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Functional Tissue Engineering of the Temporomandibular Joint Disc

by

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Abstract

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Temporomandibular joint (TMJ) disorders arise from disease or trauma and may result in degeneration of the soft tissues. Tissue engineering may provide a solution to disorders of the TMJ without the side effects seen with artificial materials, such as improper incorporation with the surrounding tissues or immunological rejection of the artificial replacement. Several experiments were completed toward the goal of creating a functional TMJ disc replacement using a cell-based approach; the cell types that were primarily examined in this work were TMJ disc cells and costal chondrocytes. Attempts were made to improve the properties of scaffolds seeded with TMJ disc cells, and while proliferation was increased for the monolayer expansion phase of the approach, improvements were not seen in the properties of the three-dimensional constructs by adding L-proline to the culture. Due to the limited success of the TMJ disc cell constructs and the donor scarcity of this cell type, alternative cell sources were investigated in a scaffoldless tissue engineering method to improve the functionality and translatability of the engineered constructs. Chief among the cell types investigated, costal chondrocytes (CCs) consistently produced constructs with considerable amounts of extracellular matrix that were relevant to regenerating TMJ disc fibrocartilage. From this initial success, other aspects in using CCs for TMJ disc tissue engineering were investigated, specifically, passaging the CCs and adding exogenous stimuli. Examining passaged costal and articular chondrocytes showed that while the process of passaging and expanding chondrocytes caused an increase in collagen type I over type II, constructs made from passaged chondrocytes had higher collagen content and tensile properties than primary chondrocyte constructs. The observation that passaged cells were just as, if not more, capable of producing functional constructs also enhanced the translatability of this method by addressing the issue of donor tissue scarcity. Therefore, CCs at a variety of passages were
examined in construct culture. Passaged CC constructs consistently produced more glycosaminoglycans per wet weight than primary cell constructs. Passaged CC constructs were then examined in the presence of exogenous stimuli to further improve their properties. At the regimens examined, hydrostatic pressure did not affect the constructs. In contrast, insulin-like growth factor-I improved construct properties over the no growth factor control. Overall, this thesis presents considerable support for the use of passaged costal chondrocytes for the purposes of improving functionality and clinical translatability of constructs for TMJ disc tissue engineering.
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Introduction

The overall objective of this thesis is to create a functional tissue engineered construct with properties approaching those of the native TMJ disc by optimizing the cell culture conditions of TMJ disc cells and exploring more clinically-relevant cell sources for fibrocartilage tissue engineering. *This two-fold objective is based on the following two hypotheses: 1) finding optimal culturing conditions for TMJ disc cells in two- and three-dimensional culture will improve the final properties of TMJ disc cell constructs, and 2) there exists a cell source that will create more mechanically and biochemically functional constructs while also improving clinical translatability.*

To examine this hypothesis-driven objective, three aims were developed:

1. **To optimize cell culturing conditions for porcine TMJ disc cells.** This aim examines the culturing conditions of TMJ disc cells in two- and three-dimensional cultures. The two phases of this aim are 1) to determine the most appropriate media additives for monolayer TMJ disc cell culture and 2) to optimize collagen production through the addition of L-proline. In the first phase, cells are examined at different passages for collagen production and gene expression with different media formulations. In the second phase, various concentrations of L-proline are added to cell-seeded poly-L-lactide scaffolds. *The specific hypotheses of this aim are that 1) each of the additives affects the cell proliferation and matrix production of porcine TMJ disc cells and using L-glutamine, sodium pyruvate, and insulin will provide the optimal conditions, and 2) higher concentrations of L-proline will increase the collagen production by the cells.* It is also expected that larger collagen production will increase the tensile properties in three-dimensional culture.

2. **To examine alternative cell sources for TMJ disc tissue engineering.** This aim, combined with aim 3, relates to the second part of the global objective, namely to explore more clinically-relevant cell sources for TMJ disc regeneration. It examines a variety of
cell sources, which have the potential to be more functional and/or more clinically translatable. Several cell sources are evaluated for their biochemical productivity in scaffoldless construct cultures; articular chondrocytes, costal chondrocytes, dermal fibroblasts, mixtures of dermal fibroblasts with both types of chondrocytes, passaged costal chondrocytes, and bone marrow derived mesenchymal stem cells are examined first. Some of the more promising cell types are then examined biochemically and biomechanically. Costal chondrocytes, dermal fibroblasts, and a mixture of costal chondrocytes and dermal fibroblasts in construct culture are compared to TMJ disc cell constructs. Costal chondrocytes are also compared to articular chondrocytes, passaged chondrocytes, and passaged articular chondrocytes for mechanical properties and biochemical content. The hypothesis of this aim is that costal chondrocytes will produce constructs that have improved biochemical content and biomechanical properties toward clinical feasibility when compared to cell types attempted previously with this scaffoldless approach.

