the reparative process. While our initial studies demonstrated that TGF-β3 can be encapsulated and released from MAMCs to stimulate the production of cartilage matrix components, improving the longevity of activity (Figure 8-7) will be necessary for an effective in vivo cartilage repair strategy. Loss of activity of biologics encapsulated within polymer systems are largely attributed to local acidification due to polymer degradation products, protein aggregation, and polymer-protein interactions due to hydrophobic or electrostatic interactions (Wang 1999; van de Weert et al. 2000). Addition of excipients to the aqueous MAMC core, including pH buffering additives, sugars (e.g. sucrose, trehalose), polymers (e.g. PEG, cyclodextrins), or free amino acids (e.g. arginine, glutamate), might improve long term stability (Wang 1999; van de Weert et al. 2000; Frokjaer et al. 2005). The inclusion of these types of stabilizing factors may improve compatibility of encapsulation for various payloads and help maintain biologic activity of the drug over extended periods of time in the in vivo environment.

8.5. Conclusion

As we work towards clinical translation of the MAMC drug delivery platform, it will be critical to expand our understanding of the MAMC property-function (release) relationships in the context of in vivo mechanical loading. Continued characterization of deformation behaviors and release thresholds for microcapsules embedded in various matrices (over
a range of microcapsule physical and matrix mechanical properties) can be used to inform finite element (FE) models that will predict MAMC rupture in complex and time evolving environments. These FE models will support the prediction of MAMC formulations that can be programmed for temporal release as a function of tissue maturation within a cartilage defect site. Mechanically-activated microcapsules are a highly tunable drug delivery system, and this work makes progress in advancing a new strategy for musculoskeletal tissue repair within the demanding mechanical environments which challenge tissue healing.
Chapter 9. Summary and Future Directions

9.1 Summary

Progressive joint degeneration in PTOA is the product of abnormal and traumatic physical events that initiate signaling cascades that irreversibly affect the structure and function of articular cartilage. While this can eventually lead to severe pain and loss of mobility at the end stages of disease, this temporal disease progression also presents therapeutic windows for targeted early intervention using biological treatments. In this dissertation, high throughput and mechano-active platforms were developed to discover and deliver new therapeutics for cartilage repair and regeneration.

While tissue engineering approaches have conventionally been used to fabricate and grow constructs that mimic native tissue properties in order to replace diseased counterparts, advances in this field have also enabled the design of in vitro, pre-clinical disease models to test drug efficacy. To build an in vitro model of PTOA using engineered cartilage, Chapter 3 focused on establishing cartilage tissue analogs (CTAs) as constructs that mature to achieve near-native functional and biochemical properties. The mechanical environment defines how tissue level applied deformations are transferred to cells in native cartilage (Alexopoulos et al. 2005), and therefore in the context of injury, it was important to use an engineered tissue that would have a similar behavior in vitro. By tracking CTA mechanical properties throughout culture duration, a minimum pre-culture period of 14 weeks was determined to be the point at which constructs were functionally mature and could be sued for subsequent injury studies.
In parallel with this work, a high throughput mechanical device was developed for two applications. First, as a testing system to rapidly evaluate the compressive mechanical properties of engineered cartilage (Chapter 4), and second, to uniformly apply mechanical injury to constructs to facilitate compound screening in an in vitro model of PTOA (Chapters 5 and 6). The device was constructed in a 48-well format to standardize the screening platform and was coupled to a standard Instron micromechanical testing system in order to control and apply step displacement to constructs via PTFE platens in each well. A force-sensitive resistor array interfaced with these platens to measure the force-response of each sample individually. As a result, the equilibrium properties of 48 samples could be measured at once in less than 2 hours; in comparison, single sample testing of 48 samples would require 16 hours of labor. Proof-of-concept studies with acellular biomaterials demonstrated the ability of the system to measure elastic and viscoelastic properties. Furthermore, dose-dependent differences in mechanical properties of engineered cartilage due to TNF-a treatment could be detected in a high manner. Together, these results supported the use of this platform as a high throughput mechanical screening tool as a first pass for materials evaluation. Complex or combinatorial studies that are not possible using single-sample methods can be carried out using this device. Potential applications include screening biomaterial properties as a function of chemistry and composition, evaluating in ‘real-time’ construct maturation, and measuring the effect of drug candidates on functional cartilage repair in vitro.

