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Exogenous Stimulation of Meniscus Cells for the Purposes of Tissue Engineering the Knee Meniscus

by

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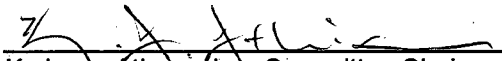

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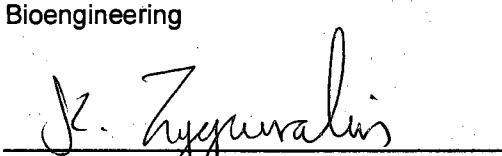

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Dedication

To: MUM and DAD

For Always Being The Wind Beneath My Wings

Abstract

Exogenous Stimulation of Meniscus Cells for the Purposes of Tissue Engineering the Knee Meniscus

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Najmuddin Juzer Gunja

Injuries to avascular regions of menisci do not heal and result in significant discomfort to patients. Current treatments, such as partial meniscectomy, alleviate the symptoms, but lead to premature osteoarthritis due to reduced stability and changes in knee biomechanics. An alternative treatment to overcome these problems involves functional tissue engineering. This thesis examined several exogenous factors to enhance the capability of meniscus cells (MCs) to synthesize relevant ECM markers and improve the functionality of constructs *in vitro*. First, the effect of passage on the phenotype of MCs in monolayer was investigated, and rapid changes were observed in collagen I, collagen II, and COMP expression. Collagen I and aggrecan protein coatings assisted in reversing expression levels of certain ECM markers; however, collagen II expression could not be reversed. Next, 3D tissue engineering studies were conducted using a cell-scaffold approach with MCs seeded on PLLA meshes. Anabolic stimuli that aided in meniscus regeneration included 1) hypoxia and bFGF, which resulted in synergistic increases in the total glycosaminoglycan content and compressive properties of constructs; 2) 10 MPa static hydrostatic pressure (HP), which resulted in increases in collagen content

and the relaxation modulus of constructs; and 3) 10 MPa static HP and TGF-β1, which resulted in additive increases in collagen content, and synergistic increases in the compressive moduli of constructs. Finally, a self-assembly, scaffoldless approach was employed for meniscus regeneration using co-cultures of MCs and articular chondrocytes (ACs). A high density of cells were seeded on non-adherent agarose molds and allowed to coalesce into a construct without a scaffold. Different co-culture ratios of MCs and ACs resulted in a spectrum of fibrocartilages that recapitulated some biochemical and biomechanical properties of the rabbit meniscus. Cell culturing conditions were optimized with the identification of a smooth 1% agarose mold that resulted in geometrically-mimetic meniscus constructs. In conclusion, this thesis quantified phenotypic changes in MCs over passage, and used scaffold-based and scaffoldless approaches to regenerate constructs with biochemical and biomechanical properties in the range of native tissue values. Successful replacement of a damaged meniscus will improve the quality of patient life and reduce the risk of osteoarthritis.

Acknowledgements

I never failed once. It just happened to be a 2000-step process.

-Thomas Edison

Five years ago, as I stood outside the Athanasiou lab, I yearned to make a contribution, however small, to the field of cartilage tissue engineering (and also, to fit the stereotype of a graduate student - yes, I longed to be part of this caffeine-infused, sleepless group of researchers). The journey, though rewarding, has been arduous at times, and I would not have been able to accomplish my research goals without the guidance and encouragement of close friends and mentors. I am glad I have this opportunity to thank them because they have given me more than a lifetime of memories.

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Introduction

The overall objective of this thesis is to identify exogenous stimuli that can enhance the biochemical and biomechanical properties of tissue engineered meniscus constructs. To achieve this objective, the thesis is divided into three sections. The first section addresses the effects of monolayer culture on meniscus cell phenotype. The second section involves the use of exogenous stimuli to enhance extracellular matrix (ECM) and biomechanical properties of meniscus cell-seeded poly-l-lactic acid (PLLA) scaffolds. The third section enhances a novel scaffoldless approach, developed in our laboratory, for meniscus tissue engineering. *The primary hypotheses of this investigation are that 1) meniscus cells will experience rapid phenotypic changes during culture, but these changes can be modulated by controlling the culturing conditions; 2) exogenous factors, individually or in combination, will allow for the formation of meniscus constructs resembling native tissue; and 3) a scaffoldless approach can be used to recreate the heterogeneities of the knee meniscus.* To test these hypotheses, three specific aims were employed:

1) **To investigate the phenotypic changes in meniscus cells with passage and examine gene expression reversal techniques.** Meniscus cells synthesize several ECM molecules including collagen I, collagen II and aggrecan that are essential for the function of the tissue. It has been shown that cell expansion in monolayer causes rapid phenotypic changes in other cartilaginous cells with variations in gene expression profiles for

important ECM markers. Corresponding studies with meniscus cells in monolayer are absent. Thus, the goal of this aim is two-fold: 1) to determine the effects of passage on important meniscus ECM markers. *The hypothesis is that, much like other cartilaginous cells, meniscus cells would exhibit phenotypic changes in monolayer*; and 2) to reverse any changes in gene expression incurred during passage by plating meniscus cells on either an aggrecan or collagen I protein coating. *The hypothesis is that, both aggrecan and collagen I will modulate the matrix forming phenotype of meniscus cells.* In this aim, meniscus cell gene expression will be studied using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

2) **To determine the effect of exogenous stimuli on meniscus cell-seeded PLLA constructs.** Prior studies in our laboratory and in the literature have shown the benefit of using exogenous stimuli such as hypoxia, bFGF, TGF-β1 and hydrostatic pressure for articular cartilage tissue engineering. Thus, the purpose of this aim is to examine these exogenous factors individually or in combination for meniscus tissue engineering and identify stimuli that enhance ECM and biomechanical properties of meniscus cell-seeded PLLA constructs. *The hypotheses of this aim are that 1) each exogenous stimulus will improve the quality of the meniscus cell-seeded PLLA constructs and 2) combinations of particular stimuli will result in additive and synergistic effects.* Several qualitative and quantitative tools will be utilized to examine the contents of the constructs

including histology, immunohistochemistry and biochemistry. The biomechanical properties of the constructs will be characterized using unconfined compression and uniaxial tension tests.

3) **To optimize cell and tissue culture conditions for scaffoldless meniscus constructs.** Previous work in our laboratory has shown that meniscus cells coalesce into scaffoldless constructs when seeded on non-adherent agarose wells. The purpose of this aim is to advance this process by investigating strategies to recreate the heterogeneities of the knee meniscus with the use of meniscus cell and articular chondrocyte co-cultures and to form geometrically mimetic meniscus-shaped constructs by altering the topology and compliance of agarose wells. *The hypotheses of this aim are that 1) co-cultures of meniscus cells and articular chondrocytes will result in a spectrum of biochemical and biomechanical properties that span the range of the inner and outer meniscus and 2) rough high compliance agarose wells will favor the formation of meniscus shaped constructs.* Constructs will be evaluated for their biochemical composition using quantitative and qualitative measurements including histology, immunohistochemistry and biochemistry. The biomechanical properties of the constructs will be evaluated using creep indentation and uniaxial tension tests.

Chapter 1 delivers an overview of the field of meniscus tissue engineering. Chapter 2 discusses a study investigating the effects of passage on gene

expression of meniscus cells and examines gene expression reversal techniques. Chapter 3 focuses on characterizing the biomechanical and degradation properties of PLLA scaffolds used in our tissue engineering studies. Chapters 4 to 7 discuss the effects of several exogenous stimuli on meniscus cell-seeded PLLA constructs. Chapter 8 and 9 discuss scaffoldless approaches for meniscus tissue engineering. Appendix A, B and C are review articles on salient biodegradable materials used in soft tissue fixation.

Chapter 1 reviews meniscus structure and function and current tissue engineering strategies. Special emphasis is placed on exogenous stimuli used to regenerate the meniscus. This chapter motivates the use of various culturing conditions, growth factors and mechanical stimuli used in this thesis.

Chapter 2 addresses work performed toward the completion of specific aim 1 of this thesis. It describes the effects of passage on the gene expression of meniscus cells. In addition, also two different protein coatings are examined to reverse these changes in gene expression.

Chapters 3-7 address work performed toward completion of specific aim 2. Chapter 3 includes a characterization study of PLLA scaffolds where the biomechanical and degradation properties are tracked over a period of three months in different medium formulations. Chapter 4 identifies a beneficial hydrostatic pressure regimen to enhance the biochemical and biomechanical

properties of meniscus cell-seeded PLLA constructs. Chapter 5 describes the beneficial effect of TGF-β1 on meniscus cell-seeded PLLA constructs and the synergistic and additive effects of combining TGF-β1 and hydrostatic pressure. Chapter 6 details the synergistic and additive effects of a combination of hypoxia and bFGF for meniscus tissue engineering. Chapter 7 investigates strategies to recreate portions of the knee meniscus via co-cultures of meniscus cells and articular chondrocytes seeded on PLLA scaffolds.

Chapters 8 and 9 address work performed toward completion of specific aim 3. Chapter 8 identifies meniscus cell and articular chondrocyte co-culture ratios most closely resembling the inner and outer meniscus. Chapter 9 investigates techniques to form meniscus shaped constructs by varying the topology and concentration of agarose wells.

Appendix A, B and C review the state-of-the-art in soft-tissue fixation devices. Appendix A provides a general overview of salient biodegradable polymers used in arthroscopic soft-tissue fixation. Appendix B reviews novel biodegradable polymers that are under investigation as musculoskeletal implants. Appendix C provides a comprehensive review of polymers and osteoconductive materials used in ACL fixation.

Chapter 1: Knee meniscus structure, function, and tissue engineering

Najmuddin J. Gunja and Kyriacos A. Athanasiou

Introduction

The knee menisci are fibrocartilaginous tissues specialized to protect the underlying articular cartilage of the femur and the tibia via load distribution and shock absorption.[1-5] Intrinsic repair capacity is limited to the peripheral region of the tissue, and injuries to other portions result in a loss of tissue functionality leading to osteoarthritic changes in the underlying articular cartilage.[6] Methods aimed at restoring native meniscus function need to be developed to prevent these long-term degenerative changes.

Meniscus composition

The knee meniscus exhibits stark regional variation in cell morphology, ECM, and biomechanical properties. For the meniscus, this variation manifests itself in the transition from a ligamentous outer region to a cartilaginous inner region.[1]

Cells and extracellular matrix

The term fibrochondrocyte or fibrocartilage cell has often been used to describe the cells of the meniscus.[7-10] However, recent characterization studies have led to the identification of different cell populations within the tissue.[1, 11] McDevitt *et al.*[11] divided the meniscal cell population into three distinct groups; fibrochondrocytes, fibroblast-like cells, and cells of the superficial zone. Fibrochondrocytes, as defined by the authors, are cells that are localized in the middle and inner meniscus and express both collagen I and collagen II. They can be identified by their round or oval shape and presence of a pericellular matrix.

The ECM in this region consists mainly of collagens I and II, in a 2:3 ratio, that are responsible for providing structural and tensile properties to the tissue.[12, 13] Fibroblast-like cells are found mainly in the outer 1/3rd of the tissue and lack a pericellular matrix. The ECM in this region is predominantly collagen I.[1, 14] Cells of the superficial zone are located below the surface of the tissue and can be identified by their fusiform shape and lack of cytoplasmic projections. In addition to the presence of collagen I and II in the inner 2/3rd of the meniscus, several other proteoglycans and glycoproteins can also be found. The major meniscal proteoglycan is aggrecan and its main function is to provide compressive properties of the meniscus, especially to the inner 1/3rd which is predominantly under compressive load.[15] COMP, a pentameric glycoprotein that influences collagen fibril formation is also present in the inner 2/3rd of the meniscus.[16] Small leucine rich proteoglycans such as biglycan and decorin, that interact with growth factors as well as influence fibrillogenesis, are present in smaller quantities as well.[10]

Biomechanical properties

Due to meniscal geometrical properties and attachments via meniscal horns, an applied physiological load not only compresses the tissue but also places it under a circumferential tensile strain. Finite element models have been used to describe the distribution of both compressive and tensile forces throughout the tissue during a loading event.[17] Results of finite element analyses have shown the inner region to be subjected to higher levels of compressive strains while

circumferential tensile strains are concentrated in the outer region. This regionally variant loading is consistent with the overall cartilaginous to fibrocartilaginous gradient present in the tissue. The biomechanical properties would be expected to correlate with this tissue gradient, suggesting that the inner region is stiffer in compression and the outer region is stiffer in circumferentially oriented tension. Characteristic of a fibrous tissue, the meniscus exhibits anisotropic tensile properties due to the large diameter, circumferentially oriented collagen fibers. Previous work has characterized both the tensile and compressive properties of leporine menisci.[18, 19] It was found that the femoral-anterior portion of the medial meniscus exhibited the highest indentation stiffness (350 ± 110 kPa), aggregate modulus (270 ± 90 kPa), and shear modulus (140 ± 40 kPa), while the tibial-posterior region exhibited the lowest indentation stiffness (170 ± 40 kPa), aggregate modulus (130 ± 30 kPa), and shear modulus (60 ± 20 kPa). Circumferential tensile tests showed that the Young's modulus was 156.6 ± 48.9 MPa, and the UTS was 21.6 ± 7.0 MPa.

Meniscus injuries

The avascularity in the inner region of the meniscus means that it is unable to intrinsically heal. The most common type of injury, a meniscal tear, can occur due to trauma to the joint or wear and tear. There are a variety of meniscal tears that have been classified depending on their location in the meniscus. For example, a split in the meniscus from the lateral to the medial rim across the

radius is known as a radial tear while tears along the length of the meniscus are called circumferential tears.

Several methods have been used to repair tears in the meniscus. In the past, menisci were considered to be functionless remains of leg muscle and were often completely removed when damaged. This technique, known as complete meniscectomy, has now been abandoned as a treatment modality as it has been shown to result in joint instability, joint damage and osteoarthritis.[20, 21] These findings led to a procedure known as partial meniscectomy where torn portions of the meniscus were removed via arthroscopic intervention. Recent research, however, has shown that this technique may also result in impaired mechanical function of articular cartilage, leading to degenerative changes and osteoarthritis.[22, 23] Biodegradable polymer materials are currently successfully used to treat meniscal tears; however, their applicability is limited only to the vascular region of the meniscus.[1] Meniscal allografts and prostheses have also been tried as replacements for the meniscus. Allografts have shown positive short-term results but there is concern regarding the possibility of disease transmission and host rejection in the joint.[24] Prostheses have been found to exhibit inferior mechanical properties when compared to native tissue.[25] In light of these problems, there has been a tremendous push down a tissue engineering pathway to develop a biological meniscal substitute that will restore normal function to the joint.

Meniscus tissue engineering

Successful tissue engineering of the meniscus will require the identification of an appropriate cell source, scaffold material and culturing condition. The following section details prior work conducted in these areas pertaining to the meniscus.

Cell source

Selection of an appropriate cell source weighs heavily on the eventual clinical translatability of a tissue engineering approach. Considering this, differentiated cells are currently used in FDA approved autologous chondrocyte transplantation, while stem cells are in the process of gaining wider clinical use for cartilage repair. Studies in our laboratory [26, 27] and others [28] have used MCs and ACs to form fibrocartilaginous tissue constructs. Another consideration for cell source selection, especially in studies culminating in an *in vivo* assessment, is the animal species from which the cells are isolated. Currently, meniscus tissue engineering *in vivo studies* have only employed an allogenic or autologous cell source as it has been traditionally believed that xenogenic constructs would elicit an immune response.[29-31] However, it has been suggested that cartilaginous tissues like the meniscus are immunoprivileged due to the dense ECM shielding the immunoreactive cells from both humoral and cell-mediated responses.[32-34] The successful application of a xenogenic tissue engineered meniscus replacement would have significant benefits over allogenic or autologous approaches, as issues associated with the low quality and quantity of donor tissues would be avoided. By using a xenogenic approach, the age of the

donor animal could be minimized, as it has been shown that aging adversely affects the ability of the cells to express mRNA corresponding to relevant ECM and produce robust fibrocartilaginous tissue.[35-37] Also, the cell expansion phase required for allogenic or autologous approaches would be avoided using a xenogenic source because of the amount of available donor tissue.

Scaffolds

Numerous studies have shown that 3-D culture is necessary for redifferentiation of expanded chondrocytes and dually functions as a means to form a tissue engineered construct. Different kinds of scaffolds have been used to tissue engineer the knee meniscus that can be broadly divided into two categories, natural and synthetic scaffolds.

Natural scaffolds that have shown the most promising results are collagen gels. Studies on these gels by Mueller *et al.*[38] have yielded encouraging results *in vitro*. However, no mechanical tests were performed to determine the integrity of the scaffolds. Several *in vivo* experiments in animal models have also been performed with collagen implants.[30, 39, 40] One particular implant collagen implant is currently in clinical trials in humans.[41-43] In spite of the relative success of these implants, insufficient tissue to host integration and inferior mechanical properties of these scaffolds are issues that remain unaddressed.

Numerous synthetic scaffolds have been used in meniscus tissue engineering experiments. One of the most popular scaffold choices in the literature is poly-glycolic acid (PGA). Non-woven PGA meshes are frequently used in tissue engineering studies.[44, 45] It has been shown that fibroblasts as well as chondrocytes readily attach and proliferate on PGA scaffolds.[46] In addition, an upregulation of protein synthesis and mechanical properties has also been observed.[46, 47] In our laboratory, knee meniscus cells produced more collagen and GAG while maintaining a higher cell number on PGA scaffolds than on agarose scaffolds.[45] Recent studies with TMJ disc fibrochondrocytes and meniscal cells have shown that seeding these cells on PGA scaffolds causes them to contract significantly within four to eight wks losing 50% of their original volume.[48-50] Furthermore, the rapid degradation of PGA is also a concern in long-term tissue engineering studies.[49, 50] Hence, a polymer with greater structural integrity and slower degradation time may be beneficial for tissue engineering studies involving fibrocartilage. One such polymer that is now gaining popularity is poly-l-lactic acid (PLLA). In recent tissue engineering pilot studies with TMJ disc fibrochondrocytes and meniscus cells in our lab, PLLA scaffolds were compared to PGA scaffolds. Over 4 wks, it was found that PLLA maintained its mechanical integrity and shape while the PGA constructs rapidly contracted and lost mechanical integrity. The PGA scaffolds could not be tested under compression or tension due to their weak structural network. In addition, both scaffolds maintained cellularity throughout the culture period while neither scaffold exhibited a significant advantage over the other in terms of collagen or

GAG content.[51] Hence, the use of PLLA in fibrocartilage tissue engineering needs to be further investigated to fully verify its potential as a scaffold material.

Scaffoldless tissue engineering

In addition to a scaffold-based approach, a novel scaffoldless approach, developed in our laboratory for articular cartilage, has been used for meniscus tissue engineering.[27, 52, 53] In this technique, a high density of cells are seeded in a non-adherent agarose mold. The cells coalesce by cadherin-mediated cell interactions as dictated by the differential adhesion hypothesis.[54-56] Over time, the cells migrate apart and secrete extracellular matrix. With this approach, anisotropic, inhomogeneous constructs mimicking the native meniscus can be created without the need for a scaffold. This approach addresses two major limitations with scaffold-based constructs: 1) scaffolds are susceptible to early or delayed degradation *in vivo*, a phenomenon which may interfere with ECM production and maturation and long-term mechanical properties [57], and 2) the random arrangements of non-woven scaffold meshes introduce a formidable challenge in producing constructs that are anisotropic like the native meniscus.

Cell culturing conditions

Current cellular approaches for meniscus tissue engineering usually involve autologous meniscus cells.[29, 30] However, primary cells from one animal are too low in number to seed on a scaffold. To overcome this, an approach often employed is to expand autologous cells in monolayer until the cell number is

sufficient for the study. A caveat with this technique is that primary cells may dedifferentiate *in vitro* in monolayer culture. This, for example, has been shown consistently with articular cartilage.[58, 59] Gene expression studies with primary chondrocytes show that they express predominantly collagen II. However after one passage, the collagen II expression drops, and the cells begin to express collagen I which is indicative of a fibroblastic phenotype.[60, 61] In an effort to reverse lost gene expression in articular cartilage and temporomandibular joint (TMJ) disc fibrochondrocytes, several growth factors, surface protein coatings and 3-D scaffolds have been investigated.[60, 62-64] However, corresponding passage and gene expression reversal studies for the meniscus are absent. Hence, understanding the state of expanded meniscal fibrochondrocytes prior to embarking on long-term tissue engineering studies may be prudent.

Recent work has suggested that low oxygen tension (hypoxia) may aid in meniscus cell (MC) and articular chondrocyte (AC) phenotype maintenance and enhancement during cell culture [65-68], with increases in expression of hypoxia inducible factor-1alpha (HIF-1α) and SOX-9. These transcription factors play important roles in collagen II synthesis.[69, 70] Furthermore, *in vivo*, cartilaginous tissues reside in hypoxic conditions where the lack of the blood supply creates a low oxygen environment (1 to 8%) for cartilage cells.[71, 72]

Growth factors

Many different growth factors have been investigated in our laboratory and others including TGFβ-1, IGF-1, PDGF-AB, bFGF, and BMP-2. [73-82] IGF-1 has been used extensively with articular chondrocytes and has been shown to increase proteoglycan synthesis.[74, 79, 81] PDGF-AB has been shown to stimulate the migration of fibrochondrocytes from the inner, middle, and outer thirds of the bovine meniscus. In addition, increased DNA synthesis was observed in cells of all three regions.[73] Of the growth factors previously investigated, TGFβ-1 has exhibited the greatest capability to enhance both collagen and GAG production, and tensile and compressive biomechanical properties. Based on previous studies using this growth factor, a concentration of 10-30 ng/mL is sufficient to obtain the maximal effects. While the results of solely applying bFGF on tissue constructs may be insufficient to warrant future usage, studies in which bFGF has been combined with other stimuli, such as hypoxia, report increases in GAG deposition and compressive properties.[65]

Mechanical stimuli

In vitro studies with meniscus explants and cells have shown that mechanical stimuli such as direct compression, tension and HP can increase extracellular matrix (ECM) expression and synthesis.[83-91] The use of HP is of particular interest in meniscus tissue engineering as it causes no macro-scale deformation to the construct, yet is responsible in stimulating cells to increase ECM synthesis, possibly by altering intracellular ion flux.[92] Such a stimulus may be favorable in

the early stages of a tissue engineering study where cells seeded on scaffolds are still in the proliferation and migration stages and are vulnerable to external stimuli.

HP studies can be broadly divided into three categories, a) 2-D monolayer studies, b) 3-D explant studies, and c) 3-D tissue engineering studies. Results from experiments in the first category are often reported in terms of gene expression. Studies on articular chondrocytes and Achilles tendon fibroblasts have shown an upregulation of aggrecan and collagen II expression in cells immediately after intermittent hydrostatic pressurization.[87, 93-95] Further upregulation of collagen and aggrecan expression was observed when the samples were tested 24 hrs post-stimulation suggesting that rest time might be an important factor to consider in long-term tissue engineering studies. TMJ disc cells have been shown to increase collagen I expression under static HP of 10 MPa and increase collagen II expression during intermittent HP stimulation of 10 MPa, 1 Hz.[96] Studies with explants have investigated the effects of HP on proteoglycan synthesis as well as MMP regulation. An experiment with articular cartilage explants showed that cyclic HP at physiological magnitudes caused an upregulation of sulfate incorporation into the explants.[97] Cyclic HP on meniscus explants has been shown to inhibit upregulation of potent effector molecules such as MMP-1, MMP-3, and COX-2.[86] Tissue engineering studies have been performed by encapsulating cells in alginate beads, using pellet cultures, seeding cells on scaffolds or using scaffoldless self-assembly techniques.[96, 98-102] Results

from these studies have varied significantly among research groups, often even when using similar cell sources. For example, although intermittent HP application has been shown to consistently increase GAG production by chondrocytes on PGA and PLGA scaffolds, as well as in pellet cultures, this has not been observed in self-assembled articular chondrocyte constructs.[98, 100, 101] Static HP has been shown to increase both collagen and aggrecan synthesis in intervertebral disc cells while only increasing collagen content in TMJ disc cells.[96, 99] Thus, specific regimens of both static and intermittent HP stimuli exhibit beneficial effects for tissue engineering purposes, even though the underlying mechanisms are still unclear.

Conclusions

Successful knee meniscus tissue engineering would be an important step toward the treatment of meniscal defects, but much work needs to be accomplished before this state can be reached. In this thesis, a variety of exogenous stimuli, reviewed above, were investigated for their abilities to enhance the biochemical and biomechanical properties of meniscus cell-seeded constructs. The ensuing chapters provide a comprehensive synopsis of the effects of these stimuli and their benefits for meniscus tissue engineering.

Chapter 2: Passage and reversal effects on gene expression of bovine meniscal fibrochondrocytes

Najmuddin J. Gunja and Kyriacos A. Athanasiou

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Abstract

The knee meniscus contains a mixed population of cells that exhibit fibroblastic as well as chondrocytic characteristics. Tissue engineering studies and future therapies for the meniscus require a large population of cells that are seeded on scaffolds. To achieve this, monolayer expansion is often used as a technique to increase cell number. However, the phenotype of these cells may be significantly different from that of the primary population. The objective of this study was to investigate changes in meniscal fibrochondrocytes at the gene expression level over four passages using quantitative real-time reverse transcriptase polymerase chain reaction. Cells from the inner two-thirds of bovine medial menisci were used. Four extracellular matrix (ECM) molecules, commonly found in the meniscus, were investigated, namely collagen I, collagen II, aggrecan and cartilage oligomeric matrix protein (COMP). In addition, primary and passaged meniscus fibrochondrocytes were placed on surfaces coated with collagen I or aggrecan protein to investigate whether any gene expression changes resulting from passage could be reversed. Collagen I expression was found to increase with the number of passages, whereas collagen II and COMP expression decreased. Collagen I and aggrecan surface coatings were shown to downregulate and upregulate collagen I and COMP expression levels, respectively, in passaged cells. However, decreases in collagen II expression could not be reversed by either protein coating. These results indicate that although monolayer expansion results in significant changes in gene expression

in meniscal fibrochondrocytes, protein coatings may be used to regain the primary cell expression of several ECM molecules.

Introduction

The meniscus is a wedge-shaped fibrocartilaginous tissue located in the knee joint. As reviewed elsewhere, it serves several mechanical functions including shock absorption, load transmission, joint stability and joint lubrication.[1, 103] Injuries to the meniscus can result in significant pain and discomfort to the patient, as well as in increasing the average stress in the knee joint, causing damage to the articular cartilage on the femoral and tibial surfaces.[2] The ability of the meniscus to heal intrinsically is limited to the vascular regions of the tissue. Thus, tissue engineering is a promising treatment modality to replace avascular sections of the meniscus.[1]

The term fibrochondrocyte or fibrocartilage cell has often been used to describe the cells of the meniscus.[7-10]. However, recent characterization studies have led to the identification of different cell populations within the tissue.[1, 11] McDevitt *et al.* divided the meniscal cell population into three distinct groups; fibrochondrocytes, fibroblast-like cells, and cells of the superficial zone.[11] Fibrochondrocytes, as defined by the authors, are cells that are localized in the middle and inner meniscus and express both collagen I and collagen II. They can be identified by their round or oval shape and presence of a pericellular matrix. The ECM in this region consists mainly of collagens I and II, in a 2:3 ratio, that are responsible for providing structural and tensile properties to the tissue.[12, 13] Fibroblast-like cells are found mainly in the outer 1/3rd of the tissue and lack a pericellular matrix. The ECM in this region is predominantly collagen I.[1, 14] Cells

of the superficial zone are located below the surface of the tissue and can be identified by their fusiform shape and lack cytoplasmic projections. In this experiment, we utilize cells from the inner 2/3rd of the meniscus; thus, the majority of cells present are fibrochondrocytes. In addition to the presence of collagen I and II in the inner 2/3rd of the meniscus, several other proteoglycans and glycoproteins can also be found. The major meniscal proteoglycan is aggrecan and its main function is to provide compressive properties of the meniscus, especially to the inner 1/3rd which is predominantly under compressive load.[15] COMP, a pentameric glycoprotein that influences collagen fibril formation can also be identified in the inner 2/3rd of the meniscus.[16] Also present, in smaller quantities, are small leucine rich proteoglycans, biglycan and decorin, that interact with growth factors as well as influence fibrillogenesis.[10]

Current cellular approaches for meniscus tissue engineering usually involve autologous meniscus cells.[29, 30] However, primary cells from one animal are too low in number to seed on a scaffold. To overcome this, an approach often employed is to expand autologous cells in monolayer until the cell number is sufficient for the study. A caveat with this technique is that primary cells may dedifferentiate *in vitro* in monolayer culture. This, for example, has been shown consistently with articular cartilage.[58, 59] Gene expression studies with primary chondrocytes show that they express predominantly collagen II. However after one passage, the collagen II expression drops, and the cells begin to express collagen I which is indicative of a fibroblastic phenotype.[60, 61] In an effort to

reverse lost gene expression in articular cartilage and temporomandibular joint (TMJ) disc fibrochondrocytes, several growth factors, surface protein coatings and 3-D scaffolds have been investigated.[60, 62-64] However, corresponding passage and gene expression reversal studies for the meniscus are absent. Hence, understanding the state of expanded meniscal fibrochondrocytes prior to embarking on long-term tissue engineering studies may be prudent.

Thus, the goal of this experiment was two-fold. The first was to determine the effects of passage on the gene expression of important ECM molecules (collagen I, collagen II, aggrecan and COMP) produced by meniscal fibrochondrocytes. The hypothesis was that, much like articular chondrocytes, meniscal fibrochondrocytes would exhibit phenotypic changes in monolayer. The second was to reverse any changes in gene expression incurred during passage by plating passaged meniscus cells on either an aggrecan or a collagen I protein coating.

Materials and methods

Cell harvesting

Medial menisci were isolated from six one wk old calf knees (Research 87 Inc.) by exposing the knee joint under aseptic conditions using scalpel blades. The procedures used were in strict accordance with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals. Ethics approval was obtained from Rice University before commencement of the study.

Each meniscus was taken to a cell culture hood, washed with autoclaved phosphate buffered saline (PBS) and transferred to a solution containing 2% penicillin-streptomycin-fungizone (PSF) (Cambrex) and culture medium. The culture medium contained 50:50 Dulbecco's modified Eagle's medium (DMEM)-F12 (GIBCO), 10% fetal bovine serum (FBS) (Mediatech), 1% non-essential amino acids (NEAA) (GIBCO), 25 µg of L-ascorbic acid (Sigma) and 1% PSF. The outer 1/3 of each meniscus was removed and the remainder was minced into small fragments (<1mm^3). Each minced meniscus was then placed in 30 mL of 2 mg/mL collagenase type II (Worthington Biochemical) and transferred to an orbital shaker to be digested overnight at 37^{0}C. Post-digestion, an equal volume of PBS was added to the mixture and centrifuged at 200 X *g*. The bulk of the supernatant was removed and more PBS was added and the mixture was centrifuged again. This process was repeated until all the collagenase was removed from the mixture leaving behind a white pellet of meniscal cells. Cell counts from each meniscus were obtained using a hemocytometer. Cell viability was assessed using a Trypan blue exclusion test and was found to be greater than 95%.

Cell culture, passage and expansion

From each meniscus, 1.3 million cells were obtained of which 0.2 million cells were placed in 1 mL of TriZol reagent (Invitrogen), 0.5 million cells were plated on T-75 flasks at approximately 25% confluence and the remaining 0.6 million cells were divided into three equal groups and placed in a 24-well non-tissue

culture plastic plate coating with either collagen 1 (Sigma), aggrecan (Sigma) or a no protein control for 24 h. Collagen I was dissolved in 0.1 M acetic acid and then diluted in water to a final concentration of 10 ug/cm^2 per 24 well plate. Aggrecan was soluble in water and was reconstituted to the same concentration. Post-plating, the 24-well plates were kept open in the cell culture hood and allowed to dry overnight. The cells were allowed to settle on the coatings for one day, and were then scraped off the bottom using a cell scraper and placed in 1 mL TriZol reagent. The cells in the T-75 flask were allowed to expand till 100% confluence and then passaged using trypsin/EDTA (GIBCO). The cells were counted using a hemocytometer and labeled as passage 1 (P1) cells. From this cell population, 0.2 million cells were placed in 1 mL TriZol reagent, 0.5 million cells were plated on T-75 flasks and 0.6 million cells were divided into three equal groups and placed in a 24-well non-tissue culture plastic plate. This process was repeated until the fourth passage. The experimental design is shown in Figure 1.

RNA isolation

Gene expression abundance of these cells was measured via qRT-PCR. In the first step, RNA was isolated from each sample that was previously placed in TriZol. Chloroform was added to each sample. The samples were then centrifuged at 12,000 rpm for 15 min. Isopropyl alcohol was added to the supernatant and the sample was centrifuged again. The RNA precipitate was washed with 75% ethanol, and dissolved in diethylpryocarbonate (DEPC) water.

The concentration and purity of RNA was determined using a spectrophotometer (Nanodrop).

Reverse transcriptase

Post-RNA isolation, the samples were normalized to 200 ng of RNA per sample suspended in DEPC water. Prior to reverse transcription, the RNA was DNase treated to eliminate any DNA contamination in our samples. The RNA was then reverse transcribed to cDNA using a Stratascript^{TM} First Stand Synthesis System (Stratagene) following the protocol set by the manufacturer. Briefly, random hexamers were added to each sample and the mixture was incubated at 65^{0}C for 5 min and then cooled to room temperature for 10 min. Finally to each sample, 10X buffer, RNase block, dNTPs, and Stratascript enzyme was added. The samples were incubated at different temperatures starting at 25^{0}C for 10 min, followed by 42^{0}C for 60 min and finally 70^{0}C for 15 min to terminate the reaction.

Polymerase chain reaction

The cDNA obtained from the previous step was then amplified using a Rotor-gene 3000 real-time PCR machine (Corbett Research). Briefly, DEPC water, 10X buffer, $MgCl_2$ dNTP, HotStar Taq, and gene-specific primers and probes were added to the cDNA sample. The samples were heated to 95^{0}C for 50 cycles, at 15 s per cycle to denature and separate the strands of cDNA. The mix was then cooled to 60^{0}C to allow the forward and reverse primers to anneal to the DNA

strand and the HotStar Taq to elongate both primers in the direction of the target sequence.

Fluorescence measurements on FAM, Cy5 and ROX channels were taken every cycle at 60^0C to provide a quantitative, real-time analysis of the PCR reaction for specific genes. The genes of interest included collagen I, collagen II, aggrecan, COMP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The forward and reverse primers and probe sequences for the above mentioned genes are shown in Table 1. The primers and probes were optimized into triplexes such that (collagen I, COMP and GAPDH) and (collagen II, aggrecan and GAPDH) genes could be detected simultaneously.

Gene expression efficiency and abundance

The efficiency of the PCR reactions was determined by taking dilutions of standard samples run in duplicate (1X, 10X, 100X and 1000X). The take off cycle (Ct) of the standard's slope was plotted against the log standards to determine the slope (S). The efficiency (E) was then determined using the following formula.[104]

$$E = 10^{(-\frac{1}{S})}$$

The abundance (A) of the gene was calculated using the determined efficiency for the reaction, as well as the take off cycle for the particular sample.[105]

$$A = (1+E)^{-Ct}$$

Statistical analyses

Statistical analysis was performed using JMP IN™ software. A one-way analysis of variance (ANOVA) was run with five treatment groups (P0, P1, P2, P3 and P4) with passage number as a factor. To compare the effects of coating, a two-way ANOVA was run with coating and passage treated as factors. Coating had four treatment groups (collagen I coating, aggrecan coating, no coating control and no coating passage) while passage had five treatment groups (P0 to P4). If significance was observed with the ANOVAs, a post-hoc Tukey's HSD test was run to pinpoint any specific differences among groups. The significant groups were further analyzed by crossing coating and passage factors to test for any specific differences observed between passages of different coating groups. A p value of less than 0.05 was considered significant for all statistical tests. All results are shown as mean ± standard deviation.

Results

GAPDH as a verification gene

For clarity, the convention mentioned in Table 2 will be used throughout the results and the discussion sections. GAPDH expression was observed in over 98% of the samples that were tested and was, thus, used as a verification gene. Samples with undetectable levels of GAPDH were not processed and considered to be part of a failed reaction. No significant difference was observed in GAPDH expression among groups over passage.

Gene expression with passage

The gene expression abundances for primary and passaged fibrochondrocytes are reported normalized to the amount of RNA per sample and are plotted for the genes of interest. These baseline passage values are shown in the upper left panels of Figure 2 (collagen I), Figure 3 (collagen II), Figure 4 (COMP) and Figure 5 (aggrecan). Over four passages, a sharp 5800-fold increase in gene expression was observed in collagen I levels [$1.1 \times 10^{-09} \pm 1.2 \times 10^{-09}$ (P0) → $6.4 \times 10^{-06} \pm 2.5 \times 10^{-06}$ (P4)] while a 70-fold decrease was observed with collagen II expression [$1.2 \times 10^{-08} \pm 2.8 \times 10^{-09}$ (P0) → $1.8 \times 10^{-10} \pm 1.6 \times 10^{-10}$ (P4)]. COMP levels decreased by 7-fold after the first passage [$6.2 \times 10^{-10} \pm 4.6 \times 10^{-10}$ (P0) → $1.2 \times 10^{-10} \pm 1.2 \times 10^{-10}$ (P1)] and then stayed relatively constant over the next three passages. Aggrecan abundance with passage did not seem to follow any particular trend. A 5-fold decrease in gene expression was observed after the first passage [$1.22 \times 10^{-06} \pm 4.17 \times 10^{-07}$ (P0) → $2.32 \times 10^{-07} \pm 1.20 \times 10^{-07}$ (P1)]. Gene expression was then up-regulated in the second passage by approximately 25-fold [2.3×10^{-07} (P1) $\pm 1.20 \times 10^{-07}$ → $5.9 \times 10^{-06} \pm 2.45 \times 10^{-06}$ (P2)] and then dipped again over the next few passages by about 1.5-fold [$5.93 \times 10^{-06} \pm 2.45 \times 10^{-06}$ (P2) → $4.76 \times 10^{-06} \pm 2.17 \times 10^{-06}$ (P4)].

Reversal attempts with protein coatings

Collagen I and aggrecan coatings were used to determine if any changes in gene expression occurring as a result of monolayer passage could be reversed. The

upper right and lower left panels of Figure 2, Figure 3, Figure 4, and Figure 5 represent the reversal behavior exhibited by these protein coatings.

Collagen I

Cells placed on collagen I and aggrecan coatings showed significantly different gene expression profiles for collagen I over passage compared to the baseline passage and the no coating groups. Both protein coatings were found to lower collagen I expression in the cells from the second to the fourth passage by 50% or greater. In addition, the gene expression in the coating groups for all passages was within 20% of the P0 baseline abundance values.

Collagen II

Contrary to expectations, the drop in collagen II expression observed over four passages was not reversed by either the collagen I or the aggrecan protein coating. In fact, both protein coatings induced a further down-regulation of collagen II expression by approximately 50% or more at most passage time points. Interestingly, even the no-coat control group showed a drop in collagen II expression as was observed with the protein coatings.

Cartilage Oligomeric Matrix Protein

Significant differences were observed between the baseline passage group and the two coating groups. COMP expression in cells plated on collagen I protein coating was up-regulated at each passage time point and had returned to

baseline P0 levels by the third passage. In contrast, the aggrecan coating group showed some signs of reversal over passage, however, the effect was not as pronounced as the collagen I coating group.

Aggrecan

None of the protein coating groups were found to have an effect on the expression of aggrecan in the passaged cells. Cells plated on the aggrecan protein coating tended to decrease aggrecan expression at all passages by at most 2-fold when compared to baseline values, however, the groups were not significantly different.

Discussion

Cartilage tissue engineering studies generally require large numbers of cells that can be attained through expansion in monolayer. However, several experiments with articular chondrocytes and TMJ disc fibrochondrocytes have shown that phenotypic changes are common when dealing with passaged cartilaginous cells.[59, 60, 67, 106] Further, gene expression reversal back to baseline (P0) passage values post-expansion has been met with minimal success.[60, 63] Since similar studies have not been performed for meniscal fibrochondrocytes, in this study the degree of dedifferentiation and subsequent phenotype reversal via protein coatings were investigated by observing gene expression changes over passage. Significant differences in gene expression were observed over four passages for collagen I, collagen II, and COMP, the first two being sensitive markers for the

differentiation state of primary meniscal fibrochondrocytes.[107] In the gene expression reversal experiments, aggrecan and collagen I protein coatings aided in reversing collagen I and COMP expression back to primary values; however, collagen II expression could not be reversed.

The morphology and phenotype of cartilaginous cells may be modulated by altering the culturing conditions. Meniscus cells cultured on alginate beads for three to four wks were found to resemble chondrocytes in morphology as well as upregulate collagen II expression.[107] Similar results have been observed with dedifferentiated chondrocytes placed in 3-D hydrogels such as agarose or alginate.[62, 67] On the other hand, meniscus cells seeded for one day in monolayer appeared to be either rounded like chondrocytes or spindle-shaped like fibroblasts. However, after one wk in monolayer, all cells spread and proliferated, exhibiting a morphology characteristic of fibroblasts.[107] It has been consistently shown in the literature that cartilaginous cells exhibiting a fibroblastic morphology express high levels of collagen I, with a down-regulation in collagen II expression.[60, 106, 108, 109] A similar result was observed in this experiment, where collagen I expression increased by 5800-fold over four passages while collagen II expression decreased by 70-fold. This observation may be attributed to dedifferentiation of meniscus cells in monolayer, analogous to dedifferentiation observed by Darling and Athanasiou.[60] However, the presence of multiple cell populations in the inner two thirds of the meniscus that can proliferate at different rates must also considered as a potential contributor to the observed

phenomenon. For instance, the rapid upregulation in collagen I expression, as normalized to total cells/sample, may be achieved by an increase in collagen I expression per cell, or in the case of multiple cell populations, an increase in the number of collagen I producing cells, or by a combination of the above.[10, 106, 107] Similarly, the observed down-regulation of collagen II may be a direct consequence of a decrease in the ratio of chondrocyte-like cells to fibroblast-like cells. Unfortunately, it is difficult to ascertain whether the passaged meniscus cells are composed of two cell populations or just one cell population expressing mainly fibroblastic genes. In future experiments examining gene expression, it will be imperative to identify whether cell populations can be clearly distinguished prior to passage and if so, isolate the different cell types and analyze their proliferative, morphological and phenotypic properties separately to gain a better understanding of their individual contributions to the observed results.

Gene expression profiles of COMP, a pentameric glycoprotein found preferentially in the pericellular and territorial matrices of meniscus cells, were found to decrease significantly with passage.[16, 110] Disruptions or mutations in the COMP structure have been linked with skeletal development disorders such as pseudochondroplasia and multiple epiphyseal dysplasia, underlining the importance of COMP in the tissue.[111, 112] A recent study with chondrocytes has shown that collagen II down-regulation (the most common chondrocytic dedifferentiation marker) during monolayer passage is accompanied by a quicker down-regulation of COMP.[59] Similar, results were obtained in this experiment,

where COMP expression dropped 7-fold after the first passage, albeit slower than collagen II expression drop (15-fold post first passage). These results are in agreement with previous studies that have determined the function of COMP to be that of maintaining the integrity and properties of the collagen II network by bridging collagen II and collagen IX fibrils.[59, 113]

In addition to culturing conditions, the effect of aging on meniscus cells is a relevant topic of interest. Behavioral differences between immature and adult animals on the level of primary cells exist, and passaged adult cells may dedifferentiate to a different phenotype when compared to the cells examined in this study. Combining the results of this study with previous literature, such differences are expected to be small and the same trends are expected to hold. For instance, a protein expression study using skeletally mature and immature rabbit fibrochondrocytes expanded in primary and secondary monolayer culture showed no significant differences in sulfated proteoglycans and cell number.[114] With respect to the increased collagen I expression and decreased collagen II expression seen in this study as a result of passaging, a more recent gene expression study by Hellio Le Graverand and associates showed that, when compared to cells from immature tissue, adult primary cells expressed higher collagen I and lower collagen II.[37] This observation, taken together with past literature on the dedifferentiation of chondrocytes and the results of this study, would indicate that adult cells are unlikely to be able to reverse this trend (i.e. begin to express more collagen II and less collagen I).[60] The practical result of

this study is thus that, as with cells from immature tissue, with adult cells, the already scarce collagen II expression is likely to be even lower with passage.

The rapid changes in gene expression of meniscus cells over passage are concerning as this has important implications for future tissue engineering studies involving passaged meniscus cells. Several techniques have been used in the past to promote gene expression reversal of passaged chondrocytes and TMJ disc fibrochondrocytes back to primary cell values. These techniques have included utilizing growth factors, 3-D hydrogels and protein coatings.[60, 62, 64, 67] For meniscus cells, experiments have focused mainly on preventing dedifferentiation and stabilizing phenotype. For example, human meniscus cells cultured in alginate beads have been shown to obtain a round chondrocytic shape as well as maintain expression of collagen II over 3 to 4 wks.[107] For most tissue engineering studies, however, the cell population needs to be expanded. Culturing cells in 3-D environments, such as alginate, has been shown to promote protein synthesis while suppressing cell proliferation.[60, 109] Unless an alternate medium that promotes both cell proliferation and phenotype retention is identified, gene expression reversal back to primary cell values of expanded meniscus cells in monolayer remains the most viable option.

We hypothesized that exposing passaged meniscus cells for 24 h to collagen I or aggrecan, proteins abundantly present in the meniscus, would mimic the *in vivo* environment and be conducive to reversing lost phenotype. It is known that cells

plated in monolayer interact with proteins present in FBS that are adsorbed to the cell culture flask.[115, 116] This results in stimuli not generally encountered *in vivo*, prompting changes in cell morphology and surface marker expression.[117]. An interesting result of the reversal study was that aggrecan coating lowered collagen I gene expression back to P0 baseline passage values. Previous studies in our laboratory have shown that insulin-like growth factor-I (IGF-I) treated dermal fibroblasts plated on an aggrecan surface coating adopted a chondrocytic phenotype and morphology, thus, initiating expression of collagen II with a down-regulation of collagen I.[118] Passaged meniscus cells contain a high population of fibroblast-like cells, thus, the observed decrease in collagen I expression was not surprising.[107] The absence of IGF-I in the culture medium, however, may have contributed to the lack of collagen II expression reversal. It is plausible that IGF-I or other growth factors are essential for the expression of collagen II on fibroblast-like cells placed on an aggrecan protein coating.[118] However, the results of this study could also be a consequence of insufficient exposure time (i.e., 24 h) to the aggrecan protein coating.

Collagen I protein coating was found to down-regulate collagen I expression and up-regulate COMP expression. The down-regulation of collagen I expression may be attributed to a collagen I saturation effect experienced by the cells via integrins on the cell surface. It is known cell-surface integrins can attach to region 1 (e.g., α2 I-domain) of collagen I surfaces with a similar homology to the von Willebrand factor.[119] In addition, integrins also aid in transmission of intracellular

signals that can regulate cell growth, differentiation and motility.[120] Thus, it is likely that similar integrins on passaged meniscus cells can sense the presence of excess collagen I in the vicinity and relay messages to the nucleus to down-regulate collagen I expression. Proliferative rates of cells may affect gene expression as well, as is commonly observed in growth plate chondrocytes.[121] It has been shown that fibroblastic cells on 3-D collagen I matrices have slower proliferative rates than chondrocytic cells on the same surface; although, the opposite is true in monolayer culture.[122, 123] As passaged meniscal cells exhibit mainly fibroblastic properties, the down-regulation of collagen I may, perhaps, be attributed to the slower proliferation rate of these fibroblast-like cells. The upregulation of COMP gene expression back to primary fibrochondrocyte levels by the third passage was another exciting finding. COMP is an important marker for the dedifferentiation state of articular chondrocytes and its upregulation may, thus, signal a resurgence of the chondrocytic population in the meniscus.[59]

In this experiment, GADPH expression stayed relatively constant with passage and may be used as a housekeeping gene for future meniscus tissue engineering studies. GAPDH has often been employed as a useful housekeeping gene in RT-PCR studies not involving other standardization techniques. It is commonly believed that within the same tissue sample, GADPH mRNA expression levels are relatively constant while they can vary considerably between tissue types.[124] Recent studies with fibrochondrocytes from the TMJ disc suggest that even though GADPH may be constant in different regions of

the disc, there is a definite change in abundance with passage, a phenomenon not observed in passaged meniscal fibrochondrocytes.[106]

Conclusions

In summary, these data indicate that the cells of the inner 2/3rd of the meniscus undergo significant changes during monolayer expansion and passage. They experience losses in major chondrocytic markers (collagen II and COMP) while simultaneously experiencing gains in fibroblastic markers (collagen I). Reversal efforts to regain lost phenotype in passaged meniscus cells via protein coatings were successful for collagen I and COMP via collagen I and aggrecan coatings. However, collagen II gene expression reversal proved to be unsuccessful. A lack of collagen II could result in structural breakdown of the tissue as well as preempt osteoarthritis.[14, 125, 126] Hence, it will be important to investigate alternate vehicles to reverse losses in collagen II expression in passaged meniscus cells. These could include studying alternate protein coatings such as collagen II and decorin, adding growth factors such as transforming growth factor-βI (TGF-βI), fibroblast growth factor-II (FGF-II), and IGF-I to the culture medium or culturing the cells in novel 2-D or 3-D environments that support proliferation while maintaining morphology.[60, 64, 65, 67, 127-130]

Figures associated with chapter 2

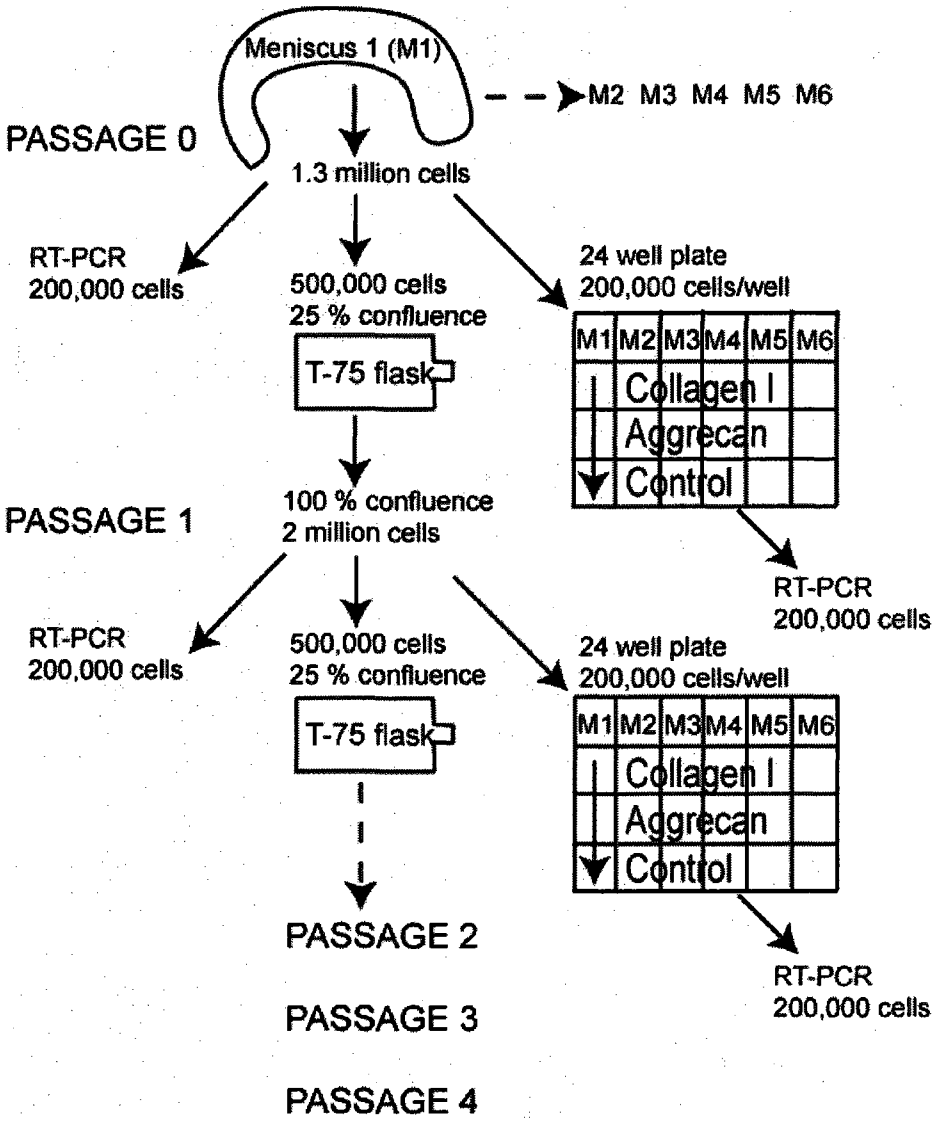

Figure 1. Experimental design
In brief, bovine meniscus cells were expanded through four passages in monolayer culture; 0.5×10^6 cells were expanded in a T-75 flask to confluence. At each passage time point, 0.2×10^6 cells were collected for RT-PCR, and 0.2×10^6 cells were plated on an aggrecan or collagen I two-dimensional surface coating or on a no coating control for 24 hrs and then subsequently processed for RT-PCR. The gene expression profiles with passage and on the different protein coatings were then determined. n = 6 was used for all gene expression abundance evaluations.

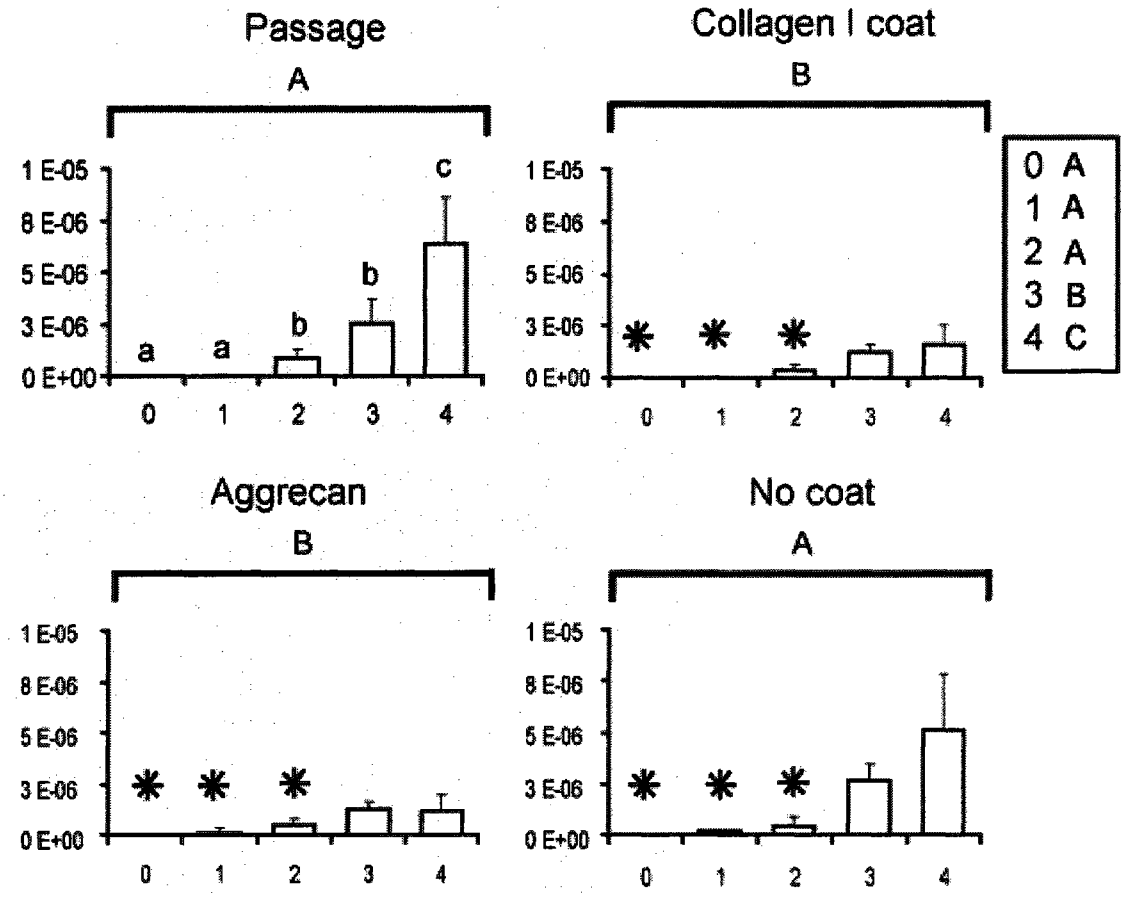

Figure 2. Collagen I gene expression profiles
The *x*-axis refers to the passage number, and the *y*-axis to the gene expression abundance (in the exponent notation used, 'E-*n*' stands for '× 10^{-n}'). Small letters denote significant differences with passage, using a one-way analysis of variance (top left). Capital letters denote significant differences between levels (passage, collagen I coating, aggrecan coating and no coating), using a two-way analysis of variance. Stars denote groups that are not significantly different from values of the primary cells (that is, the P0 value in the top left panel), using an interaction term between the two factors.

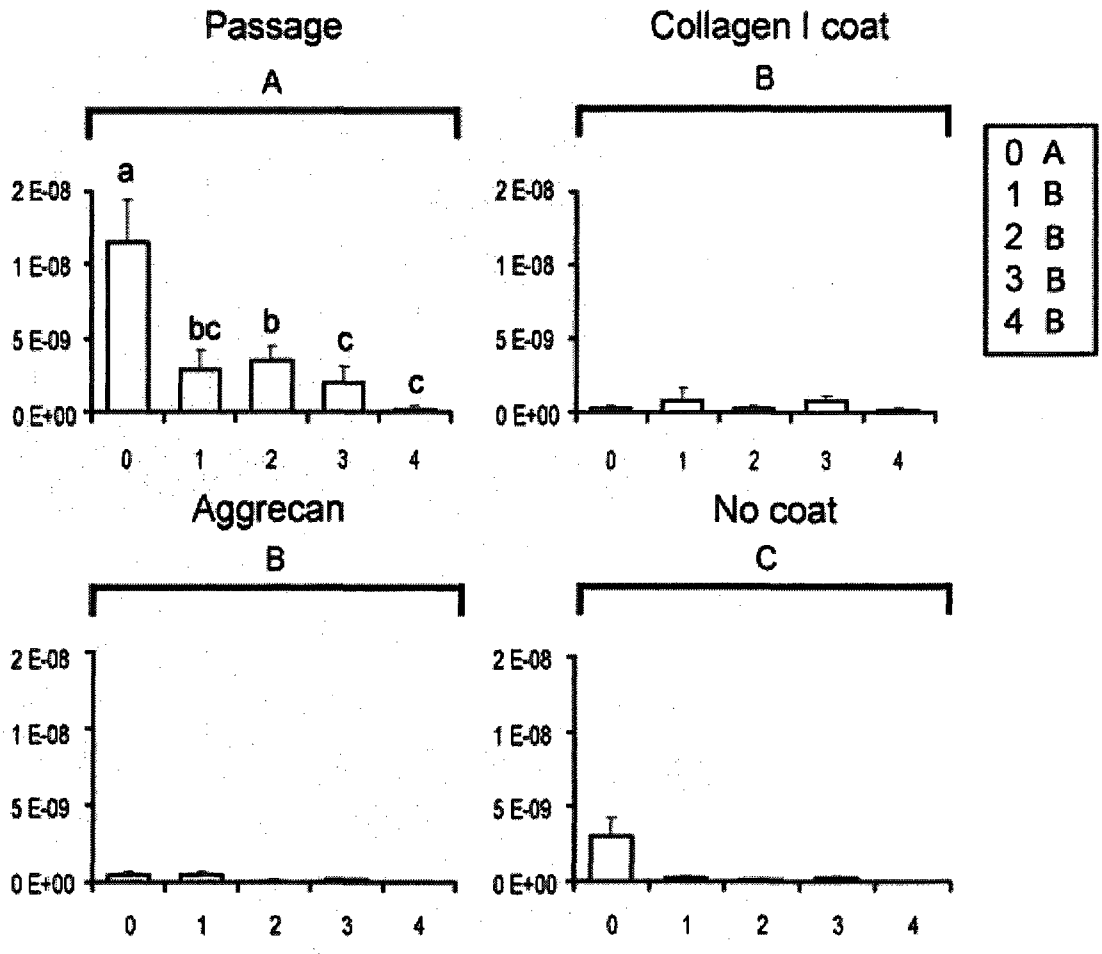

Figure 3. Collagen II gene expression profiles
The *x*-axis refers to the passage number, and the *y*-axis to the gene expression abundance (in the exponent notation used, 'E-*n*' stands for '× 10^{-n}'). Small letters denote significant differences with passage, using a one-way analysis of variance (top left). Capital letters denote significant differences between levels (passage, collagen I coating, aggrecan coating and no coating), using a two-way analysis of variance.

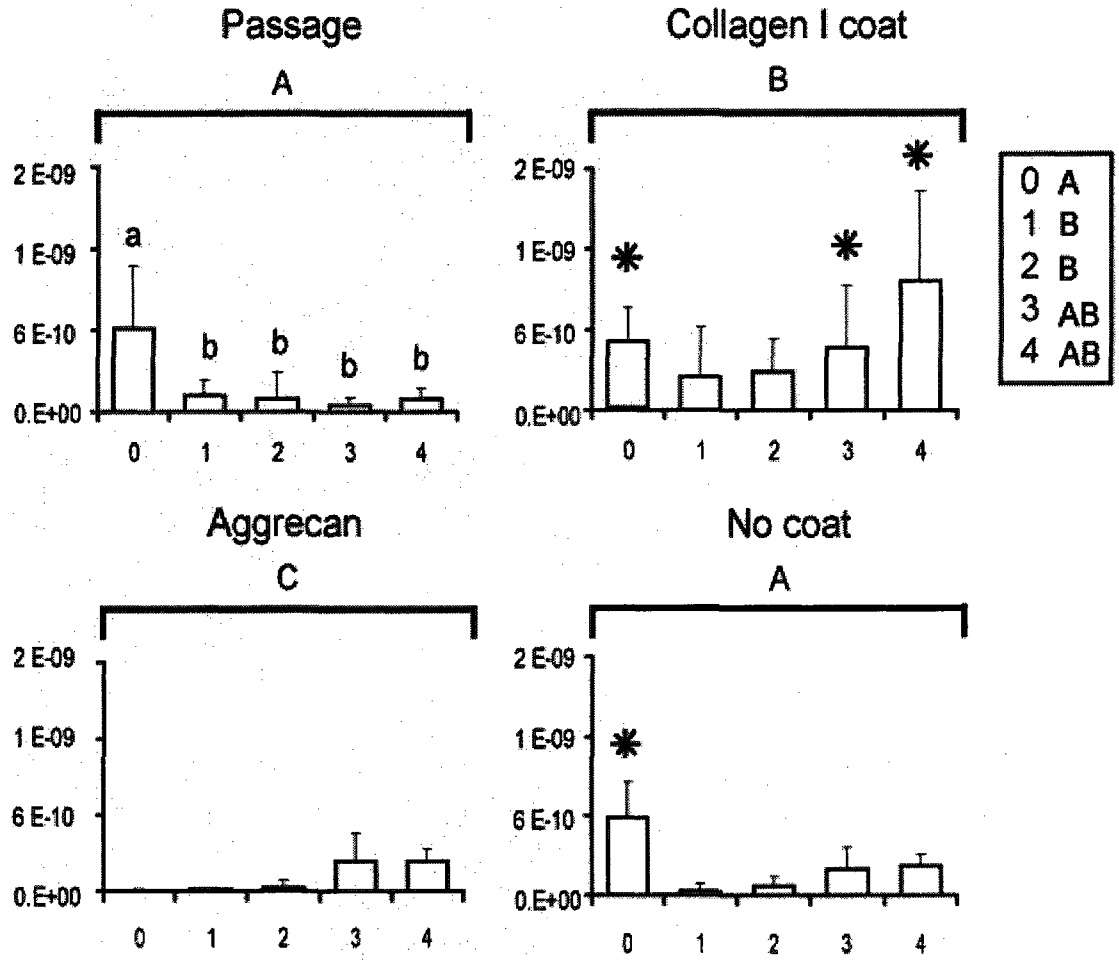

Figure 4. COMP gene expression profiles
The *x*-axis refers to the passage number, and the *y*-axis to the gene expression abundance (in the exponent notation used, 'E-*n*' stands for '× 10^{-n}'). Small letters denote significant differences with passage, using a one-way analysis of variance (top left). Capital letters denote significant differences between levels (passage, collagen I coating, aggrecan coating and no coating), using a two-way analysis of variance. Stars denote groups that are not significantly different from values of the primary cells (that is, the P0 value in the top left panel), using an interaction term between the two factors.

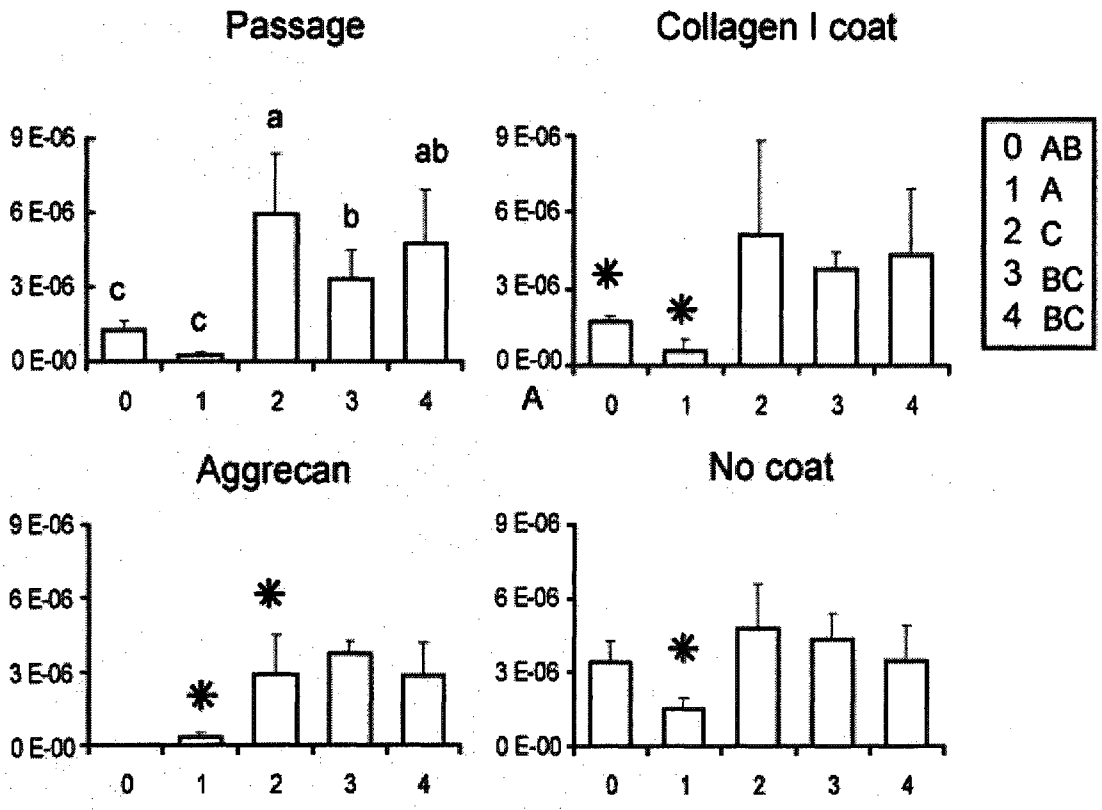

Figure 5. Aggrecan gene expression profiles
The *x*-axis refers to the passage number, and the *y*-axis to the gene expression abundance (in the exponent notation used, 'E-*n*' stands for '× 10^{-n}'). Small letters denote significant differences with passage, using a one-way analysis of variance (top left). Capital letters denote significant differences between levels (passage, collagen I coating, aggrecan coating and no coating), using a two-way analysis of variance. Stars denote groups that are not significantly different from values of the primary cells (that is, the P0 value in the top left panel), using an interaction term between the two factors.

Table 1: Primer and probe sequences of desired genes

Target gene (GenBank accession number, product size)	Forward primer (5′ - 3′)	Reverse primer(5′ - 3′)
Collagen I (NM-174520, 97bp)	CATTAGGGGTCACAATGGTC	TGGAGTTCCATTTTCACCAG
Collagen II (X02420, 76bp)	AACGGTGGCTTCCACTTC	GCAGGAAGGTCATCTGGA
Aggrecan (U76615, 76bp)	GCTACCCTGACCCTTCATC	AAGCTTTCTGGGATGTCCAC
COMP (X74326, 72bp)	TCAGAAGAGCAACGCAGAC	TCTTGGTCGCTGTCACAA
GAPDH (U85042, 86bp)	ACCCTCAAGATTGTCAGCAA	ACGATGCCAAAGTGGTCA

Target gene (GenBank accession number, product size)	Probe (5′ - 3′)
Collagen I (NM-174520, 97bp)	ATGGATTTGAAGGGACAGCCTTGG
Collagen II (X02420, 76bp)	ATGACAACCTGGCTCCCAACACC
Aggrecan (U76615, 76bp)	TGACGCCATCTGCTACACAGGTGA
COMP (X74326, 72bp)	CAGAGGGATGTGGACCACGACTTC
GAPDH (U85042, 86bp)	CCTCCTGCACCACCAACTGCTT

Table 2. Explanation of different passage numbers and surface coating groups

Passage		Coating	
P0	Unpassaged cells	**Baseline passage**	Cells from P0 to P4 on T-75 flasks
P1	Cells undergone one passage	**Col1 coat**	Cells from P0 to P4 on collagen I coat
P2	Cells undergone two passages	**Agc coat**	Cells from P0 to P4 on aggrecan coat
P3	Cells undergone three passages	**No coat**	Cells from P0 to P4 on a no coat control
P4	Cells undergone four passages		

Chapter 3: Temporal effects of culture media on unseeded non-woven PLLA meshes for meniscus tissue engineering

Najmuddin J. Gunja and Kyriacos A. Athanasiou

I would like to acknowledge Dr. Elizabeth Christenson and Sue Anne Chew for assisting with the GPC and ^{1}H-NMR assays, respectively

Abstract

A variety of scaffolds are currently used for meniscus tissue engineering including agarose, alginate and PLLA. Of these, PLLA has shown particular promise in supporting meniscus cell adhesion and proliferation on the construct, as well as ECM deposition on the construct. However, the temporal effects of aqueous medium on the mechanical and degradation properties of PLLA scaffolds used in our tissue engineering approach have not been studied. In this experiment, three culturing conditions were examined (PBS, medium + 10% FBS and serum free medium) and scaffolds were cultured for 0, 4, 8, or 12 wks. Compressive biomechanical and tensile tests were performed at each time point for each culturing condition. In addition, GPC and proton NMR-spectroscopy were performed to examine molecular weight (MW) and ester degradation over time. No significant differences were observed for compressive and tensile properties among culturing conditions at each time point and over time. GPC results showed that the MW of our scaffolds ranged from 108 kDa ± 12 kDa at t = 0 wks to 95 ± 15 kDa at 12 wks, although this drop was not significant. Proton-NMR spectroscopy analyses showed a typical PLLA curve with consistent peaks between culturing conditions and between time points suggesting that degradation did not take place over the 12 wk culture period. Overall, these results confirm that unseeded PLLA scaffolds do not degrade substantially or lose mechanical properties over 12 wks in culture.

Introduction

Tissue engineering of the meniscus requires consideration of several factors including cell source, culturing conditions and animal model. Of particular importance is the scaffold material where the cells can proliferate and secrete extracellular matrix (ECM). Natural scaffolds such as collagen, small intestinal submucosa, perichondrial tissue and the periosteum have been studied for meniscus tissue engineering and a summary of the results obtained with these scaffolds has been reviewed elsewhere.[131] Synthetic polymer scaffolds, such as poly-urethane, poly-l-lactic acid (PLLA), poly-glycolic acid (PGA), poly-caprolactone (PCL) are also gaining popularity.[131] The mechanical and degradation properties of these scaffolds can be tailored depending on the manufacturing or fabrication process.[57] Of these synthetic materials, some warrant no further research, while others such as PLLA which are amenable to cell attachment and proliferation and support ECM production need to be further characterized.

In the present study, we sought to characterize the degradation and mechanical properties of unseeded non-woven PLLA scaffolds used in the laboratory for meniscus and tempormandibular joint (TMJ) disc tissue engineering. Cells can quicken the degradation of polymers such as PLLA by releasing factors and enzymes that can quicken ester hydrolysis, the mechanism known to degrade PLLA.[57] PLLA itself undergoes bulk degradation over time in aqueous medium which results in a loss of molecular weight.[132] Bulk degradation is the

predominant process of degradation in poly-lactides and glycolides. A recent study has shown that bovine serum albumin, an important component of fetal bovine serum, can quicken PLLA degradation.[133] Several tissue engineering studies utilize FBS as an important component of the culture medium and, thus, its effect of PLLA degradation may influence construct properties.

The objective of this study was to ascertain that the scaffold used in our meniscus tissue engineering studies does not degrade prematurely, potentially impeding ECM production. To isolate the effects of culture medium and time, we examined unseeded PLLA constructs at multiple time points ranging from 0 to 12 wks. In addition, three different culture media were utilized, phosphate buffered saline (PBS), medium with FBS and serum free medium. It was hypothesized that: a) scaffolds cultured in medium with FBS would degrade to a greater extent than those cultured in other groups; and b) the mechanical properties and MW of the scaffolds would decrease over time in aqueous solution but not substantially.

Materials and methods

Scaffold culture

Cylindrical 2 mm thick and 3 mm wide non-woven PLLA scaffolds (Biomedical structures) were obtained from a PLLA sheet using a 3 mm dermal punch. The porosity of the scaffolds was over 90% with fiber diameters approximately 25 μm. The scaffolds were sterilized using ethylene oxide. They were then pre-wetted with 70% ethyl alcohol, and subsequently washed twice with phosphate buffered

saline (PBS). The scaffolds were housed in individual wells of 12 well plates that were previously coated with 0.5 ml of 2% sterile molten agarose and incubated overnight in culture medium. 60 scaffolds per group were cultured in one of three aqueous conditions: a) PBS b) Dulbecco's Modified Eagle's Medium (DMEM) and c) DMEM and 10% FBS. The culture medium was changed once every 3 days for a period of 12 wks. At 0, 4, 8 and 12 wks, 15 scaffolds from each group were removed and processed for biomechanical testing, proton-nuclear magnetic resonance (H-NMR) and gel permeation chromatography (GPC).

Compressive tests

The viscoelastic compressive properties of samples from each group (n = 5) were tested at t = 4 wks using a set up described previously.[134] Briefly, an incremental stress relaxation test at 10%, 20% and 30% strain was designed and implemented after determining the height of the construct. Each sample was held at the chosen strain level for 20 min with a 10% strain step. The strain rate was kept constant throughout at 0.5 mm/s. Data obtained from each test were fitted using MATLAB to an incremental stepwise viscoelastic stress relaxation solution for a standard linear solid.[135] The parameters obtained were converted to instantaneous modulus (E_i), relaxation modulus (E_r) and coefficient of viscosity (μ) for each strain level.

Tensile tests

Five samples were used for tensile tests at t = 4 wks. Tests were performed using an Instron 5565 with a 50 N load cell. To ensure testability, samples were carefully carved into a dog-bone shape with a 2 mm gauge length. The ends of the samples were glued to paper tabs using cyanoacrylate. After a 0.05 N tare load was applied, the constructs were pulled to failure at a constant strain rate of 0.02 s^{-1}. Stress-strain curves generated from the test yielded the tensile stiffness and the ultimate tensile strength (UTS) of each construct.

Proton - NMR spectroscopy

Post-compression, two PLLA samples from each group were processed for ^{1}H-NMR spectroscopy to analyze chemical structure and to probe for potential ester degradation to the polymer. Samples were dissolved in $CDCl_3$ and NMR spectra were acquired on a Bruker AC-250 spectrophotometer.

Gel permeation chromatography

Three samples from each group were combined and weighed at each time point. Chloroform was added to the samples at 20mg/ml. Molecular weight distributions were determined using gel permeation chromatography. Phenogel columns and guard columns (50×7.8 mm, 5 μm, mixed bed, Phenomenex, Torrance, CA) were used to elute samples at 1 ml/min flow rate. Known molecular weights of polystyrene were used to form calibration curves.

Statistical analyses

The two factors (culturing condition and time) were compared using a two-way analysis of variance (ANOVA). If significant differences were observed, a Tukey's *post hoc* test was performed to determine specific differences among groups. A significance level of 95% with a p value of 0.05 was used in all statistical tests performed. All values are reported as mean ± standard deviation.

Results

Gross morphology

At all time points, samples appeared to retain their shape and structure with no observable signs of degradation. Temporally, no significant differences were observed in the thickness and diameter of constructs cultured in the three types of media.

Compressive tests

Compressive viscoelastic tests were performed at three different strain levels, 10%, 20% and 30% and the instantaneous modulus, relaxation modulus and coefficient of viscosity were determined at each strain level (Figure 6). No significant differences were observed among groups for the three tested parameters at all strain levels. In addition, no temporal differences were observed among groups for the compressive properties. Values for the instantaneous modulus ranged from 10-15 kPa, the relaxation modulus ranged

from 6-9 kPa and the coefficient of viscosity ranged from 0.4 to 0.55 MPa-s. These values persisted at later time points as well.

Tensile tests

The highest tensile stiffness and strength values were recorded in the serum free medium group at t = 0 with 400 ± 130 kPa and 220 ± 120 kPa, respectively (Figure 7). The lowest tensile stiffness and strength values were recorded in the medium + FBS groups at t = 12 wks with 380 ± 100 kPa and 190 ± 100 kPa. Although a general downward trend was observed in tensile properties over time, the differences among groups and time points were not significant.

Proton - NMR spectroscopy

^{1}H-NMR spectra for PLLA scaffolds in all groups and at all time points were unremarkable (Figure 8). Specifically, evidence of ester degradation in scaffolds cultured for longer time points was not observed with spectra of scaffolds at t = 0 similar to that of scaffolds cultured at t = 12 wks. In addition, the presence of FBS was not found to enhance degradation of ester bonds or interfere with the peaks.

Gel permeation chromatography

Molecular weights ranged from 108 ± 12 kDa (t = 0, PBS) to 95 ± 15 kDa (t =12 wks, medium + FBS), however no significant differences were observed among groups for both factors (culturing condition (p = 0.53) and time (p = 0.11) (Table 3).

Discussion

Scaffold degradation profiles can influence the success of a tissue engineering study. The scaffold should be able to retain sufficient mechanical properties early on and allow cells to produce relevant ECM. Aliphatic polymeric scaffolds, such as PLLA, degrade via ester hydrolysis, which implies that the aqueous nature of the culture medium may play a crucial role in degradation. To isolate the effects of culture medium, in this experiment, unseeded PLLA scaffolds in three types of media (serum free medium, medium with FBS, and PBS) were examined for 0, 4, 8 and 12 wks. Compressive and tensile properties, and molecular weights of the scaffolds were not found to significantly differ among groups cultured in different media and over time. ^{1}H-NMR spectra did not indicate ester degradation over three months in culture with consistent peaks observed at all time points. Overall, the culture medium did not significantly affect PLLA mechanical and degradation properties over three months confirming the potentially non-deleterious role of the scaffold in tissue engineering studies.

The mechanical properties of non-woven PLLA meshes were examined at various time points with no significant differences observed for the instantaneous modulus, the relaxation modulus, and the coefficient of viscosity. In addition, no obvious strain dependencies were observed; although, samples compressed at 30% strain trended towards the highest mechanical properties. Our results agree with a prior study showing only minor changes in the tensile properties of PLLA fibers cultured in PBS.[136] In that study, PLLA was cultured up to 35 wks,

approximately three times longer than our culture period. The molecular weight of the scaffolds decreased with time; however, the decrease was not significant. This was contrary to reports in the literature that suggest molecular weight drops consistently with time in culture.[132, 136] These differences may be attributed to pre-culturing factors such as the fabrication technique, original molecular weight, degree of crystallinity, thermal history, inherent viscosity and porosity of the polymer that can influence the polymer's overall behavior.[132]

FBS contains a variety of growth factors and chemicals that are beneficial for cell proliferation and growth. However, a recent study showed that BSA, an important component of FBS, enhances the degradation of PLLA films as a result of activity resembling the enzyme esterase that is responsible for ester degradation.[133] However, in this experiment we were unable to find any differences in degradation profiles of the scaffolds with time using ^{1}H-NMR analysis.

Conclusions

Overall, we were able to show that the scaffolds utilized in our tissue engineering approach can retain their mechanical properties and MW, and do not degrade up to at least 12 wks in serum-free or serum containing media. Future studies will have to examine whether the presence of cells accelerates potential degradation and subsequent drop in biomechanical properties.

Figures associated with chapter 3

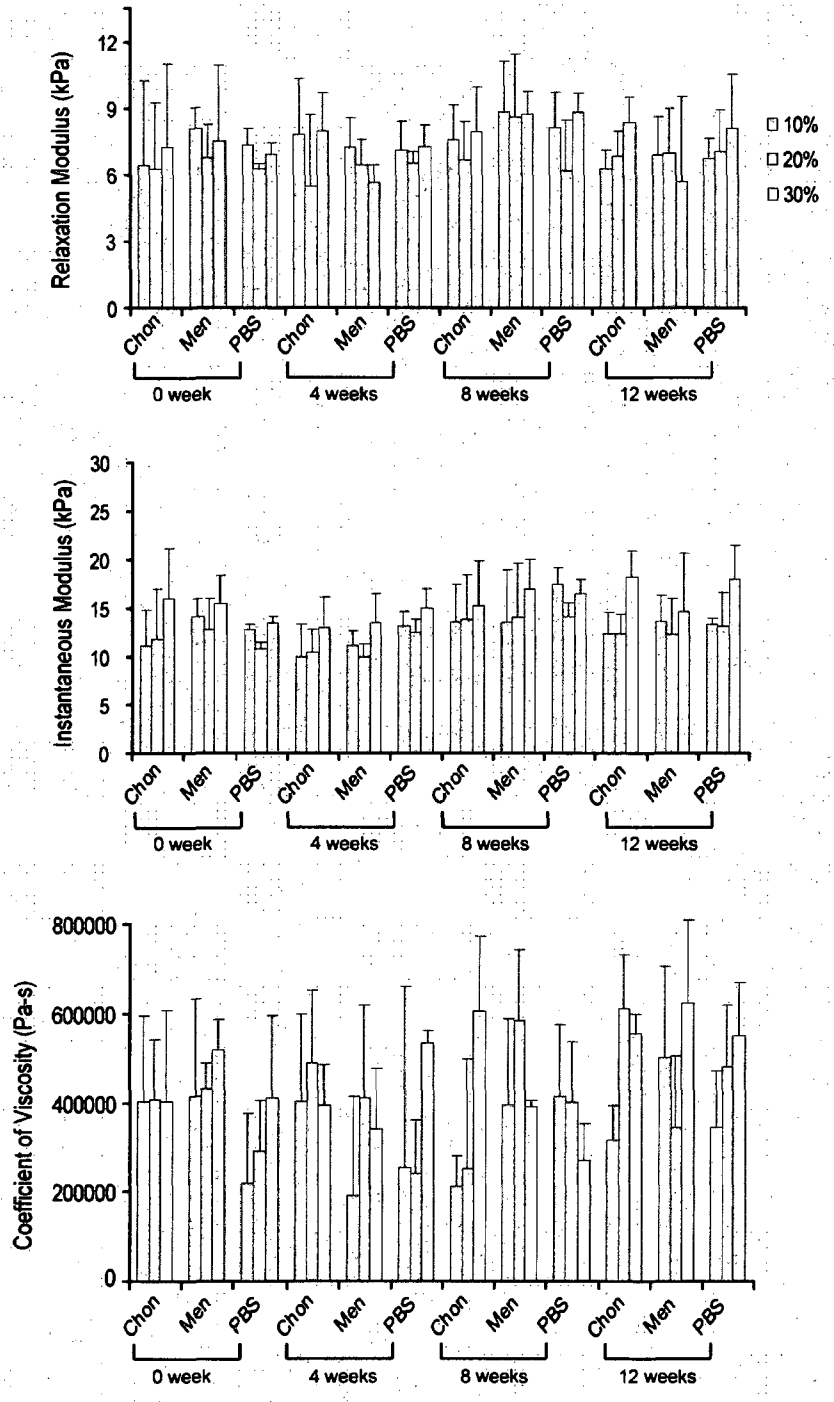

Figure 6. Compressive properties of unseeded PLLA constructs
No significant differences were observed among culturing conditions and time points at all strain levels for the compressive properties of the constructs. All values are reported mean ± SD.

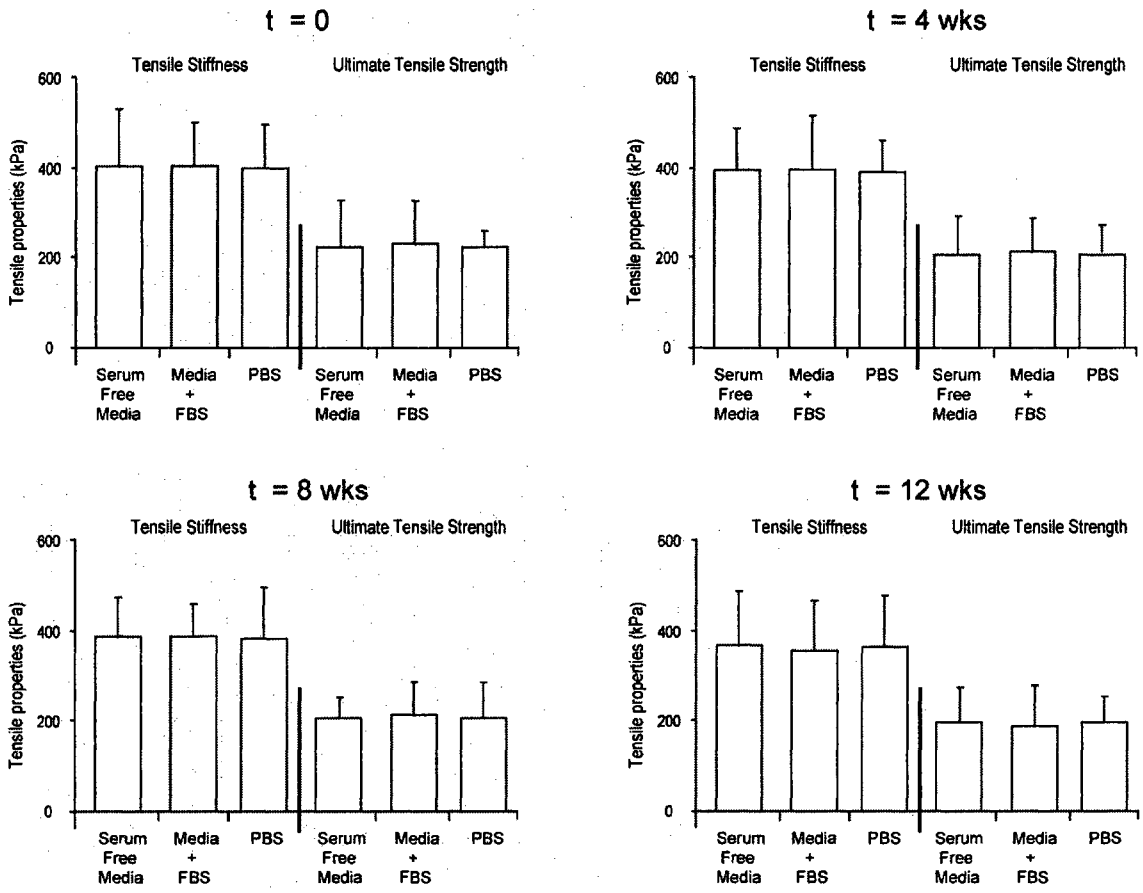

Figure 7. Tensile properties of unseeded PLLA constructs
No significant differences were observed among culturing conditions and time points for the tensile stiffness and UTS of the constructs. All values are reported mean ± SD.

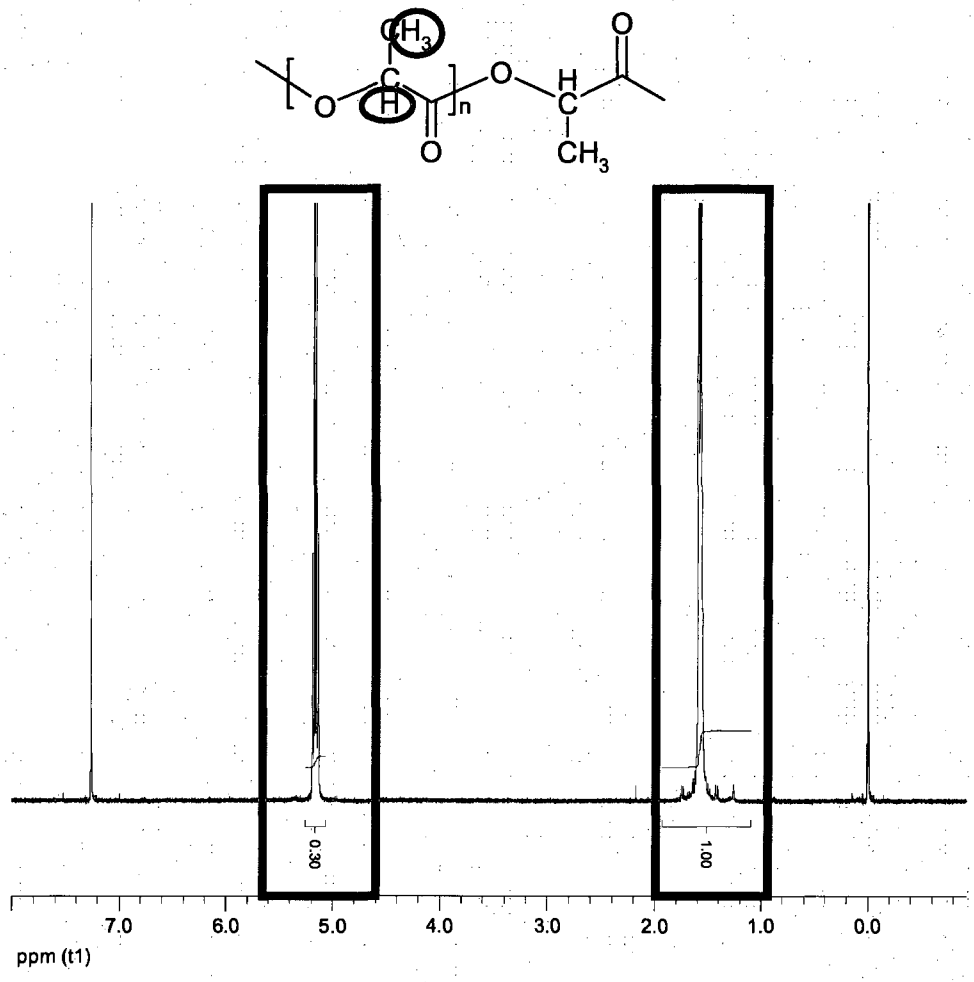

Figure 8. Representative ^{1}H-NMR spectra of PLLA
The structure of PLLA is shown on the top of the PLLA spectrum. A shift in the hydrogen peak would represent ester degradation. However, peaks did not shift as a result of the culture conditions or over time.

Table 3. Weight-average molecular weight of PLLA constructs at different time points

Experimental groups	t = 0 kDa	t = 4 wks kDa	t = 8 wks kDa	t = 12 wks kDa
Serum-free medium	105 ± 11	103 ± 10	101 ± 8	99 ± 11
Medium + FBS	103 ± 15	105 ± 11	97 ± 13	95 ± 15
PBS	108 ± 12	104 ± 9	99 ± 14	97 ± 18

Chapter 4: Effects of hydrostatic pressure on leporine meniscus cell-seeded PLLA scaffolds

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Chapter accepted as: Gunja NJ and Athanasiou KA. Effects of hydrostatic pressure on leporine meniscus cell-seeded PLLA scaffolds. Biomedical Materials Research Part A.

Abstract

Hydrostatic pressure (HP) is an important component of the loading environment of the knee joint. Studies with articular chondrocytes and TMJ disc fibrochondrocytes have identified certain benefits of HP for tissue engineering purposes. However, similar studies with meniscus cells are lacking. Thus, in this experiment, the effects of applying 10 MPa of HP at three different frequencies (0, 0.1 and 1 Hz) to leporine meniscus cell seeded-PLLA scaffolds were examined. HP was applied once every 3 days for 1 hr for a period of 28 days. Constructs were analyzed for cellular, biochemical and biomechanical properties. At t = 4 wks, total collagen/scaffold was found to be significantly higher in the 10 MPa, 0 Hz group when compared to other groups. This despite the fact that the cell numbers/scaffold were found to be lower in all HP groups when compared to the culture control. Additionally, the total GAG/scaffold, instantaneous modulus, and relaxation modulus were significantly increased in the 10 MPa, 0 Hz group when compared to the culture control. In summary, this experiment provides evidence for the benefit of a 10 MPa, 0 Hz stimulus, on both biochemical and biomechanical aspects, for the purposes of meniscus tissue engineering using PLLA scaffolds.

Introduction

The knee menisci, fibrocartilaginous tissues that lie on the tibial plateau, are involved in several important biomechanical processes, including load transmission, shock absorption, and lubrication of the knee joint.[137, 138] The menisci are subjected to a variety of forces *in vivo*, including tension, compression, shear, and hydrostatic pressure (HP).[15, 86, 137] The ability of the meniscus to withstand these forces may, to a large extent, be attributed to the unique collagen fiber alignment and orientation within the structure.[1, 15] Abnormalities or complications in the structure of the meniscus can lead to degeneration of the tissue as well as precipitate osteoarthritis.[22, 23] A promising modality to overcome this problem is functional tissue engineering which serves to replace a damaged meniscus with an engineered construct with desired biomechanical and biochemical properties.

The past decade has seen a marked increase in efforts to engineer the meniscus using classical tissue engineering strategies involving cells, growth factors and scaffolds. The concept of using mechanical stimulation as an additional element to tissue engineer the meniscus is now gaining popularity.[15] The importance of the mechanical environment for developing musculoskeletal tissues cannot be understated, as mechanical forces are known to influence these tissues' performance. For example, numerous experiments on disuse of musculoskeletal tissues like cartilage, muscle, and bone have been correlated to apoptosis within the tissues, followed by their subsequent atrophy.[139-141] Additionally, *in vitro*

studies with meniscus explants and cells have shown that mechanical stimuli such as direct compression, tension and HP can increase extracellular matrix (ECM) expression and synthesis.[83-91] The use of HP is of particular interest as it causes no macro-scale deformation to the construct, yet is responsible in stimulating cells to increase ECM synthesis, possibly by altering intracellular ion flux.[92] Such a stimulus may be favorable in the early stages of a tissue engineering study where cells seeded on scaffolds are still in the proliferation and migration stages and are vulnerable to external stimuli.

HP studies can be broadly divided into three categories, a) 2-D monolayer studies, b) 3-D explant studies, and c) 3-D tissue engineering studies. Results from experiments in the first category are often reported in terms of gene expression. Studies on articular chondrocytes and fibrocartilaginous metaplasia of Achilles tendon fibroblasts have shown an upregulation of aggrecan and collagen II expression in cells immediately after intermittent hydrostatic pressurization.[87, 93-95] Interestingly, a further upregulation of collagen and aggrecan expression was observed in the fibrocartilaginous metaplasia study when the samples were tested 24 hrs post-stimulation suggesting that rest time might be an important factor to consider in long-term tissue engineering studies. TMJ disc cells have been shown to increase collagen I expression under static HP of 10 MPa and increase collagen II expression during intermittent HP stimulation of 10 MPa, 1 Hz.[96] Studies with explants have investigated the effects of HP on proteoglycan synthesis as well as MMP regulation. An experiment with

articular cartilage explants showed that cyclic HP at physiological magnitudes caused an upregulation of sulfate incorporation into the explants.[97] Cyclic HP on meniscus explants has been shown to inhibit upregulation of potent effector molecules such as MMP-1, MMP-3, and COX-2.[86] 3-D studies for tissue engineering purposes have been performed by encapsulating cells in alginate beads, using pellet cultures, seeding cells on scaffolds or using scaffoldless self-assembly techniques.[96, 98-102] Results of these studies have varied significantly between research groups, often even when using similar cell sources. For example, although intermittent HP application has been shown to consistently increase GAG production by chondrocytes on PGA and PLGA scaffolds, as well as in pellet cultures, this has not been observed in self-assembled articular chondrocyte constructs.[98, 100, 101] Static HP has been shown to increase both collagen and aggrecan synthesis in intervertebral disc cells while only increasing collagen content in TMJ disc cells.[96, 99] Thus, specific regimens of both static and intermittent HP stimuli exhibit beneficial effects for tissue engineering purposes, even though the underlying mechanisms are still unclear.

In this experiment, a traditional tissue engineering approach was employed using meniscus cells seeded on PLLA scaffolds. Non-woven meshes of high molecular weight PLLA (>100kDa) degrade slowly over time and are, thus, advantageous for long term applications over the extensively studied PGA, which degrades rapidly in aqueous environments.[45, 57, 142, 143] In addition to the longer half-life of the polymer, data from our laboratory using meniscus cells and TMJ disc

fibrochondrocytes suggest that PLLA scaffolds adequately promote cell proliferation and ECM synthesis and are comparable to data published with PGA scaffolds.[45, 96, 144] Thus, PLLA was chosen as the scaffold material for the experiment.

The objective of this study was to determine the effects of periodic and intermittent HP on leporine meniscus cell-seeded PLLA scaffolds. Several different loading regimens were tested where the frequency of loading and the pressure applied were controlled. The hypothesis was that both intermittent and static HP would increase the production of collagen and GAG molecules on the scaffolds as well as enhance the mechanical integrity of the scaffolds as a result of matrix deposition.

Materials and methods

Cell harvesting

Medial and lateral menisci were isolated under aseptic conditions from 1 to 2 yr old New Zealand white rabbits, sacrificed by a local rabbit breeder on the day of harvest. Each meniscus was taken to a cell culture hood, washed with autoclaved phosphate buffered saline (PBS) and transferred to culture medium containing 2% penicillin-streptomycin-fungizone (PSF) (Cambrex). The culture medium contained 50:50 Dulbecco's modified Eagle's medium (DMEM)-F12 (Invitrogen), 10% fetal bovine serum (FBS) (Mediatech), 1% non-essential amino acids (NEAA) (Invitrogen), 25 µg of l-ascorbic acid (Sigma) and 1% PSF. The

menisci were minced into small fragments (< 1 mm^3) and digested overnight at 37°C in 2 mg/ml collagenase type II (Worthington Biochemical). Vascular portions of the meniscus were discarded prior to mincing. Post-digestion, an equal volume of PBS was added to the collagenase digest and the mixture was centrifuged at 200 *g*. The bulk of the supernatant was removed, more PBS was added, and the mixture was centrifuged again to wash the pellet of collagenase. This process was repeated twice leaving behind a white pellet of meniscus cells. Cell counts were obtained using a hemocytometer. Cell viability was assessed using a Trypan blue exclusion test and was found to be greater than 95%. The cells were then resuspended in culture medium supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO) and frozen at -80°C for up to a month.

Cell culture and passage

Cells from four rabbits were pooled together after thawing, and the viability was found to be over 90%. The cells were then plated on T-225 flasks at 25% confluence and allowed to expand. At full confluence, the cells were passaged using trypsin/EDTA (Invitrogen) and counted with a hemocytometer.

Scaffold, spinner flask preparation and cell seeding

High molecular weight (100 kDa) non-woven PLLA scaffold sheets (~90% porosity) (Biomedical Structures) were cut into cylinders 1.5 mm in thickness and 3 mm in diameter using a 3 mm dermal punch. Each scaffold was strung on a stainless steel wire, alternating with beads to avoid scaffold-scaffold contact. The

scaffolds were sterilized using ethylene oxide. These were then pre-wetted in 70% isopropyl alcohol, washed twice with PBS, and placed in an autoclaved spinner flask filled with regular culture medium. Cells, passaged once, were seeded into the spinner flask at a density of 0.7 million cells/scaffold corresponding to approximately 50,000 cells/mm^3 of scaffold by volume. Stirrer bars placed at the bottom of the spinner flask were rotated at 60 RPM post-seeding for 3 days. The scaffolds were left in the spinner flask for an additional 4 days without stirring to allow cells to fully adhere to the polymer. After 1 wk, the steel wire was removed and the cell-seeded constructs were transferred to agarose-coated six-well plates. The agarose coatings on the wells were prepared by adding 1.5 ml of 2% sterile molten agarose to each well. Each well housed six cell-seeded scaffolds.

Experimental groups

Post-static culture, the scaffolds were randomly assigned into HP or control groups (n = 6 per group). Three different HP groups (Figure 9) were tested. Scaffolds in the first group were exposed to static HP of 10 MPa for 1 hr every 3 days for 4 wks. Semantically, a static HP regimen implies one continuous application throughout the entire culture duration; however, since the application occurred repeatedly over a period of 4 wks with non-pressurized rest in between, the term periodic HP will be used throughout the results and the discussion section to describe this group. The second and third groups were intermittent HP groups where 10 MPa at 0.1 Hz or 10 MPa at 1 Hz of HP was applied for the

same period as the periodic HP group. These particular regimens were chosen based on their benefits observed in prior HP experiments.[87, 96, 145] Additionally, during the 1 hr of stimulation, a duty cycle of 60 s on, 60 s off was employed for the intermittent HP groups. This was employed based on a study with dynamically stimulated articular cartilage explants where increased radiolabeled sulfate uptake was observed if a rest time of 100 s or less was employed between each stimulation.[146] Two control groups were utilized in this experiment (Figure 9). Scaffolds in the first control group remained in six-well plates for the duration of the experiment (culture control). The second control group consisted of unpressurized scaffolds that underwent the same manipulation as pressurized scaffolds including loading into the pressure chamber, except no pressure was applied (HP control).

HP preparation and application

The cell-seeded scaffolds in the HP groups were placed in histology cassettes (Fisher Scientific) that were previously cleaned with 70% isopropyl alcohol and sterilized with ethylene oxide. The cartridges were then transferred to individual heat sealable sterile bags (Ampac). To each bag, 30 ml of culture medium with 1% FBS (supplemented with 10mM Hepes) was added, and the bag was tapped lightly to remove air bubbles from the medium and from the cartridges. The bag was then heat-sealed without any bubbles inside.

The HP set-up used in this experiment has been described previously.[96, 101] Briefly, the control specimens were placed in an open unpressurized chamber while the pressurized specimens were placed in a water filled stainless steel chamber (Parr Instrument Company) that connected to an Instron 8871 via a water-driven stainless steel piston (PHD Inc.). Both chambers rested in a water bath set at 37°C. Displacement was controlled by an Instron WaveMaker program that also recorded pressures. Post-stimulation, the bags containing the scaffolds were cleaned with 70% isopropyl alcohol and transferred back into the culture hood and unsealed. The scaffolds were then removed from the cartridges and placed back into six-well plates with fresh medium.

Histology

Post-stimulation (t = 4 wks), one sample from each group was frozen and sectioned at 12 μm and placed on adhesive slides (Instrumedics). Ultraviolet light was used to crosslink the section to the slide. Harris hematoxylin and eosin (H&E) stains were performed to confirm presence of ECM. Safranin-O / fast green stains were performed to examine the distribution of glycosaminoglycans in the section.[147] Picrosirius red was used to determine the presence of collagen.[148]

Biomechanics

The viscoelastic compressive properties of samples from each group (n = 5) were tested post-stimulation (t = 4 wks) using an Instron 5565 set-up described

previously.[134] Incremental stress relaxation curves were obtained at 10%, 20%, and 30% strain for 5 min each. Data were fitted using MATLAB to an incremental stepwise viscoelastic stress relaxation solution for a standard linear solid.[135] Fitted parameters obtained were converted to instantaneous modulus (E_i), relaxation modulus (E_r) and coefficient of viscosity (μ).

Biochemistry

Biochemical tests were performed on samples at t = 0 wks and t = 4 wks. The 4 wk samples were obtained post-biomechanical testing. All samples were digested at 65ºC overnight with 125 µg/ml papain (Sigma) in 50 mM phosphate buffer (pH = 6.5) containing 2 mM N-acetyl cysteine (Sigma) and 2 mM EDTA. A picogreen cell proliferation assay kit (Molecular Probes) was used to determine total DNA content in each sample. It was assumed that each cell contains 7 picograms of DNA content, as reported in the literature.[149] Samples were also tested for total GAG present by a dimethymethlylene blue (DMMB) assay.[150] A modified hydroxyproline assay was used to determine total collagen in the scaffold.[151]

Statistical analyses

The quantitative biochemical and biomechanical data were compared using a one-way analysis of variance (ANOVA) where HP was the single factor. If a statistical difference was observed, a Tukey's *post hoc* test was performed to determine specific differences among groups. A significance level of 95% with a

p value of 0.05 was used in all statistical tests performed. All values are reported as mean ± standard deviation (SD).