Validation of this device as a measurement tool naturally supported its adaptation to a high throughput mechanical injury (HiTMI) platform for an in vitro model of PTOA, as described in Chapter 5 and 6. Compression at high strains (50 to 75%) and strain-rate
(50%/s) induced an injury response in mature CTAs that mimicked the acute hallmarks of mechanical damage (cell death and proteoglycan loss) observed in in vitro explant culture and in vivo models. As a step towards validating this platform for drug screening, putative therapeutics reported in the literature (ZVF, NAC, and P188) were also tested and showed similar levels of efficacy in CTAs in attenuating either cell death or matrix loss. This work enabled the next step in the process, which was to conduct un-biased screens of small molecule libraries to identify compounds that modulate non-canonical or unknown pathways in PTOA. A total of 118 compounds were screened that modulated apoptotic signaling pathways or those relevant to cartilage development. Out of 20 ‘hits’ identified, 7 strongly reduced LDH release (a marker for cell damage and death) alone or concurrent with reduced GAG loss. Among these was again ZVF, but also a number of JAK inhibitors (e.g. WHI-P154, Filgotinib, Tofacitinib Citrate) that may modulate catabolic activity and have been explored for the treatment of rheumatoid arthritis (Milici et al. 2008). Secondary screens in ‘donor-specific’ CTAs showed variable efficacy across donors, however, suggesting that differences in cell phenotype (or patient characteristics) may play an important role in developing clinically-translatable therapeutics.

As described in the previous chapters, the scope of the in vitro model of PTOA was to develop a platform for therapeutic screening in order to target the acute effects of mechanical overload. Yet, long-term cartilage degeneration is mediated by the inflammatory environment that persists within the joint space. Towards strategies for cartilage repair for patients with end-stage OA, Chapter 7 focused on characterizing cell source-dependent sensitivity to pro-inflammatory cytokines. Given that chondrocytes and MSCs are often considered for tissue-engineered replacements, the goal of this study was to understand how these two cell types tolerate and survive an inflammatory challenge.
Compared to chondrocytes, MSC-derived constructs treated with either IL-1β or TNF-α exhibited a greater reduction in mechanical properties and matrix content in a dose-dependent manner. This response was observed despite elevated levels of catabolic mediators (NO and MMPs) for chondrocyte-derived constructs, indicating that chondrocytes may have an inherent resistance to inflammation and therefore, an advantage over MSCs in successfully repairing cartilage in vivo.

Finally, to facilitate the delivery of newly identified therapeutics and improve tissue engineering approaches for cartilage repair across the spectrum of degeneration in PTOA, mechanically activated microcapsules (MAMCs) were developed as described in Chapter 8. The rupture and release characteristics of MAMCs were characterized in both 2D and 3D environments. Under direct compression in 2D, as the microcapsule shell thickness-to-diameter ratio increased, the resistance to failure also increased. Given that MAMCs were fabricated using PLGA, polymer degradation kinetics also impacted the mechano-activation in a predictable fashion. While fast-degrading MAMCs (PLGA 50:50) rapidly showed a marked increase in sensitivity to applied loads (<1 week), more slowly-degrading MAMCs (PLGA 85:15) maintained a stable mechano-activation profile for up to 10 weeks. To further assess MAMC behavior in a 3D context, microcapsules were embedded in hydrogels analogous to engineered cartilage with mechanical properties that spanned the range of cartilage maturity. For both static step compression and dynamic loading, MAMC deformation and mechano-activation increased with increasing matrix stiffness. Together, these results highlighted the potential of MAMCs to be embedded within biomaterials and implanted in vivo, wherein drug release could be initiated in an ‘on-demand’ fashion as a function of tissue maturity and loading conditions to stimulate
cartilage repair over an extended time frame. In order to demonstrate the ability of this system to encapsulate and deliver active agents, TGF-β3 was assessed as model therapeutic. In an engineered cartilage model, TGF-β3 released from ruptured MAMCs induced chondrogenesis as measured by increased GAG content and matrix staining. While TGF-β plays an important role in ECM production, it is only one of a host of biofactors that could be encapsulated within MAMCs in order to promote or sustain cartilage repair. These include additional growth factors (e.g. IGF-1, BMP-2), anti-inflammatory drugs (e.g. IL-1RA, dexamethasone), and small molecules (e.g. Kartogenin) that have been experimentally and clinically used to treat OA.