Results

Gross morphology and histology

Post-seeding, cells on the PLLA scaffold appeared elongated with multiple protrusions attached to the PLLA fibers. In addition, clusters of rounded cells were also observed lodged between fibers of the non-woven PLLA mesh. At t = 4 wks, the presence of translucent tissue was observable in all groups (Figure 10), however, this was more prominent in the controls and the periodic HP group (10 MPa, 0 Hz) where holes created by the stainless steel wire through the centers of scaffolds prior to seeding were filled with ECM. The holes were still partially visible in the intermittent HP groups. Though structural integrity was maintained over 4 wks in all constructs, the scaffolds exposed to intermittent HP (10 MPa, 0.1 and 1 Hz) were less uniform around the periphery; i.e., strands of single or multiple fibers projected outwards when compared to the scaffolds in the other groups. At t = 4 wks, construct diameter in the culture control and the intermittent HP groups was 3.0 ± 0.1 mm while in the HP control and the HP 10 MPa 0 Hz groups the diameter was higher, 3.1 ± 0.1 mm and 3.1 ± 0.8 mm, respectively. The thickness of the constructs varied from 1.4 ± 0.2 mm for the 10 MPa, 1 Hz group to 1.7 ± 0.2 for the 10 MPa, 0 Hz group. No significant differences, however, were observed among groups for the construct diameter ($p = 0.71$) and thickness ($p = 0.27$). H&E stains confirmed the presence of ECM in all groups

(Figure 11). Safranin–O / fast green exhibited little staining for proteoglycans, correlating with low GAG production observed in all groups (Figure 11). Picrosirius red staining was positive, with stronger staining observed on the periphery of the scaffolds (Figure 11).

Biochemistry

Dry and wet weights of the scaffolds at t = 4 wks are shown in Table 4. The dry weight of scaffolds exposed to a pressure of 10 MPa at 1 Hz was approximately 1.3 ± 0.3 mg, half the weight of scaffolds in other groups ($p = 0.009$). The wet weight of the scaffolds ranged from 12.4 ± 0.5 to 15.7 ± 2.9 mg; however, no significant difference was observed among groups ($p = 0.37$). A significant difference was observed in cell number/scaffold among groups ($p < 0.0001$) (Table 4). Cells were originally seeded at 0.7 million cells/scaffold. At t = 0, the cell count was measured at 0.62 ± 0.17 million cells/scaffold (efficiency > 80%) and by the 4^{th} wk, the number of cells on the control scaffolds increased to 0.8 million cells/scaffold, although increase over t = 0 was not significant. However, an inter-group comparison showed that the cell numbers on scaffolds in all HP groups (<= 0.5 million cells/scaffold) were fewer than in the culture control at t = 4 wks. No detectable collagen or GAG was observed at t = 0. At t = 4 wks (Figure 12), the total collagen/scaffold was found to be 2 times higher in the periodic HP group (27 µg ± 5 µg) than in all other HP groups and controls ($p = 0.0006$). At t = 4 wks, low amounts of GAG (Figure 12) were detected in all groups (< 8 µg/scaffold, $p = 0.03$). A significant difference in GAG/scaffold was only observed

between the 10 MPa, 0 Hz group and the culture control (~1.8 times higher). Collagen and GAG data were also normalized to cell number (Figure 12). In both cases, all three HP groups (10 MPa 0, 0.1, and 1 Hz) were found to exhibit significantly higher collagen levels/cell when compared to the controls. The highest collagen content/cell ($p = 0.003$) was exhibited in the 10 MPa, 0 Hz group with collagen values 3 times greater than the culture control. For GAG content/cell ($p = 0.008$), a 3 time greater GAG accumulation was observed in 10 MPa, 0 Hz and 10 MPa, 1 Hz groups when compared to the culture control.

Biomechanics

The viscoelastic parameters, E_r, E_i and μ, were determined at 10%, 20%, and 30% strain via incremental stress relaxation tests. No apparent strain dependencies were identified for any of the parameters. Values of E_r, E_i, and μ at 30% strain are shown in Figure 13. In general, the instantaneous modulus ranged from 31-88 kPa while the relaxation modulus ranged from 8-30 kPa among groups. The periodic HP group was found to be significantly stiffer than the culture control and 10 MPa, 1 Hz groups for the instantaneous moduli (~ 1.5 times stiffer, $p = 0.005$) and relaxation moduli values (~ 1.75 times stiffer, $p = 0.02$). The coefficient of viscosity ($p = 0.05$) was found to be greatest for the periodic HP group (730 ± 167 Pa-s) and lowest for the culture control (241 ± 190 Pa-s). Similar differences were also observed for these parameters at 10% and 20% strain.

Discussion

HP was used as a mechanical stimulus to coax meniscus cells seeded on PLLA constructs to upregulate relevant ECM matrix *in vitro*, as well as to enhance the biomechanical properties of the constructs. The results of this study show that HP does, indeed, have a beneficial effect for meniscus tissue engineering purposes. It was found that the application of periodic HP (10 MPa, 0 Hz) for 1 hr every 3 days resulted in constructs with greater collagen deposition when compared to the other examined groups. In addition, the compressive mechanical properties of scaffolds in this group were found to be significantly higher than scaffolds in the culture control and the 10 MPa, 1 Hz group.

Physiologic pressures in the knee joint range from 3-18 MPa.[152, 153] To our knowledge there is scant information about pressures experienced by fibrochondrocytes and chondrocytes. Several mechanical tests have shown that chondrocytes in articular cartilage experience 7 to 10 MPa of hydrostatic pressure during normal activities.[154] In addition, a frequency ranging from 0.6 to 1.09 Hz is observed during normal walking rhythm of an adult human.[155] Thus, the goal in this experiment was to apply physiologically relevant pressures to knee meniscus constructs. Several tissue engineering studies with articular chondrocytes and TMJ disc fibrochondrocytes have shown beneficial effects for a loading regimen of 10 MPa [94, 96, 156] and for frequencies between 0 and 1 Hz.[96, 101, 157] As a first step, we sought to use these beneficial regimens for a meniscus tissue engineering strategy. Thus, a pressure of 10 MPa at three frequencies (0,

0.1 and 1 Hz) was investigated with clear benefits for the 10 MPa, 0 Hz group. In future studies, we will examine alternative loading regimens and frequencies to further optimize and enhance the biochemical and biomechanical properties of meniscus cell-seeded constructs.

The cell numbers per scaffold in each group were analyzed to determine whether a particular HP stimulus resulted in cell proliferation on the scaffold. Contrary to our hypothesis that HP would increase cell proliferation, cells numbers/scaffold in all HP groups, including the HP controls, were found to be significantly lower than those of the unpressurized culture control at t = 4 wks, as well as lower than the original seeding density of approximately 0.7 million cells/scaffold. These results are consistent with a previous study using TMJ disc fibrochondrocytes seeded on PGA scaffolds where the average cell number/scaffold in pressurized groups was four times lower than the day 0 control.[96] However, unlike that study, we did not observe a drop in cell number in our unpressurized culture control. This leads us to believe that the manipulation involved in bagging the constructs was a major factor in inducing cell loss. Furthermore, our experiment was conducted over a long duration (4 wks), with samples pressurized once every 3 days, thereby increasing the probability of cell dislodgment from the scaffolds. In addition to manipulation of the constructs for prolonged periods, the HP stimulation itself might have contributed to cell dislodgment from the scaffold. Although HP stimulation should not cause volumetric changes to the construct, theoretical models for the effect of HP on composite polymers, such as PLLA, have shown

that increasing HP can increase the bulk modulus and Poisson's ratio of the polymer slightly while decreasing its specific volume.[158] Thus, these effects might result in extremely small displacements on the scaffold causing cells to dislodge. Despite observations of cell loss, however, HP was still shown to be beneficial for constructs in terms of their biochemical and biomechanical composition.

Meniscus cells were pooled from medial and lateral menisci which may have introduced some variability in our experiment. The meniscus contains two major cell types, fibroblast-like cells and fibrochondrocytes, which are capable of producing different types of collagens *in vivo* and *in vitro*.[1, 37, 159, 160] Although specific collagens present in our constructs were not examined, it is expected that both collagen I and collagen II would be present. A study in the literature has shown that meniscus cells seeded on scaffolds produced both collagen I and collagen II as seen by immunohistochemical analysis.[160] The focus of this experiment was to identify a hydrostatic pressure regimen that enhanced the total collagen and GAG content and improved the mechanical properties of the cell-seeded PLLA constructs. In future studies, we will investigate whether hydrostatic pressure influences specific collagen levels, akin to a study conducted with inner and outer fibrosus cells where significant differences were observed in collagen I and collagen II content between inner and outer cells exposed to hydrostatic pressure.[102]

Biochemical tests were conducted after 4 wks of pressurization to determine 10 MPa HP effects at varying frequencies (0, 0.1 and 1 Hz). HP was found to be a significant factor, and the 10 MPa, 0 Hz group showed the greatest collagen content/scaffold. This result is in agreement with a previous study using TMJ disc cells that showed significantly higher levels of collagen/construct under a 10 MPa static HP regimen than under an intermittent regimen of 10 MPa, 1 Hz.[96] If total collagen and GAG content are normalized to cell number, constructs in all three HP groups (10 MPa 0, 0.1 and 1 Hz) result in significantly higher collagen and GAG deposition per cell than in the controls, suggesting that both intermittent and periodic HP increase the cells' capability to synthesize ECM. This finding is consistent with gene expression studies on articular chondrocytes where intermittent HP results in upregulation of collagen II and aggrecan expression.[68, 87, 93, 94] Although the mechanism to explain these results remains elusive, several theories have been proposed in the literature. In chondrocytes, intracellular ionic and osmotic variations as a result of HP stimulus have been shown to alter the activity of membrane transport pathways that affect matrix synthesis.[92] In cultured mouse calvariae and periosteal cells, intermittent HP has been shown to stimulate the release of activated TGF-β.[161] TGF-β1 has been shown in several experiments to upregulate collagen expression and synthesis *in vitro* in meniscus cells.[143, 162] HP has also been shown to regulate matrix metalloproteases (MMPs) and tissue inhibitors of matrix metalloproteases (TIMPs) in meniscus cells which would affect matrix breakdown and accumulation. A recent study showed that removing rabbit menisci from the knee joint results in an upregulation of MMP-1

and MMP-3 in the explant, which could then be down-regulated by HP.[86] Further, meniscus cells embedded in alginate beads down-regulated MMP-1 and MMP-13 when exposed to static HP and upregulated TIMP-1 and TIMP-2 when exposed to intermittent HP.[163] Thus, we believe that collagen regulation may be affected both directly due to HP mechanotransduction on the cells, as well as indirectly via the regulation of TGF-β and MMP pathways which in turn may enhance collagen synthesis or prevent collagen breakdown.

Some interesting observations can be made about the compressive mechanical properties of the scaffolds in relation to their biochemical properties. Constructs in the 10 MPa, 0 Hz group were significantly stiffer in compression than the culture control and the 10 MPa, 1 Hz group. In addition, the intermittent HP regimens were neither detrimental nor beneficial for the formation of robust tissue when compared to the controls. *In vivo*, articular cartilage and, to a lesser extent, the knee meniscus exhibit high compressive properties due to the presence of highly negatively charged GAGs that attract water and hydrate the tissue.[13] A significant increase in GAG/scaffold at t = 4 wks for the periodic HP group over the culture control correlated well with the biomechanical data and suggested that periodic HP stimulation resulted in mechanically superior scaffolds.

Rest time between HP stimulation may be an important factor to consider in tissue engineering studies. A short-term gene expression study with fibrochondrocytes showed that ECM gene profiles were upregulated to a greater

extent 24 hr post HP stimulation as opposed to directly after pressurization.[95] A delayed gene expression response for ECM molecules would likely translate to delays in ECM synthesis as well. Based on this working hypothesis, we utilized a rest time of 2 days after each HP stimulus to allow cells seeded on scaffold to upregulate gene expression of important ECM components and provide sufficient time for matrix synthesis and organization prior to the next pressurization cycle. Other loading regimens of 5 day on, 2 days off or 2 days on, 1 day off have been employed in the past by researchers conducting HP tissue engineering studies on articular chondrocytes and TMJ disc cells.[96, 101] However, these studies and ours examined only one rest time and so conclusions about their beneficial effect may be premature. Nonetheless, it will be prudent in future experiments to examine rest time as an experimental factor to determine an optimal condition that favors greater ECM synthesis such as the study on dynamically compressed articular cartilage explants where the authors discovered that a rest time of 100 s or less between stimulation increased the radiolabeled sulfate uptake in the explants.[146]

Conclusions

The experimental data provide significant evidence for the benefit of periodic HP (10 MPa, 0 Hz) for the purposes of meniscus tissue engineering using PLLA scaffolds. The increase in total collagen in the periodic HP constructs, despite the drop in cell number, is a significant finding and an investigation on the influence of HP on the metabolic pathways of meniscus cells might shed some light on the

underlying mechanism for this observation. The compressive stiffness of our constructs was found to increase concomitantly with increases in GAG deposition; however, the values (31-88 kPa) are still below those of native tissue (100-400 kPa).[164] To further improve the structural integrity, the use of growth factors can be employed. Previous research has shown that growth factors, such as TGF-β1 and b-FGF, are responsible for upregulating collagen I, collagen II, and proteoglycan synthesis in cartilaginous cell cultures, explants, and cell-seeded scaffolds.[65, 75, 143, 162, 165] Further, static HP alone has been shown to upregulate TGF-β1 expression in a characterized human chondrocyte cell-line.[166] Thus, a myriad of possibilities can be investigated to better understand the effects of HP at the cellular and tissue level, as well as create cartilaginous tissue engineered constructs with biochemical and biomechanical properties approaching those of native tissue.

Figures associated with chapter 4

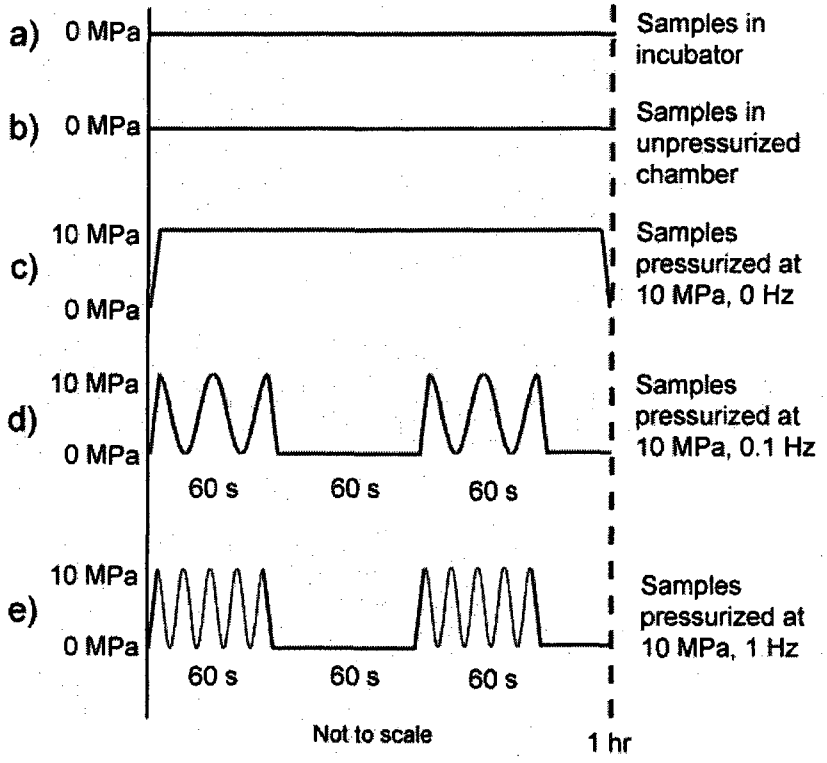

Figure 9. Experimental groups
a) Culture controls b) HP controls c) Periodic HP (10 MPa, 0 Hz) d) Intermittent HP (10 MPa, 0.1 Hz) e) Intermittent HP (10 MPa, 1 Hz). Samples were stimulated once every three days for one hr for a period of 28 days.

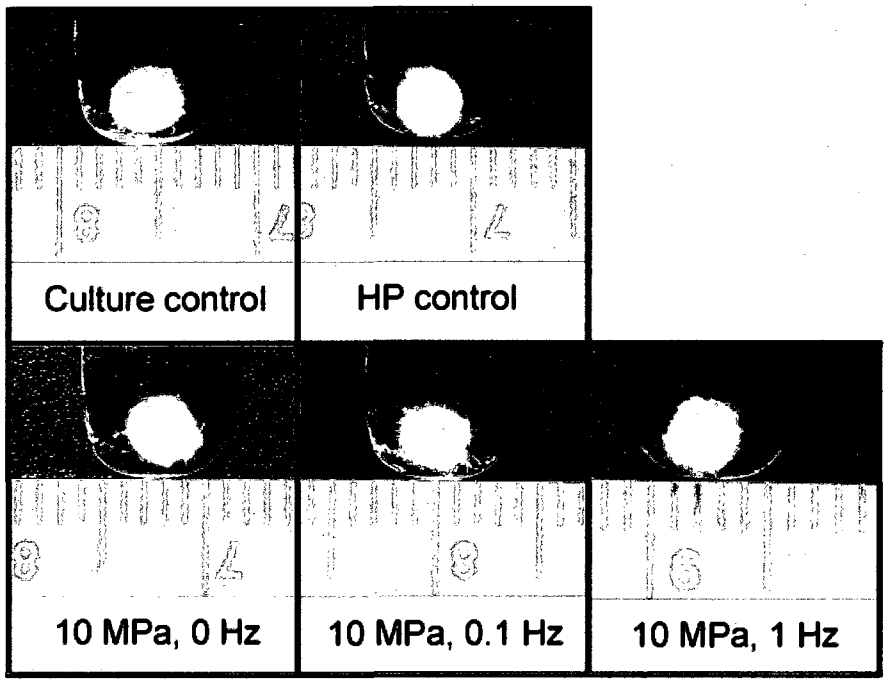

Figure 10. Gross morphology of pressurized and unpressurized scaffolds
Top left and right pictures represent unpressurized controls. Bottom from left to right: constructs pressurized at 10 MPa and 0 Hz, 10 MPa and 0.1 Hz, and 10 MPa and 1 Hz.

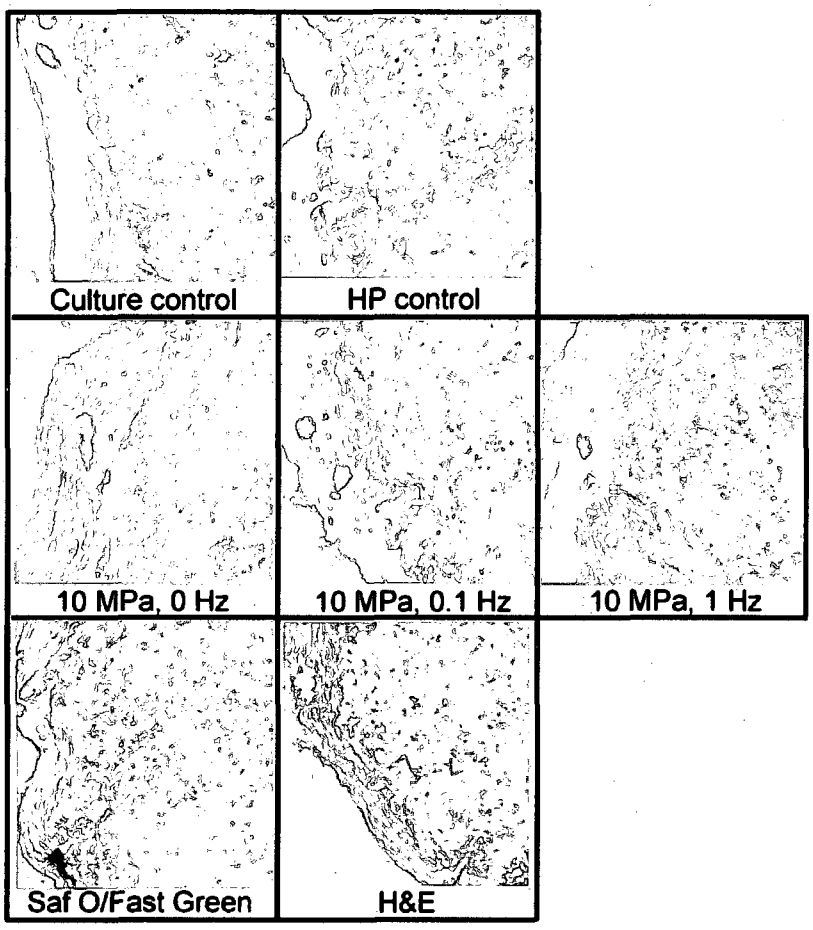

Figure 11. Histological stains of constructs
Top from left to right: Picrosirius red stains of unpressurized constructs (original magnification, 200X). Middle from left to right: Picrosirius red stains of constructs pressurized at 10 MPa and 0 Hz, 10 MPa and 0.1 Hz, and 10 MPa and 1 Hz. Bottom from left to right: Representative images of constructs stained with Safranin–O / fast green and H&E.

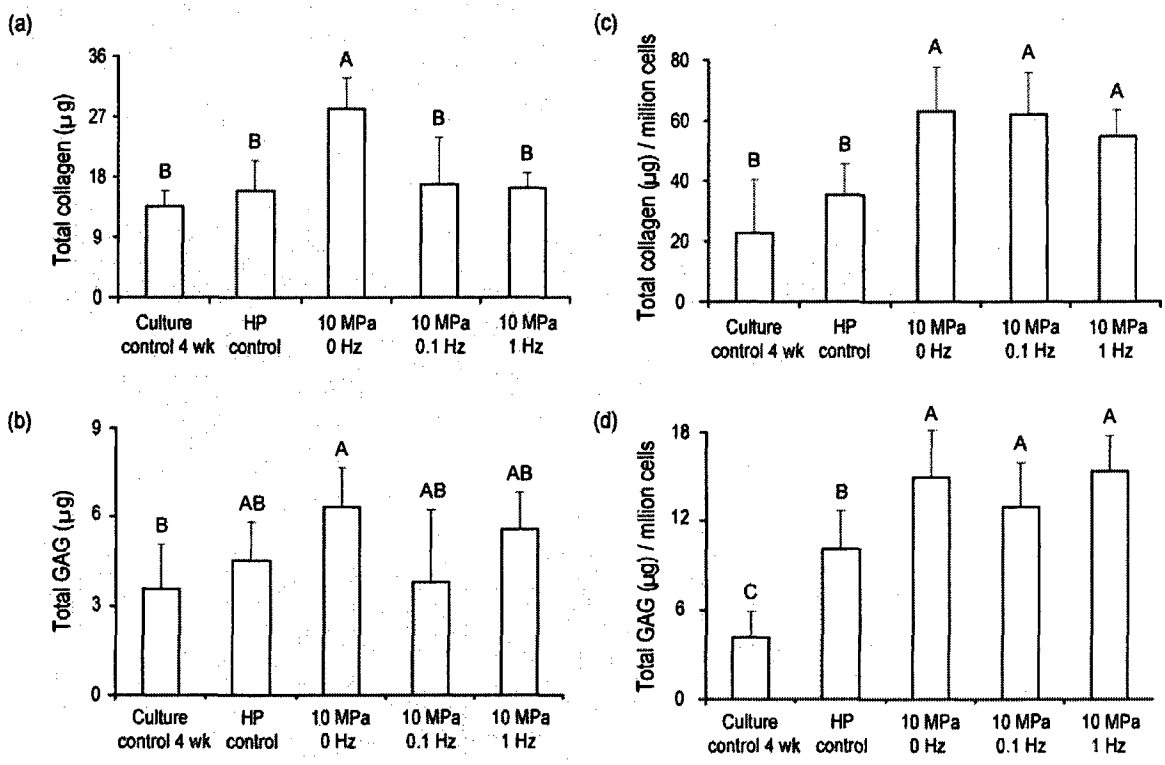

Figure 12. Biochemical data
At t = 4wks (a) Collagen/scaffold (p = 0.0006) (b) GAG/scaffold (p = 0.03). (c) Collagen/million cells (p = 0.003). (d) GAG/million cells (p = 0.008). All values reported as mean ± SD. Groups with the same letter are not significantly different from each other.

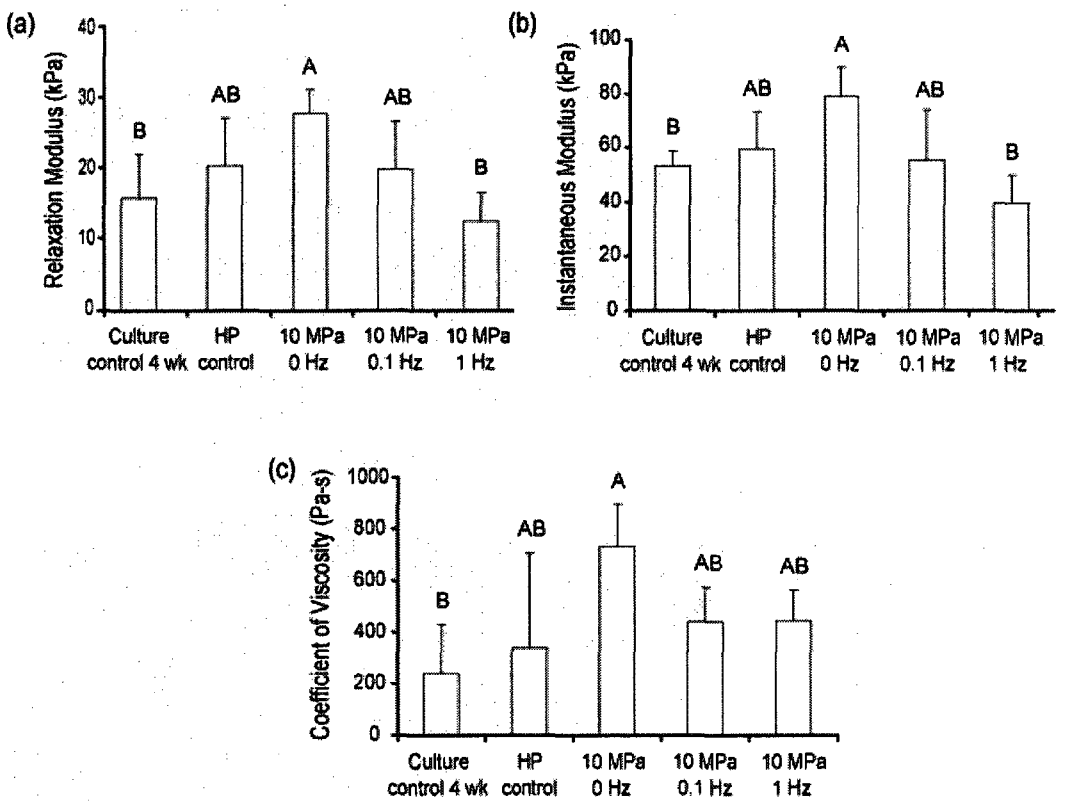

Figure 13. Biomechanical data at 30% strain
At t = 4wks (a) Relaxation modulus (p = 0.02) (b) Instantaneous modulus (p = 0.005) (c) Coefficient of viscosity (p = 0.05). All values reported as mean ± SD. Groups with the same letter are not significantly different from each other.

Table 4. Dry weight, wet weight and cell number of scaffolds

Treatment	Dry weight (mg)	Wet weight (mg)	Cell number (millions)
Culture control	2.2 ± 1.1	15.7 ± 2.9	0.8 ± 0.1
HP control	2.8 ± 0.2	12.4 ± 0.5	0.5 ± 0.1
10 MPa, 0 Hz	2.3 ± 0.5	13.8 ± 1.4	0.5 ± 0.1
10 MPa, 0.1 Hz	1.3 ± 0.3	13.1 ± 2.7	0.3 ± 0.1
10 MPa, 1 Hz	2.9 ± 0.8	13.7 ± 1.4	0.4 ± 0.1
p value	0.009	0.37	< 0.0001

Chapter 5: Effects of TGF-β1 and hydrostatic pressure application on meniscus cell-seeded scaffolds

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Abstract

The combinatorial effects of TGF-β1 and hydrostatic pressure (HP) were investigated on meniscus cell-seeded PLLA constructs using a two-phase sequential study. The objective was to identify potentially synergistic effects of these stimuli toward enhancing the biomechanical and compositional characteristics of the engineered constructs. In Phase I, the effects of TGF-β1 were examined on the ability of meniscus cells to produce ECM. In Phase II, meniscus cell-seeded PLLA constructs were cultured for 4 wks with a combination of TGF-β1 and HP (10 MPa, 0 Hz or 10 MPa, 0.1 Hz). TGF-β1 was found to increase collagen and GAG deposition in the scaffolds 15-fold and 8-fold, respectively, in Phase I. In Phase II, the combination of TGF-β1 and 10 MPa, 0 Hz HP resulted in 4-fold higher collagen deposition (additive increase), 3-fold higher GAG deposition and enhanced compressive properties (additive and synergistic increases), when compared to the unpressurized no growth factor culture control. Though significant correlations were observed between the compressive properties (moduli and viscosity), and the GAG and collagen content of the constructs, the correlations were stronger with collagen. This study provides robust evidence that growth factors and HP can be used successfully in combination to enhance the functional properties of *in vitro* engineered knee meniscus constructs.

Introduction

The knee meniscus is a fibrocartilaginous structure that cushions the tibia and the femur.[1] Damage to the meniscus can lead to significant pain around the knee joint and result in osteoarthritis. Current arthroscopic meniscal treatments, such as partial meniscectomy, provide short term relief; however, longer term studies have shown that meniscectomy quickens the onset of osteoarthritis.[167] Thus, other treatment modalities, such as tissue engineering, are widely researched including the use of growth factors and mechanical stimuli, as reviewed elsewhere.[1]

A wide range of growth factors including transforming growth factor beta-1 (TGF-β1), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), platelet-derived growth factor-AB (PDGF-AB), epidermal growth factor (EGF), and hepatocyte growth factor (HGF) have been studied for their potential use in exogenously stimulating meniscus cells to increase production of extracellular matrix (ECM).[65, 73, 75, 143, 168, 169] Of these, TGF-β1 has shown the most promise in terms of production of glycosaminoglycans (GAGs) and collagen in alginate and PGA scaffold studies with meniscus cells.[75, 143] TGF-β1 is also found in varying quantities (2.7 ± 0.37 to 20.9 ± 2.2 ng/ml) in fetal bovine serum (FBS), a vital component of cell culture medium involved in aiding cell proliferation and ECM formation.[170] To examine effects of exogenous TGF-β1 on meniscus cells would, therefore, require removal of FBS from the medium. Pilot studies in the laboratory, however, have shown that cell proliferation decreases and

subsequent cell death ensues in meniscal cells grown in basal culture medium devoid of FBS. To overcome this, one may consider the use of FBS, but perhaps at a lower concentration (1%), when compared to the current standard (10%). Lowering of FBS concentration may also be advantageous as it increases clinical translatability by reducing the risk of disease transmission as a result of serum.[171]

In addition to growth factors, the application of mechanical stimuli, such as hydrostatic pressure (HP) or direct compression (DC), has been shown to induce positive changes in gene expression and ECM synthesis in meniscal explants and cells.[84, 86, 163, 172] During every day activity, the knee joint is exposed to 7-10 MPa HP with a normal observed frequency ranging from 0 to 1.1 Hz.[155, 173] HP is also of particular interest as it results in no macro-scale deformation to the construct while the stimulus is applied. Experiments in the literature focusing on articular chondrocytes and temporomandibular joint (TMJ) disc fibrochondrocytes have shown benefits for both static and intermittent HP regimens.[68, 87, 96] For example, static HP of 10 MPa applied for 1 wk for 4 hr using a 2 day on, 1 day off regimen was shown to upregulate collagen synthesis on PGA scaffolds seeded with porcine TMJ disc fibrochondrocytes.[96] Intermittent HP of 6.87 MPa (5 s pressurized, 15 s unpressurized) has been shown to increase collagen and sulfated GAGs on PGA scaffolds seeded with equine articular chondrocytes.[174] Recent growth factor-HP combination studies have shown that TGF-β3 can enhance expression of relevant cartilaginous proteins in human mesenchymal

stem cells grown in pellet culture and the addition of intermittent HP of 10 MPa, 1 Hz can further increase gene expression of these proteins.[175, 176]

The goal of this experiment was two-fold. In Phase I, we investigated the effects of FBS concentration and TGF-β1 on the ability of meniscal cells to proliferate and produce ECM on poly-l-lactic acid (PLLA). It was hypothesized that TGF-β1 would increase ECM production on the constructs and that lowering FBS concentration would not be detrimental to ECM formation. In Phase II, we examined whether the combination of HP (static or intermittent) and TGF-β1 would enhance the biochemical and biomechanical properties of tissue-engineered constructs. It was hypothesized that an additive or synergistic effect would occur between the HP treatment and the growth factor.

Materials and methods

Cell harvesting, culture and passage (Phase I and II)

Lateral and medial meniscus cells from ten skeletally mature New Zealand white rabbits were harvested using aseptic technique. All procedures used were in strict accordance with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals. Ethics approval was obtained from Rice University before commencement of the study. The tissue was minced and digested overnight with 0.2% collagenase (Worthington, Lakewood, NJ). The isolated cells were then counted using a hemocytometer, pooled and stored at -80 °C. At the start of the experiment, meniscus cells were thawed, and the cell

viability was calculated to be over 90%. The cells were plated on T-225 flasks at 25% confluence and allowed to expand in culture medium containing 50:50 Dulbecco's modified Eagle's medium (DMEM)- Ham's F12 (Gibco, Grand Island, NY), 10% FBS (Biowhittaker, Walkersville, MD), 1% non-essential amino acids (NEAA) (Invitrogen, Grand Island, NY), 25 μg of l-ascorbic acid (Sigma, St Louis, MO) and 1% penicillin-streptomycin-fungizone (PSF) (Sigma, St Louis, MO). At approximately 90% confluence, the cells were passaged using trypsin/EDTA (Sigma, St Louis, MO) and counted with a hemocytometer. All cells used in the experiment were passaged once.

Scaffold, spinner flask preparation and cell seeding (Phase I and II)

For phase I and II of the experiment, non-woven PLLA (Biomedical Structures, Warwick, RI) scaffold sheets of molecular weight 100 kDa were cut into cylinders 2 mm in thickness and 3 mm in diameter using a 3 mm dermal punch. The scaffolds were sterilized by treatment with ethylene oxide. They were then pre-wetted with 100% ethyl alcohol, washed twice with phosphate buffered saline (PBS) and seeded into a spinner flask filled with medium + 10% FBS using a technique described elsewhere.[177] Briefly, 30 scaffolds were cultured in each spinner flask containing 400 ml of medium. A seeding density of 1 million cells/ml was utilized. Stirrer bars at the bottom of the spinner flask were rotated at 60 RPM post-seeding for 3 days. The scaffolds were left in the spinner flask for an additional 4 days to allow cells to adhere to the polymer. After 1 wk, the

constructs were transferred to agarose-coated six-well plates. The coatings were prepared by adding 1.5 ml of 2% sterile molten agarose to each well.

Tissue culture and experimental groups (Phase I)

Each agarose coated well plate contained 5 ml of culture medium. The culture medium was supplemented with 1% or 10% FBS in the presence or absence of 10 ng/ml TGF-β1 (PeproTech Inc., Rocky Hill, NJ) (n = 8 per group). Constructs (five per well) were housed in static culture and medium was changed once every three days for a period of four wks. At t = 4 wks, constructs were collected for histological and biochemical analysis.

HP preparation, application and experimental groups (Phase II)

Post-seeding, phase II constructs were placed in agarose coated wells containing culture medium supplemented with 1% FBS for an additional wk to increase cell-scaffold adhesion and improve cell migration into the scaffold. The scaffolds were then randomly assigned into HP or control groups and housed in sterile customized histology cartridges capable of holding up to nine constructs (n = 8 per group). Four different HP groups were tested. Constructs were pressurized at 10 MPa, 0 Hz or 10 MPa, 0.1 Hz in the presence or absence of 10 ng/ml TGF-β1 once every 3 days for 1 hr. The growth factor dose and stimulation regimens were chosen based on previous studies that highlighted their benefit.[143, 178] Four control groups were utilized in the experiment. Constructs in the presence or absence of TGF-β1 were housed in the incubator (culture

controls) for the experiment duration or manipulated for HP stimulation (HP controls) without receiving any HP stimulus.

Prior to HP stimulation, the cartridges with cell-seeded scaffolds were transferred to individual heat sealable sterile bags (Ampac, Cincinnati, OH) containing 30 ml of culture medium supplemented with 10 mM Hepes (Fisher Scientific, Pittsburg, PA). The bag was tapped lightly to remove air bubbles from the medium and from the cartridges, and heat-sealed. The HP set-up used in this experiment has been described previously.[96] Briefly, control specimens were placed in an open unpressurized chamber while the pressurized specimens were placed in a water-filled stainless steel chamber that connected to an Instron 8871. Post-stimulation, the bags were transferred back to the culture hood and unsealed. The cartridges were then placed back into six-well plates with fresh medium. At t = 4 wks, samples were collected for histological, immunohistological, biochemical and biomechanical analyses.

Histology (Phase I and II)

At t = 4 wks, one sample from each group was frozen using HistoPrep (Fisher Scientific, Pittsburg, PA) and then sectioned at 14 μm. Safranin-O / fast green stains were performed to examine the distribution of GAGs in the section.[147] Picrosirius red was used to determine the presence of collagen.[148]

Immunohistochemistry (IHC) (Phase II)

Collagen I and collagen II presence was determined using IHC, as described previously.[179] Briefly, the sectioned samples were fixed in chilled acetone at 4 °C for 20 min and then rinsed with IHC buffer. They were then quenched of peroxidase activity with hydrogen peroxide/methanol for 30 min and blocked with horse serum (Vectastain ABC kit). Each slide was then exposed to mouse anti-COL 1 (1:1000 dilution) (Accurate Chemicals, Westbury, NY) mouse or anti-COL 2 (1:1000 dilution) (Chondrex, Redmond, WA) antibodies for 1 hr. After incubation with the primary antibodies, a secondary mouse IgG antibody (Vectastain ABC kit) was added for 30 min at the dilution specified by the kit protocol and color was developed using the Vectastain ABC reagent and DAB (Vector Labs, Burlingame, CA) for 8 min.

Biochemistry (Phase I and II)

Biochemical tests were performed on samples at t = 0 and t = 4 wks for both phases. The 4 wk samples were obtained post-biomechanical testing for phase II of the experiment. All samples were digested at 65 °C overnight with 125 µg/ml papain (Sigma, St Louis, MO) in 50 mM phosphate buffer (pH = 6.5) containing 2 mM N-acetyl cysteine and 2 mM EDTA. A picogreen cell proliferation assay kit (Molecular Probes) was used to determine total DNA content in each sample. Total GAG was quantified using the Blyscan GAG Assay kit.[150] A modified chloramine-T hydroxyproline assay was used to determine total collagen in the construct.[151]

Biomechanics (Phase II)

The viscoelastic compressive properties of samples from each group in Phase II were obtained at t = 4 wks post-stimulation using an Instron 5565 set-up described previously.[134] Incremental stress relaxation curves were obtained at 10%, 20%, and 30% strain. Data were fitted using MATLAB to an incremental stepwise viscoelastic stress relaxation solution for a standard linear solid.[135] Fitted parameters obtained were converted to instantaneous modulus (E_i), relaxation modulus (E_r) and coefficient of viscosity (μ).

Statistical analyses

Quantitative biochemical and biomechanical data were compared using analysis of variance (ANOVA). If significant differences were observed, a Tukey's *post hoc* test was performed to determine specific differences among groups. For phase II, univariate regression analysis was conducted to determine whether biochemical data correlated with biomechanical data. The presence of synergy between TGF-β1 and HP in our system was examined using the interaction term obtained from the 2-way ANOVA between the culture control (with and without TGF-β1) and each individual HP group (with and without TGF-β1).[180] A significance level of 95% with a p value of 0.05 was used in all statistical tests performed. All values are reported as mean ± standard deviation. JMP IN statistical software was used for all statistical analyses.

Results

Phase I – Effect of serum and TGF-β1 on PLLA scaffolds

Gross morphology and histology (n = 3)

Figure 14 shows histology sections of constructs at t = 4 wks. Morphologically, white translucent tissue was observed in all groups. Groups treated with TGF-β1 exhibited a higher density of ECM when compared to the untreated samples, at both 1% and 10% FBS concentrations. Collagen and GAG staining was positive in all groups, with more intense staining observed in the TGF-β1 treated groups.

Biochemistry (n = 5)

At t = 4 wks, the total number of cells per construct increased by 100% in the TGF-β1 + 1% FBS group when compared to the 1% FBS control, while no significant difference was observed between the TGF-β1 + 10% FBS and the 10% FBS group ($p = 0.02$) (Figure 14). A 15-fold and 8-fold increase was observed in total collagen and total GAG/construct, respectively, in TGF-β1 treated samples when compared to their non-treated controls for the 1% and 10% FBS groups ($p < 0.001$) (Figure 14). No significant difference was observed, however, between the 1% FBS + TGF-β1 and 10% FBS + TGF-β1 groups for collagen or GAG content. Thus, for phase II of the experiment, a concentration of 1% FBS was utilized in the culture medium.

Phase II – Effect of TGF-β1 + HP on PLLA scaffolds

Gross morphology, histology and IHC (n = 3)

On gross examination, all constructs showed presence of ECM. However, constructs cultured with TGF-β1 appeared denser than the constructs cultured without TGF-β1, HP application notwithstanding. At t = 4 wks, construct thickness and diameter ranged from 1.8 ± 0.4 mm to 2.2 ± 0.1 mm and 2.8 ± 0.2 mm to 3.2 ± 0.1 mm, respectively; however, no significant difference was observed between any of the scaffolds for culturing condition ($p = 0.43$, $p = 0.63$) or for growth factor treatment ($p = 0.09$, $p = 0.07$) (Table 5). Weak staining for collagen and GAG was observed for constructs cultured without TGF-β1 using picrosirius red and safranin–O / fast green stains (Figure 15). The staining was more intense in TGF-β1 treated groups, especially around the periphery of the scaffolds. IHC staining showed presence of collagen I in all groups, with weak staining for collagen II (images not shown).

Biochemistry (n = 5)

Wet weight of the constructs ranged from 11.3 ± 2.5 mg to 15.9 ± 1.4 mg (Table 5). The growth factor treatment was found to be a significant factor ($p = 0.004$) using a 2-way ANOVA while the culturing condition was not ($p = 0.35$). Dry weights of the constructs ranged from 1.8 ± 0.1 mg to 2.1 ± 0.1 mg, with a significant difference observed in samples treated with TGF-β1 over the untreated groups, ($p = 0.03$) but not when comparing different culturing conditions ($p = 0.06$) (Table 5).

At t = 0, the cell seeding density was found to be approximately 0.61 ± 0.1 million cells/scaffold (shown by the dotted line in Figure 16A). By t = 4 wks, the cell number/scaffold increased to 0.71 ± 0.1 million cells/scaffold in the culture control group. A drop in cell number (approximately 20%) was observed in all HP treated groups when compared to the culture control; however, the cell numbers were not significantly different from the seeding density at t = 0. The addition of TGF-β1 significantly increased cell number in all groups when compared to their respective no-GF groups by at least 15%.

In terms of total collagen/scaffold, at t = 4 wks both the type of stimulus applied and growth factor treatment were found to be significant factors ($p = 0.01$, and $p = 0.004$, respectively) (Figure 16B). Specifically, samples stimulated with 10 MPa, 0 Hz induced a two-fold increase in collagen content/construct when compared to the culture and HP control. If normalized to cell number, this effect was even higher with a three-fold increase observed in collagen/cell number. TGF-β1 alone was found to significantly increase collagen deposition on the scaffold up to 4-fold when compared to respective no-GF groups. TGF-β1 was found to further enhance collagen deposition on constructs exposed to HP (10 MPa, 0 Hz) by approximately 1.6 fold. This resulted in constructs with the highest overall collagen/construct with 76 ± 22 μg. If normalized to cell number, this effect was even higher with a 2-fold increase in collagen deposition over the culture control + GF control. Overall, an additive effect was observed in constructs exposed to TGF-β1 and HP, 10 MPa, 0 Hz when compared to the

culture control group suggesting a beneficial interplay between the applied chemical and mechanical stimuli.

Both treatment type and growth factor application were found to be significant factors when analyzing total GAG/scaffold ($p = 0.03$ and $p = 0.01$, respectively) (Figure 16C). Without TGF-β1 treatment, constructs in the 10 MPa, 0 Hz group contained at least 50% more GAG than when compared to other groups. If normalized to cell number, a 7-fold increase in GAG content was observed in the 10 MPa, 0 Hz group when compared to the culture control. In the presence of TGF-β1, GAG production increased 4-fold in the culture control group when compared to its no-GF group. A significant increase in GAG was not observed, however, in the 10 MPa, 0 Hz group when compared to its no GF group. Despite this, the highest GAG content was observed in the 10 MPa, 0 Hz + TGF-β1 group (28 ± 6 μg). These results suggest that although the individual application of TGF-β1 and HP enhanced the GAG formation on constructs, their combination did not result in an additive or synergistic effect.

Biomechanics (n = 5)

At all strain levels (10, 20 and 30%), the type of stimulus and growth factor were found to be significant factors for all three tested parameters. TGF-β1 was found to increase the compressive properties of all unpressurized and pressurized constructs. Its effect was most pronounced for the coefficient of viscosity where a 2.3 fold increase was measured in constructs exposed to the combination of

TGF-β1 and 10 MPa of HP when compared to the HP group alone. At 10% strain, the 10 MPa, 0 Hz + TGF-β1 group exhibited an instantaneous modulus, relaxation modulus , and coefficient of viscosity of 104 ± 16 kPa, 40 ± 5 kPa, and 1403 ± 193 Pa-s, respectively; these values were statistically higher than the other tested groups (Figure 17 A, B and C). An additive effect of TGF-β1 and HP 10 MPa, 0 Hz group was observed for the instantaneous modulus. A synergistic increase in the relaxation modulus and coefficient of viscosity was observed in the 10 MPa, 0 Hz group when compared to the culture control group.

Correlation between biochemical and biomechanical data

The three compressive properties obtained from incremental stress relaxation curves were correlated with the two measured ECM components, GAG and collagen at each strain level strain. At 10% strain, univariate regression analysis showed a significant correlation between instantaneous modulus and GAG/construct (r^2 = 0.42, $p < 0.001$), and collagen/construct (r^2 = 0.56, $p < 0.001$) (Figure 18 A, B). Similar results were obtained when correlating the relaxation modulus and GAG/construct (r^2 = 0.24, $p = 0.002$), and collagen/construct (r^2 = 0.45, $p < 0.001$) (Figure 18 C, D). Further, the coefficient of viscosity correlated significantly with GAG/construct (r^2 = 0.16, $p = 0.01$), and collagen/construct (r^2 = 0.52 $p < 0.001$) (Figure 18 E, F) as well. Similar significant correlations were obtained at 20 and 30% strain levels (data not shown).

Discussion

This study investigated the effects of TGF-β1 and HP at low serum concentrations on the ability of meniscus cells to produce relevant ECM and enhance construct biomechanical functionality. TGF-β1 was found to significantly increase collagen and GAG deposition in PLLA scaffolds in phase I of the study. In addition, no significant differences were observed in the biochemical composition of PLLA constructs cultured with TGF-β1 at 1% or 10% FBS. In phase II, an HP regimen of 10 MPa, 0 Hz was found to significantly increase collagen and GAG deposition on the scaffold over the controls and the 10 MPa, 0.1 Hz group. ECM formation and compressive properties were further enhanced when TGF-β1 was added in conjunction with 10 MPa, 0 Hz HP, with additive increases in collagen deposition on the scaffold and the instantaneous modulus. Synergistic increases in the relaxation modulus and coefficient of viscosity were observed in this group as well over the culture control.

In traditional cell culture, a high concentration of FBS (~10%) is used in the medium to enhance cell attachment and proliferation. Unfortunately, this introduces the risk of disease transmission in clinical settings.[171] The ability to use a defined culture medium in lieu of FBS increases the safety of engineered meniscus constructs and may help to reduce construct variability due to changes in serum components originating from different animals. Defined culture medium like knock-out serum replacement (KOSR) or Nutridoma, have been successfully used in articular cartilage engineering while serum-free medium with bFGF has

been reported effective in self-assembling meniscus cells and co-cultures of meniscus cells and articular chondrocytes.[181-183] However, for scaffold culture, elimination of serum during meniscus cell culture on scaffolds significantly lowers the ability of cells to proliferate and produce ECM (data not reported). In this experiment, it was found that lowering FBS concentration from 10% to 1% resulted in a significant drop (~60%) in cell number per scaffold, as well as ~50% less collagen and GAG deposition on the scaffold. However, when both groups were supplemented with 10 ng/ml of TGF-β1 the effect of FBS concentration was overshadowed, resulting in increased cell proliferation and a 15-fold increase in collagen and a 8-fold increase in GAG over the no-growth factor controls. This suggests that lowering FBS concentration to 1% does not have a detrimental effect on the cells' ability to proliferate on PLLA scaffolds and produce ECM, so long that the medium is supplemented with 10 ng/ml of exogenous TGF-β1.

Our experiment showed a dramatic effect of TGF-β1 on ECM formation by adult meniscal cells *in vitro* that has not been shown before. Several studies have investigated the effects of TGF-β1 in monolayer culture and on scaffolds using fibroblasts, articular chondrocytes and immature meniscus cells.[28, 143, 166, 184, 185] In fibroblasts, TGF-β1 has been shown to increase fibronectin, thrombospondin and collagen mRNA levels.[186] In chondrocytes, TGF-β1 has been implicated in increasing cell proliferation, and collagen II and sulfated GAG synthesis [129, 186-188]. TGF-β1 may directly influence GAG synthesis by accelerating glucose transport in human articular chondrocytes via protein kinase C and extracellular

signal-regulated kinase-dependent signaling pathways.[189] A study with human chondrocytes showed that TIMP mRNA was upregulated in the presence of TGF-β1 suggesting that the growth factor also played a protective role once ECM was formed.[166] In all biochemical and biomechanical tests conducted in this study, the growth factor was found to be significant. In Phase I, for example, a dramatic increase was observed in collagen (15-fold) and GAG (8-fold) deposition when TGF-β1 was added. In addition, TGF-β1 was also responsible in increasing cell proliferation on the PLLA scaffolds as determined from picogreen assays. Thus, the results support the hypothesis that TGF-β1 is a powerful modulator of ECM synthesis.

This study showed benefits for both static and intermittent hydrostatic pressure, although static hydrostatic pressure resulted in a greater enhancement of construct biochemical and biomechanical properties. In the absence of TGF-β1, constructs stimulated with static HP at 10 MPa were found to have the highest collagen and GAG content when compared to control and intermittent HP groups. This result is in agreement with previous studies applying static HP in 3-D where increases in protein production were observed for important cartilaginous markers.[190, 191] If the total collagen and GAG production per construct were normalized to cell number, however, both static and intermittent HP regimens were found to significantly enhance collagen and GAG production per cell when compared to the controls. This is consistent with prior studies conducted in monolayer culture with TMJ disc fibrochondrocytes and articular chondrocytes

where enhances in collagen and aggrecan gene expression were observed under static and cyclic HP conditions.[87, 93, 94, 96]

The hypothesis that the combination of HP and TGF-β1 would result in additive or synergistic effects in the biochemical properties was confirmed. In a previous study, we highlighted the benefit of static and intermittent HP for meniscus tissue engineering purposes.[178] In the current study, we examined the effect of adding a growth factor, TGF-β1 in concert with the HP stimulus on the biochemical and biomechanical properties of the construct. We found that the combination of 10 ng/ml of TGF-β1 and 10 MPa, 0 Hz of HP was responsible in generating PLLA constructs with the highest overall biochemical and biomechanical properties. Additive increases were observed in collagen deposition/construct and the instantaneous modulus, while synergistic increases were observed in the relaxation modulus and coefficient of viscosity for our 10 MPa, 0 Hz constructs exposed to TGF-β1.

The combination of HP (static or intermittent) and TGF-β1 may stimulate similar down-stream pathways leading to adjunctive, additive or synergistic effects. For example, both HP and TGF-β1 have been shown to increase TIMP expression in meniscus cells and chondrocytes.[86, 166] HP is known to alter conformations of cell membrane ion transport pathways resulting in altered intracellular ion flux, thereby influencing other downstream processes.[92] A recent study has shown that intracellular Ca^{2+} mobilization can significantly affect the cells' response to

the TGF-β1 by controlling the Smad-TGF-β pathway.[192] Certainly, we may expect several different cell-signaling pathways with feedback loops either complementing or antagonizing the final down-stream effect. Though the exact mechanism is yet to be elucidated, we can conclude that for the pressure and dosage combination we chose, i.e., 10 MPa, 0 Hz and 10 ng/ml of TGF-β1, there was a positive effect. It is possible that other HP regimens or TGF-β1 doses may foster alternative responses. For example, a recent study from our laboratory using TGF-β1 (30 ng/nl) and 10 MPa static HP showed synergistic increases in collagen/wet weight and additive increases in aggregate and Young's modulus in self-assembled articular cartilage constructs.[193] Interestingly, the same result could not be reproduced when static HP of 5 MPa was applied in the presence of TGF-β1. This suggests that cells respond differently to varying HP regimens in 3-D environments, possibly by stimulating different downstream pathways that can affect eventual gene expression and protein production levels. This might explain why additive and synergistic increases in construct biochemical and biomechanical properties under static culturing conditions could not be achieved under the particular intermittent conditions that we examined (10 MPa, 0.1 Hz).

Our compressive biomechanical data (instantaneous modulus, instantaneous modulus and coefficient of viscosity) showed significant correlations with GAG/construct ($p < 0.05$) and collagen/construct ($p < 0.05$) data. Traditionally, compressive properties are linked with GAG production since GAGs sequester water molecules which provide the bulk of the resistance to compressive

forces.[13] These results suggest, however, that interplay between GAG molecules and collagen fibers might be responsible for the overall compressive biomechanical properties of the constructs, possibly via smaller proteoglycans such as decorin and biglycan which are known to affect collagen diameter and alignment.[194] A detailed analysis on the composition of the proteoglycans, as well as collagen, needs to be performed in the future.

After 4 wks in culture, the instantaneous moduli in the 10 MPa, 0 Hz group was approximately 105 kPa. This result is exciting since as it approaches native values; for example, the aggregate modulus of the tibial posterior portion of the medial leporine meniscus is 130 ± 30 kPa.[18] One needs to be cautious, however, in directly comparing results from the biphasic model to derive the aggregate modulus to a viscoelastic model to derive the instantaneous modulus. A comparison between the two techniques shows that values for instantaneous moduli are generally slightly lower than the aggregate moduli.[195] In a prior study, an HP regimen of 10 MPa, 0 Hz significantly increased construct compressive properties.[178] The inclusion of TGF-β1 in this study in conjunction with the HP stimulus resulted in additive and synergistic increases to the instantaneous modulus, relaxation modulus and the coefficient of viscosity. This effect was observed at all tested strain levels (10, 20 and 30% - data not shown for 20 and 30% strain). In addition, to ensure that the mechanical properties obtained were not those of the PLLA scaffold, separate biomechanical tests were performed on

unseeded constructs at t = 0 and t = 4 wks in regular culture medium with an obtained instantaneous modulus ranging from 10-15 kPa.

Conclusions

It was found that 90% of the FBS content in the standard tissue culture medium could be successfully replaced with addition of 10 ng/ml TGF-β1 in knee meniscus tissue engineering, yielding significant increases in ECM production and cell proliferation. Furthermore, a combination of HP and TGF-β1 led to additive and synergistic increases in the biochemical and compressive properties of tissue engineered meniscal constructs. To produce constructs mimicking native tissue, additional combinatorial stimulation regimens will need to be tested in future experiments to maximize the effect of the applied biochemical and biomechanical stimuli.

Figures associated with chapter 5

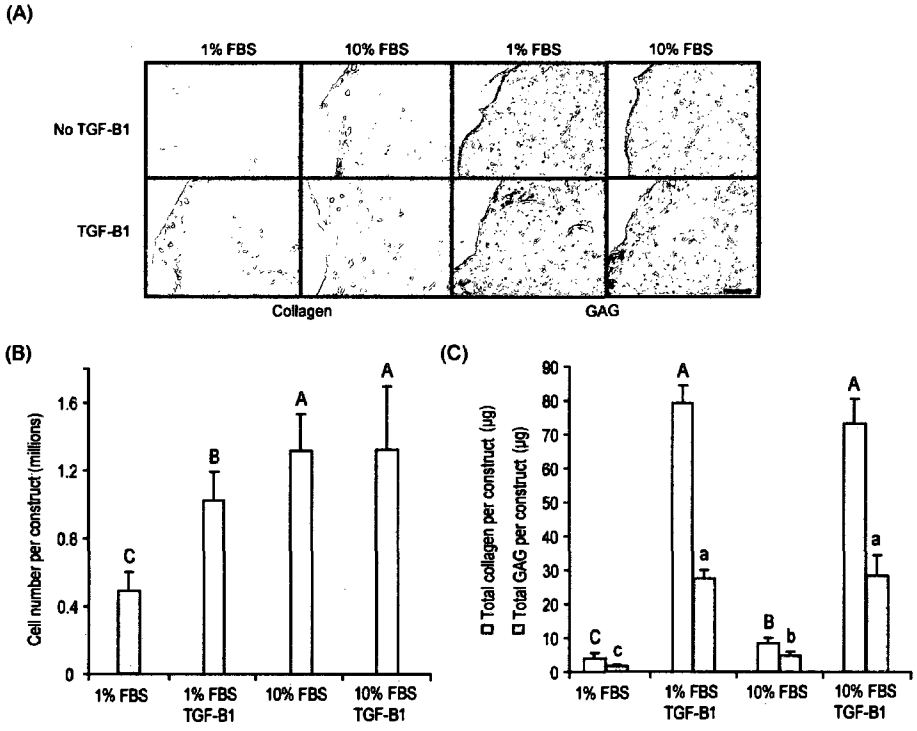

Figure 14. Phase I data
(A) Histological sections of scaffolds. Collagen and GAG presence is observed, especially on the periphery of the scaffolds. Scale bar: 200 µm. (B) Cell number/construct. Both 10% FBS and 10 ng/ml of TGF-β1 were responsible in increasing cell number on the scaffold. (C) GAG and collagen/construct. The inclusion of TGF-β1 significantly increased collagen and GAG deposition on the construct in both the 1% FBS and 10% FBS groups. 1-way ANOVAs were performed followed by a Tukey's post hoc analysis to determine significant differences between groups for collagen/construct and GAG/construct. Groups with the same letter (capital or small) are not significantly different from each other. All values are reported as mean ± SD. An n = 5 was used for all biochemical tests.

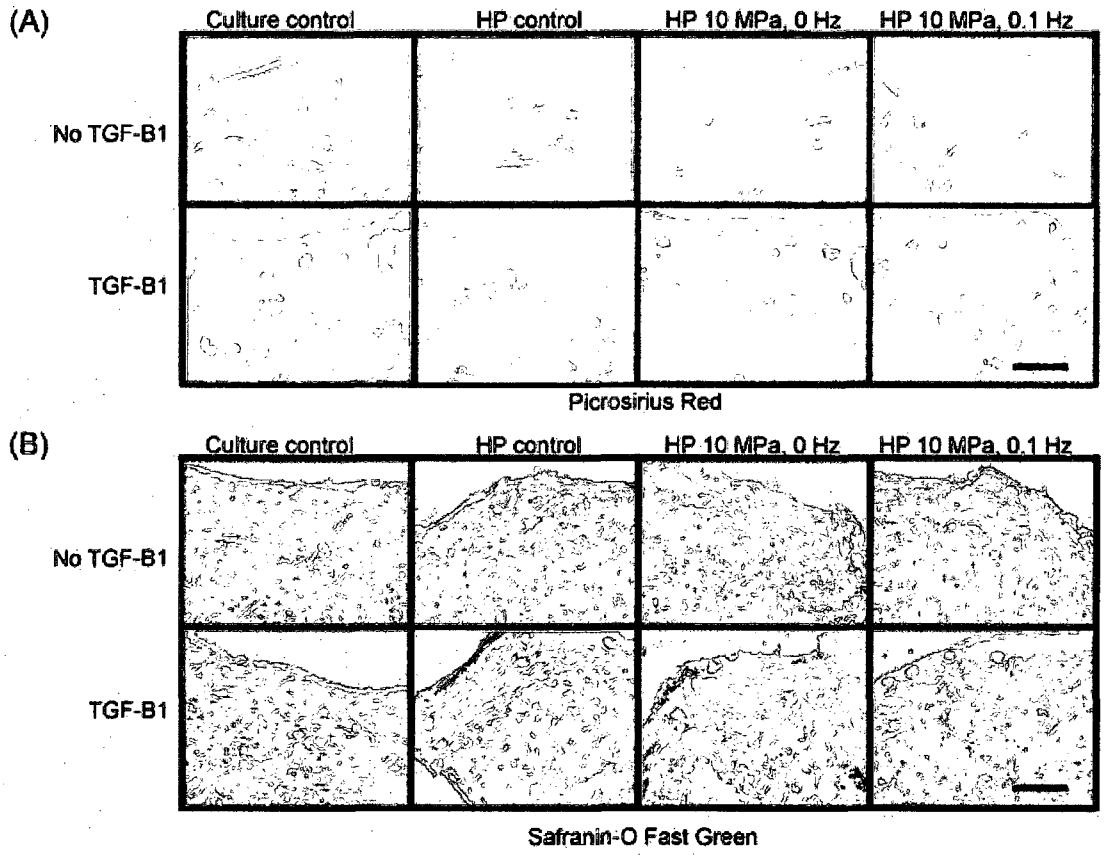

Figure 15. Phase II - histological sections of scaffolds
(A) Picrosirius Red. (B) Safranin-O / fast green. TGF-β1 was found to increase intensity in collagen staining in all groups. In the 10 MPa, 0 Hz + TGF-β1 group, strong staining for collagen and GAG was observed throughout the construct. Scale bar: 200 μm

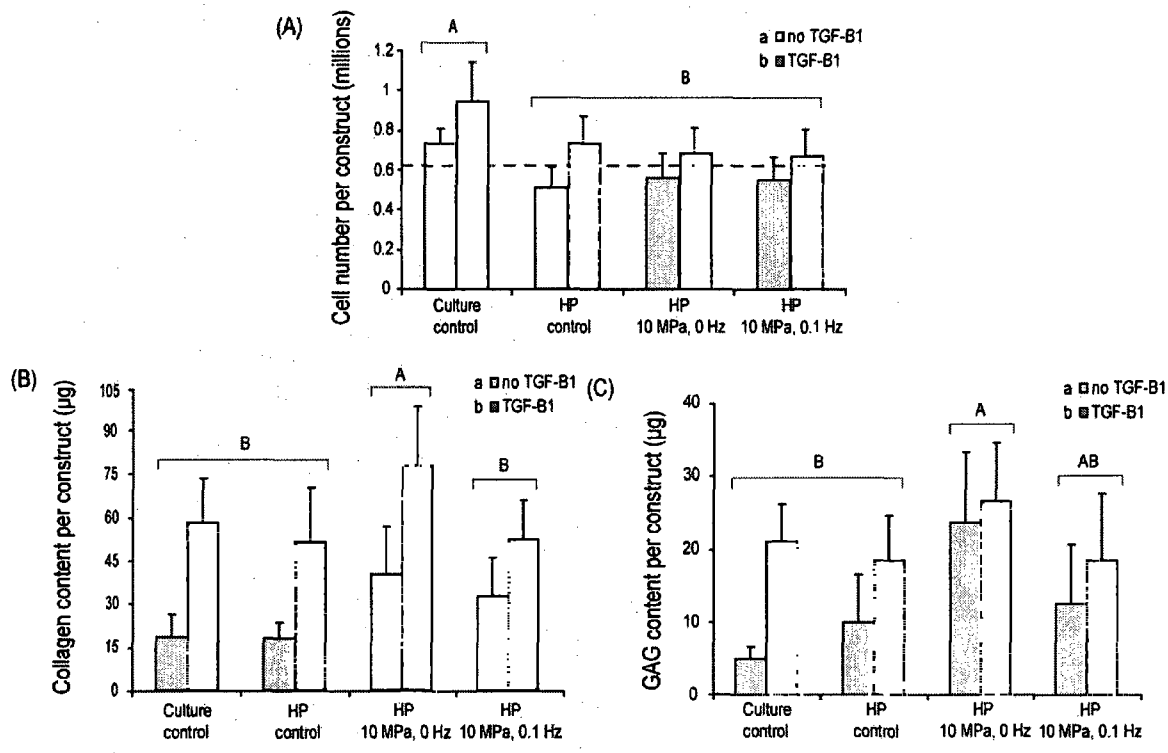

Figure 16. Phase II – Biochemical analysis
A) Cell number/scaffold. The static culture control was found to contain more cell/construct than all other groups at t = 4 wks. Dotted line signifies original seeding density of 0.61 million cells/scaffold. B) Collagen/scaffold. A significant increase in collagen deposition is observed in constructs exposed to 10 MPa and 0 Hz hydrostatic pressure. In addition, a significant increase is also observed in all groups treated with growth factor over the untreated groups C) GAG/scaffold. A significant increase in GAG deposition is observed in constructs exposed to 10 MPa and 0 Hz hydrostatic pressure. In addition, a significant increase is also observed in all groups treated with growth factor over the untreated groups. Statistics were conducted using a 2-way ANOVA. Groups with the same letter (capital or small) are not significantly different from each other. All values are reported in mean ± SD. An n=5 was used for all biochemical tests.

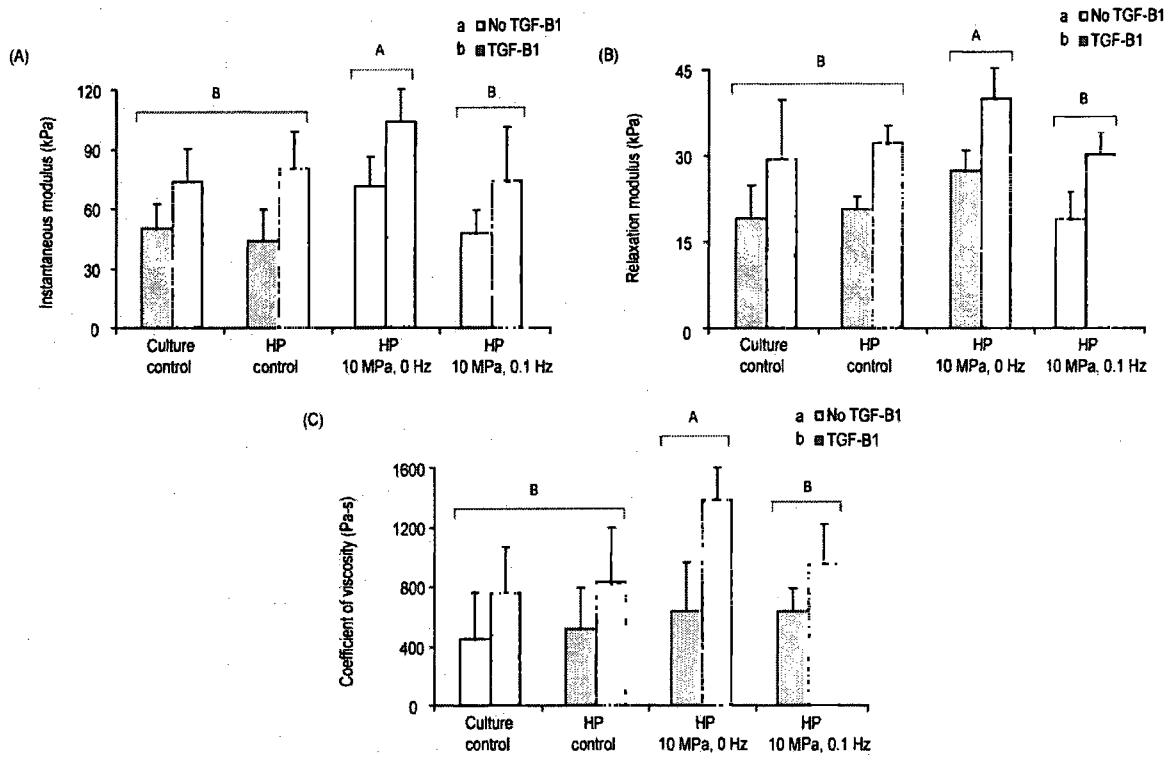

Figure 17. Phase II - compressive properties of the constructs (10% strain) (A) Instantaneous modulus (B) Relaxation modulus (C) Coefficient of viscosity. Statistics were conducted using a 2-way ANOVA. The instantaneous modulus, relaxation modulus and coefficient of viscosity of constructs stimulated at 10 MPa, 0 Hz was significantly high than constructs in other groups. Growth factor treatment was significant for all measurements Groups with the same letter (capital or small) are not significantly different from each other. All values are reported in mean ± SD. An n=5 was used for all biomechanical tests.

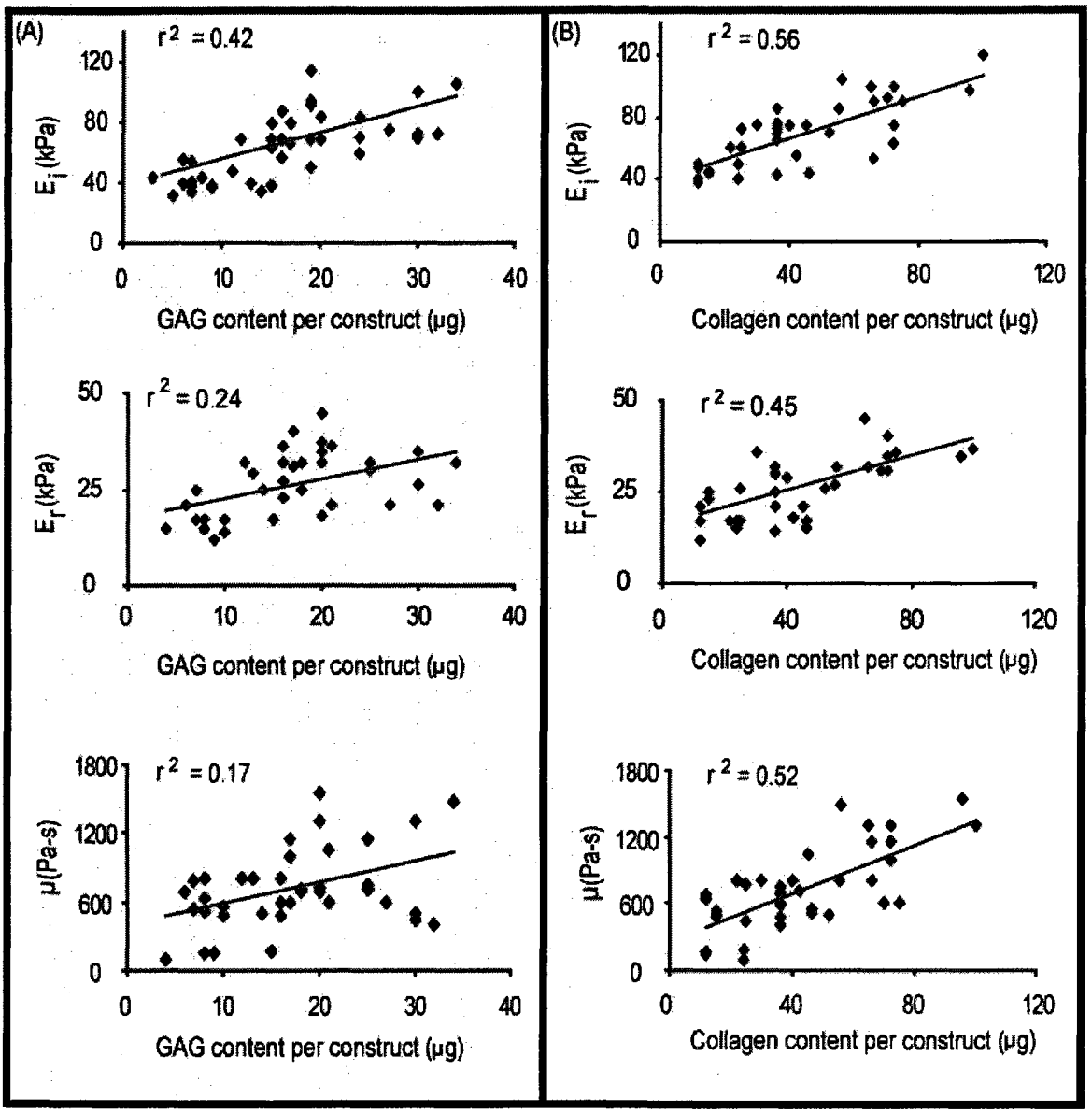

Figure 18. Correlation analysis between compressive properties (10% strain) and biochemical properties

(A) Correlation to GAG content/construct (B) Correlation to collagen content/construct. Significant correlations were observed for all parameters between the compressive properties and the two ECM components. 40 data points from TGF-β1 and no-TGF-β1 treated groups were used in each comparison.

Table 5. Phase II - Wet weight, dry weight, thickness and diameter of constructs

Growth factor application was found to significantly increase the wet and dry weight of the constructs. No significant differences were observed among groups for the wet weight, dry weight, thickness and diameter of the constructs

Groups	Wet weight		Dry weight		Thickness		Diameter	
	No TGF-β1 (mg)	TGF-β1 (mg)	No TGF-β1 (mg)	TGF-β1 (mg)	No TGF-β1 (mm)	TGF-β1 (mm)	No TGF-β1 (mm)	TGF-β1 (mm)
Culture control	11.3 ± 2.5	13.1 ± 1.0	1.8 ± 0.1	2.0 ± 0.1	1.9 ± 0.2	2.2 ± 0.1	2.8 ± 0.2	3.0 ± 0.2
HP control	11.2 ± 2.9	13.0 ± 2.1	1.9 ± 0.2	2.0 ± 0.2	2.0 ± 0.1	2.0 ± 0.3	2.9 ± 0.2	3.1 ± 0.3
HP 10 MPa, 0 Hz	13.2 ± 2.5	15.3 ± 3.3	1.8 ± 0.3	2.1 ± 0.1	1.8 ± 0.4	2.2 ± 0.1	2.8 ± 0.4	3.2 ± 0.1
HP 10 MPa, 0.1 Hz	12.7 ± 1.6	15.9 ± 1.4	1.8 ± 0.2	2.0 ± 0.1	2.0 ± 0.1	2.1 ± 0.2	3.0 ± 0.1	2.9 ± 0.5
p value – growth factor	0.004		0.03		0.07		0.09	
culture condition	0.35		0.06		0.43		0.63	

Chapter 6: Additive and synergistic effects of bFGF and hypoxia on leporine meniscus cell-seeded PLLA scaffolds

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Chapter submitted as: Gunja NJ and Athanasiou KA. Additive and synergistic effects of bFGF and hypoxia on leporine meniscus cell-seeded PLLA scaffolds. Biomaterials.

Abstract

Injuries to avascular regions of menisci do not heal and result in significant discomfort to patients. Current treatments, such as partial meniscectomy, alleviate these symptoms in the short-term, but lead to premature osteoarthritis as a result of compromised stability and changes in knee biomechanics. Thus, functional tissue engineering of the meniscus may provide an alternate treatment modality to overcome this problem. In this experiment, a scaffold-based tissue engineering approach was utilized to regenerate the meniscus. Meniscus cells were cultured on poly-l-lactic acid scaffolds in normoxic (~21% oxygen) or physiologically relevant hypoxic (~2% oxygen) conditions in the presence or absence of the growth factor, basic fibroblast growth factor (bFGF) for 4 wks. Qualitative and quantitative tests were performed to examine the morphological, biochemical and biomechanical properties of the cell-seeded constructs in each group. At t = 4 wks, histological sections of constructs showed presence of collagen and glycosaminoglycan (GAG) in all groups. Immunohistochemical staining showed presence of collagen I in all groups, and collagen II in groups cultured in hypoxic conditions. The presence of bFGF in the culture medium significantly increased cell number/construct by 25%, regardless of culturing condition. No significant differences were observed among groups for collagen/construct. For GAG/construct, synergistic increases were observed in constructs cultured in hypoxic conditions and bFGF (2-fold) when compared to constructs cultured in normoxic conditions. Viscoelastic compressive tests showed synergistic increases in the relaxation modulus and coefficient of

viscosity, and additive increases in the instantaneous modulus for constructs cultured in hypoxic conditions and bFGF when compared to constructs cultured in normoxic conditions. This was observed at all tested strain levels (10, 20 and 30% strain). Overall, these results demonstrate that bFGF and hypoxia can significantly enhance the ability of meniscus cells to produce GAGs and improve the compressive properties of tissue engineered meniscus constructs *in vitro*.

Introduction

Injuries to avascular portions of the knee meniscus do not heal and can result in significant pain, swelling and loss of range of motion to patients. Current arthroscopic treatments to remove all or part of the meniscus alleviate these symptoms but lead to premature osteoarthritis as a result of compromised stability and changes in knee biomechanics.[167] Functional tissue engineering of the meniscus may provide an alternative treatment modality to overcome this problem. To obtain high cell numbers necessary in cartilage tissue engineering experiments, cells are usually passaged multiple times in standard Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS). The expansion of these cells, however, results in phenotypic loss with rapid drop in cellular collagen II and COMP expression.[60, 106, 159] Researchers have employed several vehicles to recover losses in gene expression by culturing cells in three dimensional alginate gels, plating cells on two dimensional surface coatings and adding growth factors to the expansion medium.[60, 61, 159, 196, 197] These techniques have been generally successful in reversing the effects of markers such as COMP and collagen I; however, collagen II expression reversal has been particularly challenging.

Recent work has suggested that low oxygen tension (hypoxia) may aid in meniscus cell (MC) and articular chondrocyte (AC) phenotype maintenance and enhancement,[65-68] with increases in expression of hypoxia inducible factor-1alpha (HIF-1α) and SOX-9. These transcription factors play important roles in

collagen II synthesi.[69, 70] Furthermore, *in vivo*, cartilaginous tissues reside in hypoxic conditions where the lack of the blood supply creates a low oxygen environment (1 to 8%) for cartilage cells.[71, 72] The tissue engineering strategy used in this experiment aims to mimic *in vivo* conditions by culturing meniscus cells on poly-l-lactic acid (PLLA) scaffolds under hypoxic conditions. Other physiologically-relevant loading environments such as direct compression and hydrostatic pressure have also been studied in the literature with beneficial results obtained at 3-D level.[86, 198, 199]

In addition to hypoxia, we also examined whether basic fibroblast growth factor (bFGF), an ubiquitous growth factor with several different functions,[200, 201] would affect extracellular matrix (ECM) production on a PLLA scaffold and influence the final biomechanical properties of the cell-seeded construct. bFGF has been shown to promote glycosaminoglycan (GAG) synthesis in costal chondrocytes and MCs and aid in maintaining their phenotype.[65, 202] bFGF is also a regular component of serum-free medium used for cartilaginous tissue engineering as well as for chondro-differentiation of mesenchymal stem cells.[203, 204]

Thus, the goal of this experiment was to investigate whether bFGF and hypoxia would influence the matrix and functional properties of MC seeded-PLLA scaffolds. It was hypothesized that the individual application of bFGF and hypoxia would enhance the biochemical and compressive biomechanical

properties of the constructs and that their combination would result in an additive or synergistic effect.

Materials and methods

Cell harvesting, culture and passage

Cells were obtained from avascular portions of lateral and medial menisci of eight skeletally mature New Zealand white rabbits less than 12 hr after slaughter using a protocol described previously.[205] Briefly, meniscus tissue was aseptically harvested from the knee joint and transferred to a sterile cell culture hood. The tissue was minced to less than 1 mm^3 sections and digested with collagenase II overnight. Cells were then pooled the following day from the rabbits to reduce animal variability and counted using a hemocytometer. Cell viability (over 95%) was assessed using a trypan blue exclusion test. Cells were then frozen at -80°C in DMEM supplemented with 20% FBS and 10% dimethyl sufloxide for one month. At the start of the experiment, cells were thawed and cell viability was determined to be 90%. The cells were plated on T-225 flasks at approximately 25% confluence in chemically defined culture medium. The medium consisted of DMEM, 4.5 g/L-glucose and L-glutamine, 40 µg/mL L-proline, 100nM dexamethasone, 1% penicillin/streptomycin/fungizone, 50 µg/mL, ascorbate-2-phosphate, 100 µg/mL sodium pyruvate, 1% ITS+ and 1% FBS. FBS was used to promote cell attachment and proliferation during monolayer expansion. Cells used in the experiment were passaged at 90% confluence using trypsin/EDTA and counted with a hemocytometer.

Cell seeding

Cylindrical 2 mm thick and 3 mm wide non-woven PLLA scaffolds (Biomedical Structures Warwick, RI) of molecular weight 100 kDa were obtained from a PLLA sheet using a 3 mm dermal punch. The scaffolds were sterilized using ethylene oxide. They were then pre-wetted with 70% ethyl alcohol, and subsequently washed twice with phosphate buffered saline (PBS). The scaffolds were housed in individual wells of 12-well plates that were previously coated with 0.5 ml of 2% sterile molten agarose and incubated overnight in culture medium. Prior to seeding, the culture medium was changed and passaged cells were injected into the scaffolds at a density of 1 million cells/scaffold. The 12-well plates were placed on an orbital shaker (80 rpm) in the incubator for 3 days. The plates were then removed from the orbital shaker and placed in static culture for an additional 2 days to allow for the cells to adhere to and infiltrate the PLLA scaffold.

Tissue culture

Post-seeding, constructs were randomly divided into four experimental groups. Constructs in the first group were housed in an incubator set at 37°C, 5% CO_2 and approximately 21% O_2 (normoxic, no bFGF group). Constructs in the second group were housed in the same incubator; however, the culture medium was supplemented with 5 ng/ml of bFGF (normoxic, bFGF group). The dose was chosen based on prior literature that showed its benefit for meniscus cell culture [65]. Constructs in the third group were housed in a Billups-Rothenberg modular incubator chamber (Billups-Rothenberg, Del Mar, CA) that contained a custom

gas mixture of 5% CO_2, 93% N_2 and 2% O_2 (hypoxic, no bFGF group). Sterile deionized water was used to humidify the chamber and the entire chamber was placed in the regular incubator housing the normoxic constructs. Constructs in the fourth group were housed in the same incubator; however, the culture medium was supplemented with 5 ng/ml of bFGF (hypoxic, bFGF group). The culture medium in all groups was devoid of FBS and was changed once every 3 days. In addition, a fresh dose of bFGF was added during every medium change to groups that were cultured with the growth factor. The custom gas mixture used in the Billups-Rothenberg incubator was flushed once every 3 days during each medium change. Constructs were kept in culture for a period of 4 wks post-seeding.

Histology and immunohistochemistry

At t = 4 wks, two samples from each group were frozen using HistoPrep and then sectioned at 14 μm. Safranin-O / fast green stains were used to determine GAG distribution.[147] Picrosirius red staining was used to qualitatively determine the presence of collagen.[148] Collagen I and collagen II distributions were determined using a Biogenex i6000 autostainer. Sectioned samples were fixed in chilled acetone and rinsed with IHC buffer. They were then quenched of peroxidase activity with hydrogen peroxide/methanol and blocked with horse serum (Vectastain ABC kit). Slides were then incubated with mouse anti-COL 1 (Accurate Chemicals) or mouse anti-COL 2 (Chondrex) antibodies. After incubation with the primary antibodies, a secondary mouse IgG antibody

(Vectastain ABC kit) was added and color was developed using the Vectastain ABC reagent and DAB (Vector Labs).

Quantitative biochemistry

At t = 0 (5 days post-seeding) and t = 4 wks, samples were digested at 65 °C overnight with 125 μg/ml papain in 50 mM phosphate buffer (pH = 6.5) containing 2 mM N-acetyl cysteine and 2 mM EDTA. A picogreen cell proliferation assay kit (Invitrogen, Carlsbad, CA) was used to determine total DNA content in each sample. Total GAG was quantified using the Blyscan Glycosaminoglycan Assay kit.[150] A modified chloramine-T hydroxyproline assay was used to determine total collagen in the construct.[151] For the 4 wk samples, compressive properties of the constructs were first determined prior to biochemical testing.

Biomechanics

The viscoelastic compressive properties of samples from each group were tested at t = 4 wks using a set up described previously.[134] Briefly, an incremental stress relaxation test at 10%, 20% and 30% strain was designed and implemented after determining the height of the construct. Each sample was held at the chosen strain level for 20 min with a 10% strain step. The strain rate was kept constant throughout at 0.5 mm/s. Data obtained from each test were fitted using MATLAB to an incremental stepwise viscoelastic stress relaxation solution for a standard linear solid.[135] The parameters obtained were converted to instantaneous

modulus (E_i), relaxation modulus (E_r) and coefficient of viscosity (μ) for each strain level.

Statistical analyses

The quantitative biochemical and biomechanical data were compared using a two way analysis of variance (ANOVA). If significant differences were observed, a Tukey's *post hoc* test was performed to determine specific differences among groups. The 2-way ANOVA was also used to determine if synergistic effects were observed among groups.[180] Specifically, the presence of synergy between the culturing condition and the growth factor was examined using the interaction term obtained from the 2-way ANOVA. A significance level of 95% with a *p* value of 0.05 was used in all statistical tests performed. All values are reported as mean ± standard deviation.

Results

Gross morphology, histology, and IHC (n = 2)

Morphological analysis at t = 4 wks showed presence of translucent cartilage-like matrix in all groups (Figure 19). Constructs retained their structural integrity over the culture period with no observable signs of scaffold degradation. No significant differences were observed for the construct diameter and thickness for the culturing condition ($p = 0.52$, $p = 0.43$) and for the growth factor treatment ($p = 0.20$, $p = 0.41$) (Table 6). Histological analysis showed presence of collagen and GAG in all constructs (Figure 19). Collagen staining was uniform throughout the

construct in groups exposed to bFGF while staining was limited to the periphery of the constructs in other groups. GAG staining was concentrated to the periphery of the construct with interspersed pockets of GAG observed in constructs cultured simultaneously under hypoxic conditions and bFGF. All constructs stained positively for collagen I (data not shown). Collagen II staining was diffuse in constructs exposed to hypoxia and absent for constructs cultured in normoxic conditions (Figure 19).

Biochemistry (n = 5)

At t = 4 wks, wet weight of constructs were significantly higher in groups cultured with bFGF ($p = 0.03$), regardless of culturing condition ($p = 0.63$) (Table 6). No significant differences were observed in the dry weight at the same time point for the growth factor treatment ($p = 0.55$) and culturing condition ($p = 0.74$) (Table 6). At t = 0, the cell number was found to be 0.60 ± 0.2 million cells/construct via picogreen analysis. At t = 4 wks, cell numbers/constructs significantly increased in constructs exposed to bFGF ($p = 0.002$), to approximately 0.74 ± 0.1 million cells/construct (Figure 20); however, the culturing condition was not found to be significant ($p = 0.82$). Thus, hypoxic conditions did not affect meniscus cell proliferation rates on PLLA constructs.

Collagen and GAG contents were undetectable at t = 0 in all groups. At t = 4 wks, the neither the culturing condition ($p = 0.81$) nor growth factor treatment ($p = 0.31$) were found to be significantly different for total collagen/construct. Values

ranged between 15 ± 7 μg for the normoxic, no bFGF group to 21 ± 3 μg for the hypoxic, no bFGF group (Figure 21). For GAG/construct, however, both the culturing condition ($p = 0.01$) and the growth factor ($p = 0.004$) were found to be significant factors. Specifically, GAG/construct was at least 2 times higher in the hypoxia + bFGF group (17 ± 5 μg) when compared to both non-bFGF treated groups (~ 8 ± 2 μg) (Figure 22). Further, it was determined that a synergistic increase was observed in the total GAG content for constructs stimulated in hypoxic conditions with bFGF when compared to constructs cultured in the normoxic condition with no bFGF.

Biomechanics (n = 5)

Compressive viscoelastic tests were performed at three different strain levels (10%, 20%, and 30%) and the instantaneous modulus, relaxation modulus, and coefficient of viscosity were determined at each strain level (Figure 23). At each strain level, the constructs exposed to hypoxia and bFGF were found to have significantly higher instantaneous modulus, relaxation modulus, and coefficient of viscosity when compared to the no growth factor control groups. At 30% strain, for example, the relaxation modulus and the instantaneous modulus for the hypoxia + bFGF group were 31 ± 7 kPa, and 80 ± 10 kPa respectively, approximately 35% and 50% higher than their corresponding normoxia – bFGF control group values. The coefficient of viscosity at the same strain for the hypoxia + bFGF group was 205 ± 58 kPa, approximately 38% higher than the normoxia – bFGF group. Further, it was determined that an additive increase was

observed for the instantaneous modulus, and synergistic increases were observed for the relaxation modulus, and the coefficient of viscosity at all strain levels.

Correlation between biochemical and biomechanical data

The compressive properties obtained from incremental stress relaxation curves were correlated with the GAG and collagen content at each strain level. At 30% strain, univariate regression analysis showed a significant correlation between instantaneous modulus and GAG/construct ($p < 0.0001$, $r^2 = 0.56$), and collagen/construct ($p = 0.002$, $r^2 = 0.24$). Similar results were obtained when correlating the relaxation modulus and GAG/construct ($p < 0.0001$, $r^2 = 0.61$), and collagen/construct ($p < 0.001$, $R^2 = 0.31$). Further, the coefficient of viscosity correlated significantly with GAG/construct ($p = 0.001$, $r^2 = 0.45$), and collagen/construct ($p = 0.01$, $r^2 = 0.32$) as well. Similar significant correlations were obtained at 20 and 30% strain levels.

Discussion

This study demonstrates the potential benefit of hypoxia and bFGF for knee meniscus tissue engineering. The biochemical content was enhanced with synergistic increases in total GAG content for constructs exposed to hypoxia and bFGF. The compressive properties of the construct also increased, with additive increases in the instantaneous modulus and synergistic increases in the relaxation modulus and the coefficient of viscosity observed for constructs

exposed to both hypoxia and bFGF. Although, significant differences were not observed for total collagen content/construct, scaffolds exposed to hypoxia and a combination of hypoxia and bFGF showed diffuse staining for collagen II, which was absent in the normoxic groups. Together, these results demonstrate that growth factors and oxygen concentrations can significantly enhance the ability of meniscus cells to produce relevant ECM *in vitro*.

Cells in the inner regions of a skeletally mature meniscus reside in an avascular, low oxygen tension environment that may induce their chondrogenic-like phenotype.[70] Studies have shown that under hypoxic conditions, MCs express HIF-1α that controls SOX-9 expression [65, 70] which has been implicated in modulating aggrecan and collagen II gene expression.[206, 207] In this experiment, a significant increase in GAG was observed in constructs cultured in hypoxic conditions suggesting increases in the production of aggrecan and other proteoglycans with sulfated GAG chains such as decorin and biglycan. Further, IHC data showed that collagen II production was enhanced relative to collagen I production in the hypoxic constructs. In a previous experiment, we showed that inner avascular portions of the meniscus that consist of mainly chondrocyte-like cells rapidly dedifferentiate during monolayer and express very low levels of collagen II.[159] Thus, the observed collagen II increase in this study was exciting and suggests that post-translational processing of collagen II relative to collagen I was increased under hypoxic conditions, even though total collagen processed remained the same.

Lateral and medial meniscus cells under hypoxic conditions have also been shown to upregulate TGF-β1 gene expression after 8 and 24 hrs of hypoxia.[208] TGF-β1 has been shown previously to be a potent regulator of GAG synthesis in meniscus cells.[143, 209] In chondrocytes, TGF-β1 has been shown to directly influence the synthesis of GAGs by accelerating glucose transport via extracellular signal-regulated kinase-dependent signaling and protein kinase C pathways.[189] Further, hypoxia and TGF-β pathways have also been shown to function synergistically,[210] and may supplement SOX-9 pathways, to enhance GAG production on the meniscus-cell seeded scaffolds.

The presence of bFGF in the medium significantly increased the GAG production in the constructs. Furthermore, in the presence of hypoxia and bFGF, a synergistic increase was observed in GAG content over the normoxic control. These results build upon a prior study that showed increases in GAG/DNA in 3-D MC aggregates in the presence of bFGF alone or bFGF and hypoxia.[65] The exact mechanism by which bFGF might modulate GAG synthesis in meniscus cells is unclear. It is known that bFGF has the ability to maintain meniscus cells in a plastic state in monolayer and0020make them more responsive to a chondrogenic stimulus.[65] In addition, bFGF also plays an important role in modulating intracellular Ca^{2+} levels through protein kinase C activity.[211] Changes in intracellular Ca^{2+} levels can affect downstream signaling pathways that influence gene expression and protein synthesis of various extracellular matrix

molecules.[192] In the vascular literature, the combination of hypoxia and bFGF has been shown to induce tube formation by human microvascular endothelial cells in fibrin clots by activating transcription factor NF-κB (p65) and increasing phosphorlyated mitogen-activated protein kinases ERK1/2 over normoxic levels [212]. NF-κB, usually associated with inflammation pathways, has also been shown to be activated in differentiated chondrocytes.[213] This suggests that the combination of hypoxia and bFGF may be responsible in enhancing cartilaginous markers, such as GAGs and collagen II, in differentiated MCs used in this experiment.

This study also showed that bFGF in the culture medium resulted in increased cell number/construct for both hypoxic and normoxic groups. This increase is likely mediated through transmembrane surface receptors with tyrosine kinase activity.[214] Other groups have also reported increases in cell number in the presence of bFGF for MCs,[143] fibroblasts,[215] chondrocytes,[216] and dedifferentiated chondrocytes.[217] It is yet unclear whether bFGF treatment results in selective proliferation of chondrocyte-like cells from the inner meniscus over fibroblast-like cells from the outer meniscus or whether it maintains both cell types in a plastic state as they proliferate and makes them more responsive to a chondrogenic stimulus. Future studies will have to examine inner and outer meniscus cells separately to isolate the effects of cell type. It is known that inner and outer meniscus cells respond differently to hypoxic treatment with stark

changes in gene expression of SOX-9 and collagen I;[70] however, whether bFGF elicits a similar response has not been shown.

The compressive properties of meniscus constructs increased when exposed to bFGF in the presence of hypoxia with synergistic increases in the relaxation modulus and coefficient of viscosity and additive increases to the instantaneous modulus. Compressive properties of the meniscus can be correlated with the concentration of GAGs in the tissues. In this study, the instantaneous modulus, the relaxation modulus, and the coefficient of viscosity were all found to be strongly correlated to GAG/construct. GAGs are negatively charged particles that attract water molecules into the tissue and increase the overall construct stiffness.[1] A weaker but statistically significant correlation was observed with collagen/construct and the instantaneous modulus, relaxation modulus, and the coefficient of viscosity. Collagen provides tensile resistance to the tissue [218] and although we did not perform tensile tests in this experiment, we hypothesize that a stronger correlation to collagen would exist with tensile properties such as Young's modulus and ultimate tensile strength when compared to compressive properties. It is of interest to note that a weak but significant correlation between the coefficient of viscosity and GAG was also observed. We are not aware of any such correlation in the literature. This result may be explained via the enhanced electrostatic forces generated by GAGs, which may in turn inhibit fluid flow and render the construct more viscous in behavior.

Conclusions

Overall, these results demonstrate the effectiveness of combining bFGF and low oxygen tension (2% O_2) to synergistically enhance matrix and functional properties of tissue engineered meniscus constructs *in vitro.* Specifically, we were able to observe significant enhancements in GAG content, collagen II content, and cell number/construct in groups exposed to hypoxia and bFGF when compared to the controls. In future studies, additional anabolic stimuli, such as hydrostatic pressure [178] or direct compression [199] can be employed in conjunction with hypoxia + bFGF to further leverage potential synergies between the systems and further enhance the overall functional properties of the meniscus constructs towards native values.

Figures associated with chapter 6

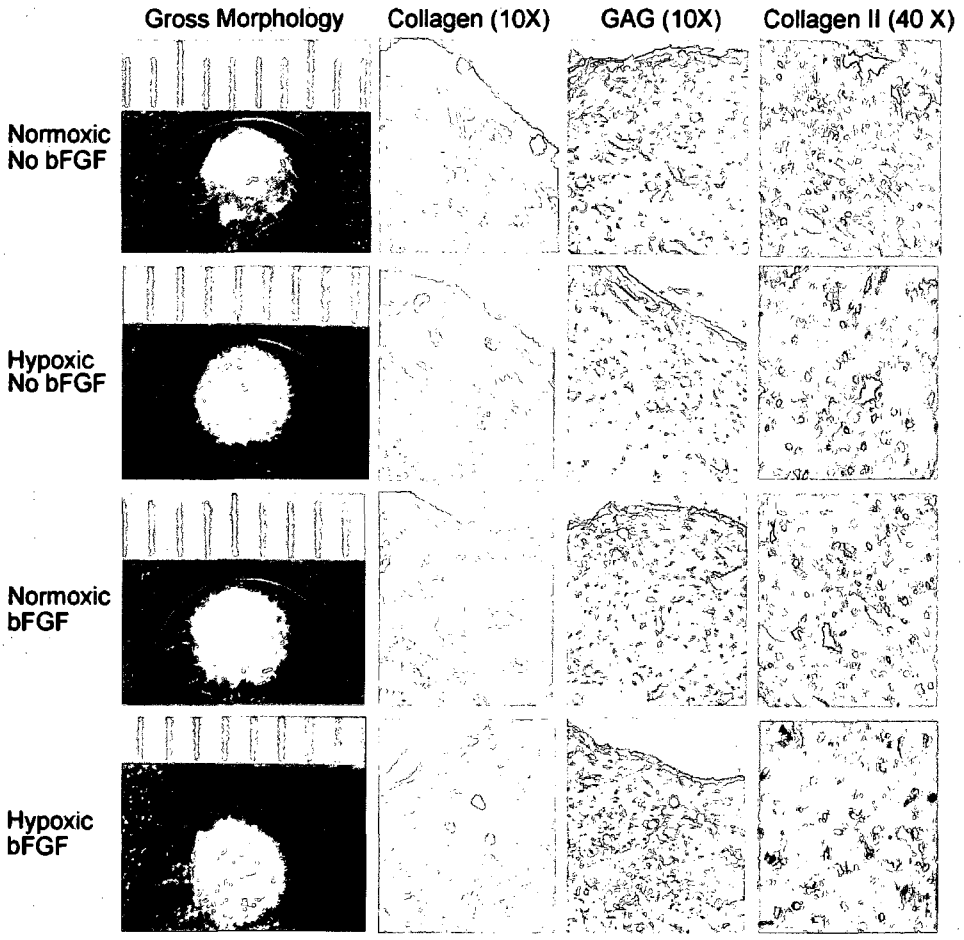

Figure 19. Gross morphological, histological and immunohistochemical sections of constructs

All constructs showed presence of translucent cartilage-like matrix. Histological sections of constructs showed presence of both collagen and GAG in all groups. Collagen staining was more uniform in constructs exposed to bFGF. GAG staining was concentrated towards the periphery of the constructs in all groups. In constructs exposed to bFGF and hypoxia, additional pockets of GAG were observed interspersed throughout the constructs. Collagen II was present in groups exposed to hypoxic conditions.

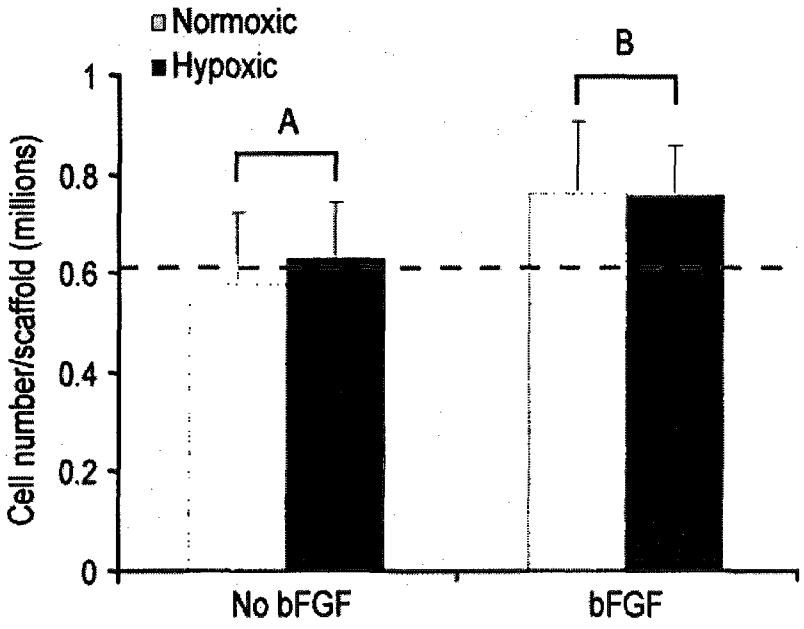

Figure 20. Cell number per construct
At t = 0, cell number per construct was found to be 0.60 ± 0.2 million cells, as indicated by the dashed line. Cell proliferation on the PLLA constructs was observed in constructs exposed to bFGF, regardless of culturing condition. A 2-way ANOVA was performed followed by a Tukey's post hoc analysis to determine significant differences within factors. Groups with different letters are significantly different from each other. All values are reported as mean ± SD. An n = 5 was used to perform the assay.

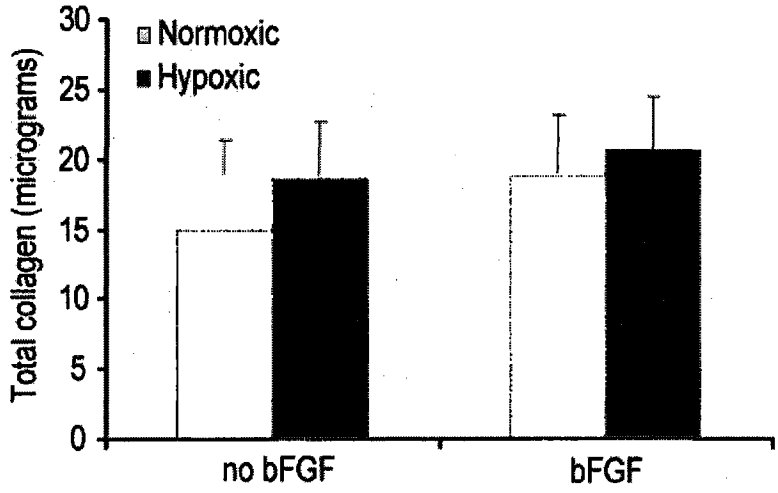

Figure 21. Total collagen per construct
At t = 4 wks, no significant differences were observed in the total collagen content/construct. Statistics were conducted using a 2-way ANOVA. All values are reported in mean ± SD. An n = 5 was used to perform the assay.

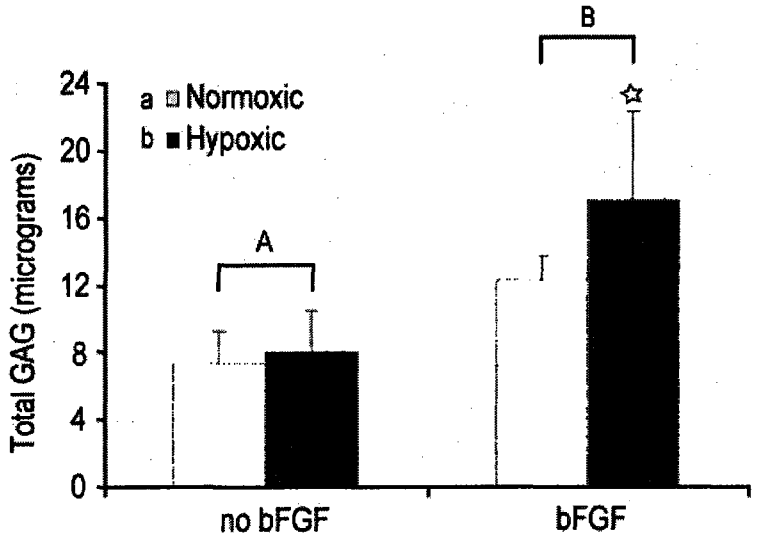

Figure 22. Total GAG per construct
At t = 4 wks, a significant increase in GAG deposition was observed in constructs exposed to bFGF and this was further enhanced in hypoxic conditions with a synergistic increased in GAG deposition in the hypoxic, bFGF group when compared to the normoxic, no bFGF group. Statistics were conducted using a 2-way ANOVA. Groups with different letters (capital or small) are significantly different from each other. Star symbol refers to a synergistic increase in GAG production. All values are reported as mean ± SD. An n = 5 was used to perform the assay.