9.2 Future Directions

While considerable progress was made in the completion of this thesis work, there of course exist additional studies and goals that warrant further exploration. Consideration of these will be discussed in the following sections, with a particular focus on screening assays and continued development of the MAMC system.

9.2.1. Secondary and Tertiary Screens for Therapeutic Discovery in PTOA

While several candidates were identified in the small molecule screens conducted using the HiTMI platform, this was only the first step in the process of clinical validation and the development of a ‘lead’ candidate. As described above, a secondary replicate screen across bovine donors was conducted to determine which ‘high performing hit’ held the most promise in attenuating cell death and proteoglycan loss. However, secondary screens also need to be performed to determine the dose-response curve (e.g. IC50) and toxicity levels in injured and control CTAs, respectively. The timing and duration of
treatment also represents a separate aspect of evaluating compound efficacy. While the studies presented here focused on the acute phase of injury, future experiments could treat CTAs after a period of delay post-injury (e.g. immediate vs. 24 hours, 48 hours, 1 week) and evaluate the injury response after extended periods of time (e.g. 1 to 4 weeks post-injury). These experiments would determine the therapeutic window in which the compound still has a beneficial effect and indicate if compounds have long-term chondro-protective potential. Outcome measures for these secondary screens could also include histology (e.g. TUNEL, Alcian Blue to confirm LDH and GAG release), as well as biochemical (proteoglycans, collagen) and soluble assays (e.g. MMP activity) to measure matrix accumulation within constructs and the presence of catabolic mediators. An additional consideration is the introduction of an inflammatory component into the model system after injury to simulate later stages of PTOA. While the studies described in Chapter 7 established the ability of IL-1β and TNF-α to induce the production of catabolic mediators in engineered cartilage, the inflamed joint microenvironment is more complex than one or two cytokines alone. One possibility is to use macrophage-conditioned medium (MCM) as previously described in an engineered cartilage model of inflammation in OA (Sun et al. 2011). MCM as compared to exogenous addition of IL-1β and TNF-α (at matched concentrations) was able to stimulate chondrocyte hypertrophy and apoptosis, two features of early OA not captured in simpler systems. Since OA is considered a ‘whole organ’ disease, including contributions from other tissues (e.g. synovium, macrophages) may improve the accuracy and predictive potential of the in vitro model in future screening studies. Lastly, although >100 compounds were tested in the work presented here, this represents only the first step in the development of a fully high throughput platform. Scale up to larger well formats (e.g. 96 well) and adaptation to robotic liquid handling platforms
would enable rapid fabrication and culture of engineered cartilage, as well as the use of larger libraries on the order of 1000’s of compounds (e.g. NINDS, Sigma LOPAC) to conduct truly un-biased screens in this *in vitro* model of PTOA.

In order to understand if the ‘lead’ candidates found in primary and secondary screens translate to attenuating pathologic changes in the complex, *in vivo* environment, the next step would be to conduct tertiary screens in an animal model of PTOA. The goal is to mimic the progression of disease in humans, from mild to moderate degeneration, but on a more rapid timeline than human OA (Christiansen et al. 2012; Little et al. 2013). Murine models, more specifically those that induce OA via surgical injury, can consistently reproduce a diseased state and be used to investigate the therapeutic efficacy of a candidate drug or molecule in an *in vivo* setting (Christiansen et al. 2012; Little et al. 2013). One of the most common models is the destabilized medial meniscus (DMM) model, in which the medial meniscotibial ligament that anchors the medial meniscus to the tibial plateau, is transected (Glasson et al. 2007). Over a period of 8 weeks, this disruption to joint loading patterns leads to GAG loss from and surface fibrillation of the articular cartilage in the medial compartment, with lesions on the medial side of the joint becoming more severe (mimicking mild to moderate OA). Due to the relatively slow progression of disease (compared to more severe ACL transection models), therapeutics can be evaluated in this setting without the injury response overwhelming the reparative potential of the compound. Previous work using the DMM model validated potential therapeutic targets using genetically modified mice, including ADAMTS (Glasson et al. 2005), MMP-13 (Little et al. 2009), and IL-1β (Chambers et al. 1997). In the context of the ‘lead’ candidates identified using the HiTMI platform, these compounds could be injected into
the joint space after destabilization induced by DMM surgery in a murine model. Similar to the proposed in vitro experiments, the timing of administration (e.g. immediately, 1 week or 2 weeks post-injury) and duration of drug efficacy could be evaluated over a period of 8 weeks as joint degeneration progresses. Histological measures at terminal time points (e.g. 2, 4 and 8 weeks) would assess the ability of the compound to protect or attenuate cartilage degeneration (loss of proteoglycans, fibrillation) post-injury.