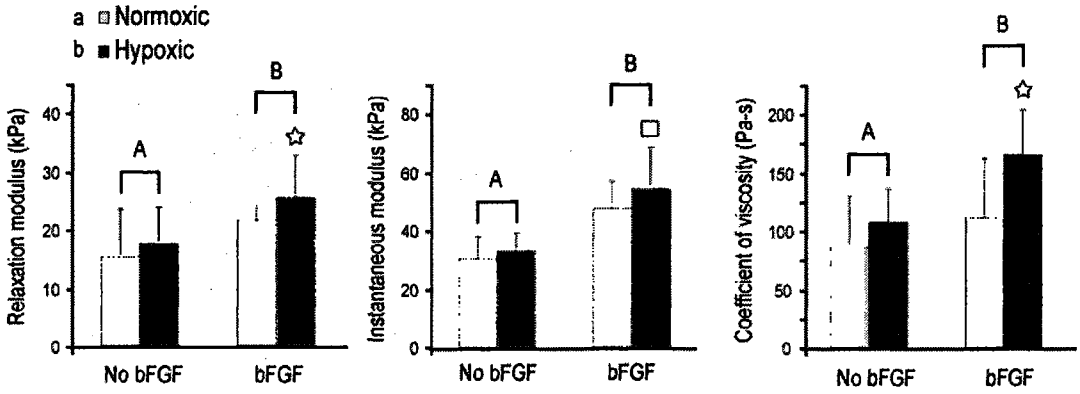

Figure 23. Compressive properties of the constructs (30% strain)
At t = 4 wks, the culturing condition and the growth factor treatment were found to be significant factors when investigating the instantaneous modulus, relaxation modulus and coefficient of viscosity. Specifically, additive increases were observed for the instantaneous modulus and synergistic increases were observed for the relaxation modulus and coefficient of viscosity in constructs exposed to both hypoxia and bFGF. Statistics were conducted using a 2-way ANOVA. Groups with different letters (capital or small) are significantly different from each other. Star symbol refers to a synergistic increase in relaxation modulus and coefficient of viscosity. Box symbol refers to an additive increase in instantaneous modulus. An n = 5 was used for all compressive tests.

Table 6. Wet weight, dry weight, thickness and diameter of constructs
Wet weight of the constructs increased in the presence of bFGF. No significant differences were observed among groups for the dry weight, thickness and diameter. A p value < 0.05 was considered significant.

Groups	Wet weight		Dry weight		Thickness		Diameter	
	No bFGF (mg)	bFGF (mg)	No bFGF (mg)	bFGF (mg)	No bFGF (mm)	bFGF (mm)	No bFGF (mm)	bFGF (mm)
Normoxic	11.4 ± 1.1	12.8 ± 1.2	0.59 ± 0.2	0.60 ± 0.1	1.9 ± 0.2	2.2 ± 0.1	2.8 ± 0.2	3.0 ± 0.2
Hypoxic	11.5 ± 1.3	13.8 ± 1.4	0.61 ± 0.2	0.62 ± 0.3	2.0 ± 0.1	2.0 ± 0.3	2.9 ± 0.2	3.1 ± 0.3
p value – culture condition	0.63		0.74		0.43		0.52	
growth factor	0.03		0.55		0.41		0.20	

Chapter 7: Effects of co-cultures of meniscus cells and articular chondrocytes on PLLA scaffolds

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Chapter submitted as: Gunja NJ and Athanasiou KA. Effects of co-cultures of meniscus cells and articular chondrocytes on PLLA scaffolds. Biotechnology and Bioengineering.

Abstract

The knee meniscus, a fibrocartilaginous tissue located in the knee joint, is characterized by heterogeneity in extracellular matrix and biomechanical properties. To recreate these properties using a tissue engineering approach, co-cultures of meniscus cells (MCs) and articular chondrocytes (ACs) were seeded in varying ratios (100:0, 75:25, 50:50, 25:75 and 0:100) on poly-l-lactic acid (PLLA) scaffolds and cultured in serum free medium for 4 wks. Histological, biochemical and biomechanical tests were used to assess constructs at the end time point. Strong staining for collagen and glycosaminoglycan (GAG) was observed in all groups. Constructs with 100% MCs were positive for collagen I and constructs cultured with 100% ACs were positive for collagen II, while a mix of collagen I and II was observed in other co-culture groups. Total collagen and GAG per construct increased as the percentage of ACs increased (27 ± 8 μg, 0% AC to 45 ± 8 μg, 100% ACs for collagen and 12 ± 4 μg, 0% ACs to 40 ± 5 μg, 100% ACs for GAG). Compressive modulus (instantaneous and relaxation modulus) of the constructs were significantly higher in the 100% ACs group (63 ± 12 kPa and 22 ± 9 kPa, respectively) when compared to groups with higher percentage of MCs. No differences in tensile properties were noted among groups. Specific co-culture ratios were identified mimicking the GAG/DW of the inner (0:100, 25:75 and 50:50) and outer regions (100:0) of the meniscus. Overall, it was demonstrated that co-culturing MCs and ACs on PLLA scaffolds results in functional tissue engineered meniscus constructs with a spectrum of biochemical and biomechanical properties.

Introduction

Knee menisci are semi-lunar shaped fibrocartilaginous discs located in between the tibia and the femur in the knee joint. They contain a heterogeneous cell population with the outer third containing predominantly elongated fibroblast-like cells and the inner two thirds containing predominantly rounded chondrocyte-like cells.[11] The outer meniscus is mainly fibrous with collagen type I, while the inner meniscus contains both collagen I and collagen II in a 2:3 ratio.[1] In addition, the meniscus also contains several glycoproteins including aggrecan, biglycan, decorin and COMP that aid in the constructs compressive and tensile properties.[219] The mechanical properties of menisci vary significantly between regions with the inner meniscus possessing higher compressive strength when compared to the outer meniscus.[164] In addition, tensile properties of the meniscus are greater in the circumferential direction when compared to the radial direction.[19] Menisci play an important role in knee mechanics and stability, but unfortunately are prone to damage, and do not heal due to low tissue vascularity. Several attempts are underway to tissue engineer the meniscus; however, much work remains before a functional construct mimicking the heterogeneities of the native meniscus is achieved.[220]

The limited supply of allogenic or autologous meniscus cells (MCs) for tissue engineering experiments necessitates the need for monolayer cell expansion to attain high cell numbers. This approach can lead to significant changes in the cells' phenotype and matrix production capability. Cartilaginous cells, such as

articular chondrocytes (ACs), MCs and tempormandibular joint (TMJ) disc fibrochondrocytes, are especially susceptible to monolayer dedifferentiation and have shown rapid changes in gene expression over passage for important cartilaginous markers.[61, 106, 159, 221] For instance, in bovine meniscal fibrochondrocytes, collagen II expression has been shown to drop 70 fold over four passages with a dramatic 5800 fold increase in collagen I expression.[159] Several different redifferentiation vehicles have been instituted to reverse losses in gene expression for cartilaginous cells with mixed results; these include exogenous growth factor addition into the medium, cell plating on surface protein coating and cell encapsulation in agarose or alginate gels.[60, 63, 65, 159, 197] Recovery of collagen II expression back to native values has posed a significant challenge, with only two studies reporting a retention of matrix phenotype in passaged MCs using a combination of hypoxia and a growth factor, bFGF.[65, 70] Both studies, however, utilized gene expression and cell aggregates to determine the matrix forming capabilities of passaged cells. Recent work has investigated the potential of using primary ACs to treat defects in the inner meniscus with the goal of increasing collagen II and glycosaminoglycans (GAGs) content.[222, 223] Primary chondrocytes express high levels of collagen II and GAGs and if co-cultured with MCs on a scaffold may result in constructs resembling various regions of the knee meniscus.[27, 60] Additionally, primary chondrocytes have been used as a re-differentiation tool for passaged chondrocytes in monolayer.[224] Such an approach, however, has not been attempted with MCs.

Thus, this experiment investigated the effects of co-culturing various ratios of passaged MCs and primary ACs on poly-l-lactic acid (PLLA) scaffolds. It was hypothesized that constructs with a spectrum of histological, biochemical and biomechanical properties would be achieved depending on the MC:AC co-culture ratio.

Materials and methods

Cell harvesting, culture and passage

ACs and MCs were aseptically harvested from the knee joint of ten skeletally mature New Zealand white rabbits less than 12 hrs after slaughter. The tissues were minced and then digested overnight using 0.2% collagenase II (Worthington, Lakewood, NJ) dissolved in Dulbecco's modified culture medium (DMEM) (Gibco, Grand Island, NY). The medium was supplemented with 10% fetal bovine serum (FBS) (Biowhittaker, Walkersville, MD), 4.5 g/L-glucose and L-glutamine (Gibco, Grand Island, NY), 1% penicillin/streptomycin/fungizone (Sigma, St Louis, MO), 1% non-essential amino acids (Invitrogen, Grand Island, NY), and 50 μg/ml L-ascorbic acid (Sigma, St Louis, MO). Post-digestion, ACs and MCs were pooled separately and counted using a hemocytometer. Cell viability was assessed using a trypan blue exclusion test and found to be over 95% for both cell types.

ACs were then placed in culture medium supplemented with 20% FBS and 10% dimethyl sulfoxide and stored in liquid nitrogen. The MCs were plated on T-225

flasks at approximately 25% confluence in chemically defined serum free DMEM medium containing 4.5 g/L-glucose and L-glutamine, 40 µg/mL L-proline, 100nM dexamethasone, 1% penicillin / streptomycin / fungizone, 50 µg/mL, ascorbate-2-phosphate, 100 µg/mL sodium pyruvate and 1% insulin-transferrin-selenium (ITS+) (BD Biosciences, San Jose, CA). MCs were expanded to the second passage and then counted using a hemocytometer. ACs were thawed post MC expansion and counted using a hemocytometer. AC viability was calculated to be over 95%.

Scaffold preparation and cell seeding

Non-woven PLLA scaffolds (molecular weight = 100 kDa) (Biomedical Structures, Warwick, RI), 2 mm thick and 3 mm wide, were obtained from a PLLA sheet using a 3 mm dermal punch. Scaffolds were sterilized using ethylene oxide, pre-wetted using ethyl alcohol (70%) and then washed twice with phosphate buffered saline (PBS). Prior to cell-seeding, scaffolds were housed in serum free medium in 12 well plates that were previously coated with 0.5 ml of 2% sterile agarose. ACs and MCs were pooled into five different co-culture ratio groups (n = 17 for each group): 1) 100% MCs; 2) 75% MCs + 25% ACs; 3) 50% MCs + 50% ACs; 4) 25% MCs + 75% ACs; and 5) 100% ACs. Cells were injected into scaffolds at a density of 1 million cells/scaffold. The 12-well plates, containing cell-seeded scaffolds were placed on an orbital shaker (80 rpm) in the incubator for 3 days followed by static culture in the incubator for an additional 2 days.

Tissue culture

Post-seeding, five constructs from each of the five co-culture groups were removed and processed for several biochemical assays to determine cell seeding numbers and total collagen and GAG levels at t = 0. The remaining constructs were transferred to 6-well plates, each well housing one scaffold. 6 ml of serum-free medium was added to each well and changed every once every two days. Constructs were kept in culture for a period of 4 wks.

Histology and immunohistochemistry

At t = 4 wks, two samples from each group were frozen using HistoPrep (Fisher Scientific, Pittsburg, PA) and sectioned at 14 μm. GAG distribution was determined using a Safranin-O / fast green stain.[147] Collagen distribution was determined using a Picrosirius red stain.[148] Collagen I and collagen II distributions were determined using a Biogenex i6000 autostainer. Briefly, samples were fixed in chilled acetone and rinsed with IHC buffer. They were then treated with hydrogen peroxide/methanol and blocked with horse serum (Vectastain ABC kit). Slides was incubated with mouse anti-COL 1 (Accurate Chemicals, Westbury, NY) or mouse anti-COL 2 (Chondrex, Redmond, WA) antibodies. Post-incubation, a secondary mouse IgG antibody (Vectastain ABC kit) was added and color was developed using the Vectastain ABC reagent and DAB (Vector Labs, Burlingame, CA)).

Quantitative biochemistry

Five samples collected at t = 0 (5 days post seeding) and t = 4 wks (33 days post seeding), were processed for biochemical analysis post compressive testing. The samples from each group at t = 4 wks were digested overnight at 65 °C with 125 μg/ml papain in 50 mM phosphate buffer (pH = 6.5) containing 2 mM N-acetyl cysteine and 2 mM EDTA. Total DNA content/sample was determined using a picogreen cell proliferation assay kit (Molecular Probes). Total GAG/sample was quantified using the Blyscan Glycosaminoglycan Assay kit.[150] Total collagen/sample was determined using a modified chloramine-T hydroxyproline assay.[151]

Compressive tests

Prior to biochemical testing of 4 wk samples, five samples from each group were subjected to biomechanical analysis. Viscoelastic compressive properties of samples from each group were examined by performing incremental stress relaxation tests at 10%, 20% and 30% strain levels using an Instron 5565.[134] Each sample was held at the chosen strain level for 20 min with a 10% strain step. The strain rate was kept constant throughout the test at 0.5 mm/s. Data obtained from each test were fitted using MATLAB to an incremental stepwise viscoelastic stress relaxation solution for a standard linear solid as described elsewhere.[135] The parameters obtained from the model were converted to instantaneous modulus (E_i), relaxation modulus (E_r) and coefficient of viscosity

(μ) for each strain level. Compressive properties of unseeded PLLA constructs at t = 4 wks after culture were also determined and served as a control.

Tensile tests

Five samples were used for tensile tests at t = 4 wks. Tests were performed using an Instron 5565 with a 50 N load cell. To ensure testability, samples were carefully carved into a dog-bone shape with a 2 mm gauge length. The ends of the samples were glued to paper tabs using cyanoacrylate. After a 0.05 N tare load was applied, the constructs were pulled to failure at a constant strain rate of 0.02 s^{-1}. Stress-strain curves generated from the test yielded the tensile stiffness and the ultimate tensile strength (UTS) of each construct. Tensile properties of unseeded PLLA constructs at t = 4 wks were also determined and served as a control.

Statistical analyses

The quantitative biochemical and biomechanical data were compared using an analysis of variance (ANOVA) test. If significant differences were observed, a Tukey's *post hoc* test was performed to determine specific differences among groups. A significance level of 95% with a p value of 0.05 was used in all statistical tests performed. All values are reported as mean ± standard deviation.

Results

Gross morphology, histology and IHC

At t = 4 wks, all constructs showed presence of ECM inside the scaffold. As the percentage of ACs increased, pockets of ECM were observed on the periphery of the scaffold (Figure 24). Construct diameter increased significantly as the percentage of ACs increased from 3.0 ± 0.4 mm for the 100% MC group to 3.2 ± 0.3 mm for the 100% AC group (Table 7) ($p = 0.02$). Similar significant increases were observed for the thickness of constructs from 2.0 ± 0.1 mm for the 100% MC group to 2.3 ± 0.1 mm for the 100% AC group (Table 7) ($p = 0.003$). Collagen and GAG staining was positive in all groups, with stronger staining for both ECM components observed in 100% AC group (Figure 24). Positive staining for collagen I was observed in constructs cultured with 100% MCs while positive staining for collagen II was observed in constructs cultured with 100% ACs. A mix of collagen I and II was observed in other co-culture groups (Figure 24).

Biochemistry

The dry weight of samples cultured with 100% ACs (1.6 ± 0.1 mg) was significantly higher than all other groups ($p = 0.002$) (Table 7); however, no significant differences were observed in the wet weight of constructs ($p = 0.31$) (Table 7). At t = 0, a cell seeding density of 0.67 ± 0.23 million cells/scaffold was obtained. Over the 4 wk culture period, no significant increases were observed among groups for cell number/construct with cell numbers ranging from 0.85 ±

0.14 to 0.68 ± 0.19 million ($p = 0.35$) (Figure 25). Total collagen (Figure 26) and GAG (Figure 27) per construct increased significantly as the percentage of ACs increased (27 ± 8 μg, 0% AC to 45 ± 8 μg, 100% ACs for collagen ($p = 0.03$) and 12 ± 4 μg, 0% ACs to 40 ± 5 μg, 100% ACs for GAG ($p = 0.006$). When collagen content was normalized to dry weight, groups with 25, 50, 75% ACs were not significantly different from each other, but were significantly higher than the 100% MC and 100% AC groups ($p = 0.03$) (Figure 26). Similar trends were observed for collagen per wet weight with 100% MC constructs (~2 μg collagen / mg DW) containing significantly less collagen than the 100% AC group (~3.5 μg collagen / mg DW) ($p = 0.03$). For GAG/dry weight, constructs exposed to 50, 75 or 100% ACs were significantly different from the other groups ($p = 0.002$) (Figure 27). GAG per WW mirrored total GAG values with significant increases observed in constructs with higher percentage of ACs ($p = 0.02$).

Compressive properties

Compressive moduli (instantaneous and relaxation modulus) and coefficient of viscosity were measured at 10, 20 and 30% strain levels. At 10% strain, the instantaneous and relaxation modulus of constructs with 100% ACs (63 ± 12 kPa and 22 ± 9 kPa, respectively) was significantly higher than the other groups ($p = 0.03$) (Figure 28). No significant differences were observed among groups for the coefficient of viscosity. Similar results were observed at 20 and 30% strain levels. Compressive properties of unseeded PLLA constructs in medium at t = 4

wks were found to be 12 ± 4 kPa for the instantaneous modulus, 6 ± 3 kPa for the relaxation modulus, and 3 ± 1 MPa-s for the coefficient of viscosity.

Tensile properties

No significant differences were observed among groups for tensile properties ($p = 0.31$) (Figure 29). Tensile stiffness ranged from 690 ± 220 kPa for the 100% MC group to 595 ± 200 kPa for the 100% AC group. UTS ranged from 500 ± 250 kPa for the 100% MC group to 300 ± 250 kPa. Tensile properties of unseeded PLLA constructs at t = 4 wks in medium were found to be 300 ± 60 kPa for the tensile stiffness and 180 ± 80 kPa for the UTS.

Correlation between biochemical and biomechanical data

The compressive and tensile properties obtained were correlated with the GAG and collagen content, respectively. For compressive properties at 10% strain, univariate regression analysis showed a significant correlation between instantaneous modulus and GAG/construct ($p = 0.0001$, $r^2 = 0.71$), and collagen/construct ($p = 0.0002$, $r^2 = 0.39$). Similar results were obtained when correlating the relaxation modulus and GAG/construct ($p < 0.0001$, $r^2 = 0.74$), and collagen/construct ($p = 0.007$, $r^2 = 0.33$). Further, the coefficient of viscosity correlated significantly with GAG/construct ($p = 0.0001$, $r^2 = 0.50$), and collagen/construct ($p = 0.0001$, $r^2 = 0.42$) as well. Similar significant correlations were obtained at 20 and 30% strain levels. For tensile properties, a significant correlation was observed between the tensile stiffness and collagen/construct (p

= 0.008, r^2 = 0.53), and GAG/construct (p = 0.002, r^2 = 0.21). In addition, the UTS was also found to correlate significantly with collagen/construct (p < 0.0001, r^2 = 0.45), and GAG/construct (p < 0.001, r^2 = 0.19)

Discussion

This study employed five different co-culture ratios of passaged MCs and primary ACs on PLLA scaffolds to identify co-culture ratios most closely resembling the biochemical and biomechanical properties of the inner and outer regions of the knee meniscus. Qualitative and quantitative tests showed clear differences between the ECM and biomechanical properties of the constructs in each group. Specifically, constructs cultured with a higher percentage of ACs had enhanced compressive properties and contained significantly higher levels of collagen, collagen II, and GAG. Constructs cultured with higher percentage of MCs contained higher levels of collagen I. Overall, it was demonstrated that co-culturing MCs and ACs on PLLA scaffolds resulted in functional tissue engineered meniscus constructs with a spectrum of biochemical and biomechanical properties.

The approach used in this tissue engineering study was unconventional in that it utilized a co-culture of second passaged MCs and primary ACs. Second passage MCs were used in this experiment to address the limitation of the number of primary rabbit MCs available and with the knowledge that MCs retain their ability to produce extracellular matrix up to the second passage.[225] Primary ACs were

judiciously chosen based on their ability to enhance collagen II and GAG content, both of which are found in abundance in the inner regions of the meniscus [1] and are down-regulated in passaged meniscus cells.[159] The IHC data showed that, as the percentage of ACs increased in the constructs, a concomitant increase in collagen II and GAG production was observed. In addition, for the first time, we were able to mimic certain compositions of the meniscus. The GAG/DW for the 100, 75 and 50% AC groups were found to be in the range of the inner region of the rabbit meniscus (~20 to 25 μg GAG/mg dry tissue) while the GAG/DW for the 100% MC group was in the range of the outer region of the rabbit meniscus (~5 to 10 μg GAG/mg dry tissue).[226] The collagen content/DW in all groups (~25 to 35 μg collagen/mg dry tissue) was found to be lower than native values (600-700 μg collagen/mg dry tissue).[226] It should be noted, however, that the dry weight data presented are likely underestimated, as approximately 65-75% of the constructs' dry weights are that of the PLLA scaffold. If the weight of the scaffold is excluded, and the data re-normalized, the new collagen per dry weights approach those of native tissue with values ranging from 70 to 100 μg collagen/mg dry tissue.

Primary ACs were also used in the experiment to aid in enhancing the capabilities of passaged MCs to produce relevant ECM. It has been shown that a low percentage of primary ACs (up to 20%), cultured with passaged ACs can aid in the reversal of gene expression of the passaged ACs.[224] Specifically, the authors observed increases in expression of chondrogenic markers, collagen II,

SOX-9 and aggrecan, and decreases in expression of the fibroblastic marker collagen I in the passaged cells. Akin to that experiment, we examined whether low percentages of ACs would significantly increase the ability of MCs to produce relevant meniscus markers, especially those pertaining to the inner meniscus, such as collagen II. IHC images of collagen II distribution, however, did not allude to possible redifferentiation of MCs towards a chondrogenic phenotype. Gene expression analyses may provide a better understanding of the cells' phenotype post co-culture. In future experiments, ACs and MCs can be separated by flow-associated cell sorting and examined for gene expression levels of relevant ECM markers.[227]

The compressive biomechanical properties were evaluated at 4 wks with increases observed in the instantaneous and relaxation modulus for constructs cultured with higher percentage of ACs. The instantaneous modulus, relaxation modulus and the coefficient of viscosity correlated strongly with the amount of GAG in the construct. GAGs are negatively charged particles that attract water molecules into the constructs and increase the overall compressive stiffness. Weaker but statistically significant correlations were observed for collagen/construct and the three tested variables for the compressive tests. The compressive properties obtained were in the range of 30 to 75 kPa, approaching those of the rabbit inner meniscus (~120 kPa).[19]

The tensile properties, UTS and tensile stiffness, at 4 wks were found to correlate strongly with the collagen content in the scaffolds. Collagen provides tensile resistance to the tissue,[218] and thus, this result was not unexpected. Weaker but statistically significant correlations were observed for the tensile properties with GAG per construct. Much work remains, however, to enhance the tensile properties of the cell-seeded constructs towards native tissue (approximately 150 MPa for the tensile stiffness and 20 MPa for the UTS).[19] Chemical agents such as chondroitinase-ABC can be utilized during culture to temporarily deplete GAGs from the constructs while enhancing collagen alignment.[228] In addition, strategies to enhance collagen content need to be employed, perhaps by the addition of growth factors such as TGF-β1 into the culture medium or by the application of mechanical stimuli, such as hydrostatic pressure.[198, 229]

In this experiment, the absence of serum did not appear to hinder ECM production or decrease cell attachment to the PLLA constructs. Previous experiments in the laboratory have shown that 1% fetal bovine serum in the culture medium is necessary for meniscus cell attachment and proliferation in monolayer culture.[229] However, to enhance the clinical translatability of our approach, we employed a serum free medium containing ITS+ and dexamethasone in this experiment. The serum free formulation obviates concerns of possible immune rejection and batch-to-batch variations in animal serum.[171, 230] This cocktail has been used previously for both meniscus and

articular cartilage tissue engineering to maintain cell phenotype and increase proteoglycan synthesis.[182, 203, 231, 232]

An interesting observation in this study was the presence of small nodules of tissue growing atop the PLLA constructs with higher percentage of ACs. Several studies have shown that chondrocytes prefer to bind to other chondrocytes when placed in a 3-D environment, possibly to minimize their surface free energy.[233] This process is likely mediated by cadherin-cadherin interactions that are described by the differential adhesion hypothesis.[56] In addition, the surface of PLLA is hydrophobic and does not possess physiologic activity which may further hinder chondrocyte cell adhesion.[234] Although, nodules were observed in constructs with a high percentage of ACs, they were conspicuously absent in groups with a higher percentage of MCs. In previous experiments, primary and passaged MCs have been shown to attach, proliferate and migrate inwards on PLLA scaffolds as evidenced by histological examination.[229] The phenotype of the cell may dictate its attachment preferences to a substrate. The elongated or spindle-shaped MCs may reduce their free surface energy by attaching to the PLLA scaffold and spreading, while the spherical chondrocytes stay rounded and form nodules.[235, 236]

Constructs in all groups stained positively for GAG and collagen. Interestingly, the intensity of staining increased as the percentage of ACs increased with stronger staining observed at the periphery of the construct and weaker staining

towards the center. The disparity in staining intensity may be an indication of cell location within the tissue. Primary ACs are known to produce GAGs and collagen II in large amounts while passaged MCs produce mainly collagen I.[159] Based on this and our histological findings, we can speculate that over the 4 wk culture period, ACs migrate towards the periphery of the construct while MCs populate the center. The differential expression of cadherins on the cell surface has been shown to drive cell sorting [237] and it is likely that passaged MCs and primary ACs express different levels of cadherins prior to and during co-culture. Further, MCs are known to exert contractile forces in scaffoldless meniscus constructs and may migrate toward the center of the scaffold.[53] In future experiments, tracking agents such as Cell Tracker Orange can be utilized to confirm whether cell migration and sorting is indeed taking place. A gradual cell separation from a fibroblastic phenotype to a chondrocytic phenotype would be ideal for a meniscus tissue engineering strategy, as it would mimic the native meniscus cell distribution.

Conclusions

In conclusion, this study used co-cultures of passaged MCs and primary ACs to form robust constructs with a spectrum of biochemical and biomechanical properties. Collagen, GAG and compressive properties increased significantly as the percentage of ACs increased. Excitingly, we were able to achieve GAG levels close to native values for the inner and outer meniscus using a variety of co-culture ratios. Collagen levels approached native meniscus as well if the dry

weight normalizations excluded the weight of PLLA. It is envisioned that this study will provide a baseline for future meniscus tissue engineering studies attempting to regenerate different regions of the meniscus.

Figures associated with chapter 7

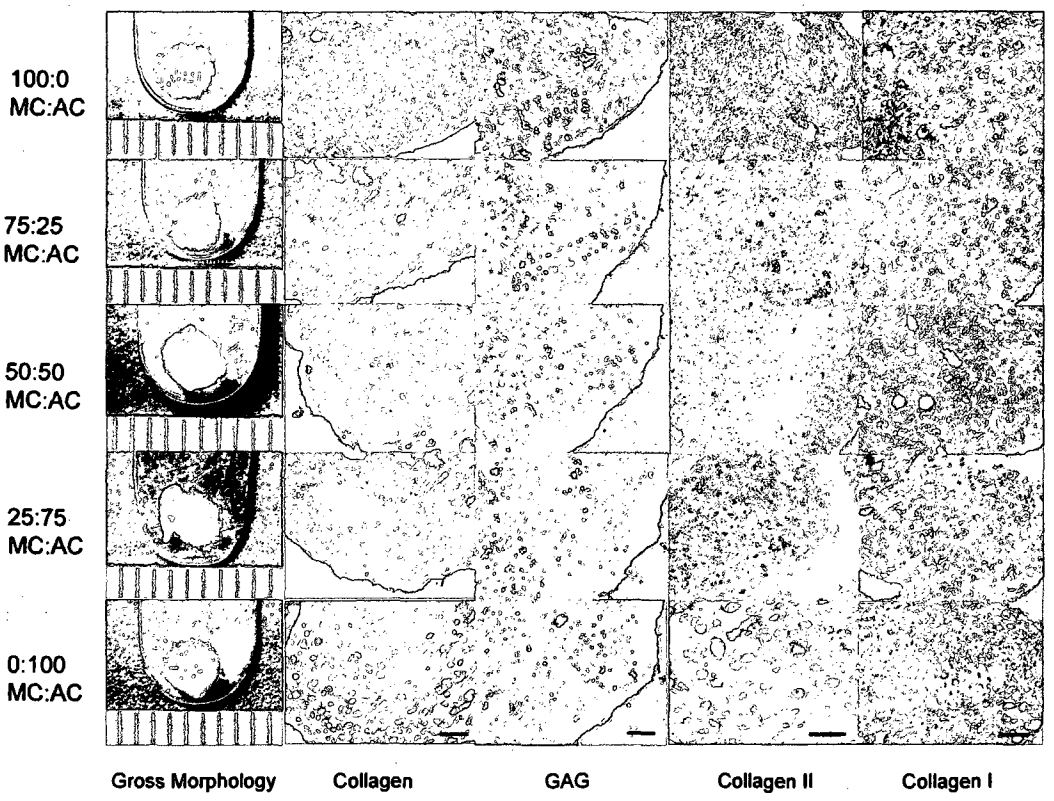

Figure 24. Gross morphological, histological and immunohistochemical sections of constructs
Strong staining for collagen and GAG was observed in all groups. Constructs with 100% MCs were positive for collagen I and constructs cultured with 100% ACs were positive for collagen II, while a mix of collagen I and II was observed in other co-culture groups. Scale bar is 200 μm.

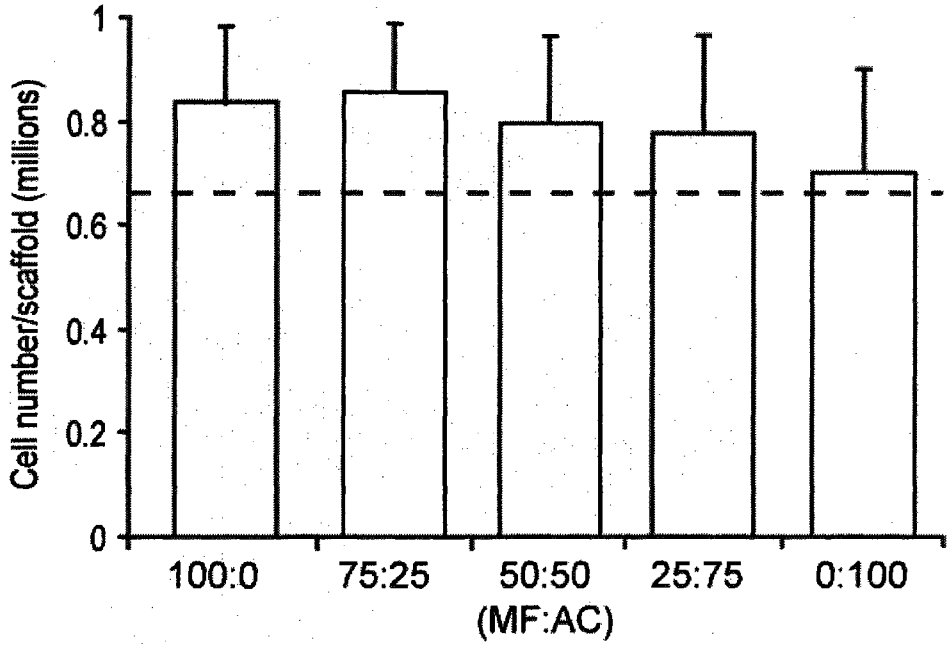

Figure 25. Cell number per construct
All values are reported as mean ± SD. An n = 5 was used to perform the assay. No significant differences were observed among groups using a one-way ANOVA. Dotted line represents initial cell seeding density.

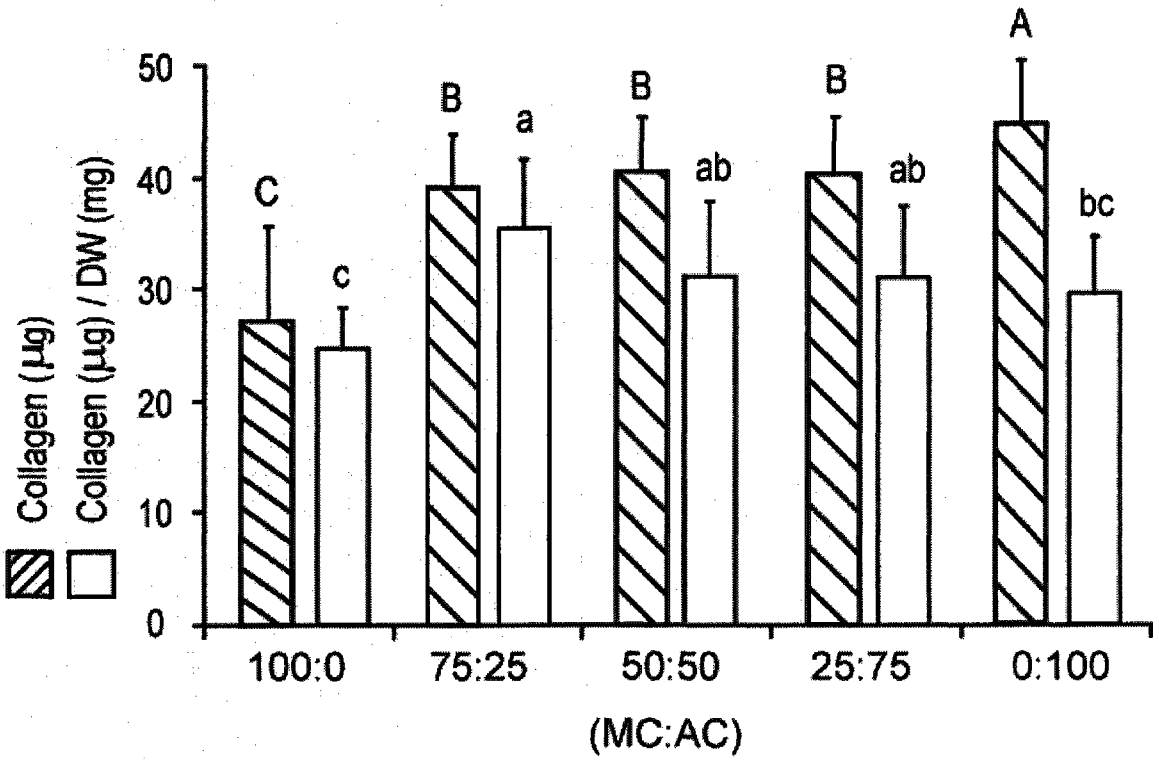

Figure 26. Total collagen per construct and collagen/DW
Total collagen per construct increased as the percentage of ACs increased. Groups with different letters are significantly different from each other. All values are reported as mean ± SD. An n = 5 was used to perform the assay.

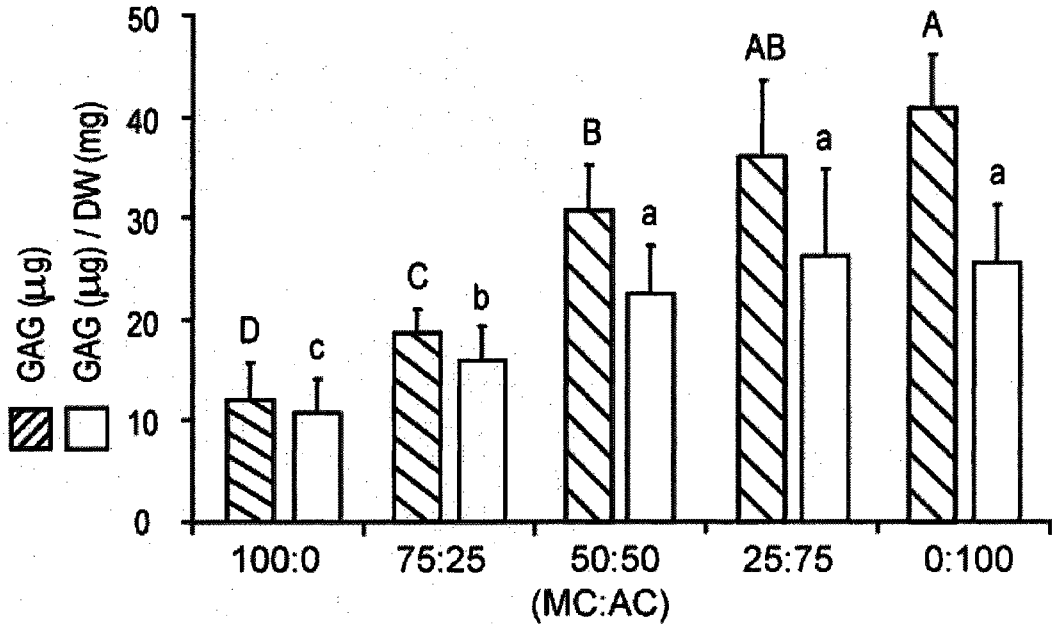

Figure 27. Total GAG per construct and GAG/DW
Total GAG per construct increased as the percentage of ACs increased. Groups with different letters are significantly different from each other. All values are reported as mean ± SD. An n = 5 was used to perform the assay.

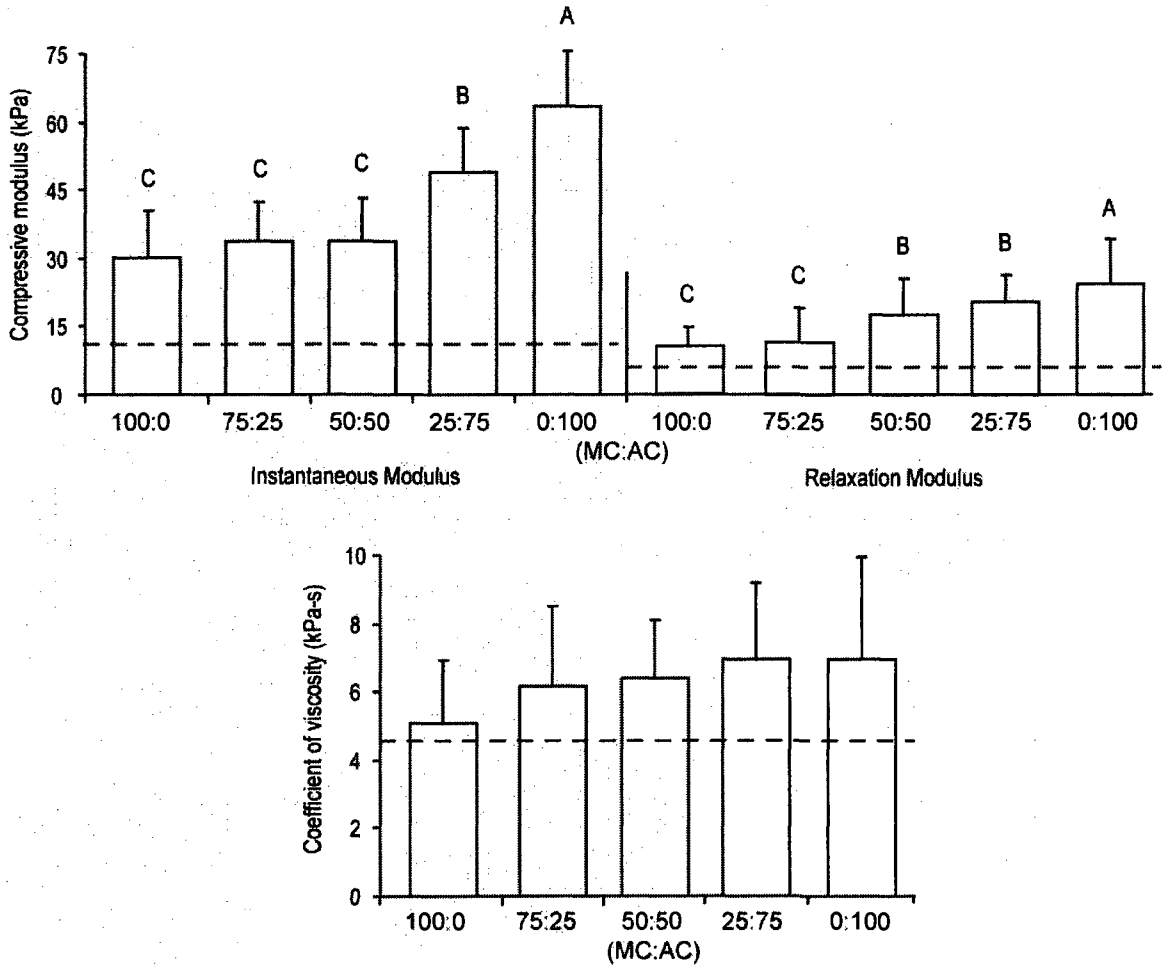

Figure 28. Compressive properties of the constructs at 10% strain
Compressive moduli of the constructs were significantly higher in the 100% ACs group when compared to groups with higher percentage of MCs. No significant differences were observed among groups for the coefficient of viscosity. Dotted line represents compressive properties of unseeded PLLA scaffolds. Groups with different letters are significantly different from each other. All values are reported as mean ± SD. An n = 5 was used to perform the assay.

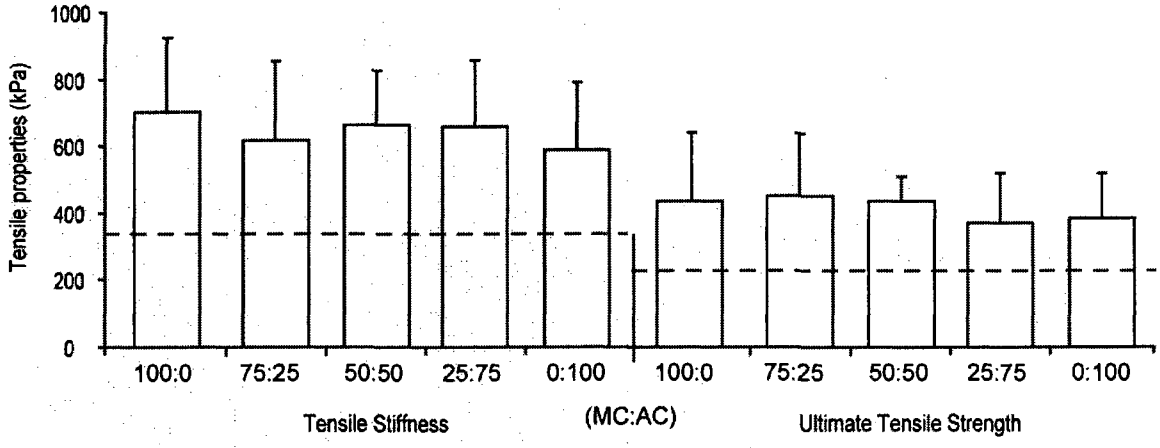

Figure 29. Tensile properties of the constructs
No differences in tensile properties were noted among groups. Dotted line represents tensile properties of unseeded PLLA scaffolds. All values are reported as mean ± SD. An n = 5 was used to perform the assay.

Table 7. Wet weight, dry weight, thickness and diameter of constructs

MC:AC	Wet weight (mg)	Dry weight (mg)	Thickness (mm)	Diameter (mm)
100:0	12.3 ± 2.4	1.3 ± 0.3 [b]	2.0 ± 0.1 [b]	3.0 ± 0.1 [b]
75:25	12.4 ± 2.4	1.3 ± 0.4 [b]	2.0 ± 0.2 [b]	3.0 ± 0.2 [b]
50:50	12.5 ± 2.2	1.3 ± 0.5 [b]	2.1 ± 0.2 [b]	3.0 ± 0.1 [b]
25:75	12.9 ± 1.7	1.4 ± 0.4 [b]	2.1 ± 0.1 [b]	3.1 ± 0.1 [b]
0:100	13.1 ± 1.5	1.5 ± 0.1 [a]	2.3 ± 0.1 [a]	3.2 ± 0.3 [a]
p value	0.31	0.002	0.003	0.02

Chapter 8: Co-cultures of bovine meniscus cells and articular chondrocytes for leporine meniscus tissue engineering

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Chapter submitted as: Gunja NJ and Athanasiou KA. Co-cultures of bovine meniscus cells and articular chondrocytes for leporine meniscus tissue engineering. Arthritis Research and Therapy.

Abstract

To regenerate the meniscus *in vitro*, a scaffoldless tissue engineering approach was utilized using co-cultures of primary bovine articular chondrocytes (ACs) and meniscus cells (MCs). Bovine cells were investigated to produce extracellular matrix (ECM) that mimics leporine menisci and to serve as a potential xenogenic cell source for future *in vivo* work. Five different MC:AC co-culture ratios (100:0, 75:25, 50:50, 25:75, 0:100) were investigated to identify the ratio most closely resembling the inner and outer regions of the leporine meniscus. Primary MCs and ACs were cultured in serum-free medium for 4 wks in non-adherent, cylindrical 3 mm agarose molds using a self-assembly, scaffoldless approach. Constructs with higher MC% contracted over the culture period. Histological examination showed presence of uniform collagen distribution in all groups. Glycosaminoglycan (GAG) staining was uniform in all groups, except the 100% MC group, which contained interspersed pockets of GAG. Biochemical tests showed that the 0% MC group contained the highest collagen per wet weight (WW) (~8%) and GAG per WW (~8.7%). The 50% MC group was found to mimic the inner meniscus in collagen II to collagen I content. Aggregate moduli for the lower MC% groups (~120 kPa) were found to be in the range of the aggregate moduli of the outer meniscus (~160 kPa). Tensile moduli and ultimate tensile strengths increased as the MC% increased; however, these values were significantly lower than native tissue. Overall, bovine MC and AC scaffoldless constructs recapitulated certain biochemical and biomechanical properties of the inner and outer regions of the leporine meniscus. Further examination of these

cells as a potential xenogenic cell source for leporine meniscus tissue engineering is warranted.

Introduction

The menisci are fibrocartilaginous discs located on the tibial plateau in the knee joint. They serve primarily a mechanical function, aiding in shock absorption, stabilization and joint lubrication.[1] Meniscal injuries may not heal, especially those in the inner avascular regions of the meniscus, and can lead to significant changes in joint function, degradation of articular cartilage, and osteoarthritis.[238] To overcome these problems, several attempts are underway to regenerate the meniscus *in vitro* and subsequently replace the damaged tissue *in vivo*.

By composition, the meniscus consists of a fluid and solid phase. The fluid phase is mainly water (75% by wet weight (WW)), while the solid phase comprises of collagen (23% by WW) and glycoproteins (2% by WW). Collagen I is the predominant fibrillar collagen in the meniscus and is present in the outer regions of the meniscus, while the inner regions contain both collagen I and collagen II in a 2:3 ratio.[1] Several glycoproteins are also present in the meniscus, including aggrecan, biglycan, decorin, and cartilage oligomeric matrix protein (COMP) that aid in the constructs' compressive and tensile properties.[219] Recent work has investigated the utilization of articular chondrocytes (ACs) for meniscus repair and tissue engineering.[27, 222, 223] ACs express high levels of collagen II and glycosaminoglycan (GAG), both of which are found in the inner regions of the meniscus. Recapitulating the heterogeneity of the inner and outer regions is a major goal of meniscus tissue engineering and may be achieved using a co-culture of meniscus cells (MCs) and ACs.

Polymeric scaffolds such as poly-L-lactic acid, poly-glycolic acid, and poly-caprolactone have been used successfully to enhance biochemical and biomechanical properties of MC-seeded constructs.[229, 239, 240] In addition to the scaffold-based approach, our laboratory is investigating a self-assembly, scaffoldless approach for meniscus regeneration using a high density of MCs and ACs.[27, 53] In this technique, cells are seeded in high density on non-adherent agarose molds and allowed to coalesce without a scaffold.[54] This approach addresses several inherent limitations of scaffolds. For instance, the random arrangement of non-woven scaffold meshes introduces a formidable challenge in producing anisotropic constructs mimicking the native meniscus. In addition, degradation times of scaffolds can vary significantly *in vivo*, a phenomenon which may interfere with extracellular matrix (ECM) production and maturation, and long term mechanical properties. A scaffoldless tissue engineering approach circumvents these issues and may be ideal for meniscus regeneration.

The animal model of choice in our tissue engineering studies is the rabbit. The leporine model has been well characterized in the literature for its biomechanical characteristics.[19] In addition, the leporine model has been commonly employed in *in vivo* meniscal repair and replacement studies.[39, 160] A disadvantage of this model, however, is the small number of cells obtainable from rabbit menisci for tissue engineering studies (~1 to 2 million per meniscus).

Leporine MCs are generally expanded in monolayer until sufficient cell numbers are obtained prior to embarking on a tissue engineering study.[229] An unintended consequence of this is the dedifferentiation of MCs towards a fibroblast-like phenotype. It has been shown that MCs increase collagen I expression and decrease collagen II expression over passage.[159] Gene expression reversal attempts are underway using serum-free media; however, alternative approaches need to be examined as well. One solution may be to use primary cells from a readily available cell source, such as immature cows cells.[52] Using this approach, meniscus tissue engineering technologies can be developed and bovine cells can be examined as a potential xenogenic cell source for leporine tissue engineering.

Thus, this experiment investigated the effects of co-culturing various ratios of primary bovine MCs and ACs using a scaffoldless approach. Bovine cells were investigated for their capacity to produce ECM on par with leporine menisci and as a potential xenogenic cell source in future *in vivo* rabbit studies. Medial leporine menisci were used as internal controls. It was hypothesized that: 1) different co-culture ratios of passaged MCs and ACs would result in constructs with significantly different biochemical and biomechanical properties; 2) a higher MC:AC bovine co-culture ratio would result in a construct more closely resembling the outer leporine meniscus while a lower MC:AC ratio would result in a construct more closely resembling the inner leporine meniscus.

Materials and methods

Cell and tissue harvest

ACs and MCs were aseptically harvested from the distal femur and the medial menisci of six 1 wk old male calves (Research 87, Boston, MA) less than 24 hrs after slaughter. The tissues were digested overnight using 0.2% collagenase II (Worthington, Lakewood, NJ) dissolved in Dulbecco's modified culture medium (DMEM) (Gibco, Grand Island, NY). The culture medium was supplemented with 10% fetal bovine serum (Biowhittaker, Walkersville, MD), 1% penicillin/streptomycin/fungizone (Sigma, St Louis, MO), 1% non-essential amino acids (Invitrogen, Grand Island, NY), 50 μg/ml L-ascorbic acid (Sigma, St Louis, MO), and 4.5 g/L-glucose and L-glutamine (Gibco, Grand Island, NY). Post-isolation, ACs and MCs were pooled separately and counted using a hemocytometer. Cell viability was found to be over 95% for ACs and MCs. In addition to bovine MCs and ACs, medial menisci were also isolated from six skeletally mature New Zealand white rabbits using sterile technique and examined for their biochemical content and biomechanical properties.

Agarose well preparation, cell seeding and tissue culture

Agarose wells were prepared on 24-well plates by using a negative polysulfone mold consisting of cylindrical prongs 3 mm in diameter and 10 mm in length. Wells were created by pressing the negative mold into 1 ml of sterile molten 2% agarose (2 g in 100 ml of phosphate buffered saline, PBS). The agarose was allowed to gel at room temperature for 1 hr with the negative mold. The negative

mold was then removed after 1 hr leaving behind a smooth agarose mold 3 mm in diameter. Serum-free medium was added once every day for 3 days to saturate the well and remove remnants of PBS. The medium consisted of DMEM, 4.5 g/L-glucose and L-glutamine, 40 µg/mL L-proline, 100nM dexamethasone, 1% penicillin/streptomycin/fungizone, 50 µg/mL, ascorbate-2-phosphate, 100 µg/mL sodium pyruvate and 1% insulin-transferrin-selenium (ITS+) (BD Biosciences, San Jose, CA). ACs and MCs were pooled into five different co-culture ratio groups (n = 12 for each group): 1) 100% MCs and 0% ACs; 2) 75% MCs and 25% ACs; 3) 50% MCs and 50% ACs; 4) 25% MCs and 75% ACs; and 5) 0% MCs and 100% ACs. Cells were seeded by adding 100 µl of the cell-suspension (2 million cells/100 µl of medium) into the mold. An additional 5 ml of culture medium was added to the wells to submerge the agarose molds. The serum-free medium was changed once every 2 days for a period of 4 wks. Constructs were left confined in the wells for the entire culture period.

Histology

At t = 4 wks, two samples from each group were frozen using cryoembedding medium and sectioned at a thickness of 14 µm. Safranin-O / fast green stain was used to determine GAG distribution. Picrosirius red stain was used to determine collagen distribution. Polarized light images were also taken to examine collagen alignment within the constructs. Two rabbit medial menisci were defrosted, placed in cryoembedding medium, frozen and then sectioned at 14 µm. Safranin-

O / fast green and picrosirius and staining was used to examine GAG and collagen distribution in the inner and outer regions of the meniscus.

Compressive tests

Five test samples from each group and six medial inner and outer leporine menisci were subjected to creep indentation testing. Briefly, 2 mm constructs and tissue samples, obtained using a 2 mm biopsy punch, were attached to a sample holder using cyanoacrylate glue and submerged in PBS. The samples were positioned under the load shaft such that they rested perpendicular to 0.5 mm flat ended, porous intender tip. A tare load of 0.2 g (0.002 N) was applied and samples were allowed to reach tare creep equilibrium. A step load of 0.7 g (0.007 N) was then applied and displacement of the sample was measured until equilibrium was reached. Three intrinsic mechanical properties were determined, the aggregate modulus, the Poisson's ratio, and the permeability using the linear biphasic theory.[241]

Tensile tests

Five test samples and six inner and outer medial leporine menisci were used for tensile tests at t = 4 wks using a previously described protocol.[26] Tests were performed using an Instron 5565 with a 50 N load cell. To ensure testability, samples were carefully carved into a dog-bone shape. The ends were fixed to paper tabs using cyanoacrylate glue. A 0.05 N tare load was applied and the constructs were pulled to failure at a strain rate of 1% of the gauge length.

Tensile stiffness and the ultimate tensile strength (UTS) of each construct were determined using stress-strain curves. Portions of the constructs not use for tensile tests were processed for biochemical analyses.

Quantitative biochemistry

At t = 4 wks, five samples from each group, and six samples from inner and outer leporine menisci were processed for biochemical analysis. Samples were lyophilized and digested with pepsin (10 mg/ml) followed by elastin (1 mg/ml) at 4°C. Total DNA content/sample was determined using a picogreen cell proliferation assay kit (Molecular Probes, Eugene, OR). Total sulfated GAG per sample was quantified using the Blyscan GAG Assay kit (Biocolor, Newtownabbey, Northern Ireland). Total collagen per sample was determined using a modified chloramine-T hydroxyproline assay.[151] Collagen I and collagen II levels were quantified using an enzyme-linked immunosorbent assay (ELISA) developed by Chondrex Inc. (Chondrex, Redmond, WA). Detailed protocols for these assays have been described previously.[26]

Statistical analysis

Biochemical and biomechanical data were compared using a one-way analysis of variance test. If significant differences were observed, a Tukey's *post hoc* test was performed to determine specific differences among groups. A significance level of 95% with a p value of 0.05 was used in all statistical tests performed. All values are reported as mean ± standard deviation. Univariate regression analysis

was conducted to probe for correlations between the biochemical and biomechanical data.

Results

Gross morphology and histology

Constructs were examined at t = 4 wks for their physical characteristics. Presence of glistening cartilage-like matrix was present in all groups. As AC% increased, constructs were found to expand radially towards the well edges while constructs in higher MC% groups appeared to contract inwards (Figure 30A). The diameter of the constructs ($p < 0.0001$) ranged from 2.2 ± 0.1 mm (100% MC) to 3.1 ± 0.1 mm (100% AC) (Table 8). Differences were also observed among groups for thickness ($p = 0.005$); constructs in the 100% MC group (0.8 ± 0.1 mm) were significantly thicker than those in other groups (0.6 ± 0.1 mm) (Table 8).

Uniform collagen staining was observed throughout the construct in all groups (Figure 30B). Polarized light images showed stronger collagen alignment in the higher MC% groups (Figure 30C). GAG staining was uniform in all groups containing ACs while in the 100% MC group, pockets of GAG were observed toward the construct periphery and were absent in the inner portions of the construct (Figure 30D). Native leporine tissue stained strongly for collagen with collagen alignment visible in the circumferential direction without polarized light

(Figure 31). GAG staining was limited to the inner regions of the rabbit meniscus (Figure 31).

Biochemistry

Values of cells per construct, WW per construct, collagen per dry weight (DW), and GAG per DW are found in Table 8. Briefly, cell number per construct was found to increase significantly with increasing MC% ($p = 0.0006$). Wet weights ranged from 4.4 ± 0.3 mg in the 100% MC group to 6.3 ± 0.2 mg in the 50 and 25% MC group ($p < 0.0001$). GAG per DW significantly increased from 11.9 ± 2.2 µg/mg in the 100% MC group to 830.5 ± 99.7 µg/mg in the 0% MC group ($p < 0.0001$). GAG per DW was found to be 38.8 ± 5.7 µg/mg and 9.6 ± 1.7 µg/mg for the inner and outer leporine meniscus, respectively. Collagen per DW varied significantly between the 100% MC group (559.8 ± 165.8) and the 0% MC group (793.6 ± 84.3) ($p = 0.007$); however, no differences were observed among the co-culture groups. Collagen per DW was found to be 698.4 ± 100.9 µg/mg and 794.3 ± 68.1 mg for the inner and outer meniscus, respectively.

Total GAG content varied significantly among groups ($p < 0.0001$) with the highest value obtained in the 0% MC group (440.5 ± 34.4 µg) and the lowest value obtained in the 100% MC group (4.8 ± 0.7 µg) (Figure 32A). When normalized to WW, the same trends were observed ($p < 0.0001$) (Figure 32B). The GAG per WW levels were 8.7 ± 1.7%, 3.2 ± 0.2%, 1.6 ± 0.3%, 0.7 ± 0.2%, and 0.1 ± 0.0% for the 0, 25, 50, 75, and 100% MC groups, respectively. GAG

per WW was found to be 0.9 ± 0.2% and 0.3 ± 0.0% in the inner and outer meniscus, respectively.

Total collagen content varied significantly among groups ($p < 0.0001$) with the highest value obtained in the 0% MC group (421.0 ± 24.9 μg) and the lowest value obtained in the 100% MC group (224.7 ± 49.5 μg) (Figure 32C). When normalized to WW, the collagen content in 0% AC group was significantly higher when compared to the other groups ($p < 0.0001$) (Figure 32D); however, no significant differences were observed among groups containing 25, 50, 75, or 100% MCs. Specifically, the collagen per WW levels were 8.3 ± 1.4%, 5.1 ± 0.2%, 4.6 ± 1.2%, 4.4 ± 1.2%, and 5.1 ± 1.1% for the 0, 25, 50, 75, and 100% MCs, respectively. Collagen per WW was found to be 15.2 ± 1.2% and 20.9 ± 1.3% for the inner and outer meniscus, respectively.

Total collagen I ($p < 0.0001$) and collagen II ($p < 0.0001$) content were found to be significantly different among groups. Collagen I content was highest in the 100% MC group and dropped with decreasing MC percentage. The collagen I levels were 101.4 ± 15.9 μg, 53.4 ± 15 μg, 39.4 ± 5.3 μg, 36.2 ± 7.8 μg, and 7.4 ± 2.0 μg, for the 100, 75, 50, 25, and 0% MC groups, respectively. Trends were reversed for collagen II content, with the highest levels observed in the 0% MC group. The collagen II levels were 18.5 ± 4.3 μg, 26.1 ± 4.9 μg, 91.6 ± 28 μg, 237.4 ± 18.4 μg, and 417.9 ± 34.0 for the 100, 75, 50, 25, and 0% MC groups, respectively. Collagen II to collagen I (or collagen I to collagen II) normalizations

showed that the 100 and 75% MC groups, and the outer meniscus contained approximately 5.9, 2.1, and 27.3 times more collagen I than collagen II, respectively. The 50, 25, and 0% MC groups, and the inner meniscus contained approximately 2.4, 6.9, 59.3, and 1.7 times more collagen II than collagen I, respectively (Figure 33).

Compressive properties

The aggregate modulus ($p < 0.0001$) (Figure 34) and Poisson's ratio ($p = 0.01$) were found to be significantly different among test groups. The 0% MC group had the highest aggregate modulus (112 ± 18 kPa), approximately three times that of the 100% MC group (34 ± 12 kPa). The aggregate modulus of the leporine inner meniscus was found to be 360 ± 90 kPa while that of the outer meniscus was found to be 160 ± 45 kPa. Poisson's ratio of the constructs were 0.1 ± 0.1, 0.1 ± 0.1, 0.1 ± 0.1, 0.1 ± 0.1, and 0.3 ± 0.1 for the 100, 75, 50, 25 and 0% MC groups, respectively. These values were significantly higher than the Poisson's ratio for the inner and outer meniscus, both of which were found to be 0.0 ± 0.0. No significant differences were observed among groups for the permeability of the constructs ($p = 0.2$). The permeability values were 2.9 ± 1.2 ($x10^{-15}$) m^4/N-s, 3.6 ± 2.6 ($x10^{-15}$) m^4/N-s, 3.5 ± 2.5 ($x10^{-15}$) m^4/N-s, 4.8 ± 3.0 ($x10^{-15}$) m^4/N-s, and 6.2 ± 2.0 ($x10^{-15}$) m^4/N-s for the 100, 75, 50, 25 and 0% MC groups, respectively. These values were all significantly higher ($p = 0.002$), however, when compared to those of the leporine inner meniscus (1.1± 0.2 ($x10^{-15}$) m^4/N-s) and outer meniscus (0.9 ± 0.2 ($x10^{-15}$) m^4/N-s).

Tensile properties

The tensile modulus ($p < 0.0001$) (Figure 35A) and UTS ($p < 0.0001$) (Figure 35B) were found to be significantly different among test groups. The highest tensile modulus was observed in the 100% MC group (1.5 ± 0.1 MPa) while the lowest tensile modulus was observed in the 25% MC group (0.8 ± 0.0 MPa). Tensile values for the inner meniscus and outer meniscus were found to be 60 ± 40 MPa and 130 ± 70 MPa, respectively. The highest UTS was observed in the 100% MC group (0.4 ± 0.1 MPa) while the lowest tensile modulus was observed in the 25% MC group (0.2 ± 0.0 MPa). UTS values for the inner and outer meniscus were found to be 12 ± 4 MPa and 23 ± 6 MPa, respectively.

Correlation between biochemical and biomechanical data

Aggregate and tensile moduli obtained from the mechanical tests were correlated with GAG and collagen values per construct and WW. Strong significant correlations were obtained between the aggregate modulus and GAG per construct ($p = 0.0002$, $r^2 = 0.7$), and GAG per WW ($p < 0.0001$, $r^2 = 0.6$). Weaker significant correlations were observed between the aggregate modulus and collagen per construct ($p = 0.01$, $r^2 = 0.3$) and collagen per WW ($p = 0.02$, $r^2 = 0.2$). Strong significant correlations were obtained between the tensile modulus and collagen per construct ($p < 0.0001$, $r^2 = 0.5$), and collagen per WW ($p < 0.0001$, $r^2 = 0.5$). Weaker non-significant correlations were observed between the tensile modulus and GAG per constructs ($p = 0.7$, $r^2 = 0.002$) and GAG per WW ($p = 0.5$, $r^2 = 0.008$).

Correlations were also examined between the mechanical and biochemical properties for the inner and outer meniscus. Strong significant correlations were observed between the aggregate modulus and the GAG per WW of the inner ($p < 0.0001$, $r^2 = 0.8$) and outer meniscus ($p < 0.0001$, $r^2 = 0.8$). Weaker but significant correlations were observed for aggregate modulus and the collagen per WW of the inner ($p = 0.002$, $r^2 = 0.4$) and outer meniscus ($p = 0.01$, $r^2 = 0.3$). Strong significant correlations were observed for the tensile modulus and collagen per WW of inner ($p < 0.0001$, $r^2 = 0.9$) and outer ($p < 0.0001$, $r^2 = 0.9$) meniscus. Weaker but significant correlations were observed for the tensile modulus and the GAG per WW of the inner ($p = 0.005$, $r^2 = 0.4$) and outer ($p = 0.002$, $r^2 = 0.4$) meniscus.

Discussion

The meniscus contains a heterogeneous ECM population which leads to differences in its biomechanical properties. Thus, recapitulating tissue heterogeneity will aid in the successful regeneration of the meniscus. This study identified co-cultures of bovine MCs and ACs that resulted in a spectrum of fibrocartilages mimicking several biochemical and biomechanical properties of inner and outer leporine menisci. The result supports the future use of a xenogenic cell source (bovine cells) for leporine tissue engineering. The results also showed clear differences among groups with higher MC% groups contracting over the culture period while lower MC% groups expanded. In addition, higher MC% groups resulted in constructs more closely resembling the

outer regions of leporine menisci, while lower MC% groups resulted in constructs more closely resembling the inner regions of leporine menisci, confirming the original hypotheses.

The use of five different MC:AC co-culture ratios (100:0, 75:25, 50:50, 25:75, and 0:100) resulted in a spectrum of biochemical properties, ranging from fibrous to cartilaginous. As the MC% decreased, collagen II levels increased and collagen I levels decreased. Excitingly, we were able to show that a 50:50 co-culture of MCs and ACs resulted in a collagen II to collagen I ratio (2.4 ± 0.8) mimicking the inner leporine meniscus (1.7 ± 0.2). Collagen I to collagen II ratio for the outer meniscus (27.3 ± 5.6), however, could not be matched by any of the co-culture groups, with the closest group comprising of 100% MCs (5.9 ± 2.4). Growth factors such as transforming growth factor-beta 1 [242] or mechanical stimuli, such as tension,[243] may be used to enhance the fibroblastic phenotype of cells.

Statistically significant changes in construct compressive properties were accompanied by concomitant changes in GAG production. As MC% decreased, significant increases in GAG per WW and aggregate modulus were observed. Moreover, univariate regression analysis showed a strong significant correlation between GAG content and aggregate modulus of the constructs. In terms of GAG per WW, the higher MC% groups (100% and 75%) were found to be equivalent to the outer meniscus. GAG levels in the inner meniscus were matched by the 75% and 50% MC groups. Despite the similarities in GAG

content between higher MC% groups and native tissue, the overall compressive properties of these constructs lagged behind native values. Interestingly, the lower MC% groups (25% and 0%) were found to exhibit the highest aggregate modulus values (~100 to 115 kPa) and these were approaching outer meniscus values (~160 kPa). One possible reason for this discrepancy may be the lack of collagen production per WW in the higher MC% groups. It is known that collagen plays an important in the final compressive properties of the construct, possibly by interacting with small leucine rich proteoglycans that can affect collagen diameter and alignment.[194] Thus, future studies will need to investigate techniques to enhance collagen deposition to further increase the aggregate modulus. This may be achieved through the use of chemical agents, such as chondroitinase-ABC, which has been shown to temporarily deplete GAGs from constructs while enhancing the collagen network.[228]

Strong correlations were observed between the tensile properties and the total collagen, as well as collagen per WW, of the constructs. The highest collagen content and highest collagen per WW were observed in the 0% MC group, while the highest tensile properties were observed in the 100% MC group. It is possible that tensile stiffening in 100% MC constructs occurred due to construct contraction which may have resulted in increased collagen alignment and cross-linking in the ECM. Polarized light images confirmed that a greater degree of alignment was observed in the 100% MC constructs with little to no alignment observed in the lower MC% groups. Regardless of these variations, the overall

tensile properties were found to be almost two orders of magnitude below native values. Collagen content per WW lagged as well in the tissue engineered groups (~5 to 8%) when compared to the inner and outer rabbit meniscus (~15 to 20%). It appears that increasing collagen content and tensile properties towards native values remains a challenging problem, even for articular cartilage tissue engineering.[54, 228] Future work will need to investigate novel methods to create constructs with preferred collagen orientations that will enhance the tensile properties. One technique may be to use ring-shaped agarose molds which have been shown to enhance collagen alignment in the circumferential direction, similar to that observed in the native tissue.[53]

The growth characteristics of the tissue engineered constructs were found to be dependent on cell type. Constructs containing a higher percentage of MCs tended to contract while constructs containing a higher percentage of ACs tended to expand. This result has been observed in a prior study investigating 50:50 co-cultures of MCs and ACs, and MCs alone cultured on cylindrical agarose molds in serum-free or serum-containing medium [26]. MCs contain a mixed population of fibroblast-like and chondrocyte-like cells. Fibroblasts have been shown in the past to generate contractile forces in scaffolds containing collagen and GAGs.[244] These contractile forces are likely caused by the reorganization of the cytoskeleton, possibly through cross-bridges between actin and myosin filaments [245] or through upregulation of alpha-smooth muscle actin (SMA).[246] These contractile forces in fibroblasts have been shown to range from

1 to 25 nN and depend on the mode of measurement and cell substrate.[244, 247, 248] Similarly, we hypothesize that MCs exhibit this contractile behavior that is characteristic of fibroblasts.

Bovine cells were used in this experiment for leporine tissue engineering to circumvent issues of cell scarcity and for possible examination as a future xenogenic cell source. A bovine medial meniscus yields 20 to 30 million MCs in comparison to a rabbit medial meniscus which yields 1 to 2 million cells. The low cell numbers obtained from the rabbit necessitate cell expansion in monolayer. This technique leads to rapid changes in the phenotype of MCs and losses in gene expression of important ECM markers, such as collagen II and COMP [159]. To preserve MC phenotype, an alternative approach may be to use primary cells from an easily available cell source, such as bovine MCs and ACs. Using these cells, meniscus tissue engineering technologies can be developed and the ability of bovine cells to produce matrix similar to that of the leporine meniscus can be assessed. If successful, a xenogenic approach may be employed in future *in vivo* studies in the rabbit. This approach may be feasible since the knee joint is considered as an immunoprivileged site due to low vascularity. A recent study has shown that pig ACs implanted into osteochondral defects of adult rabbits do not illicit a noticeable immune response.[249] In addition, immune response was limited when rabbit ACs, embedded in fibrin glue, were implanted in a goat full thickness articular cartilage defect.[250] Success with these studies provides

excitement for the use of xenogenic cells and may diminish fears of a major immune rejection in the joint.

Although the inner and outer leporine menisci were characterized primarily to serve as internal controls for our tissue engineering groups, it is also interesting to examine the biochemical and biomechanical characterization results to other animal models in the literature. GAG per DW levels were found to be slightly higher in the inner meniscus of the rabbit when compared to human menisci.[251] A possible explanation for this may be due to high loads placed on the inner portions of the meniscus due to the flexed knee-resting stance of the rabbit and its propensity to jump. Collagen content in human and porcine menisci have been measured in terms of hydroxyproline levels.[219, 251-253] If our collagen data is converted to hydroxyproline using a conversion factor of 10 [254], the obtained hydroxyproline values are slightly lower (~70 to 80 μg/mg DW) than those observed in human and porcine tissue (~100 to 140 μg/mg DW). This was unexpected and the animal model is most likely the source of this discrepancy. Both, the aggregate and tensile moduli were found to be in the same range of values obtained with larger animal models.[251, 255-260] Overall, the similarities in the biochemical and biomechanical properties between the results obtained in this study and those of larger animal models justified the investigation of the leporine model as a first step towards tissue engineering the knee meniscus.

Conclusions

This work examined five different co-culture ratios of bovine ACs and MCs for leporine meniscus tissue engineering. Lower MC% groups approached the compressive moduli of the outer regions of native tissue and collagen per WW of the inner regions of native tissue. Higher MC% groups more closely mimicked the GAG per WW of both the inner and outer regions of the meniscus. The 50% MC group was found to mimic the inner meniscus in collagen II to collagen I content. Tensile properties of native tissue could not be matched by any of the test groups. Based on these data, a 50% MC group may be suitable to recapitulate biochemical characteristics of the inner regions of the tissue. For the outer regions, a lower MC% group with higher compressive strength may be suitable from a functional tissue engineering viewpoint. Finally, further examination of bovine cells as a potential xenogenic cell source is warranted based on their ability to produce constructs approaching those of the leporine meniscus.

Figure associated with chapter 8

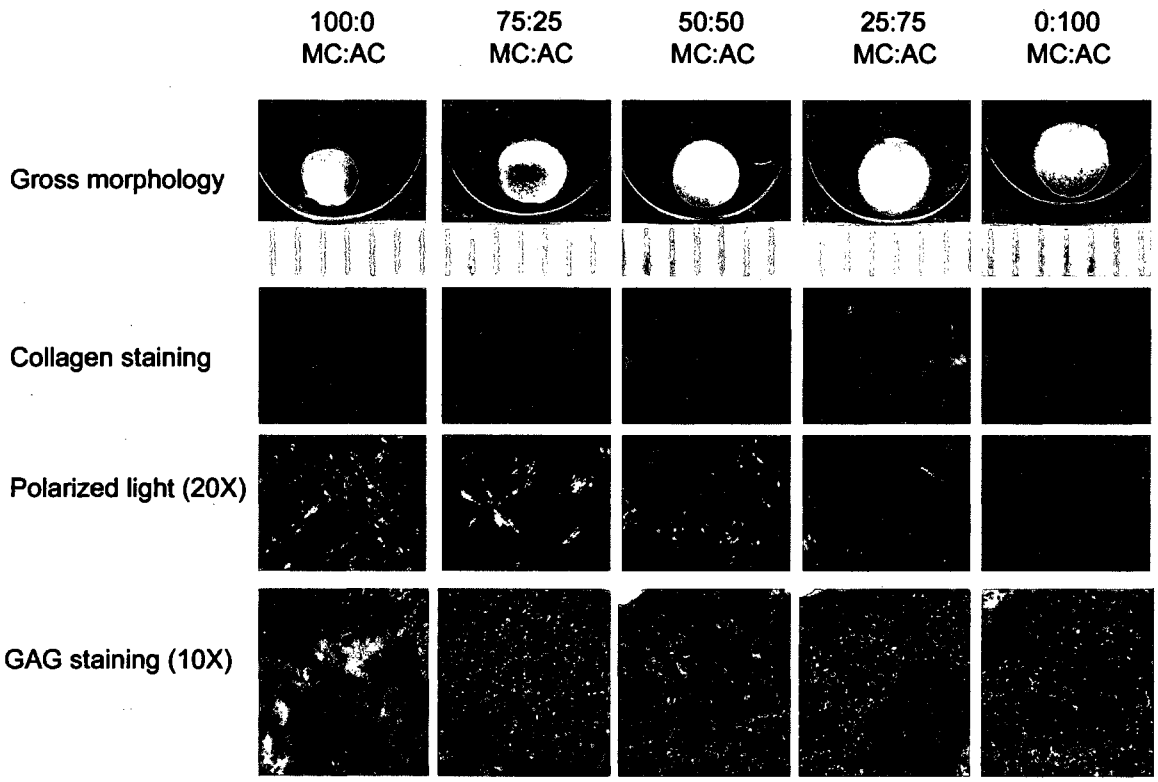

Figure 30. Gross morphological and histological sections of constructs Constructs contracted with increasing MC%. Uniform collagen staining (20X) was observed in all groups. Polarized light images (20X) showed greater degree of collagen alignment in higher MC% groups. Uniform GAG staining (10X) was observed in all groups, except the 100% MC group.

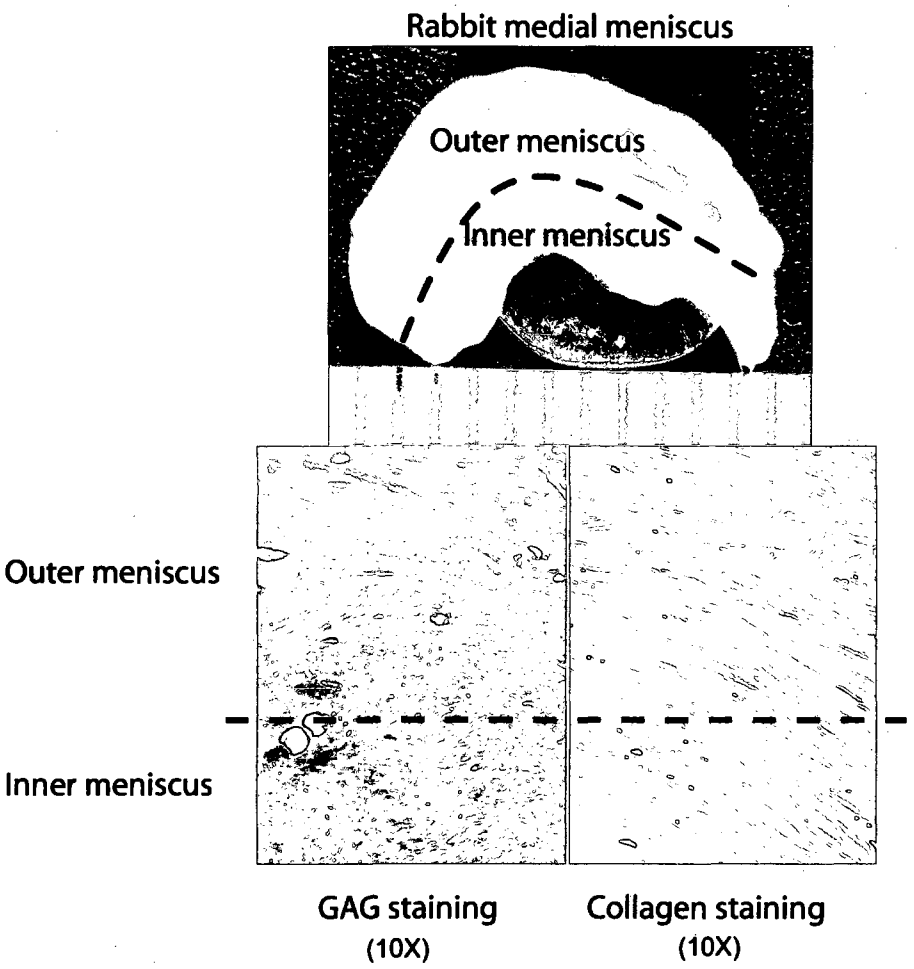

Figure 31. Gross morphology and histology of the leporine meniscus
Inner and outer regions of the native tissue (designated by the dashed line) were examined separately for their biochemical and biomechanical characteristics. Strong GAG staining was observed in the inner regions of the meniscus. Strong collagen alignment was visible in all regions of the meniscus in the circumferential direction.

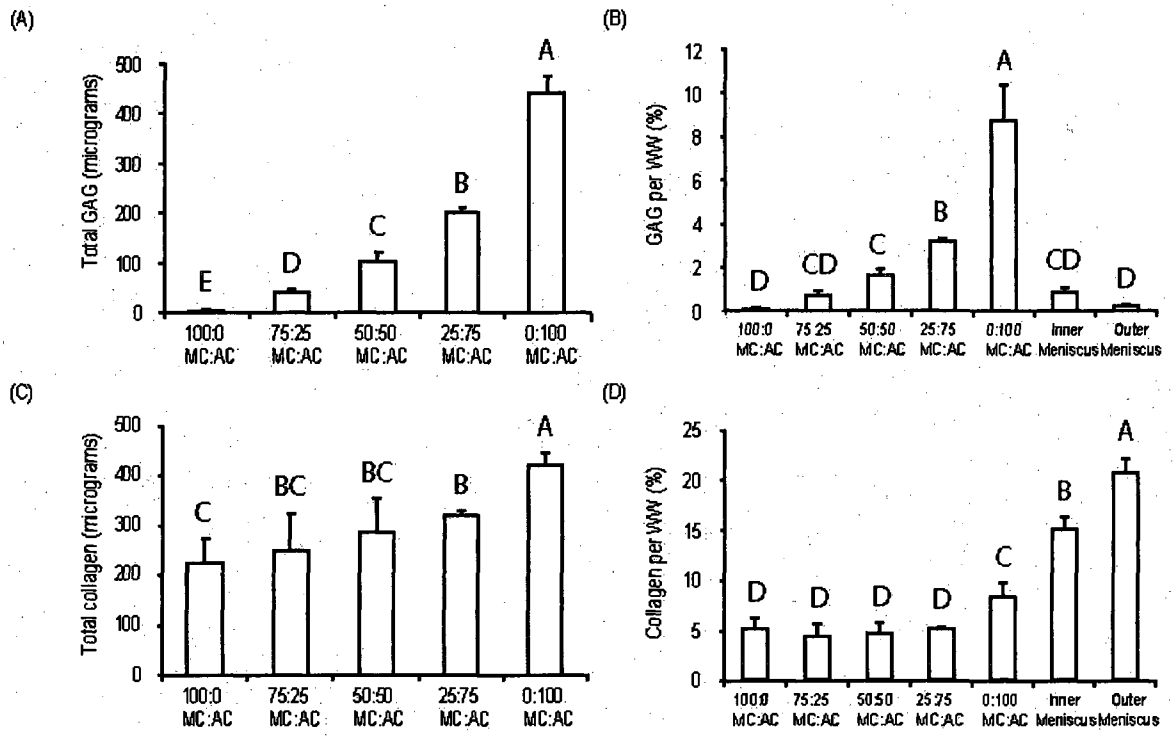

Figure 32. Biochemical properties of constructs and native tissue
(A) Total GAG per construct. (B) GAG per wet weight (constructs and native tissue). (C) Total collagen per construct. (D) Collagen per wet weight (constructs and native tissue). Groups with different letters are significantly different from each other. All values are reported as mean ± SD. An n = 5 (constructs) and n = 6 (for native tissue) was used to perform the assays.

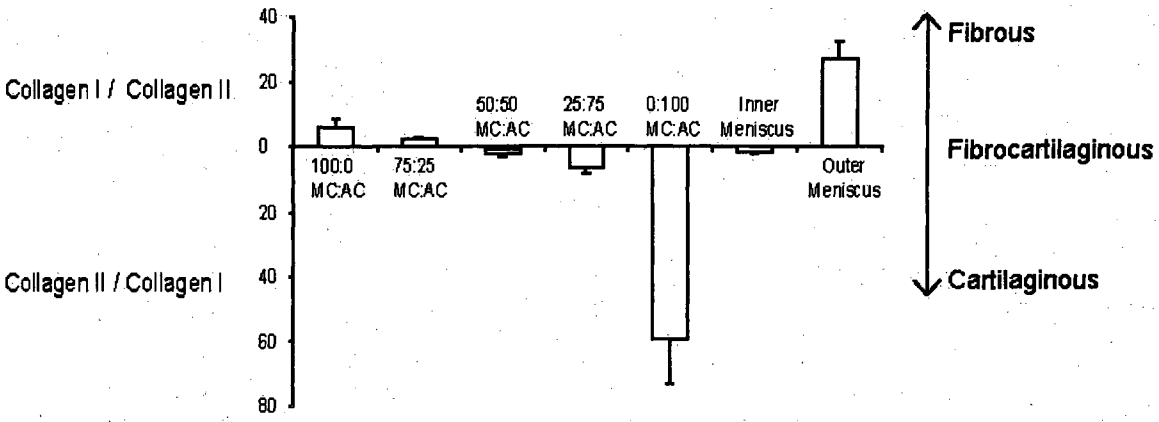

Figure 33. Spectrum of fibrous to cartilaginous nature of constructs and native tissue

Groups with higher collagen I to collagen II ratio (fibrous) are shown on the upper panel, while groups with higher collagen II to collagen I (cartilaginous) ratio are shown in the lower panel.

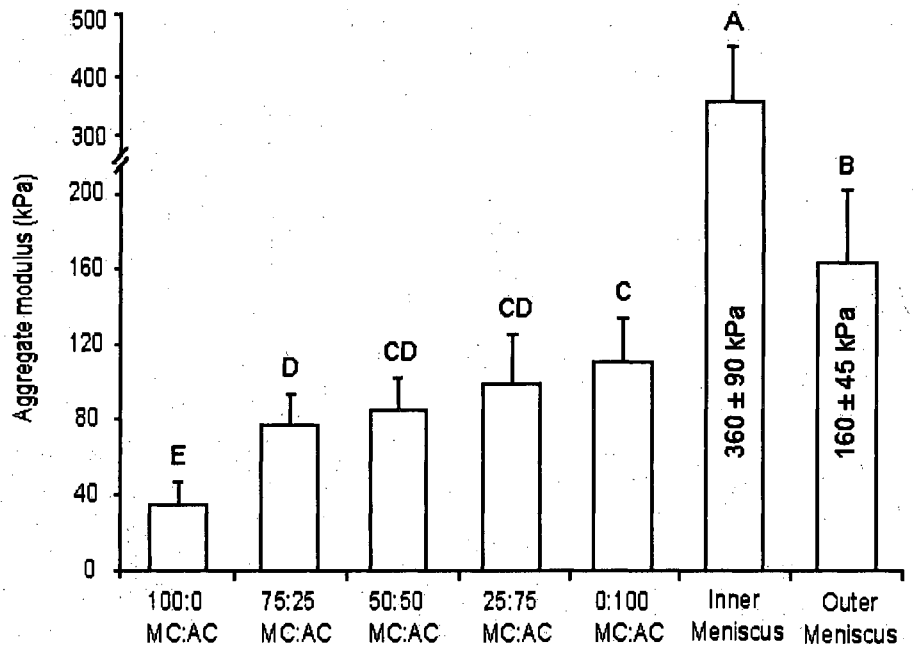

Figure 34. Aggregate modulus of constructs and native tissue
Groups with different letters are significantly different from each other. All values are reported as mean ± SD. An n = 5 (constructs) and n = 6 (for native tissue) was used to perform the assays.

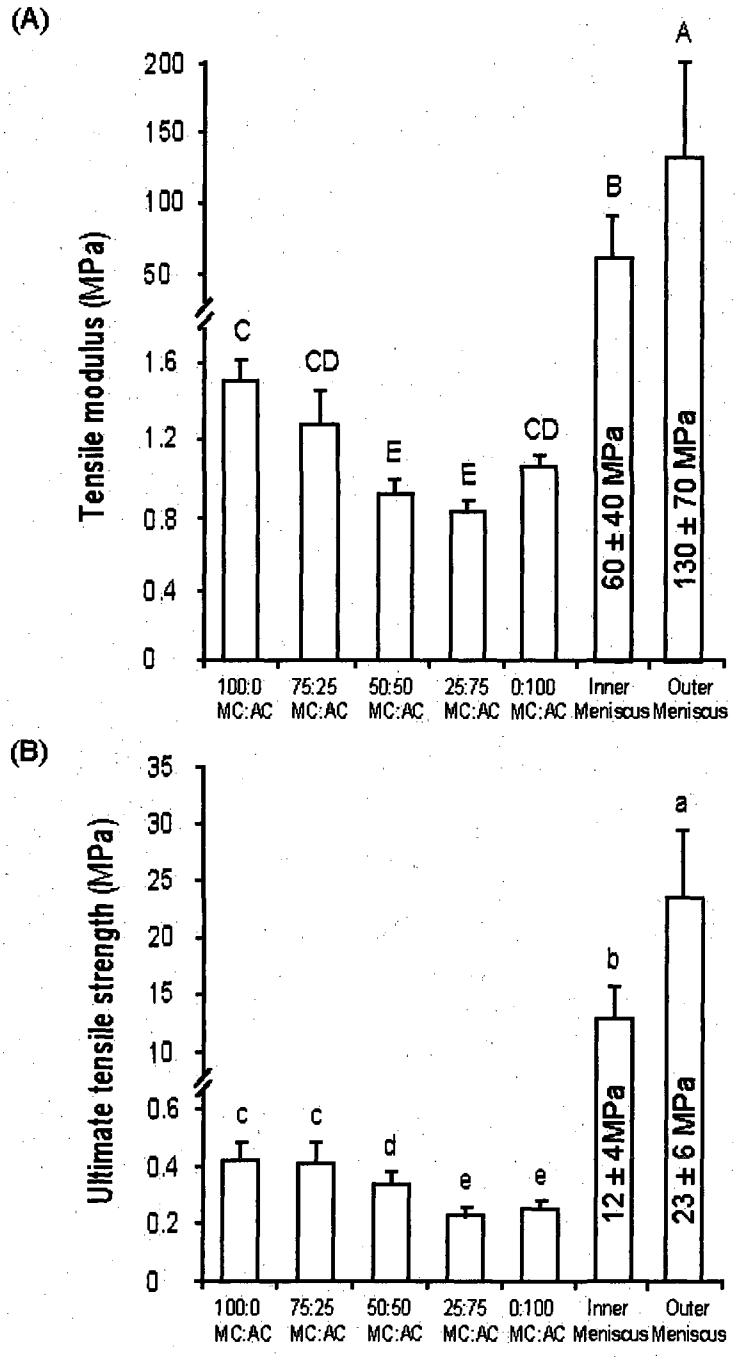

Figure 35. Tensile properties of constructs and native tissue
(A) Tensile moduli. (B) Ultimate tensile strength. All values are reported as mean ± SD. An n = 5 (constructs) and n = 6 (for native tissue) was used to perform the assays.

Table 8. Biochemical and gross morphological properties of constructs and native tissue

The star symbol denotes that inner and outer meniscus groups were only compared to tissue engineered construct groups for the GAG and collagen per DW.

Groups	GAG / DW (µg/mg)	Collagen / DW (µg/mg)	Cell number (millions)	WW (mg)	Diameter (mm)	Thickness (mm)
100:0 (MC:AC)	11.9 ± 2.2^{f}	559.8 ± 165.8^{b}	2.2 ± 0.2^{a}	4.4 ± 0.3^{d}	2.2 ± 0.1^{d}	0.8 ± 0.1^{a}
75:25 (MC:AC)	80.8 ± 16.1^{d}	521.8 ± 180.4^{b}	2.1 ± 0.3^{ab}	5.7 ± 0.3^{bc}	2.7 ± 0.1^{c}	0.6 ± 0.1^{b}
50:50 (MC:AC)	191.3 ± 32.7^{c}	534.1 ± 108.9^{b}	1.8 ± 0.3^{abc}	6.3 ± 0.2^{ab}	2.7 ± 0.1^{c}	0.6 ± 0.1^{b}
25:75 (MC:AC)	374.7 ± 17.4^{b}	599.7 ± 55.6^{ab}	1.7 ± 0.2^{bc}	6.3 ± 0.2^{a}	2.9 ± 0.1^{b}	0.6 ± 0.1^{b}
0:100 (MC:AC)	830.5 ± 99.7^{a}	793.6 ± 84.3^{a}	1.6 ± 0.2^{c}	5.1 ± 0.6^{c}	3.1 ± 0.1^{a}	0.6 ± 0.1^{b}
*Inner meniscus**	38.8 ± 5.7^{e}	698.4 ± 100.9^{ab}				
*Outer meniscus**	9.6 ± 1.7^{f}	794.3 ± 68.1^{a}				
p value	< 0.0001	0.007	0.0006	< 0.0001	< 0.0001	0.005

Chapter 9: Effects of mold compliance and surface roughness on scaffoldless meniscus-shaped constructs

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Abstract

The meniscus is a fibrocartilaginous tissue that is critically important to the loading patterns within the knee joint. If the meniscus structure is compromised, there is little chance of healing due to limited vascularity in the inner portions of the tissue. Several tissue engineering techniques to mimic the complex geometry of the meniscus have been employed. Of these, a self-assembly, scaffoldless approach employing agarose molds avoids drawbacks associated with scaffold use while still allowing formation of robust tissue. In this experiment two factors were examined, agarose percentage and mold surface roughness, in an effort to consistently obtain constructs with adequate geometric properties. Co-cultures of ACs and MCs (50:50 ratio) were cultured in smooth or rough molds composed of 1% or 2% agarose for 4 wks. Morphological results showed that constructs formed in 1% agarose molds, particularly smooth molds, were able to maintain their shape over the 4 wk culture period. Significant increases were observed for the collagen II to collagen I ratio, total collagen, GAG, and tensile and compressive properties in smooth wells. Cell number per construct was higher in the rough wells. Overall, it was observed that the topology of an agarose surface may be able to affect the phenotypic properties of cells that are on that surface, with smooth surfaces supporting a more chondrocytic phenotype. In addition, wells made from 1% agarose were able to prevent construct buckling potentially due to their higher compliance.

Introduction

The meniscus is a fibrocartilaginous tissue that is situated between the femur and tibia within the knee joint. Due to its location, geometry, and composition, the meniscus protects the underlying articular cartilage from excessive stresses via force distribution and shock absorption.[3, 261] However, if the meniscus structure is compromised, its ability to perform these critical functions is lost.[20, 262] Furthermore, injuries to the inner regions of the meniscus do not heal due to limited vascularity in that region. Partial meniscectomy is the most common treatment for meniscal tears and can alleviate short term symptoms of pain and temporarily restore joint function.[263] In the long term, the loss of meniscal cartilage leads to degeneration of the underlying articular cartilage and quickens the onset of osteoarthritis.[238] Thus, tissue engineering may be a viable option to repair or replace injured tissue.

The meniscus contains a heterogeneous cell and ECM population.[235] The inner region contains chondrocyte-like cells and the ECM is composed of a network of collagen II and collagen I fibers in a 3:2 ratio, and the proteoglycan aggrecan. *In vivo*, the inner meniscus is loaded with an axial compressive force. The outer region contains fibroblast-like cells with circumferentially oriented collagen I fibers. Due to the wedge-shaped profile and attachments of the meniscus, the compressive load experienced by the inner meniscus is partially converted into circumferential tensile forces in the outer regions. Furthermore, the wedge-shaped profile of the meniscus corrects the incongruity between the curved

femoral condyles and flat tibial plateau allowing distribution of the compressive forces over a larger area of the articular surfaces.[264, 265] Finite element models have shown that geometrical aspects of the meniscus, particularly the radius of curvature to match the femoral condyle, are critically important to the ability of the meniscus to absorb and distribute loading.[17] Thus, for an engineered meniscal substitute, recapitulation of geometry and composition will be critical to achieve successful tissue replacement.

Most attempts at creating meniscus-shaped constructs have involved producing scaffolds by pouring a liquid polymer solution into a mold and allowing it to set or by adding a cross-linking agent.[30, 266, 267] Other attempts have used solid freeform fabrication [268] or physical shaping.[160] These attempts at shape-mimicking have highlighted a number of issues that must be addressed before a meniscus-specific construct can be realized, including shape retention and obtaining adequate biochemical and biomechanical properties.

Although widely used in tissue engineering studies, scaffold materials can present several drawbacks. These include toxicity associated with scaffold degradation byproducts, lack of cellular mechanotransduction due to stress shielding, and irregular tissue ingrowth to scaffold degradation rates.[52] A self-assembly, scaffoldless approach developed in our laboratory circumvents these issues, and allows for the creation of shape-specific geometries. Previous work with this approach has shown that meniscus cells (MCs) and articular

chondrocytes (ACs) can be co-cultured in ring-shaped molds.[53] However, with increasing AC percentage, the constructs expand radially, contact the well edge and slide up the smooth wells or buckle in response to the force placed on the construct by the agarose well. With increasing MC percentage the constructs contract into a torus. Thus, by using various ratios of the two cell types, the expansive contribution of ACs and contractile contribution of MCs can be modulated to form different shaped constructs.

Utilizing data from a previous study,[53] and from pilot studies in the laboratory, it has been determined that a 50:50 co-culture ratio of ACs and MCs, grown in a ring-shaped mold, in the presence of serum most closely resembles the native meniscus. However, when switching to a serum-free approach, enhanced expansive growth is noted as the constructs expand radially.[26] To address this issue, the compliance of the agarose well was studied to determine if a well that provided less resistance to construct impingement would allow for proper construct geometry. To this end, two different agarose percentages (1% and 2%) were examined. In addition, topology of the well walls was also altered (rough and smooth wells) to examine whether roughness would prevent buckling of the constructs by increasing the frictional contact between the radially expanding constructs and the well wall. It was hypothesized that 1) buckling would be reduced by increased compliance of lower agarose-content (1%) wells; and 2) buckling would be reduced by increased frictional contact in wells with rough edges.

Materials and methods

Well-maker fabrication

Pictures of rabbit medial menisci were taken to obtain meniscus geometric properties. Using AutoCAD 2007 (Autodesk, San Rafael, CA), information from these pictures was used to create an idealized elliptical shape with a curved, wedge profile which closely matched the meniscus geometry (Figure 36A). In order to obtain two molds with similar geometrical properties but differing in surface roughness, two different solid-freeform fabrication machines were employed. To create the smooth well-maker (i.e., the negative of the idealized meniscus geometry), a ZPrinter 310 (Z Corporation, Burlington, MA) was used. This machine employs 3D printing technology to place a drop of binding solution into a basin of powder to create a part with a layer thickness of 76 μm. Although the layer thickness alone was sufficient to obtain a smooth surface, the well-maker was dipped in latex solution to ensure smoothness and to prevent agarose from infiltrating the porous material (Figure 36B). The rough well-maker was created using a Dimension 768 SST (Stratasys, Eden Prarie, MN) which utilized fused deposition modeling technology to build the part layer by layer using a thin acrylonitrile butadiene styrene (ABS) plastic cord (Figure 36C). This method of rapid prototyping imparted an inherent anisotropic roughness to the well-maker due to the large layer thickness dictated by the use of a 245 μm diameter ABS cord. The average roughness value (Ra) was estimated based on the B46.1-2002 ASME standard by assuming the surface was a waveform composed of semi-circles with no gaps in between. Images of the surface of the well-maker

were analyzed using Image J (NIH, Bethesda, MD) to determine the diameter of the semi-circular protrusions. The surface roughness, Ra, was measured using the following formula.

$$Ra = \frac{1}{L}\int_0^L |Y(x)|dx$$

Y(x) is the function describing the surface of the well-maker with the horizontal axis set midway between the highest and lowest amplitude of the waveform and L is the length over which roughness is measured. Roughness was not present in the direction corresponding to the long axis of the ABS cord but was present in both the horizontal and vertical directions emanating from the center of an individual elliptical well-maker post.

Well fabrication

Agarose wells (1% and 2%) were created by pouring molten agarose into the wells of a 24-well plate (BD, Franklin Lakes, NJ). The well-makers were placed into the agarose and left until the agarose hardened. The well-makers were then removed and the agarose molds were transferred to 12-well plates (BD, Franklin Lakes, NJ). Chemically-defined serum-free medium was allowed to infiltrate the agarose hydrogel for 4 days in an incubator before seeding. The medium consisted of Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L-glucose and GlutaMAX (Invitrogen, Carlsbad, CA), 100 nM dexamethasone, 1% fungizone (Sigma, St Louis, MO), 1% penicillin/streptomycin (BD Biosciences, Bedford, MA), 1% ITS+ premix, 50 mg/mL ascorbate-2-phosphate, 40 mg/mL L-proline, and 100 mg/mL sodium pyruvate (Fisher Scientific, Pittsburgh, PA).

Cell isolation and seeding

The knee joints of skeletally immature calves (Research 87, Boston, MA) were dissected to obtain the medial menisci, and the tibial and femoral articular cartilage. In a cell culture hood, the vascular outer third of the meniscus was removed and the remainder was diced into small ~1mm pieces. The articular cartilage was shaved from the femoral and tibial surfaces. Meniscus and articular cartilage fragments were digested separately in a solution of 0.2% collagenase II (Worthington, Lakewood, NJ) in serum-free medium. After a 14-18 hr digestion, MCs and ACs were isolated through sequential dilution of the tissue/collagenase solution with phosphate buffered saline (PBS), centrifugation of the mixture, and removal of the supernatant. When the cells were sufficiently separated from the digested tissue, they were passed through a 70 μm cell strainer, counted using a hemocytometer, and frozen at -80°C. The freezing medium consisted of the same medium described above in addition to 20% fetal bovine serum and 10% dimethyl sulfoxide (Sigma, St. Louis, MO). Two wks after harvest, primary bovine ACs and MCs were thawed and re-suspended in serum-free medium. The cells were then combined in a 50:50 ratio and 20 million cells were seeded into the meniscus-shaped mold. Culture medium was changed every other day for the 4 wk duration of the study. At 4 wks, constructs were removed from the agarose wells and assessed via histological, biochemical, and biomechanical tests.

Histology

Constructs were cut in half along the major elliptical axis and a cylindrical 2 mm section of neo-tissue was taken from each half-ellipse using a 2 mm biopsy punch. Care was taken to note the orientation of the sample relative to the overall construct. The samples were frozen using HistoPrep (Histo Prep, Fisher Scientific, Pittsburgh, PA) and sectioned at 14 μm. Safranin-O / fast green was used to examine GAG distribution and picrosirius red was used for qualitative examination of collagen content. Slides stained with picrosirius red were also viewed under polarized light to examine collagen orientation.

Biochemistry

Samples were weighed to determine total wet weights and then processed for tensile, compressive, and histological tests. The remainder of the construct was then reweighed, lyophilized for 48 hrs, and then dry weights were measured. Samples were digested in pepsin (10 mg/ml) for 1 day at 4°C followed by elastase (1 mg/ml) for 5 days at 4°C. Biochemical assays performed were dimethylmethylene blue (DMMB) (sulfated-GAGs), hydroxyproline (collagen), PicoGreen (cell number), and ELISA (collagen I and collagen II). The DMMB assay for sulfated GAGs was completed using a commercially available Blyscan GAG Assay Kit (Biocolor, Newtownabbey, Northern Ireland). A modified hydroxyproline assay was used to determine total collagen in each construct.[151] Each sample was hydrolyzed in 4 M NaOH at 121°C for 1 hr. The samples were then neutralized and placed in buffer. The collagen content was then determined

by combining the samples with dimethyl aminobenzaldehyde and chloramine T allowing for a colorimetric comparison. The PicoGreen assay (Molecular Probes, Eugene, OR) was used to determine the DNA content of scaffoldless constructs. Collagen II and collagen I amounts were quantified using ELISA protocols described previously.[26] Briefly, for collagen II, Chondrex reagents and protocols were employed (Chondrex, Redmond, WA). For collagen I, a similar protocol was employed with antibodies from US Biological.

Uniaxial tension

Tensile tests were performed using an Instron 5565 with a 50 N load cell. To ensure testability, samples were taken from the longer axis of the ellipse and carved into a dog-bone shape. The ends were fixed to paper tabs using cyanoacrylate glue. A 0.05 N tare load was applied and the constructs were pulled to failure at a strain rate that was equivalent to 1% of the gauge length. Stress-strain curves were created from the load-displacement curves and the cross-sectional area of the samples; tensile stiffness and ultimate tensile strength were calculated from the linear region of each stress-strain curve. Construct thickness was measured using digital calipers.

Creep indentation

Disc-shaped constructs, obtained using a 2 mm biopsy punch, were evaluated with an indentation apparatus. The specimens were loaded with a tare mass of 0.4 g (0.004 N), using a 0.5 mm-diameter rigid, flat-ended, porous indenter tip.

When tare equilibrium was reached, a step mass of 2.3 g (0.02 N) was applied. Displacement of the sample surface was measured until equilibrium was reached. At that time, the step load was removed, and the displacement recorded until equilibrium was again reached. The aggregate modulus, Poisson's ratio and permeability of the samples was then determined using the linear biphasic theory.

Statistical analyses

Biochemical and biomechanical assessments were performed on all constructs ($n = 6$). A single factor ANOVA was used to analyze the samples, and a Tukey's *post hoc* test was used when warranted. Significance was defined as $p < 0.05$. Univariate regression analysis was also conducted to determine whether the biochemical properties correlated with the biomechanical properties.

Results

Well-maker roughness

The average roughness value (Ra) was estimated by assuming the surface was entirely composed of semi-circles. Image J (NIH, Bethesda, MD) was used to estimate the diameter of these semi-circles (~281 μm). Using the roughness formula, the average roughness (Ra) of the rough well-maker was calculated to be 44.7 ± 4.0 μm.

Gross morphology

Within 48 hrs of seeding, the cells coalesced into an elliptical shaped construct that grew in size over the 4 wk culture period (Figure 37). At t = 4 wks, the constructs cultured in the smooth agarose wells were extracted with a spatula by maneuvering the construct up the central post. Constructs cultured in the rough agarose wells were more difficult to extract and had pieces of agarose adhered to the construct. The adhered agarose was carefully removed using a spatula prior to histological and biochemical examination. Although translucent cartilage-like tissue was present in all groups, stark differences were observed in the morphology of the constructs in each group. Constructs cultured in the smooth wells exhibited a smooth surface topology while those cultured in rough wells exhibited an uneven topology. In addition, buckling was observed in constructs cultured in 2% agarose wells but was absent in constructs cultured in 1% agarose wells. Each elliptical construct was divided down the major axis to yield two meniscus-shaped-constructs. No significant differences were observed among groups in height ($p = 0.70$) and thickness ($p = 0.38$) of the meniscus-shaped constructs (Table 9).

Histology

Uniform collagen and GAG staining was observed in all constructs (Figure 37). Polarized light micrographs showed presence of collagen alignment in the circumferential direction and the alignment was most prominent in constructs cultured in smooth wells with 1% agarose (Figure 37).