9.2.2. Characterizing MAMC Release in Complex Loading Environments In Vitro and In Vivo

In the work presented here, MAMC rupture and release in vitro was characterized within homogeneous matrices and under uniform compressive dynamic loading. However, in vivo, tissue maturation within constructs is likely to be less homogeneous and the loading environment more complex. To better predict deformation and rupture in inhomogeneous matrices, MAMCs could be embedded in tri-layer PEGDA hydrogels produced with depth dependent mechanical properties more similar to engineered and native cartilage (Farrell et al. 2012). MAMC release under dynamic loading would be tracked spatially and as a function of the properties of each layer. Data from these experiments could be subsequently used to develop finite element models to predict MAMC stress, strain, and failure in complex and time-evolving 3D environments (Figure 9-1). To further demonstrate the influence of tissue maturation, hydrogel constructs (e.g. methacrylated hyaluronic acid, MeHA) could be fabricated with encapsulated cells (e.g. MSCs) and TGF-β3-MAMCs together. Based on MAMC physical properties and mechano-activation profiles (as well as the predicted construct maturation profile (Erickson et al. 2012; Fisher et al. 2014), different sets of MAMCs could be embedded in constructs to program a time
course of release. The first set could be designed to completely rupture in an immature matrix (5 to 50 kPa) to jumpstart chondrogenesis at the onset of dynamic loading. The second set could achieve partial to complete rupture as the construct matures (300 to 600 kPa). Finally, a third set could be designed to not rupture, and serve as an intact, positive control. At various time points during physiologic dynamic loading (e.g. 10% strain, 1Hz, 3 hours per day, 5 days per week, 8 weeks (Mauck et al. 2000; Kelly et al. 2006; Huang et al. 2010)), constructs could be harvested to evaluate matrix deposition and function properties. Comparisons between TGF-β3-MAMC constructs and controls (e.g. growth factor free, free swelling, and exogenous TGF-β3) would determine if MAMCs could be used to accelerate the rate of engineered cartilage maturation in a mimic of the in vivo environment.

Figure 9-1. Finite element (FE) model of a MAMC-hydrogel composite to predict von Mises stress with hydrogel deformation. FE analysis could be used to predict stress in and around microcapsules, and enable variation of geometry, boundary conditions, and properties of the shell and local matrix.
Should these in vitro experiments validate 3D mechano-activation, the next step would be to demonstrate that MAMCs implanted in vivo remain structurally intact and sensitive to load. To understand if MAMC mechano-activation profiles are altered due to exposure to the in vivo milieu, MAMC-hydrogel composites could be implanted subcutaneously in a rat model. After 2, 4 or 8 weeks, implants could be harvested and dynamically loaded to evaluate rupture and release as a function of cycle number (i.e. loading duration), similar to the experiments described in Chapter 8. In vivo constructs would then be compared to naïve constructs (pre-implantation) and those cultured in vitro for the same length of time to determine the MAMC integrity and mechanical stability. The appearance of the surrounding tissue would also be documented and sampled for histological analysis to determine if MAMCs induce an inflammatory response. A separate subset of studies could also be conducted to determine how long biofactor activity can be retained in vivo. For example, TGF-β3-MAMC constructs could be harvested at specific time points, homogenized, and evaluated for bioactivity using an ELISA assay. These experiments would inform the choice of of payload to be encapsulated within MAMCs and estimate the efficacy of a drug in stimulating functional repair long-term.