Biochemistry

At t = 4 wks, wet weights of the meniscus-shaped constructs were significantly different among groups ($p = 0.0004$) and ranged from 38.8 ± 3.3 mg (1% agarose rough) to 32.7 ± 1.8 mg (1% agarose smooth) (Table 9). Cell number/construct increased significantly ($p = 0.02$) from 21.8 ± 2.1 million cells/construct in the 1% smooth agarose group to 25.2 ± 1.9 million cells/construct in the rough 1% agarose group (Figure 38A). Values for the total collagen, GAG, collagen I, and collagen II per construct are shown in Table 1. Significant differences were observed among groups for total collagen per WW ($p = 0.0008$) (Figure 38B), GAG per WW ($p = 0.0003$) (Figure 38C) and collagen II to collagen I levels in the constructs ($p < 0.0001$) (Figure 38D). Specifically, collagen content per WW ranged from 8.9 ± 1.9% in the rough 1% agarose group to 12.9 ± 1.7% in the smooth 1% agarose group, while GAG content per WW ranged from 3.4 ± 0.9% in the rough 1% agarose group to 6.4 ± 0.7% in the smooth 2% agarose group. Collagen II to collagen I ratios in the constructs were highest in cells cultured in smooth wells with values ranging from 9.8 ± 2.3 in the rough 1% agarose group to 27.4 ± 5.3 in the smooth 1% agarose group.

Biomechanics

Samples were tested under tension with significant differences observed among groups for the tensile modulus ($p = 0.008$) and the UTS ($p = 0.03$) (Figure 39A). Specifically, samples cultured in the smooth 1% agarose wells exhibited the highest tensile modulus (1018 ± 259 kPa) and the highest UTS (252 ± 53 kPa).

These values were approximately one and half times (tensile modulus: 534 ± 201 kPa, UTS: 151 ± 66 kPa) those of constructs cultured in the rough 1% agarose wells.

Meniscus-shaped constructs were evaluated under conditions of creep indentation with significant differences observed among groups for the aggregate modulus ($p = 0.002$) (Figure 39B). The highest aggregate modulus was found in constructs cultured in the smooth 2% agarose wells (116 ± 38 kPa), approximately two and a half times the aggregate modulus of constructs cultured in rough 2% agarose wells (45 ± 12 kPa). No significant differences were observed among groups for the permeability ($p = 0.46$) and the Poisson's ratio ($p = 0.91$). The values for permeability for the 1% agarose (smooth and rough), and the 2% agarose (smooth and rough) were 4.5 ± 3.2 (x 10^{-15}) m^4 / N-s, 7.1 ± 5.1 (x 10^{-15}) m^4 / N-s, 4.5 ± 1.6 (x 10^{-15}) m^4 / N-s, and 6.7 ± 3.0 (x 10^{-15}) m^4 / N-s, respectively. The values for the Poisson's ratio for the 1% agarose (smooth and rough), and the 2% agarose (smooth and rough) were 0.2 ± 0.1, 0.2 ± 0.1, 0.2 ± 0.1 and 0.2 ± 0.2, respectively.

Correlation between biochemical and biomechanical data

Compressive and tensile moduli obtained from the biomechanical data were correlated to the total GAG and collagen content in the constructs. Univariate regression analysis showed a significant correlation between tensile modulus and collagen/construct ($r^2 = 0.44$, $p = 0.0007$) but not for GAG/construct ($r^2 =$

0.0001, p = 0.95). Significant correlations were also observed between aggregate modulus and GAG/construct (r^2 = 0.45, p = 0.0004) but not for collagen/construct (r^2 = 0.005, p = 0.74).

Discussion

The results presented here demonstrate that agarose percentage and topography of the mold can significantly influence the final shape of meniscus constructs cultured using a 50:50 ratio of primary bovine MCs and ACs. Specifically, it was found that constructs cultured in 1% agarose wells retained the shape of the original meniscus mold and did not buckle as a result of growth in the radial direction, unlike what was observed with constructs cultured in 2% agarose wells. Interestingly, the biochemical and biomechanical properties of the resulting constructs were also significantly altered. The highest tensile properties and total collagen were observed in the smooth 1% agarose wells, while the highest compressive modulus and total GAG were observed in the smooth 2% agarose wells. Further, collagen II production relative to collagen I production was significantly enhanced in groups cultured in smooth wells.

In this experiment, an elliptical shaped mold was designed that would closely mimic the curved wedge-shaped cross section of the meniscus. The curved shaped profile facilitates the translation of axial compressive forces in the inner regions to circumferential hoop stresses in the outer regions of the meniscus. This provides the meniscus with its shock absorption and load transfer

capabilities. In addition, the wedge-shaped profile of the meniscus maintains knee stability by preventing the rounded surface of femur from sliding off the flat surface of the tibia. In a previous experiment examining various co-culture ratios of ACs and MCs seeded in circular ring-shaped molds, we observed that the 50:50 co-culture ratio resulted in a construct approaching the geometry of the native meniscus.[53] To more accurately depict the overall geometry of the meniscus, the mold design was optimized from a ring to an ellipse and the bottom of the mold was fashioned with a changing concave slope to mimic the variation in cross-sectional area of the meniscus. These modifications resulted in constructs more closely mimicking native tissue, especially those cultured in smooth 1% agarose wells.

Two different agarose percentages (1% and 2%) were examined in this experiment to determine their effect on construct geometry during the culturing phase. In previous work, meniscus constructs grown in a ring-shaped mold expanded radially and buckled as they reached the confining walls of 2% agarose wells. Compared to 1% agarose wells, the 2% agarose wells exhibit lower compliance and exert greater inward forces onto the constructs as they expand. Mechanical properties of agarose gels have been shown to be concentration dependent with an equilibrium aggregate modulus of 5 kPa for 1% w/v agarose gels and approximately 20 kPa for 2% w/v agarose gels.[269] We hypothesized that buckling may be reduced by utilizing a lower percentage agarose well with increased compliance. This was confirmed by our results

where constructs cultured in 1% agarose wells formed meniscus-shaped constructs with no buckling, while constructs cultured in 2% agarose wells buckled.

In addition to agarose percentage, the effect of well topography was also examined on the final geometrical shape of the meniscus construct. Studies have shown that cells are sensitive to the surface texture of the material and can alter their attachment, phenotype and matrix deposition patterns on different topographies.[270-272] For instance, microgrooves on a surface have been shown to alter morphology of osteoblasts and influence matrix deposition.[273] In this experiment, constructs in smooth agarose wells were easily removed using a spatula at 4 wks. Interestingly, constructs cultured on rough surfaces exhibited resistance upon removal and appeared attached to the bottom of the agarose wells. Surface roughness may have provided the means for interdigitation of the construct into the biomaterial. We hypothesize that portions of the developing matrix were anchored into the microgrooves of the molds. As the construct expanded to the well edge, buckling was prevented due to construct anchorage to the well bottom. However, reactionary tensile forces on the constructs, as a result of non-homogeneous interdigitation with agarose, led to overall uneven construct topology and impeded construct extraction from the well. Thus, although the original hypothesis that rough surfaces would eliminate buckling due to increased frictional contact was proven, the geometry of the final construct did not mimic the meniscus due to its high degree of unevenness.

An interesting result of this study was that cell number was significantly higher in constructs cultured on rough surfaces. One potential reason for this may be dedifferentiation and subsequent proliferation of primary ACs and MCs as a result of tensile forces exerted due to interdigitation of the construct with the agarose mold. The ECM is capable of transducing external mechanical stimuli into changes in cell function through mechanotransduction. At the cellular level, mechanical forces can stretch protein-cell surface integrin binding sites, deform gap junctions containing calcium sensitive stretch receptors, alter ion channel permeability, and lead to activation of anabolic or catabolic factors.[274, 275] These cellular changes can trigger intracellular pathways that can influence cell division as well as the regulation of genes that synthesize ECM molecules.[274] In this experiment, tensile forces appeared to dedifferentiate MCs and ACs cultured in rough wells towards a fibroblastic lineage, a phenomenon that has been observed previously with mesenchymal stem cells.[243] Increases were observed in collagen I relative to collagen II production and decreases were observed in GAG production in rough wells when compared to ECM produced in smooth wells. It has been previously shown with TMJ disc fibrochondrocytes that dedifferentiation of cells can also lead to enhanced cell division and proliferation [106]. Thus, cell dedifferentiation due to the external mechanical stimulus likely contributed to increased cell number and decreased cartilaginous matrix production in constructs cultured on rough surfaces.

This experiment utilized a co-culture of primary bovine MCs and ACs. Through this approach, large quantities of collagen II and GAG were obtained, both of which are important ECM molecules present in the inner regions of the rabbit meniscus. Primary cells were chosen since passaged MCs and ACs dedifferentiate in monolayer and express high levels of collagen I, and low levels of collagen II and aggrecan.[159, 276] Bovine cells were judiciously chosen to recreate the rabbit meniscus since they are an easily available cell source that can be harnessed to develop meniscus tissue engineering technologies. In addition, bovine cells may also be considered as a potential xenogenic cell source for leporine meniscus tissue engineering. For this approach to be successful *in vivo*, a battery of tests will need to be conducted *in vitro* and *in vivo* to ensure the cells do not elicit a humoral immune response. This approach may be feasible so long as the cells are shielded by a scaffold or ECM.[250]

To enhance the clinical translatability of our approach, as well as to modulate the phenotype of cells during culture, this experiment utilized a serum-free medium to grow meniscus-shaped constructs. Previous studies have shown that serum-free medium containing additives such as ITS+ and dexamethasone can significantly enhance GAG production in ACs.[231, 277] We have also obtained similar results in a previous study comparing serum-containing and serum-free medium.[26] Co-cultures of MCs and ACs (50:50), grown in cylindrical molds using a similar scaffoldless approach, were found to contain higher levels of GAG in constructs in serum-free medium when compared to those cultured in serum-

containing medium. In addition, total collagen was increased and the collagen I to collagen II ratio was decreased when comparing serum-free culture to serum-containing culture. This showed that the matrix was more cartilaginous in nature when cultured in serum-free conditions. Our results utilizing the elliptical mold design were even more dramatic, with greater amounts of total collagen II produced than collagen I. Specifically, collagen II levels surpassed collagen I levels by at least ten times in constructs cultured in rough wells and up to 27 times in constructs cultured in smooth wells. This was an exciting result, especially since the inner region rabbit meniscus contains a high amount of collagen II. In future work, AC to MC ratios can be modified to attain the correct levels of each type of collagen in the construct.

While recapitulating the geometric shape of the meniscus is an important component of a meniscus tissue engineering strategy, the functional properties of the engineered construct are also an effective assessment tool. Examination of the structure-function relationships of the constructs showed that increased GAG levels resulted in increased compressive stiffness of the constructs, while increased collagen content resulted in increased tensile stiffness of the construct. Excitingly, we were able to achieve GAG levels per wet weight (~5 to 6%) similar to those of native rabbit meniscus values (~3-4%). Collagen levels per wet weight ranged from 8 to 13%, lower than native values (~23%).[1] Compressive stiffness of constructs cultured in 2% agarose smooth wells (~120 kPa) mimicked those of the outer regions of the rabbit meniscus (~120 kPa), although the compressive

properties of the inner meniscus are four times higher (~510 kPa). The highest tensile stiffness (~1 MPa) and UTS (~0.25 MPa) were observed in the 1% agarose smooth wells. These results were corroborated by polarized light micrographs showing most prominent collagen alignment in this group. In spite of this, the tensile values obtained were two orders of magnitude below those of native tissue.[19] Thus, future work will need to investigate strategies to enhance the tensile properties of the constructs without comprising their compressive properties. This could be achieved by using exogenous factors, such as chondroitinase ABC, which temporarily remove GAGs from the matrix and allow enhancement of the collagen network.[228]

Conclusions

A smooth mold made of 1% agarose was able to create geometrically-mimetic meniscus constructs for meniscus tissue engineering using a scaffoldless approach. Rough wells inhibited construct buckling but resulted in uneven shaped constructs. Stark changes were observed in ECM components among groups, with smooth wells enhancing cartilaginous markers such as GAG and collagen II and rough wells enhancing the fibroblastic marker collagen I. Overall, this work presents an effective strategy for knee meniscus tissue engineering using a self-assembly, scaffoldless process to create constructs approaching the geometrical, biochemical, and biomechanical properties of the native meniscus.

Figures associated with chapter 9

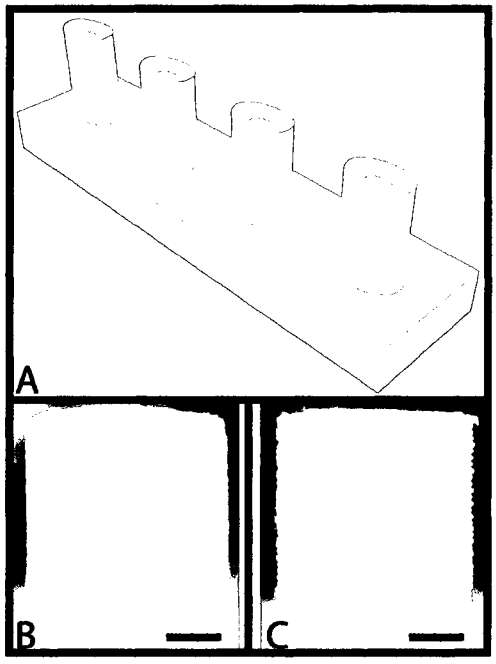

Figure 36. Meniscus well-maker
(A) AutoCAD drawing of the elliptical well-maker (B) Well-maker fabricated with smooth topology (scale bar = 2 mm). (C) Well-maker fabricated with rough topology (scale bar = 2 mm).

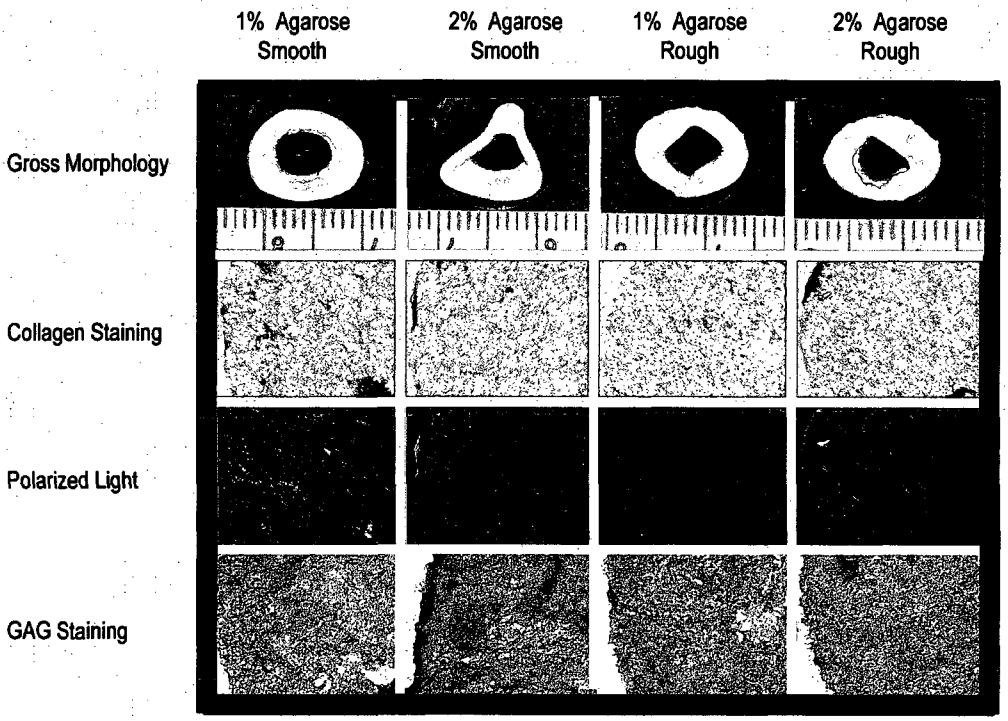

Figure 37. Gross morphology and histology of constructs
Picrosirius red (collagen), polarized light and safranin–O / fast-green staining (GAG) of constructs at 10X magnification.

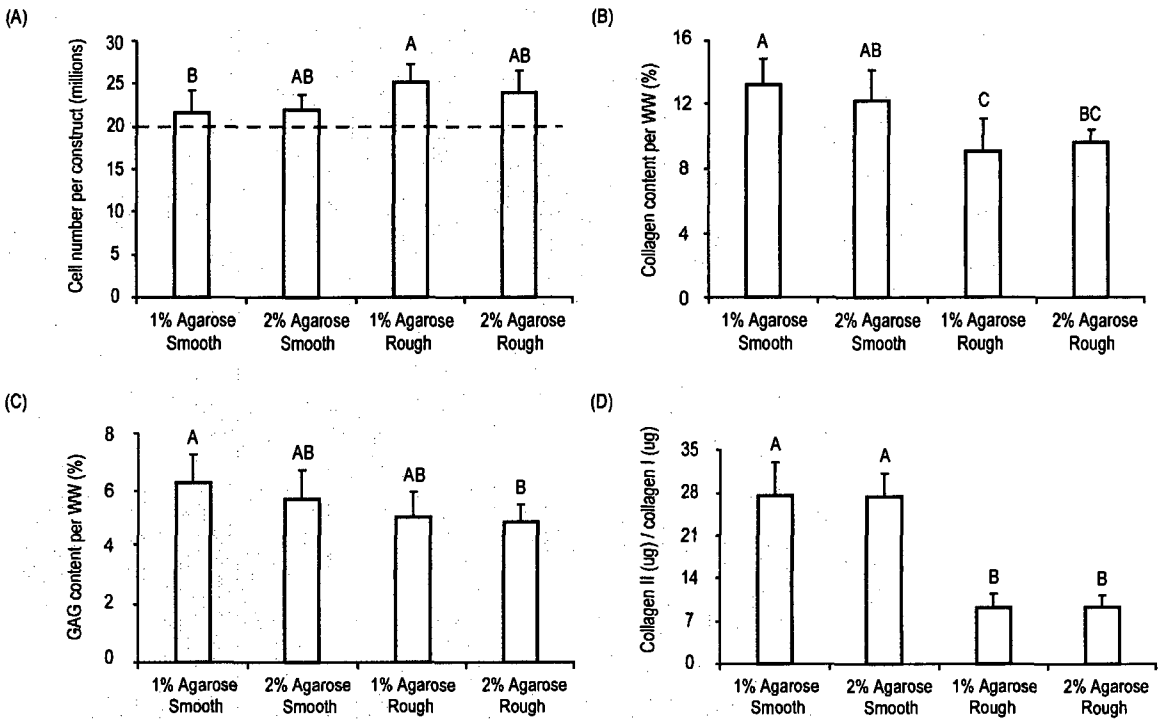

Figure 38. Biochemical characterization of constructs
(A) Cell number per construct. Dashed line indicates original cell seeding density of ~20 million cells per construct. (B) Collagen content normalized to construct wet weight. (C) GAG content normalized to construct wet weight. (D) Collagen II per construct normalized to collagen I per construct. Data presented as mean ± SD with significance among groups labeled with different letters ($p < 0.05$).

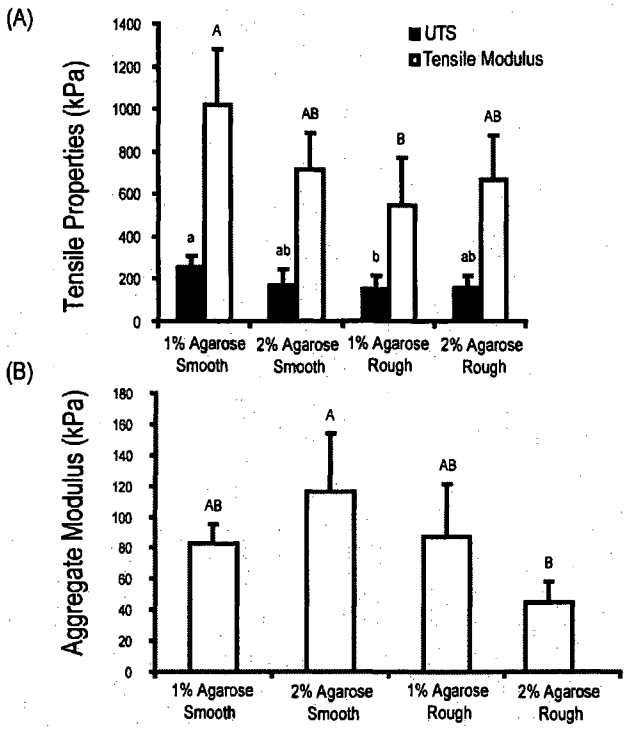

Figure 39. Biomechanical characterization of constructs
(A) Tensile modulus and ultimate strength of constructs. (B) Aggregate modulus determined from creep indentation testing. Data presented as mean ± SD with significance between groups labeled with different letters ($p < 0.05$).

Table 9. Gross morphological and biochemical results
Data presented as mean ± SD with significance between groups labeled with different letters ($p<0.05$).

Group	Average thickness (mm)	Average height (mm)	Wet weight (mg)	Total GAG (mg)	Total collagen (mg)	Collagen I (µg)	Collagen II (mg)
1% agarose smooth	3.5 ± 0.1	1.0 ± 0.1	32.7 ± 1.8 [b]	1.5 ± 0.3 [a]	4.2 ± 0.6	22.5 ± 1.7 [b]	0.6 ± 0.1 [a]
2% agarose smooth	3.7 ± 0.1	1.0 ± 0.1	32.0 ± 2.7 [b]	2.0 ± 0.2 [ab]	3.8 ± 0.7	22.1 ± 4.6 [b]	0.6 ± 0.1 [a]
1% agarose rough	3.6 ± 0.2	1.0 ± 0.0	38.8 ± 3.3 [a]	1.3 ± 0.3 [b]	3.4 ± 0.5	45.5 ± 8.9 [a]	0.4 ± 0.1 [b]
2% agarose rough	3.7 ± 0.2	1.0 ± 0.1	36.0 ± 1.9 [ab]	1.3 ± 0.4 [b]	3.4 ± 0.3	45.8 ± 7.4 [a]	0.4 ± 0.1 [b]
***p* value**	0.38	0.70	0.0004	0.002	0.05	< 0.0001	0.003

Conclusions

The overall objective of this thesis was to identify exogenous stimuli that could enhance the biochemical and biomechanical properties of tissue engineered meniscus constructs. To achieve this objective, the thesis was divided into three aims. In aim 1, meniscus cells (MCs) were characterized *in vitro*, and the effects of monolayer on the gene expression of important ECM markers were quantified. In aim 2, a scaffold-based approach using non-woven PLLA meshes was used to tissue engineer the meniscus. Several anabolic stimuli were utilized including growth factors and mechanical forces. In aim 3, a self-assembly, scaffoldless approach was utilized for meniscus tissue engineering. In this approach, a high density of MCs and articular chondrocytes (ACs) were seeded on non-adherent agarose molds and allowed to coalesce into constructs with varying biochemical and biomechanical properties, and geometries.

The global hypotheses of this investigation were that 1) MCs would experience rapid phenotypic changes during culture, but these changes could be modulated by controlling the culturing conditions (aim 1); 2) exogenous factors, individually or in combination, would allow for the formation of meniscus constructs resembling native tissue (aim 2); and 3) a scaffoldless approach could be used to recreate the heterogeneities of the knee meniscus (aim 3). The results obtained through various tissue engineering experiments confirmed our original hypotheses and are presented in chapters 2 to 9 of this thesis.

Aim 1 of this thesis was to investigate the phenotypic changes in MCs with passage and examine gene expression reversal techniques (chapter 2). It was found that MCs undergo significant changes during monolayer expansion and passage. They experienced losses in major chondrocytic markers (collagen II and COMP) while simultaneously experiencing gains in fibroblastic markers (collagen I). The rapid changes in gene expression of MCs over passage was concerning as this had important implications for future tissue engineering studies involving passaged meniscus cells. Several techniques have been used in the past to promote gene expression reversal of passaged chondrocytes and TMJ disc fibrochondrocytes back to primary cell values. These techniques have included utilizing growth factors, 3-D hydrogels and protein coatings.[60, 62, 64, 67] In this experiment, collagen I and aggrecan protein coatings were used successfully to regain lost phenotype in passaged MCs for collagen I and COMP genes; however, collagen II gene expression reversal proved to be unsuccessful. A lack of collagen II could result in structural breakdown of the tissue as well as preempt osteoarthritis.[14, 125, 126] To overcome this, several exogenous stimuli were investigated in aim 2 (serum-free medium, bFGF, hypoxia and ACs) to modulate MC phenotype in culture and enhance collagen II formation on meniscus cell-seeded PLLA constructs.

Aim 2 of this thesis was to investigate exogenous stimuli to enhance the biochemical and biomechanical properties of meniscus constructs. This aim consisted of five studies (chapters 3 to 7). In chapter 3, the effects of long term

culture of PLLA scaffolds in PBS, serum-containing medium, or serum-free medium were examined. The objective was to determine whether the scaffold used in our meniscus tissue engineering studies did not degrade prematurely, potentially impeding ECM production. It was shown that the PLLA scaffolds retained their mechanical properties and MW, and did not degrade up to at least 12 wks in PBS, serum-free or serum-containing media. Future studies will have to examine whether the presence of cells accelerates potential degradation and subsequent drop in biomechanical properties.

In chapters 4 and 5, the effects of hydrostatic pressure and the combination of hydrostatic pressure and TGF-β1 were examined. The objective of the first study (chapter 4) was to identify a HP regimen that enhanced the properties of MC-seeded PLLA constructs. Physiologic pressures in the knee joint range from 3-18 MPa.[152, 153] In addition, a frequency ranging from 0.6 to 1.09 Hz is observed during normal walking rhythm of an adult human.[155] Thus, a physiologically relevant HP pressure of 10 MPa was applied on MC-seeded PLLA constructs at several frequencies (0, 0.1 and 1 Hz). The experimental data provided significant evidence for the benefit of static HP (10 MPa, 0 Hz) with increases in the total collagen and compressive moduli of the constructs. Since HP does not result in macroscopic construct deformation, it was postulated that it may directly affect cell transmembrane ion transporters that can activate signal transduction cascades that lead to enhances in gene expression of relevant ECM markers.

To further improve the structural integrity of our constructs, a growth factor, TGF-β1, was used alone and in combination with the static hydrostatic pressure regimen identified from the previous chapter (chapter 5). TGF-β1 alone was found to significantly increase collagen and GAG deposition in PLLA scaffolds. TGF-β1 may directly influence GAG synthesis by accelerating glucose transport in human articular chondrocytes via protein kinase C and extracellular signal-regulated kinase-dependent signaling pathways.[189] The combination of static HP and TGF-β1 led to additive increases in the collagen deposition and instantaneous modulus, and synergistic increases in the relaxation modulus and coefficient of viscosity of the PLLA constructs. As mentioned in the previous paragraph, HP is known to alter conformations of cell membrane ion transport pathways. This results in altered intracellular ion flux, thereby influencing other downstream processes.[92] A recent study has shown that intracellular Ca^{2+} mobilization can significantly affect the cells' response to TGF-β1 by controlling the Smad-TGF-β pathway.[192] Certainly, several different cell-signaling pathways with feedback loops either complementing or antagonizing the final down-stream effect may affect our system. Future work will need to identify and examine these pathways more closely to better understand the mechanisms involved in this process.

In chapter 6, the effects of hypoxia and bFGF were examined on MC-seeded PLLA constructs. The objective was to investigate whether a hypoxic environment and bFGF would significantly enhance constructs' ECM and

functional properties. The results demonstrated the effectiveness of combining bFGF and low oxygen tension (2% O_2) to synergistically enhance matrix and functional properties of tissue engineered meniscus constructs *in vitro*. Specifically, we observed significant enhancements in GAG content (synergistic increase), collagen II content, and cell number/construct in groups exposed to hypoxia and bFGF when compared to the controls. In addition, the compressive properties of the construct also increased, with additive increases in the instantaneous modulus, and synergistic increases in the relaxation modulus and the coefficient of viscosity. bFGF has been shown to play an important role in modulating intracellular Ca^{2+} levels through protein kinase C activity.[211] Changes in intracellular Ca^{2+} levels can affect downstream signaling pathways that influence gene expression and protein synthesis of various extracellular matrix molecules.[192] MCs under hypoxic conditions have been shown to upregulate TGF-β1 expression which has been implicated as a potent ECM modulator. Further, hypoxia and TGF-β pathways have also been shown to function synergistically,[210] and may supplement SOX-9 pathways, to enhance GAG production on the meniscus-cell seeded scaffolds. In future work, combinations of identified stimuli from chapters 4, 5 and 6 may be synergized or applied in a temporally coordinated fashion to maximize the functional and organizational properties of MC-seeded PLLA constructs.

In chapter 7, five different co-culture ratios of primary ACs and passaged MCs were seeded on PLLA scaffolds and cultured in serum-free medium. The

objective of this study was to identify co-culture ratios most closely resembling the biochemical and biomechanical properties of the inner and outer regions of the knee meniscus. Qualitative and quantitative tests showed clear differences between the ECM and biomechanical properties of the constructs in each group. Specifically, constructs cultured with a higher percentage of ACs had enhanced compressive properties and contained significantly higher levels of collagen, collagen II, and GAG. Constructs cultured with higher percentage of MCs contained higher levels of collagen I. Excitingly, GAG levels close to native values for the inner and outer meniscus were achieved using this approach. Collagen levels approached native meniscus as well if the dry weight normalizations excluded the weight of PLLA. Overall, we were able to demonstrate that co-culturing MCs and ACs on PLLA scaffolds resulted in functional tissue engineered meniscus constructs with a spectrum of biochemical and biomechanical properties. It is envisioned that this study will provide a baseline for future meniscus tissue engineering studies attempting to regenerate different regions of the meniscus.

Aim 3 of this thesis was to optimize cell and tissue culture conditions for scaffoldless meniscus constructs. This aim consisted of two studies (chapters 8 and 9). In chapter 8, five different co-culture ratios of primary bovine MCs and ACs were investigated for leporine meniscus tissue engineering. The objective was to determine co-culture ratios that mimic the inner and outer regions of the leporine meniscus. Medial leporine menisci were used as internal controls.

Bovine cells were utilized since they are an easily available cell source, unlike leporine cells, and are ideal to develop meniscus tissue engineering technologies. In addition, bovine cells were also investigated for their capacity to produce matrix that mimics leporine menisci and for consideration as a potential xenogenic cell source in future *in vivo* studies in the rabbit. The results of this study demonstrated the ability of this co-culture system to create a spectrum of fibrocartilages that mimic several biochemical and biomechanical properties of leporine menisci. Higher MC% groups more closely mimicked the GAG per wet weight of both, the inner and outer regions of the meniscus. The 50% MC group was found to mimic the inner meniscus in collagen II to collagen I content. Tensile properties of native tissue could not be matched by any of the test groups. Based on these data, a 50% MC group may be ideal to recapitulate biochemical characteristics of the inner regions of the tissue. For the outer regions, a lower MC% group with higher compressive strength may be ideal from a functional tissue engineering standpoint.

In chapter 9, two different agarose mold surfaces and concentrations were examined for their ability to produce geometrically-mimetic meniscus shaped constructs. The results demonstrated that agarose percentage and topography of the mold can significantly influence the final shape of meniscus constructs cultured using a 50:50 ratio of primary bovine MCs and ACs. Specifically, it was found that constructs cultured in 1% agarose wells retained the shape of the original meniscus mold and did not buckle as a result of growth in the radial

direction, unlike what was observed with constructs cultured in 2% agarose wells. In addition, constructs cultured on rough surfaces resulted in uneven construct formation and appeared attached to the agarose at the bottom of the wells. We hypothesized that portions of the developing matrix were anchored into the microgrooves of the molds. As the construct expanded to the well edge, buckling was prevented due to construct anchorage to the well bottom. However, reactionary tensile forces on the constructs, as a result of non-homogeneous interdigitation with agarose, led to overall uneven construct topology and impeded construct extraction from the well. Interestingly, the biochemical and biomechanical properties of the resulting constructs were also significantly altered. The highest tensile properties and total collagen were observed in the smooth 1% agarose wells, while the highest compressive modulus and total GAG were observed in the smooth 2% agarose wells. Further, collagen II production relative to collagen I production was significantly enhanced in groups cultured in smooth wells. This was exciting since the inner regions of the meniscus contain higher levels of collagen II when compared to collagen I. Overall, a smooth mold made of 1% agarose was able to create geometrically-mimetic meniscus constructs for meniscus tissue engineering using a scaffoldless approach.

The work described in this thesis has made several important advances to the field of meniscus tissue engineering. Salient achievements of this thesis include: 1) the characterization of meniscus cell (MC) dedifferentiation over passage and the identification of suitable protein coatings (collagen I and aggrecan protein

coatings) to recover losses in gene expression; 2) the identification of specific anabolic stimuli (hypoxia, bFGF, TGF-β1, and hydrostatic pressure) that can enhance the functional and biochemical properties of meniscus cell-seeded PLLA scaffolds; 3) the demonstration of synergistic and additive increases in biochemical and biomechanical content via the combination of two types of stimuli (hypoxia and bFGF, static hydrostatic pressure and TGF-β1); 4) the recreation of heterogeneities of the knee meniscus using a co-culture of articular chondrocytes (ACs) and MCs, via a scaffold-based and scaffoldless approach; and 5) the optimization of the self-assembly scaffoldless approach to grow regionally variant meniscus-shaped constructs. Future work in the meniscus field will need to focus on further enhancing the biochemical and biomechanical properties of the constructs, especially the tensile properties. This may be accomplished by improving scaffold alignment in the circumferential direction by techniques such as electrospinning [278] or by using exogenous factors such as chondroitinase-ABC to temporarily deplete GAGs while enhancing the collagen matrix.[228]

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Appendix A: Biodegradable materials in arthroscopy

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Abstract

The use of biodegradable materials as implants has revolutionized the way medicine is practiced today. This review provides a general description of salient biodegradable polymeric materials currently used in arthroscopy. These materials include polyglycolic acid, self-reinforced polyglycolic acid, poly-L-lactic acid, self-reinforced polylactic-L-acid, poly-D-L-lactic acid, copolymer of poly-D-L-lactic acid polyglycolic acid, and polyglyconate. The mechanical strength, degradation properties, and widespread use of these materials, especially in the knee and shoulder, are discussed individually. Also discussed are the relatively few complications that are related to these materials' arthroscopic use. Future directions in biodegradable materials, including smart polymers, are also considered. In the future, novel techniques to identify the ideal polymer for a particular application will need to be developed to minimize the risk for implant complications.

Introduction

Despite the relative success of metallic implants in surgery, rapid advances in science and technology have allowed researchers to explore other avenues that can resolve some of the inherent disadvantages that metals present. For instance, the permanency of metallic implants makes it difficult for the body to recapitulate its native state, a major goal in medicine. In addition, failure of the implants due to corrosion, infection, or stress shielding mismatches requires removal of the device leading to additional surgeries.[279, 280] Metallic implants are also known to contraindicate imaging applications such as magnetic resonance imaging (MRI).[281, 282] Additionally, chromium and nickel coatings on stainless steel surfaces, applied to prevent corrosion may even be genotoxic and harmful to DNA molecules.[283, 284]

To address these problems in the past three decades there has been a gradual focus shift from metallic implants to biodegradable ones. Biodegradable implants are natural, synthetic or biosynthetic polymers that are biocompatible with the body and do not elicit a foreign body response. Today, more than 40 types of polymers are used as implants in both soft and hard tissue applications ranging from arthroscopy to tissue engineering.[285]

This review serves as an overview of biomaterials primarily used in arthroscopic soft tissue applications. These include polyglycolic acid (PGA), polylactic acid enantiomers (PLA), polyglyconate, self-reinforced polyglycolic acid (SR-PGA)

and polylactic acid (SR-PLLA), and copolymers of lactic and glycolic acid (PDLLA-co-PGA). The *in vivo* and *in vitro* mechanical strength and degradation properties of these polymers are described. Although the polymers have been successfully used as degradable implants, there have been few reports of complications, the most recent of which are discussed. Special emphasis is placed on knee and shoulder, both joints that are most commonly associated with arthroscopic surgery. Future directions in biodegradable materials involving smart polymers are also addressed.

Mechanical strength and degradation of polymers

The ability of biodegradable polymers to withstand high loading early in the healing process and then gradually degrade while preserving some of their properties is critical for rebuilding healthy tissue. It is therefore essential to understand how polymeric mechanical properties change over time when placed in a physiological environment. One of the basic parameters used to describe the mechanical properties of polymers is mechanical strength. The mechanical strength of a polymer implant is largely determined by its molecular weight (MW) and degree of crystallinity. Polymeric devices with desired MW and crystallinity can now be fashioned by using commercially available fabrication techniques such as injection molding, compression molding, extrusion, solution casting, solvent casting particulate leaching or gel casting.[286, 287] The choice of fabrication technique is often influenced by the physical and thermal properties of the polymer as well as its final desired morphology. For instance, PLLA fibers with

high MW ranging from 300-500 kiloDaltons (kDa) can be extruded at 110^0C while low MW PLLA fibers (below 300kDa) can be extruded at 180^0C.[287] Three dimensional scaffolds and foam can be fabricated by solvent-casting particulate leaching.[288] Internal fixation devices like screws, rods and plates can be fabricated from polymer blocks by injection molding. However, the final desired mechanical properties can vary depending on variations in the molding process.[289]

There are several ways to characterize the mechanical strength of a material. Stress-strain deformation curves provide valuable information on the tensile properties of a polymer, including the tensile strength, elastic modulus and the yield strength. Flexural and shear strength are often reported in the literature as they provide explanations of the polymer's ability to resist bending and angular deformation. The strength of a polymer can also be enhanced by a technique called self-reinforcement. In this technique, fibers of the desired polymer made from the same material as the polymer matrix are sintered together at high temperatures and pressures.[290-292] Fatigue behavior of a polymer is also important in applications involving cyclic dynamic strain.

The mechanical properties of an implanted material can change dramatically *in vivo* as it degrades. Results from *in vitro* and *in vivo* degradation studies have provided us with a basic understanding of how different polymers degrade in different environments.[293] For example, it is known that most polyesters,

including PGA, PLLA, polyglyconate and copolymers of PGA-PLA, degrade via non-specific hydrolytic clipping of ester bonds.[294, 295] The susceptibility of these polymers to hydrolysis is a function of their chemical structure, morphology and the harsh *in vivo* environment of the body.[287, 296, 297] The mechanical properties and degradation times of the above mentioned polymers are described below and listed in Table 10 and Table 11 respectively.

Polyglycolic Acid (PGA) and Self-Reinforced Polyglycolic Acid (SR-PGA)

PGA has been used extensively as a biodegradable material as its byproduct is a natural metabolite, glycolic acid.[298] PGA implants of varying MW have been fabricated by compression and injection molding.[299, 300] The melting temperature of crystalline PGA ranges from 225-230^{0}C with a glass transition temperature of 36^{0}C.[300] Polymers at temperatures below their glass transition temperature are rigid or glassy while those at temperatures greater than their glass transition temperature exhibit rubbery characteristics.

In bulk form, the tensile strength of PGA has been reported to be around 57-70 MPa.[301, 302] The shear and flexural strength of injection molded PGA rods have been found to vary between 95 MPa and 220 MPa.[303] Radiolabeled PGA in animal metabolism studies showed that less than 1% of the implant was present at the site four months after implantation.[304] Self reinforced PGA implants have been shown to exhibit vastly improved mechanical properties.[290] High initial flexural strengths varying between 370-405 MPa and initial shear strengths

varying between 250-255 MPa, respectively, have been reported by several groups.[303, 305, 306] The high initial strength provided by the reinforcement process however is not retained for a long period of time *in vivo*. Several investigators have discovered that, *in vivo*, the implants rapidly lose their flexural and shear strength.[306-309] A complete loss of flexural and shear strength of SR-PGA screws over six wks was observed by one group.[306] Another group observed a 50% drop in flexural strength of SR-PGA rods over three wks.[307] Histology on implanted SR-PGA screws in lambs showed evidence of fragmented screws in four to six wks and complete resorption in three months.[308] Similar results have been observed with SR-PGA pins implanted in a canine femoral canal.[309]

Polylactic Acid (PLA) Enantiomers and Self-Reinforced Polylactic-L-Acid (SR-PLLA)

PLA is a semi-crystalline polymer exhibiting thermoplastic properties. Due to its asymmetric shape, it can exist in two enantiomeric forms poly-L-lactic acid (PLLA) and poly-D-lactic acid (PDLA). PLLA is more commonly used as a polymeric implant as its degradation byproduct yields a naturally occurring stereoisomer of lactic acid which enters the Krebs cycle and is converted to carbon dioxide and water.[310] Copolymers of these two enantiomers (PDLLA) can exhibit varying physical and chemical properties depending on amount of each polymer present in the polymer chain.[311, 312] Crystalline PLLA can vary in MW from 50 to 300 kDa.[313] High MW PLLA (>100 kDa) has a melting temperature

ranging from 173-178°C and a glass transition temperature of about 58°C.[300] The MW of PDLLA implants range from 21 to 550 kDa.[313]

The tensile strength of PLLA and PDLLA has been reported between 11 MPa-3.2 GPa whereas the flexural strength has varied between 45-145 MPa.[285, 302, 313-321] The shear strength of PLLA rods of high MW (1,800 kDa) was reported to be around 45-47MPa.[319, 322] PLLA implants have been shown to provide stable fixation for at least six months.[319, 323, 324] PLLA rods placed in phosphate buffered saline (PBS) for nine wks showed no change in the shear strength or any shrinkage in diameter. Further, the MW dropped by less than 20% over 18 wks.[319] PDLLA on the other hand is less stable and degrades faster than PLLA.[324] A clinical degradation study comparing PLLA and PDLLA interference screws for knee ligament reconstruction showed large fragments of PLLA screws present *in vivo* 20 months post operation. However, the PDLLA screws degraded completely within 10 to 14 months.[324]

The self-reinforcement of PLLA increases its mechanical strength and is thus used in applications requiring higher loads such as cortical bone fixation.[325] The flexural strength of SR-PLLA has varied between 89-210 MPa while the shear strength was found to be around 143 MPa.[319, 326, 327] An experiment comparing SR-PLLA and metallic screws implanted in sheep showed that although both provided suitable fixation, SR-PLLA proved more advantageous as its lower rigidity prevented stress-shielding and mechanical weakening of bone.[328] The

degradation rates of SR-PLLA and PLLA *in vivo* were found to be fairly similar. SR-PLLA screws implanted in goat medial menisci were found to be present *in toto* at six months.[329] Similar results were observed in SR-PLLA screws implanted in bone osteotomies. Complete degradation of the screws was observed two yrs post-implantation.[308]

Copolymer of Poly-D-L-Lactic Acid and Polyglycolic Acid (PDLLA-co-PGA)

Copolymers of PDLLA and PGA have often been used in soft tissue applications for bone-tendon-bone grafts and anterior cruciate ligament (ACL) reconstruction. Nakafuku and Takehisa[330] investigated the mechanical properties of PLLA and PDLLA-co-PGA blends at various monomer ratios of PDLLA and PGA. The glass transition temperature for pure PDLLA-co-PGA was found to be 38°C. In terms of mechanical properties of 75/25 PDLLA-co-PGA, it was found that the stress-strain curves varied dramatically at various temperatures around the glass transition temperature. At 35°C, a maximum tensile strength of 10 MPa was reported while the maximum tensile strength at 40°C dropped to 5 MPa. At 10°C, however, the polymer blend became brittle with a maximum tensile strength of 45 MPa. For clinical applications, however, the mechanical properties of the polymer at body temperature (37°C) could be useful. Although the authors did not report the stress-strain properties at body temperature, an extrapolation from the graph would indicate the maximum tensile strength of a 75/25 PDLLA-co-PGA at 37°C would be about 7-8 MPa. In an experiment to compare the pull out force of PDLLA-co-PGA interference screws to titanium screws implanted in calf tibia, it

was found that the force ranged from 246 N to 737 N and was found to be much lower than for titanium screws (605 N to 971 N).[331] A pullout force of 565 N has been recorded for interference screws used in patellar bone-tendon-bone grafts.[332] Degradation times for PDLLA-co-PGA constructs have varied between one and five yrs depending on application.[324, 329]

Polyglyconate

Polyglyconate implants are block copolymers of glycolic acid and trimethylene carbonate. They are used extensively in the form of sutures, tacks, screws and anchors for internal fixation applications. Literature suggests that polyglyconate implants lose their mechanical integrity over six wks *in vivo* but usually are resorbed six to 12 months post-implantation.[295, 333-335] This absorption profile has been shown to mirror the healing response of soft tissue to bone.[336]

Mechanical tensile tests on injection molded polyglyconate of varying MW showed a maximum initial yield strength of about 62 MPa while the ultimate tensile strength was about 57 MPa. When placed in PBS however, the tensile strength dropped down to about 4 MPa in one month.[295] Another group compared the pull-out strength of polyglyconate interference screws to titanium screws implanted in calf tibia.[331] Pull-out forces for the polyglyconate screws ranged from 469 N to 914 N. However, no significant difference was observed between the pull-out strength of polyglyconate and titanium screws. For tendon to bone suture applications in rotator cuff repairs, monofilament polyglyconate

sutures were compared with PGA braided sutures.[337] The authors found that even though polyglyconate sutures failed at higher loads than PGA, their lower stiffness properties resulted in considerable elongation *in vitro* even at lower loads. It was concluded that the combined ultimate tensile strength and stiffness of braided PGA sutures were more advantageous than polyglyconate sutures for this particular application.

Clinical applications and complications

Arthroscopic techniques are used to treat a variety of disorders in the knee, shoulder, wrist, elbow and ankle. The relatively large size of the knee and shoulder joints renders them amenable to arthroscopic surgery and it is, therefore, not surprising that the majority of the articles published in the literature focus on knee and shoulder fixation. The following section discusses different biodegradable polymers implanted in these two joints as well as reports of some complications that have been observed. It should be emphasized, however, that complications have been noted only in low percentages of patients. Some of these complications involving polymer implants include premature implant breakage, delayed implant degradation, superficial wound formation, deep infections, effusions, and sinus formations around the implant.[282, 338]

Biodegradable implants for the knee

Soft tissues in the knee such as the meniscus and the ACL can be easily injured during sports requiring sudden changes of speed or side-to-side motion. Several

types of implants including interference screws, arrows, tacks and sutures are currently used clinically to repair meniscal tears or to reconstruct the ACL.[323, 335, 339-352]

The first biodegradable implants used to repair the meniscus were meniscus arrows. Their simple implantation procedure has reduced surgery time and the possibility of neurovascular complications, thus becoming an increasingly popular clinical treatment modality.[346, 347] Early meniscal arrows were made of self-reinforced 96L/4D PLA, a polymer that was found to be capable of providing strength retention for at least six months.[349] *Ex vivo* studies on self-reinforced meniscal arrows showed that their biomechanical properties (pullout strength and stiffness) were found to be comparable to horizontal suture loops but were mechanically inferior to vertical suture loops.[340, 347, 348, 353, 354] Despite their mechanical inferiority to vertical sutures, meniscal arrows have been shown to still provide stable fixation to heal a meniscal tear.[355] To improve the pull-out strength of the meniscal arrow, a novel arrow made of self-reinforced 80L/20D PLA was fabricated and compared to its older counterpart. Mechanical tests showed that both arrows exhibited similar stiffness properties, but the new arrow showed significantly higher fixation.[349] Jones *et al.*[350], however, have recently reported device migration and local soft tissue inflammation in 32% of the patients implanted with PDLLA meniscus arrows to treat meniscal tears.

Meniscal screws have also been used as a mode to fix meniscal tears. Tsai and colleagues[341] implanted cannulated PLLA screws in 25 patients with meniscal tears. Follow-up interviews were conducted with 18 patients two yrs post-surgery. Using modified Tegner and Lysholm scores, rating systems used in knee injury evaluations, the authors concluded that the screws were safe and provided suitable fixation for meniscal repair applications. A similar study using SR-PLLA screws has yielded positive results in patients.[344] However, no follow-up arthroscopy was performed in both cases to determine if there were any morphological changes in the knee joint. A more recent study has shown that implanting SR-PLLA meniscal screws in goat menisci resulted in significant chondral lesions in all the tested goats.[329] In a case report, a patient was treated with eight screws to treat a meniscal tear.[342] An arthroscopy, 32 months post surgery, showed several broken screw fragments near the medial collateral ligament but the MW of the screw had dropped only 35.3% in almost three yrs. Other groups have also reported delayed degradation of meniscal screws.[343, 356] This delayed degradation of screws is certainly of some concern, and suggests that the true degradation time of meniscal screws *in vivo* is still unknown and needs to be further investigated.

The effectiveness of non-absorbable sutures in meniscal repair has also been compared to that of absorbable sutures.[357] Magnetic resonance images of menisci from mongrel dogs were obtained and histology was performed one and three months post surgery. The authors discovered that the menisci repaired with

the absorbable sutures showed significantly greater changes in structure than the non-absorbable sutures. This finding suggests perhaps that non-absorbable sutures are better suited for meniscal repair than absorbable sutures.

Metallic interference screws have been commonly used for ACL reconstruction.[324] However with the advent of biodegradable materials, there has been considerable research on a variety of polymeric interference screws. Stahelin et al.[324] evaluated the biocompatibility and biodegradability of PLLA, 85/15 PDLLA-co-PGA and PDLLA interference screws for ACL reconstruction in six patients. In all cases, there was little to no foreign body reaction to the screws. PDLLA screws in patients showed no degradation for the first six wks but were completely degraded by 10 months. A second arthroscopy in a patient with PLLA screws 20 months post initial surgery showed that the PLLA screw had not degraded and there was no sign of any bony ingrowth. Other researchers have also reported delayed degradation of PLLA interference screws for ACL and PCL reconstruction which caused severe cartilage damage to one patient and extreme pain and swelling to another.[352, 358, 359] Patients treated with 85/15 PDLLA-co-PGA showed complete degradation of the screw in one yr and presence of bony tissue in the location of the screws.[324] On the other hand, Lajtai et al.[351] reported that 85/15 PDLLA-co-PGA interference screws took five yrs to completely degrade in patients. There have also been several reports of effusions in patients implanted with PDLLA-co-PGA screws used in ACL reconstruction.[324, 360] In all cases, however, the effusions disappeared over time

after the screws completely degraded. Polyglyconate interference screws have also been tested in patients for graft fixation in ACL reconstruction. Complete degradation of the screws was observed one yr post surgery without any serious complications or osteolysis.[335] Benedetto *et al.*[361], however, reported that about 19% of the 67 patients implanted with cannulated polyglyconate screws for ACL reconstruction developed complications which included effusions (5%), infections (2%) and cysts (2%). Thus, current literature suggests that PDLLA interference screws may be prime candidates for ACL reconstruction as they completely degrade in a yr and have shown low morbidity.

Although meniscal arrows have traditionally been used to treat vertical, longitudinal bucket handle tears of the meniscus, researchers have attempted to expand their applicability to other knee related disorders as well. For example, self reinforced 96L/4D PLA meniscal arrows were tested *in vitro* to determine their effectiveness in the treatment of osteochondritis dissecans (OD).[362] OD is a disorder resulting from the separation of a loose piece of bone and cartilage from the end of the bone due to a lack of blood supply.[363] Pull-out tests of the arrows showed a good initial hold in bone and could be potentially used in the treatment of OD.[362] Tuompo *et al.*[364] implanted SR-PGA and SR-PLLA rods in 24 patients with OD. Patients were analyzed over a 7 yr period and the authors reported good clinical and subjective results. In another study by the same group, SR-PGA rods were implanted in 1879 patients to treat OD. The authors observed a

clinical tissue response in 8% of the cases. Complications included effusions (5%) and sinus formation (3%) in the knee joint.[365]

Biodegradable implants for the shoulder

The development of biodegradable implants such as suture anchors and tacks has provided surgeons with a new arsenal to effectively treat shoulder instability.[366] Today, these implants are used in rotator cuff repair, shoulder reconstruction, biceps anchor lesions and biceps tenodesis.[367-369]

Cannulated polyglyconate tacks have been used by several researchers to repair Bankart lesions (labral tears in the shoulder) and more recently for rotator cuff repair.[370-374] Bankart lesions can only be treated by polyglyconate tacks however, if the labrum has not completely degenerated, a point that was first noted by Resch *et al.*[371] In their study, 18 patients were treated with the polyglyconate tacks. Follow-up at four to 18 months post-surgery showed that almost 80% of the patients had regained full motion of their shoulder.[371] In another study, 25 patients with Bankart lesions were treated with polyglyconate tacks. The Rowe point score, a clinical rating scale for the shoulder, was utilized as a follow-up test to determine whether the surgery was successful. About 95% the patients reported good to excellent results and were able to return to normal activity within two yrs, confirming that polyglyconate tacks were effective in treating Bankart lesions. Burkart *et al.*[375] reported massive synovitis in shoulders of all patients implanted with cannulated polyglyconate tacks. The foreign body reaction was

believed to be caused by loose screw fragments that migrated into the joint cavity. Other groups have also reported high failure rates (>10%) of cannulated polyglyconate tacks used to treat anterior shoulder instability.[376-378]

Recently a group reported the use of polyglyconate tacks to treat rotator cuff injuries.[372] Although rotator cuff repairs are usually performed with suture anchors, the authors argued that arthroscopic implantation of tacks would facilitate the surgery by eliminating the need to ensure proper suture anchor placement, suture passing and knot tying. In their experiment, 53 patients were implanted with polyglyconate tacks and rated using three different shoulder rating systems pre- and post-operatively. All rating systems showed a significant improvement in patient pain relief and function after the surgery. Although the authors felt confident that tacks could be used successfully for this application, the risk of tissue necrosis beneath the head of the implant is high if proper care is not taken during the surgical process.