Finally, MAMC behavior could be characterized in an in vivo load bearing environment as a step towards clinical translation. Previous work has shown the clinical relevance (Kuster et al. 1997; Gomoll et al. 2006) and utility of a Yucatan minipig model of focal cartilage defect repair for evaluating tissue engineered approaches (Belkin et al. 2013; Fisher et al. 2015; Kim et al. 2015; Pfeifer et al. 2015; Fisher et al. 2016); therefore, this model system may be appropriate for measuring MAMC mechano-activation in situ. Prior to embarking on a large animal study, an ex vivo model of joint loading could be used to test final MAMC
formulations based on *in vitro* and subcutaneous data. Defects could be created in the trochlear groove of an isolated patella, and filled with a MAMC-hydrogel composite to various heights. ‘Fully-filled’ defects would contain a hydrogel flush with the cartilage surface, whereas 'half-filled' defects would be filled to only half the depth. While the majority of MAMCs in ‘fully-filled’ defects would be expected to rupture, those in ‘half-filled’ defects would be minimally affected due to the lack of load transfer. The femur and patella could then be potted and aligned, with the potential addition of a Tekscan force-sensor between the two surfaces to ensure consistent distribution of contact pressures during loading (Figure 9-2). Axial, cyclic compressive loading would be applied (e.g. 1.5x body weight at 1 Hz for 1, 6 or 12 hours), after which MAMC rupture would be quantified by image analysis.

**Figure 9-2.** Pilot testing of *in situ* pressure mapping of patella and trochlear groove contact during axial compressive loading in a 3D printed model constructed using
**mini-pig MRI scans.** Higher cyclic loads (in the range of expected physiological forces, 1 to 1.5x body weight) resulted in higher peak pressure readouts by the force sensor.

*Figure 9-3. Schematic for *in vivo* characterization of MAMC mechano-activation using a bilateral trochlear groove cartilage defect model in a mini-pig.* (Top) Exposure of the trochlear groove with one defect site filled. (Bottom) Depiction of hydrogel and MAMC groups to be tested in the mini-pig model. Under each group, the associated hypothesis (H) is described as minimal (min.) or full rupture of MAMCs within the defect, or for the induction of chondrogenesis (chondro.).

If MAMC mechano-activation is responsive in the *ex vivo* loading model, then an *in vivo* model could be initiated. Using a bilateral trochlear groove full-thickness cartilage defect
model (4 defects per knee), a total of four hydrogel groups could be evaluated: ‘fully-filled’ defects containing a PEGDA hydrogel (1) alone, (2) with BSA-MAMCs (inactive protein), (3) with TGF-β3-MAMCs (e.g. 100ng/defect), and (4) ‘half-filled’ defect containing a repeat of condition (2) (Figure 9-3). In addition, autologous porcine MSCs (harvested and expanded prior to the start of the study, 60 million cells/mL) would be labelled (fluorescent cell tracker) and encapsulated in each hydrogel. Animals would be euthanized at various time points corresponding to physical activity level: 2 days (no activity, pigs are lying down, minimal weight bearing), at 1 week (partial return to activity), and at 4 weeks (return to pre-surgery activity levels) following surgery (Qu et al. 2016). Each of the implant sites (surrounding cartilage and bone included) could then be harvested and analyzed for both MAMC rupture and release (via multi-photon microscopy), as well as TGF-β3 induced chondrogenesis in defects (via histological analysis) to determine if MAMCs have clinical potential as a drug delivery system.

9.3 Conclusion
Collectively, these studies establish a new micro-engineered system for drug screening to identify novel compounds that could influence the response of articular cartilage to injury, as well as an innovative mechanically activated drug delivery system that could be used to guide tissue formation after injury has progressed to the need for surgical intervention. While considerable effort and numerous studies are needed to realize these platforms, this thesis work sets the stage for such future endeavors. Identification and delivery of newly discovered therapeutics has the potential to transform current paradigms for the treatment of post-traumatic osteoarthritis and expand the impact of regenerative medicine solutions for cartilage repair.
Bibliography