Several *in vivo* tests have been conducted comparing the effectiveness of biodegradable suture anchors to metallic suture anchors.[379, 380] In one study comparing biodegradable PDLLA suture anchors with titanium suture anchors in goats, no significant difference was observed in the fixation strength over time between the two anchors.[379] In another study, biodegradable polyglyconate suture anchors or non-absorbable implant-grade polyacetyl anchors were implanted in humans with Bankart lesions. High Rowe scores were recorded in both groups post-surgery indicating that both the degradable and non-degradable

suture implants were successful.[380] PLLA suture anchors have also been successfully implanted in patients undergoing Bankart repair, with high Rowe scores reported in follow-up interviews with more than 90% of the patients.[381] These findings suggest that degradable suture anchors are equivalent to metallic suture anchors in terms of function and performance. A recent study, however, showed that PDLLA suture anchors implanted in shoulder capsules resulted in osteolysis in almost 50% of the cases.[382]

Future directions

Recently, a new generation of "smart polymers" is being given considerable attention. These polymers have the unique property of changing their shape upon application of an external stimulus such as change in temperature or lighting. Different materials such as polyurethanes, polynorbornene and hydrogels have been shown to exhibit thermal induced shape memory.[383]

An application where the smart polymers have been successfully used is in wound management. Lendlein and Langer[384] described a smart suture system that exhibited different mechanical properties with small changes in chemical structure. An incision was made into the belly of a euthanized rat. The polymer was loosely sutured into the wound site by elongating fibers to 200% strain at a controlled stress. When the temperature was raised above the transition for the polymer, the fibers shrank forming a tight knot around the wound. Using this concept, degradable smart polymers could be placed at the correct position at

the implant site through arthroscopy. Increase in temperature would cause the material to form the appropriate shape required for that particular application.[383]

As biodegradable polymers continue to gain popularity in clinical applications, the need to understand the biology and mechanics of the devices in vivo, as well as to determine factors that can affect subsequent clinical outcomes, is crucial. With an eclectic variety of degradable biomaterials available to clinicians, it can often be a challenge to determine the right choice of implant for a particular application. For example, suture anchors made from PDLLA, PLLA and polyglyconate and tacks made from polyglyconate have all been used successfully to treat Bankart lesions in the shoulder.[374, 381] Which polymer and type of implant should the clinician choose? To address such a problem, McFarland et al.[367] recently published a review on suture anchors and tacks used in shoulder surgery. The group reported a set of 16 questions that clinicians can address to account for the multiple variables that could affect the biological and biomechanical performance of the biomaterial. Some of the questions posed are straightforward, e.g. asking whether the implant is absorbable or not. Others address more specific topics, for example a question is asked whether the biodegradable implant has been tested in cyclic loading and if so which part of the device failed and at what strength. In similar fashion, standardized procedures need to be developed for each application to assist clinicians in deciding appropriate biomaterials to implant in patients. Only when clinicians can confidently address the issue of the ideal polymer for an application in a

particular patient will the risk of implant complications decrease dramatically and the true effectiveness of biodegradable materials be realized.

Figures associated with appendix A

Table 10. Mechanical properties of polymers

Polymer	Type of implant	Tensile strength	Flexural strength	Shear strength	Pull out force
PGA					
Christel *et al.*[302]	Plates	57 MPa			
Tormala *et al.*[303]	Rods		90-110 MPa	205-235 MPa	
SR-PGA					
Vainionpaa *et al.*[305]	Rods		370 MPa	250 MPa	
Tormala *et al.*[303]	Rods		220-405 MPa	156-255 MPa	
Vasenius *et al.*[306]	Screws		~200 MPa		
PLLA					
Engelberg *et al.*[313]		28-48 MPa			
Haltia *et al.*[319]	Rods			47 MPa	
Claes[322]	Rods			45 MPa	
PDLLA					
Engelberg *et al.*[313]		29-35 MPa			
Christel *et al.*[302]	Plates	45.6 MPa - 3.2 GPa			
SR-PLLA					
Casper *et al.*[326, 327]			89-193 Mpa		
Haltia *et al.*[319]	Rods		210 MPa	143 MPa	
PDLLA-co-PGA					
Nakafuku and Takehisa[330]		7-8 MPa			
Weiler *et al.*[323]	Screws				246-737 N
Johnson and vanDyk[332]	Screws				565 N
Polyglyconate					
Farrar *et al.*[295]		57 MPa			
Weiler *et al.*[331]	Screws				469-914 N
Gerber *et al.*[337]	Sutures				67 N

Table 11. Polymer degradation times

Polymer	Degradation Time	Type of implant
PGA		
Frazza *et al.*[304]	4 months	Sutures
SR-PGA		
Peltoniemi *et al.*[308]	3 months	Screws
Andriano *et al.*[309]	3 months	Pins
PLLA		
Stahelin *et al.*[324]	> 20 months	Screws
Willcox *et al.*[342]	> 32 months	Screws
PDLLA		
Stahelin *et al.*[324]	10-14 months	Screws
SR-PLLA		
Peltoniemi *et al.*[308]	2 yrs	Screws
PDLLA-co-PGA		
Stahelin *et al.*[324]	1 yr	Screws
Lajtai *et al.*[351]	5 yrs	Screws
Polyglyconate		
Fink *et al.*[335]	12 months	Screws

Appendix B: Perspectives and possibilities for degradable polymers for skeletal implants

Ying Deng, Najmuddin J. Gunja and Kyriacos A. Athanasiou

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Abstract

The use of degradable polymers for surgery in skeletal repair is becoming more frequent. Numerous biocompatible, biodegradable polymers are now available for both experimental and clinical use. Despite this, there is a need to further develop polymers with a spectrum of mechanical properties especially for musculoskeletal applications where loading conditions vary depending on location. This chapter reviews some of the prominent biodegradable polymers used in medicine today, such as poly-lactic acid, poly-glycolic acid, poly-caprolactone, polydioxanone, and poly-trimethylene carbonate, and includes strategies to improve their mechanical strength through co-polymerization. The use of smart polymers, such as energy storing polyesters for skeletal implants, is also discussed in addition to state-of-the-art computational modeling techniques that might significantly expedite characterization of novel polymers.

Introduction

Historically, metallic implants have been used to treat injuries in load-bearing areas of tissues since their high mechanical strength and rigidity allow for the healing process to occur in non-collapsed fracture sites. However, concerns relating to stress shielding, corrosion, and radiologic imaging have given rise to a new generation of biodegradable implants that can resolve the inherent problems that metals present.[283, 385-388] In addition to being non-toxic and biocompatible, the polymer degradation byproducts, in most cases, can be converted to naturally occurring compounds and excreted from the body. Further, biodegradable polymers can also act as carriers of cells and bioactive agents for controlled drug delivery applications. To date, degradable polymers made from lactic acid, glycolic acid, and dioxanone, as well as copolymers of these materials, are the most commonly studied and are used clinically in fracture fixation, bone replacement, articular cartilage and meniscus repair, and ligament fixation applications.[160, 389-397]

Although polymers are successfully used as biodegradable implants, there have been a few reports of complications arising as a result of premature implant breakage, delayed implant degradation and foreign body reactions to the implant.[359, 398, 399] To avoid complications resulting from the first two factors, a more detailed understanding of the factors influencing the physical and mechanical properties of the polymers is required. Two such identified factors are the molecular weight (MW) and degree of crystallinity that largely determine the

polymer physical properties, such as the glass transition temperature (T_g) and melting point (T_m), and polymer mechanical properties, such as stiffness and tensile strength. These factors can be controlled during polymer fabrication using commercially available techniques like injection molding, extrusion, and compression molding.[400]

In this chapter, salient biomedical polymers such as poly-lactic acid (PLA), poly-glycolic acid (PGA), poly-caprolactone (PCL), polydioxanone (PDO), and poly-trimethylene carbonate (PTMC) are reviewed along with strategies to improve their mechanical and degradation properties using self-reinforcement and copolymerization. Contributions of smart polymers such as polyhydroxyalkanoate (PHA) are also reviewed. In addition, a promising new technology that utilizes computer modeling to easily and quickly characterize polymers is discussed.

Overview of common biodegradable polymers

Significant advances in materials research have changed the perception of degradable polymers and have elevated them to the forefront of surgical fixation procedures. The most commonly used biomaterials today are PGA and PLA, first approved by the FDA for clinical use as sutures in 1969 and 1971, respectively.[401, 402] Since then, these polymeric biomaterials and others have been fabricated into a variety of fixation devices such as screws, plates and rods to be used in a myriad of musculoskeletal applications. A brief overview of the most common biopolymers follows.

Polyglycolic acid (PGA)

PGA is the simplest linear aliphatic polyester used as a biodegradable implant material. It is a hard, tough, crystalline molecule that is more hydrophobic than its counterpart PLA. The crystallinity of PGA may vary from 35 to 75% with a molecular weight (MW) ranging from 40 to 210 kDa depending on the fabrication technique.[299, 403] The T_m and T_g for PGA may vary between 185-230 °C and 25-65 °C, respectively.[300, 404] PGA exhibits high tensile properties (57-69 MPa), and, thus, has been used effectively as a braided suture in rotator cuff repair.[294, 337] In an aqueous environment, PGA has been shown to rapidly degrade in two wks and lose about 50% of its mechanical strength.[405]

Polylactic acid (PLA)

PLA is a semi-crystalline asymmetric polymer with two optical isomers. The D-isomer, PDLA, while readily produced, is not widely used because of its poor biological activity. In contrast, the L-isomer, PLLA, is biologically active and, thus, used frequently in implant materials. The MW of PLLA can range from 50 to 300 kDa depending on fabrication technique.[313] High MW PLLA (>100 kDa) has a T_m that ranges between 170 to 180 °C and a T_g that ranges between 58-65 °C.[300, 404] PLLA exhibits high tensile (11.4-82.7 MPa) and flexural (45-145 MPa) strength suggesting that it could be suitable for load-bearing applications.[294, 313, 406-408] PLLA implants have been shown to retain their mechanical properties for at least six months *in vivo*, however in some cases complete resorption in the joint site can take up to five yrs.[324, 406, 409]

Polycaprolactone (PCL)

PCL is a semi-crystalline hydrophobic aliphatic polyester studied for long term drug delivery systems and implants. The melting point and glass transition temperatures of PCL are unusually low, approximately 58 to 63 °C and -60 to 65 °C, respectively.[404, 410] The tensile strength and elongation of PCL has been shown to vary between 21-35 MPa and 300-500%, respectively.[404] PCL's slow degradation rate (> 2 yrs) makes it an excellent candidate for long-term implantable systems, when compared to PGA or PLLA implants.[411]

Polydioxanone (PDS)

PDS is a crystalline polymer (55% crystallinity) with a melting point of approximately 115 °C and a glass transition temperature of -10 °C; thus, the polymer is usually processed at the lowest possible temperature to prevent depolymerization back to monomer.[412] *In vitro* studies have shown that tensile strengths of PDS sutures can range from 296-358 MPa, with a percent elongation of 43.7-58.1%.[413] An experimental study of PDS implants in rabbits showed that the degradation time *in vivo* ranged from 16 to 25 wks.[414, 415]

Poly(trimethylene carbonate) (PTMC)

PTMC is a soft, rubbery polymer that, until recently, had not been considered for biomedical applications owing to its tackiness and weak mechanical properties. However, recent studies have shown that amorphous PTMC of high MW (~500 kDa and molecular number > 200,000) exhibits rubber-like properties with a

Young's modulus of 6 MPa and a tensile strength of 12-16 MPa.[416, 417] The T_m and T_g of PTMC can vary from 30 ºC to 50 ºC and -17 ºC to -19 ºC, respectively depending on the fabrication technique.[416] Degradation occurs mostly enzymatically and to a lesser extent by hydrolysis since autocatalysis, a phenomenon observed in polyesters like PLLA, is absent in PTMC.[418] *In vivo* studies in rabbit tibia and femur have shown that high MW PTMC rods (~450 kDa) degrade rapidly losing about 60% of their mass by the 8th wk.[418]

In addition to the above mentioned polymers, numerous other materials have been developed and used experimentally in recent yrs, including poly(ortho-esters),[419] poly(3-hydroxybutyrate),[420] and poly(3-hydroxybutyrate-co-3-hydroxyvalerate).[421, 422]

Challenges in the development of degradable polymers

For a polymer to function as an efficient skeletal implant, it must exhibit several properties, biocompatibility notwithstanding. A polymeric implant should be able to withstand the harsh loading environments *in vivo* early in the healing process. At the same time, the implant must provide a suitable anchorage site to cells toward integration with the surrounding native tissue. The implant should also be expected to gradually degrade over time while transferring the load to healing tissues in the surrounding area. These properties are largely influenced by the chemical stability of the polymer backbone and the polymer processing technique which play a large role in determining the polymers' hydrophilicity, crystallinity,

melt and glass-transition temperatures, and MW.[423-425] In addition, the presence of residual monomers or additives can also significantly affect the properties of the polymer.[424] A polymer scientist working with biodegradable materials must evaluate each of these variables and determine the appropriate properties for a specific load-bearing skeletal implant.

The stability of the polymer backbone is an important factor to consider when choosing a particular polymeric biomaterial for a load-bearing application. Common degradable polymers with ester, anhydride, orthoester and amide functional groups have hydrolytically unstable linkages in their backbone, and exhibit time-dependent properties resulting in viscoelastic behavior. Polymer degradation is accelerated by greater hydrophilicity in the backbone or end groups and greater reactivity among hydrolytic groups in the backbone.[426, 427] Thus, the biomaterial of choice must maintain adequate mechanical properties over time in an aqueous environment; i.e., not degrade too rapidly or slowly, while healing occurs. The mechanical properties of a polymer implant may be manipulated by the use of different processing techniques. Some of the current techniques to fabricate polymers include extrusion, injection molding, compression molding, gel casting, solution casting, solvent casting particulate leaching, electrospinning and self-reinforcement.[285, 291, 407] The choice of technique depends largely on the desired properties and morphology of the final product. For instance, the MW of a polymer might be controlled by varying the temperature at which the extrusion process takes place.[57] Nano-fibrous polymers

can be created using electrospinning to enhance cell migration and adhesion on a scaffold.[428] The strength of a polymer can be increased by self-reinforcement, where polymer fibers made from the same material as the desired matrix are sintered at high pressures and temperatures.[291, 429, 430] For instance, the flexural strength of PGA can be increased from 100 MPa to 400 MPa upon self-reinforcement.[303] Thus, determining a polymer with the appropriate chemical and mechanical properties is crucial to the survival and success of an implant *in vivo*.

Co-polymers

In addition to the techniques mentioned above to manipulate the mechanical properties of individual polymers, an alternate approach is to chemically synthesize two or more different monomers to form a co-polymer. By varying the individual amounts of each polymer, the mechanical and degradation properties of the copolymer can be controlled, thereby allowing for greater flexibility in choosing an appropriate implant material for a particular application (see Table 12). A general description of some of the salient copolymers is given below.

Poly-DL-lactic acid (PDLLA)

PDLLA is an amorphous copolymer with a random distribution of the two enantiomers of PLA (PDLA and PLLA). A combination of PLLA and PDLA serves to disrupt the crystallinity of PLLA and accelerate the degradation process. Thus, PDLLA has a lower tensile strength (27.6-41.4 MPa), higher elongation (3-10%), and quicker degradation time (12-16 months) than PLLA, making it a more

attractive option in a load-bearing system requiring shorter fixation times. [404, 431, 432]

Polylactic acid–polyglycolic acid (PLLA-PGA)

The combination of two distinct polymers, PLLA and PGA offers to extend the range of mechanical properties not provided individually by either homopolymer. Experiments have shown that a non-linear relationship exists between the copolymer composition and the mechanical and degradation properties of the individual homopolymers. For instance, a copolymer implant of 50% PGA and 50% PLLA degrades faster than either homopolymer. Copolymers of PLLA with 25 to 70% PGA are amorphous due to the disruption of the regularity of the polymer chain by the other monomer.[311, 433] A copolymer of 90% PGA and 10% PLLA, developed as an absorbable suture material, degrades within three to four months but has a slightly longer strength-retention time.[434]

Poly-L-lactic acid-hydroxyapatite (PLLA-HA)

HA, an inorganic component of bone, has been shown to facilitate and promote bone formation.[435] HA is highly brittle and exhibits low fracture toughness upon mechanical loading. To overcome this issue, researchers have combined HA and degradable polymers (e.g., PLLA) using techniques such as electrospinning to produce more robust scaffolds that can aid in bone regeneration.[435] The crystallinity of PLLA-HA can be increased by increasing the HA content in the co-polymer. Accordingly, the mechanical properties of the PLLA/HA composites vary

with HA content with increased levels of HA resulting in increased bending modulus from 2 to 7.4 GPa and strength from approximately 3 to 114 MPa.[436]

Poly-L-lactide-β–tricalcium phosphate (PLLA-TCP)

β-TCP is a ceramic with osteoconductive properties that degrades via hydrolysis into phosphate and calcium ions.[437] Similar to HA, TCP is brittle and exhibits low fracture toughness. Thus, the addition of β-TCP to PLLA creates a mechanically superior osteoconductive and biocompatible material that facilitates bone healing and regeneration. The Young's modulus and yield strength of PLLA-TCP scaffolds have been shown to be about 22 MPa and 1.5 MPa, respectively.[438]

Future of biodegradable polymer implants

The market for biodegradable orthopedic implants in the US and Europe exceeded over 250 million in revenues in 2006 with growth rates expected to increase further.[439 2006] As the popularity of biodegradable implants continues to grow, researchers are looking to develop novel low cost smart polymers that can complement the polymers already in use. An excellent example of this is the synthesis of energy-storing aliphatic polyesters, such as polyhydroxyalkanoates (PHA), by microorganisms. Two PHA materials, polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) have been researched extensively in the field of biodegradable plastics.[440-442] More recently, PHB has been studied for use in drug delivery systems, artificial skin grafts and as bioabsorbable sutures.[443] The sutures produced by recombinant DNA technology are FDA approved.[444] The

PHB homopolymer is highly crystalline (~80%) and brittle, and its major degradation byproduct, hydroxybutyric acid, is found naturally in human blood. The T_m of PHB is around 177 °C, with a recorded tensile and flexural strength of 40 MPa and 3.5 GPa, respectively.[440] The brittleness of the polymer can be overcome by copolymerizing it with PHV (80:20 PHB-PHV) to reduce the crystallinity to 35% and increase failure strain from 8 to 50%.[440, 445-447]

The advent of video assisted minimally invasive surgery has resulted in less pain for patients, reduced scarring and tissue injury, shortened hospital time and increased accuracy of the procedure. The major disadvantage with the technique, however, is that the smaller work area for the surgeon may increase the challenge in implanting a larger implant or knotting a suture. Groundbreaking research in material science has allowed for the development of degradable elastic shape memory polymers that can take a certain shape upon thermal or light induction.[384, 448] These polymers may be introduced into the body in a compressed form prior to application of the appropriate stimulus to regain the desired shape. Several different biodegradable polymers are currently investigated for their potential as shape memory polymers including PLLA-co-PCL (PCLA), PDLLA/hydroxyapatite composites, 82:18 PLA-PGA, tert-butyl acrylate (tBA)/poly ethylene glycol dimethacrylate (PEGDMA), and copolyester-urethane networks.[449-452]

Advances in computational modeling techniques utilizing high throughput screening may now eliminate the need for detailed characterization of individual polymers. The traditional mode of materials design begins with the synthesis of a new material, followed by its characterization, and eventual identification of a suitable application. The goal of the computational approach is to accurately predict the behavior of polymers under different conditions and create virtual biomaterial libraries containing thousands of individual compositions.[453] Using these libraries, prediction of material characteristics such as elastic modulus, glass transition temperature, hydrophobicity and degradation time will be facilitated.[453, 454] Several design factors can be controlled to ensure that the implants will survive in the host and provide appropriate fixation. These include shape, type of implant-abutment mating, presence of threads, thread design, surface topography and chemical composition of the material.[455, 456] In addition, customized implants can now be created directly from computer data by using solid freeform fabrication [457-460] and combinatorial polymer scaffold libraries.[461] Solid freeform fabrication is a technique that allows for the three dimensional printing of custom designed objects created using a CAD drawing. The uniqueness of this technique stems from the ability to add and bond materials in layers. This provides the user with precision control and the option to create an object with multiple materials. Combinatorial polymer scaffold libraries provide the user with a tool to determine the appropriate cell-scaffold variations that are most likely to increase tissue formation on the construct for tissue engineering applications. With a plethora of biomaterials currently being researched for tissue

engineering of load bearing tissues, such as articular cartilage and the knee meniscus, a predictive tool such as this could be used to screen cell-polymer combinations to streamline the tissue engineering process.

Conclusion

We can speculate that advancements in polymer science will continue to fuel the development of novel biocompatible polymeric implants, with or without the incorporation of bioactive agents, toward fixation as well as healing and repair of the injured tissue. Using techniques such as copolymerization and self-reinforcement, implant tensile, flexural and shear strengths, crystallinity, and *in vivo* degradation profiles can be controlled. In addition, computational modeling techniques can now be used to predict polymer properties prior to implantation. Finally, novel biodegradable elastic smart polymers, that can change shape by triggering changes in the immediate environment, may potentially be used in minimally invasive surgeries as fixation devices.

Figures associated with appendix B

Table 12. Some FDA approved polymers and their mechanical contributions in a co-polymer system

Polymer	Major properties	Contribution to implants
PLLA	Hydrophobic Slow degradation	Provides strength
PDLA	Disrupts crystallinity	Provides flexibility
PGA	Hydrophilic Quick degradation	Lowers stiffness
TMC	Subzero glass transition temperature Rubbery at room temperature	Improves degradation rate Enhances malleability and toughness

Appendix C: A biomechanical survey of salient biodegradable ACL interference screws

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Introduction

Anterior cruciate ligaments are composed of fascicles of dense collagenous tissue that function primarily to stabilize the knee joint by preventing the femur and tibia from sliding onto each other.[462] The ACL is exposed to a variety of axial and tensile forces *in vivo* resulting from its complex ultrastructure and positioning in the knee joint. Biomechanical characterization of the ACL, conducted in humans, has shown that the ultimate tensile load and linear stiffness of the human femur-ACL-tibia complex vary depending on age. In younger cadaveric knees (20 - 35 yrs), the ultimate tensile load and stiffness have been shown to be 2160 N ± 157 N and 242 N ± 28 N/mm, respectively, while in older cadaveric knee (60 - 97 yrs) the force and stiffness are lower, 658 N ±129 N and 180 N ± 25 N, respectively.[463]

Today, the ACL is the most commonly studied ligament in the knee joint as a result of its high incidence of injury.[464] Large mechanical forces caused by sudden twisting movements can result in ACL tears. Several different approaches are investigated to treat ACL injuries including the use of autograft and allograft tissue to replace the torn ACL and more recently tissue engineering strategies are also being considered.[465] Patellar tendon and hamstring muscle tendon autografts and allografts, however, remain the gold standard with over a 90% success rate in restoring function and stabilizing the knee joint.[466, 467] To achieve effective fixation of these replacements, several different devices have been tested including buttons, washers, cross-pins, sutures, staples, and

interference screws.[468] The following chapter reviews the material and biomechanical properties of the most common class of fixation devices used in ACL reconstruction, i.e., ACL interference screws.[469]

The past few decades have seen a gradual shift toward the use of biodegradable polymeric implants at the expense of metallic implants, especially for soft tissue applications. This trend is expected to continue, as novel polymers are developed with a wide range of mechanical properties that can be modulated to prevent stress shielding mismatches observed with metals. In addition, biodegradable devices do not interfere with imaging techniques, do not need to be removed for revision surgery, and rarely illicit a foreign body response.[57] Today, over 40 different polymer formulations are used in different applications ranging from ligament reconstruction to tissue regeneration.[57] For ACL reconstruction in particular, the vast majority of the fixation implants are variants of poly-lactic acid (PLA) or copolymers of PLA and poly-glycolic acid (PGA). To enhance bone regeneration as the screw degrades, several different osteoconductive materials are currently being investigated such as tri-methylene carbonate (TMC), calcium carbonate (CC), β-tricalcium phosphate (β-TCP), hydroxyapatite (HA), and biphasic calcium phosphate (BCP) in conjunction with the base polymer.[437, 470-472]

Mechanical strength, degradation and pH

The mechanical strength of a polymer is dependent on several different factors including molecular weight (MW), degree of crystallinity and choice of fabrication technique. The MW of the polymer can be altered by changes in mechanical stress, temperature and pressure. Polymeric fixation devices *in vivo* must be designed to account for these changes, since drops in MW can significantly decrease the mechanical properties of the implant and jeopardize fixation.[296] The degree of crystallinity, i.e., percentage of the polymer that is represented by an ordered structure, is dependent on the molecular chain structure and molecular chemistry of the polymer. Polymers never exhibit 100% crystallinity and, usually, the greater the crystallinity of the polymer, the greater are its stiffness and density. Polymers can range from amorphous to highly crystalline depending on the temperature and cooling rates utilized during the fabrication process.[286] A variety of fabrication techniques, including solution casting, solvent-casting particulate leaching, gas saturation, phase separation, extrusion and injection molding, can be utilized to generate polymers with specific properties.[287] For example, poly-l-lactic acid (PLLA) fibers of high MW (300 – 500 kDa) can be extruded at 110°C while PLLA fibers of low MW (< 300 kDa) can be extruded at 180°C.[287]

The mechanical strength of a polymer can be characterized using stress-strain curves to obtain tensile properties including the ultimate tensile strength (UTS), yield strength, and the elastic modulus or stiffness. Flexural and torsional

strengths can also be determined to examine the ability of polymers to resist bending. Cyclic loading to determine fatigue behavior of the polymer can also be conducted.[57]

Degradation of PGA and PLLA molecules via ester hydrolysis results in the formation of acidic byproducts, glycolic acid and lactic acid, respectively.[296] These acids enter the Krebs cycle where they are broken down into water and carbon dioxide prior to excretion. Materials composed of PGA and PLLA undergo bulk degradation which results in loss of MW when placed in an aqueous solution. However, the mass of the polymer remains until molecular chains are small enough to diffuse out of the polymer matrix. This phenomenon leads to accelerated degradation of the polymer that occurs suddenly wks or months post-surgery, resulting in an acidic environment with non-physiologic pH levels. To overcome this problem, researchers have used calcium compounds, such as calcium carbonate, and calcium hydroxyapatite to act as a buffer for the degrading acidic byproducts.[132] Thus, a variety of factors can influence the fate of an implant *in vivo*. In general, a successful implant must be able to withstand high stresses early in the process and degrade gradually while healing occurs.

Biodegradable materials for ACL interference screws

A variety of biodegradable interference screws are currently produced by several companies with varying mechanical and chemical characteristics. Some of the major biodegradable polymers and compounds used to manufacture ACL screws

include PLLA, PGA, poly-lactide carbonate (PLC), poly-dl-lactic acid (PDLLA), β-TCP, TMC, HA, BCP and their combinations (Table 13 and Table 14). The following paragraphs describe i*n vitro* or *in vivo* experiments conducted to characterize the mechanical and chemical properties of interference screws. Also discussed are clinical studies in patients along with any observed complications:

Poly-L-lactic acid (PLLA)

PLLA, an enantiomer of PLA, is the most commonly used biodegradable polymer in ACL fixation. Its degradation by-product yields a naturally occurring stereoisomer of lactic acid which can be eliminated from the body via the Krebs cycle.[310] The MW of PLLA can vary depending on manufacturing technique; high MW PLLA (>100 kDa) has a melting temperature ranging from 173°C to 178°C with a glass transition temperature of 58°C.[300] Although PLLA is generally semi-crystalline, injection molding of PLLA results in non-crystalline products, leading to a biomaterial whose modulus drops above the glass transition temperature.[473] To increase the mechanical properties of PLLA, a technique called self-reinforcement can be used where fibers of the desired polymer, made from the same material as the polymer matrix, are sintered together at high pressures and temperatures.[291] Several studies in the literature have examined the degradation profiles and foreign body responses associated with PLLA *in vivo*. It has been shown that PLLA interference screws implanted in patients persisted 20 to 30 months after surgery with little to no foreign body response observed.[324, 474]

However, in some cases cartilage damage and effusions around the knee joint have been observed with delayed PLLA degradation.[352, 358, 359]

The mechanical properties of PLLA interference screws have been extensively characterized by performing load to failure experiments and pull-out tests with a range of properties obtained depending on the material characteristics of the product and the testing protocol used. The fixation strength of PLLA interference screws (Arthrex, Inc.) have been compared to titanium (Ti) interference screws using bone-patellar tendon bone (BPTB) grafts. No significant differences were observed between the ultimate failure loads of the PLLA (680 to 995 N; average 805.2 N) and Ti screws (544 to 1094 N; average 768.6 N).[475] Delta tapered bio-interference screws® (Arthrex, Inc., non-crystalline PLLA) have been mechanically tested for quadruple hamstring tendon graft fixation post-dynamic loading. The authors found that the interference screws failed at 647 ± 200 N with a displacement at failure of 10.91 ± 4.4 mm. In addition the stiffness of the screws was calculated to be 64.54 ± 22.1 N/mm.[476] Biomechanical properties of the RetroScrew® (Arthrex, Inc., PLLA) for tibialis anterior graft-tibial tunnel fixation have also been obtained. During cyclic testing (more information), a screw displacement of 1.8 ± 0.5 mm was observed with a stiffness of 114.1 ± 23.3 N/mm. When load-to-failure tests were conducted, the maximum load at failure was found to be 787 ± 177.5 N with a pullout displacement of 5.3 ± 2 mm and a pullout stiffness of 204.4 ± 52.9.[477] Additional research conducted by Arthrex, Inc with the RetroScrew® suggested that screw placement during

fixation of whipstitched human anterior tibialis bundles in a porcine model significantly affected the pull-out stiffness of the screws.[478]

A study using BioScrew® interference screws (Conmed Linvatec, PLLA) in cadavers for fixation of BPTB grafts showed that the mean load to failure was 418 ± 118 N.[479] Fixation strength of fibrillated Biofix® screws (Bioscience, Ltd., SR-PLLA) in a BPTB graft was found to be 1211 ± 362 N with an elastic modulus of 189 ± 47.4 in the lower load range (< 500 N) and 304 ± 71.8 in the higher load range (> 500 N).[480] These values were not significantly different from two other metallic screws that the authors investigated. BIORCI® interference screws (Smith & Nephew, PLLA) have been mechanically evaluated for femoral fixation of soft tissue grafts. The UTS of the screws was found to be 643.5 ± 148.4 N with a stiffness of 315.7 ± 38.9 N/mm.[481] Universal wedge interference screws® (Stryker Endoscopy, PLLA) have been tested for hamstring ACL reconstruction at the central and peripheral locations on the tibia. Screws placed in the central location were found to be significantly stiffer, with a greater yield load and UTS suggesting that central interference screw fixation may allow greater confidence in rehabilitation and reduce the risk of failure in *vivo*.[482] Absolute interference screws® (DePuy Mitek, Inc., PLLA) have been compared to RigidFix® biodegradable pins (Ethicon, Inc.) for BPTB graft fixation strengths in bovine knees. The authors found that the interference screw stiffness was 168 ± 42 N/mm, with yield strength of 402.7 ± 143.9 N and UTS of 515.7 ± 168.5 N. In

addition, it was found that the use of two pins provided the same mechanical stability as one interference screw.[483]

Poly-DL-lactic acid (PDLLA) and co-polymers of PDLLA, PLLA and PGA

Copolymers of the two enantiomers of PLA, PLLA and poly-d-lactic acid (PDLA), result in PDLLA which can exhibit a variety of mechanical properties depending on the percentage of each enantiomer present.[311, 312] PDLLA is typically amorphous with a glass transition temperature of 56°C.[484] *In vivo*, PDLLA interference screws have been shown to survive at least 6 wks without degradation, but are known to resorb completely by 10 months.[324] Copolymers of PDLLA, PGA and PLLA offer to extend the range of mechanical properties not offered by either material individually. For example, the glass transition temperature of a 50:50 PDLLA-PGA blend is around 30°C. As the ratio of PDLLA:PGA increases from 65:35 to 75:25, the glass transition temperature increases as well from 33.5°C to 38°C.[330] Combining PLLA and PGA in an 82:18 ratio (Lactosorb®, Biomet Sports Medicine, Inc.) can result in a non-crystalline co-polymer with a T_g of 55.3°C. It has been shown that a 2°C rise in temperature can dramatically increase the hydrolysis rate of Lactosorb® by 20-25%.[485]

Phusiline® interference screws (Phusis, 98/2 PDLLA-PLLA), have been used for fixation of patellar tendon autografts in patients. Results at an average follow up time of 2 yrs showed that the screws had degraded with observable bony-ingrowth observed, earlier than what is generally observed with PLLA screws.

Clinical tests and MRI examinations showed no adverse effects as a result of screw degradation.[396] The most common PDLLA polymerization technique involves tin octoate as an initiator; however, to reduce any potential immune response, Phusiline® screws are polymerized using ring opening polymerization in the presence of zinc, a less toxic material.[486] Ring opening polymerization is a technique where cyclic monomers join to form a large polymer chain through ionic interactions. This results in more hydrophilic interference screws with quicker degradation rates.[396]

Sysorb® screws (Centerpulse Medical AG, 50/50 PDLLA) have been used for BPTB graft fixation in 25 patients. In only case, tibial bone enlargement and a subcutaneous cyst was observed eight months post-surgery with no bone formation in the tibial tunnel.[487] Biologically quiet interference screws® (Instrument Makar, Inc., 85/15 PDLLA/PGA) have been used for ACL reconstruction in patients with complete degradation, bone remodeling and neo-bone formation observed at the implantation site at an average of 5.2 yrs post surgery. In addition, MRI images showed no cystic or osteolytic changes at the site with minimal edema.[351]

Mechanical testing with Sysorb® screws for BPTB graft fixation in human cadavers showed that the load to failure was 544 ± 109 N with stiffness 162 ± 27 N/mm. Cyclic elongation tests showed that at three cycles, 1-5, 5-20 and 20-1500, the observed elongation was 1.4 ± 0.5 mm, 0.14 ± 0.06 mm and

4.1 ± 2.7 mm respectively.[488] SmartScrew® interference screws (Conmed Linvatec, SR 96/4 PDLLA) have been examined for human semitendinosus-gracilis tendon graft fixation. Single cycle to load failure tests and cyclic loading tests were conducted. The yield load for the single cycle test was found to be 665 ± 201 N while the stiffness was 115 ± 34 N/mm. For the cyclic loading test, a residual displacement of 3.8 ± 1.5 mm was observed at 1500 cycles.[489] MegaFix® interference screws (Karl Storz - Endoscope, 70/30 PDLLA) of three different screw diameters 6 mm, 7 mm and 8 mm were mechanically evaluated in porcine knees. The results showed that zero 6 mm screws, three 7 mm screws and all 8 mm screws survived the cyclic loading protocol. An elongation of 8.36 ± 0.5 mm was observed in the 7 mm screws and 4.26 ± 1.5 mm in the 8 mm screws after 1000 loading cycles. Maximum load, yield load and stiffness were also determined for the 7 mm (245.4 ± 76.5 N, 199.1 ± 37.2 N and 98.6 ± 20.2 N) and 8 mm screws (567.7 ± 177.0 N, 465.9 ± 126.3 N and 151.0 ± 26.4 N).[490]

Polymers with trimethylene carbonate (TMC)

TMC is a mechanically weak material with elastic properties resembling rubber.[491] In combination with polymers such as PGA, PLLA and PDLLA, however, a range of biomechanical properties can be obtained to suit a particular application. For example, polyglyconate is an A-B-A block copolymer of PGA and TMC in a 2:1 ratio. The letters A and B represent homopolymer sub-units that are linked by a covalent bond. The copolymer provides better flexibility than PGA alone. *In vitro* tests have shown that the MW of non-irradiated polyglyconate,

cultured in PBS, drops from 124.1 kDa to 18.6 kDa over a period of 31 days. Additionally, UTS of the samples dropped from 51.7 MPa to 5.7 MPa over 3 wks.[295] *In vivo*, polyglyconate implants have been shown to lose their mechanical integrity over 6 wks but usually take about 6-12 months to resorb.[295, 334, 335]

An *in vivo* study was carried out in 20 patients comparing Ti screws and Endofix® absorbable interference screws (Smith & Nephew, PGA-TMC) for femoral bone block fixation. Clinical assessments and computer tomography scans were conducted at 3, 6, 12 and 24 months. No complications were observed with the graft fixation in either group. At 12 months, complete screw degradation was observed; however, neo-bone formation was not observed with the polyglyconate screws up to three yrs post-surgery.[335] Inion Hexalon® ACL screws (Inion Ltd., PDLLA-TMC) have been shown to retain 70-90% of their initial strength for up to 12 wks, with observable degradation from 18-36 wks and complete screw degradation within 2 yrs.[471, 492, 493] MRI imaging at 2 yrs showed that the bone tunnels post-screw degradation were filled with fibrous-like tissue; however, the authors could not conclude the exact tissue type since histological sections were not taken. Complications occurred in only two out of 29 patients tested with tunnel enlargement or cyst observable 2 yrs post surgery.[471]

The mechanical properties of Inion Hexalon® screws have been characterized using mature porcine cadaver tibiae (please refer to Chapter 52). add reference The initial fixation strength of the Inion Hexalon® interference screws were

measured in three separate experiments by comparing to 1) metal interference screw (via soft tissue graft fixation) 2) SR-PDLLA SmartScrew® (Conmed Linvatec) (via soft tissue graft fixation) 3) PLLA BioScrew® (Conmed Linvatec) (via bone-tendon-bone graft fixation). The yield loads for the Inion Hexalon® in each experiment were found to be 491 ± 154 N, 501 ± 122 N and 901 ± 262 N, respectively while the maximum failure loads were 548 ± 130 N, 563 ± 109 N and 926 ± 259 N, respectively.[471] The authors found fixation strengths of the Inion Hexalon® to be similar to the other tested metallic and polymer screws. In a previous study, torsional strength of the Inion Hexalon® was also characterized using a previously established protocol.[494] Briefly, screws were mounted in a polyurethane resin to mimic screw failure observed *in vivo*. The mean maximum insertion torque was found to be 2.4 ± 0.3 Nm. Additionally screw failure was not observed up to 5 Nm. Higher clinically irrelevant torques (> 5 Nm) did not cause screw breakage but resulted in bending of the metallic screw driver shaft.[471] Based on these results, the authors concluded that the Inion Hexalon® screws equaled or surpassed other commercially available screws in torsional strength.[494]

Polymers with calcium carbonate (CC)

CC is an inorganic component of bone that can be used to form other calcium salts found in bone. When combined with other polymers, an osteoconductive interface is achieved with enhanced screw degradation properties and quickened neo-bone regeneration.[495] Calcium salts have also been shown to provide a pH

buffer for the acidic products released during polymer degradation.[132] An example of this blend is the Calaxo® interference screw (Smith & Nephew), which is a polylactide carbonate (PLC) composed of 85:15 PDLLA–co-PGA (65%) and calcium carbonate (35%).

A recent *in vitro* study investigating the MW and pH of Calaxo® screws in PBS (pH = 7.36) over 12 wks found an 83.5% drop in MW of Calaxo® with a drop in pH to 6.86.[496] The authors noted that the rapid degradation of the screw possibly overwhelmed the buffering effect of calcium carbonate. The Calaxo® screw has also been tested in 41 sheep for ACL fixation. No inflammatory reactions were observed at 6, 12, 26 or 52 wks. At t = 26 wks, the screw was partially replaced with new bone and at t = 52 wks, complete screw degradation had occurred with total bone replacement. Ultimate tensile loads to failure at 6 and 12 wks were approximately 70 N and 225 N, respectively.[495] In humans, one case report highlighted the presence of tibial cysts and intra-articular granuloma formations in a patient implanted with Calaxo® screws 6 months post-surgery.[497] According to a recent review, "a vast number of Calaxo® screws have been implanted without incident"; however, the complication described in the case report was "not unique in human subjects" prompting product withdrawal from the market by the manufacturer.[498]

Polymers with β-tricalcium phosphate (β-TCP)

Several companies manufacture screws with PLLA, PLGA or PDLLA as the base polymer and β-TCP as the osteoconductive material. β-TCP is a brittle ceramic with low fracture toughness, meaning cracks in the material have low ability to resist fracture. It exhibits osteoconductive properties and degrades via hydrolysis into phosphate and calcium ions.[437] The addition of β-TCP into PLLA screws, results in enhanced mechanical properties and faster degradation, which quicken bone healing and regeneration. PLGA, which degrades faster than PLLA, in conjunction with β-TCP may provide an optimized degradation profile in addition to osteoconductive properties due to TCP. A description of the salient β-TCP based screws follows.

Bio-Intrafix® system (DePuy Mitek, Inc., 30% β-TCP – 70% PLLA) consists of two bio-absorbable components, the expansion sheath and the tapered screw. The components are manufactured using Micro Particle Dispersion, a proprietary technology of DePuy Mitek, Inc., which allows for a homogeneous blend between PLLA and β-TCP.[499] A study investigating the Bio-Intrafix® system in patients showed no complications in patients 2 yrs post surgery. Additionally, a pull-out strength of approximately 1067 N was determined for the system.[500]

The Milagro® interference screw (DePuy Mitek, Inc., 30% β-TCP – 70% PLGA) is composed of Depuy Mitek's proprietary composite material Biocryl Rapide®, a homogeneous blend of PLGA and TCP particles. Pre-clinical *in vivo* studies in

beagles have shown that Biocryl Rapide® degrades completely when placed in a defect in cortical femoral bone and is replaced by new bone by 24 months.[501] A recent *in vitro* study investigating the effects of PBS (pH = 7.36) on Milagro® screws showed that minor dimensional changes occurred to the screws 12 wks post-culture.[496] MW was found to drop by 66.9% in that time period; however, the pH stayed relatively constant (pH = 7.34 at 6 and 12 wks) suggesting that β-TCP successfully created a buffering effect to acidic degradation products released as a result of screw breakdown.[132] Milagro® screws have also been used to study tibial bone-tunnel enlargements during double-bundle ACL reconstruction in patients. Tibial fixation in 25 patients was achieved with two Milagro® screws and MRI images and radiographs were obtained approximately 1 yr post-surgery. A mean tunnel enlargement of 43% was observed in the tibia. The authors, however, did not provide details on the degradation profiles of the implanted screws.[502] Another study in 70 patients, comparing single bundle and double bundle ACL reconstruction, utilized Milagro® screws for tibial fixation. However, radiographs taken an average of 19 months post-surgery in patients could not reliably detect the interference screws and sizes of bone tunnels. Two patients were treated with antibiotics for infections post-surgery and in two patients cyclops lesions were debrided using arthroscopy. The authors did not, however, attribute a possible cause for these complications.[503] Another interference screw, Biocryl®, manufactured by Mitek utilizing PLLA instead of PLGA, (30% β-TCP-70% PLLA) was studied in mature sheep and was shown to yield superior bone to tissue osseo-integration when compared to an HA-PLLA composite screw.[504]

Bilok® interference screws (ArthroCare Corporation, 25% β-TCP- 75% non crystalline PLLA) have been tested *in vitro* for their degradation properties over 12 wks in PBS (pH = 7.36). At t = 12 wks, minor morphological changes were observed with a 44.7% decrease in MW. The pH of solution was also found to be in the physiologic range (pH = 7.28 at 12 wks).[496] A study in 41 patients, investigating the clinical effectiveness of Bilok® interference screws for patellar tendon autograft ACL fixations, showed that 3 yrs post-operatively the screws were completely replaced by bone with no observable osteolysis. A quicker degradation rate of Bilok® interference screws was observed when compared to degradation rates of PLLA interference screws.[505, 506] In another study involving patellar tendon graft ACL fixation in 20 patients, Bilok® interference screws did not degrade completely 24 months after implantation.[507] In a few cases, fracture of Bilok® interference screws has been observed during insertion into the tibial or femoral tunnel.[505, 507, 508] However, in each study, the authors modified their technique of screw insertion to resolve the problem.

Polymers with hydroxyapatite (HA)

HA is an inorganic component of bone which has high biocompatibility and bio-affinity and has been used extensively in the past for bone regeneration.[470] In addition, HA also acts as a buffer to the acidic byproducts of PLLA during degradation.[132] Similar to β-TCP, HA exhibits low fracture toughness upon loading and is highly brittle. The elastic modulus of HA can range from 80-100 GPa with a compressive strength of 500-1000 MPa.[509] When blended with PLLA,

the composite's mechanical properties can be substantially altered to mimic those of bone.[435] In addition, HA also promotes bone regeneration by increasing osteoblast attachment and proliferation on the composites.[510]

Biosteon® (Stryker, HA-PLLA) interference screws are composites of non-crystalline PLLA and HA. *In vitro* studies on Biosteon® screws cultured in PBS (pH = 7.36) showed that no significant gross changes occurred to the screws over a period of 12 wks. In addition, the MW of the screws dropped by 22.9% with a minimal drop in pH from 7.36 to 7.32.[496] Experiments by Stryker, comparing PLLA and Biosteon® screws have shown that the latter exhibited superior strength retention *in vivo* over 24 wks and mimicked the body's natural healing response. In addition, the modulus of Biosteon® implants was shown to be similar to that of bone circumventing the issue of possible stress shielding resulting in bone density reduction.[511] BIORCI-HA® interference screws (Smith & Nephew, HA-PLLA) were used for patellar tendon graft fixation in 20 patients. Two yrs post-operatively, no inflammation was visible via MRI around the graft in patients; however, screw degradation was minimal with little observable bone integration. In one patient, screw breakage was observed during insertion.[507]

Polymers with biphasic calcium phosphate (BCP)

Biphasic calcium phosphate consists of a mixture of HA and β-TCP in varying ratios.[512] HA and β-TCP are known osteoconductive materials and their combination has been shown to increase osteoblast adhesion and proliferation

over each individual material, as described on the Arthrex, Inc. website. By determining the appropriate ratios leading to a more stable phase of HA and a more soluble β-TCP, researchers have been able to achieve controlled degradation and promote osteogenesis.[472, 512] Combining polymers such as PDLLA with BCP serves to improve the mechanical properties of the composite material. BioComposite interference screws (Arthrex Inc., 70/30 PDLLA-BCP) are manufactured by blending and binding the two materials together to form a homogeneous porous implant. The macro and micro-pores in the screws aid in promoting bone cell adhesion and proliferation. To maximize the insertion torque and prevent screw breakage during insertion, a stepped-taper design is employed.

Conclusions and future directions

Biodegradable polymeric interference screws are now commonly used in surgery in lieu of metallic interference screws. Several studies comparing the two have found that metals provide no significant mechanical advantage over their polymeric counterparts.[513-516] In fact, metallic screws release metal ions into the joint space, cause stress shielding which lowers bone density and contraindicate imaging applications such as MRI.[57] Screws made out of aliphatic polyesters, such as polylactides and polyglycolides, overcome these inherent problems that metals present; however, the majority of studies in humans have shown that these polymers are susceptible to delayed degradation *in vivo* and the absence of neo-bone formation post screw degradation.[358, 359, 487] The inclusion of

osteoconductive materials, such as HA, CC, TMC, β-TCP, and BCP, along with the base polymer appears to quicken screw degradation, enhance osseous screw replacement and result in few complications.[471, 502, 505]

Other osteoconductive agents such as growth factors (TGF-β and IGF), and bone morphogenic proteins (BMP I-7), have also shown potential in enhancing bone formation.[517] By incorporating these agents into the polymer matrix during the fabrication process, the interference screw could serve a dual role of fixation device and drug delivery vehicle. As screws degrade *in vivo*, the bioactive agents released into the surrounding area could aid in neo-bone formation. Injectable osteoconductive agents may also be used to enhance and accelerate bone growth and healing post ACL reconstruction. For example, calcium phosphate cement has been used in a rabbit model to augment tendon-bone healing.[518, 519] A recent study in dogs using injectable TCP to fill tunnel parts that were not filled by the graft showed greater tendon-bone adherence and superior pullout strength 12 wks post surgery when compared to the controls.[520]

Xenogenic implants have been used in the past for various bone-graft sites with varying clinical success.[521] Regeneration Technologies Inc. has developed a novel bovine interference screw (Sterling® interference screw) which consists of decellularized cortical bone to prevent immune responses while still maintaining the bone's mechanical properties. Similar to osteoconductive agents discussed previously, the goal of this product is to increase bone regeneration at the

implant site. Tests conducted by Regeneration Technologies Inc. have shown that the pull-out strengths of this screw (538 ± 268 N) were on par with that of metallic and biodegradable screws tested by other groups in the literature.[331, 513, 522] In addition, a study with 17 patients showed no inflammatory responses or screw rejection 7 months post-operatively, with neo-bone formation observed around the graft site. Allograft screws (Regeneration Technologies Inc., CorIS®) have also been studied for ACL fixation in patients. A study found that 24 months post-surgery, the screws were completely degraded and the defect was filled with neo-cancellous bone.[507]

The geometric design of interference screws can also affect graft fixation and influence the mechanical properties of the ACL screw-graft. For example, screw size, screw tapering and thread angle have been shown to affect graft fixation and screw engagement with the bone tunnel.[499, 523-525] Although, the general rule of equal screw diameter and bone tunnel is advocated, several experiments have investigated using smaller tunnel diameters with larger screws to achieve enhanced graft-tunnel fixation.[526] Indeed, it is expected that geometrically equivalent screws made from different biodegradable and bioactive materials will respond differently *in vitro* and *in vivo* owing to intrinsic differences in the material properties of the screws. Thus, to ensure use of an appropriate screw, more tests need to be conducted, both *in vitro* and *in vivo*, to determine potential differences in mechanical and degradation properties and prevent screw breakage upon insertion into the bone tunnel as has been observed in several clinical studies.[505,

[507, 508] In addition, state-of-the-art computer modeling programs and combinatorial polymer scaffold libraries that can predict the properties of the screws prior to implantation may also be used as a selection tool to identify configurations and combinations of polymers that may achieve the desired fixation levels required *in vivo*.[453, 461]

Figures associated with appendix C

Table 13. Salient polymer and co-polymer formulations used in ACL screws

Polymer	Company	Commercially available screws
PLLA	Arthrex	Delta tapered bio-interference screw Round delta tapered bio-interference screw Full thread bio-interference screw Biocortical screw Retro screw
	Conmed Linvatek	Bioscrew
	Depuy Othropedic Technologies	Phantom absorbable screw
	Mitek	Absolute screw
	Scandius	Bio-straTis
	Smith and Nephew	BIORCI
	Stryker	Universal wedge interference acrew
SR-PLLA	Bioscience	Biofix
PDLLA	Sulzer Medica	Sysorb
	Storz	MegaFix
SR-PDLLA	Conmed Linvatek	SmartScrew
PLLA-co-PGA	Biomet	Gentle threads BioCore
PLLA-co-PDLLA	Phusis	Phusline interference screw
PGA-co-PDLLA	Instrument Makar	Biologically quiet interference screw

Table 14. Novel osteoconductive ACL screws

Filler	Polymer	Company	Commercially available screws
β-Tri-calcium phosphate (β-TCP)	PLLA	ArthroCare	Bilok
		Mitek	Bio-Intrafix Biocryl TCP/PLA
	PGLA	Mitek	Milagro
	PDLLA	Conmed Linvatek	Osteo ACL Screw
	SR-PDLLA	Conmed Linvatek	Matryx Interference Screw
Tri-methylene carbonate (TCP)	PGA	Smith and Nephew	EndoFix
	PDLLA	Inion	Inion Hexalon
Calcium carbonate	PGA-co-PDLLA	Smith and Nephew	Calaxo
Hydroxyapatite (HA)	PLLA	Smith and Nephew	BIORCI-HA
		Stryker	Biosteon