3. To improve translatability and functionality of engineered constructs using passaged costal chondrocytes and external stimuli. This aim employs passaged costal chondrocytes in a scaffoldless approach and attempts to improve upon previous results by adding growth factors and applying hydrostatic pressure. First, the passage number (0, 1, 3, and 5) is examined to elucidate the effects of passage on the biochemical and mechanical properties of the costal chondrocyte constructs. Hydrostatic pressure of 10 MPa at 0 Hz and 10 MPa at 1 Hz is applied to passaged costal chondrocyte constructs, and the samples are examined for changes in biomechanical properties and biochemical content. This aim also examines five growth factors: insulin-like growth factor-I, transforming growth factor-β1, epidermal growth factor, platelet-derived growth factor-BB, and basic fibroblast growth factor for their effects on the constructs. The hypotheses of this aim are: 1) higher passage chondrocytes will produce less cartilage-like tissue and more fibrous-like tissue, and 2) external stimuli will improve
the biochemical and biomechanical properties of the passaged costal chondrocyte constructs.

Chapters 1 and 2 review the relevant background of interest for this thesis. Chapter 1 compares the tissue characteristics and tissue engineering design criteria for articular cartilage, the TMJ disc, and tendon. Characteristics of interest include quantitative and qualitative collagen and glycosaminoglycan content, mechanical properties, cellular characteristics. This review also discusses the motivation for tissue engineering and a broad overview of the tissue engineering approaches for each type of tissue. Chapter 2 examines these aspects in greater depth for the TMJ disc.

Chapter 3 encompasses all the work included in Aim 1. Phase 1 of this chapter/aim examines the monolayer gene expression and collagen production of TMJ disc cells exposed to various culture media. In phase 2, TMJ disc cells are seeded on poly-L-lactide scaffolds, and these constructs are examined after 0, 3, and 6 wks for collagen, glycosaminoglycan, and cell quantities, compressive and tensile properties, and extracellular matrix distribution throughout the scaffold.

Chapters 4, 5, and 6 address the goals and hypotheses in Aim 2. Chapter 4 takes a broad look at a variety of cell types for their ability to produce collagen and glycosaminoglycans in scaffoldless constructs. The cell types initially examined in this approach are bone marrow derived mesenchymal stem cells, dermal fibroblasts, articular chondrocytes, costal chondrocytes, passaged costal chondrocytes, and mixtures of dermal fibroblasts with both types of primary chondrocytes. Chapters 5 and 6 examine with greater scrutiny some of the cell groups from chapter 4, with a similar method. The constructs in both these chapters are examined for specific collagen types and mechanical properties, as well as the quantitative biochemical assays used previously. Chapter 5 looks at costal chondrocyte constructs compared to TMJ disc cell constructs, dermal fibroblast constructs, and costal chondrocyte/dermal fibroblast 50/50 cell
mixture constructs. Chapter 6 compares primary costal chondrocytes to primary articular chondrocytes, passaged costal chondrocytes, and passaged articular chondrocytes.

Chapters 7, 8, and 9 examine the passaged costal chondrocyte constructs in greater detail toward the achievement of Aim 3. Chapter 7 examines the biochemical and biomechanical properties of constructs created from costal chondrocytes at passages 0, 1, 3, and 5. In chapter 8, passaged costal chondrocyte constructs are then examined after hydrostatic pressure stimulation. Constructs are exposed to 10 MPa hydrostatic pressure at 0 or 1 Hz and compared to non-loaded and bagged controls. Biomechanical and biochemical quantities are examined immediately following 4 days of pressure (t = 10 days) or after 21 days of total culture. In chapter 9, passaged costal chondrocyte constructs are cultured in media with or without growth factors (insulin-like growth factor-I, transforming growth factor-β1, epidermal growth factor, platelet-derived growth factor-BB, and basic fibroblast growth factor) for 6 wks. After 3 wks and 6 wks, constructs are evaluated for biomechanical properties and biochemical content.
Chapter 1: Design characteristics for TMJ disc tissue engineering: Learning from tendon and articular cartilage

Abstract

Tissue engineering of chondrocytic or fibroblastic musculoskeletal tissues has been relatively well studied compared to that of the temporomandibular joint (TMJ) disc. Early attempts at tissue engineering the disc have been misguided due to a lack of understanding of the composition and function of the TMJ disc. The objective of this review is to compare the TMJ disc to a chondrocytic tissue (hyaline articular cartilage) and a fibroblastic tissue (tendon) to better understand the properties of this fibrocartilaginous tissue. The TMJ disc has 25 times more GAG per dry weight than tendon but half that of articular cartilage. The disc's tensile modulus is 6 times more than cartilage, but orders less than tendon. The GAG content and tensile modulus suggest that the TMJ disc is characterized as a tissue between hyaline cartilage and tendon, but the disc appears more tendon-like when considering its collagen makeup and cell content. Like tendon, the TMJ disc contains primarily collagen type I at 85% per dry weight, while articular cartilage has 30% less collagen, which is type II. Knowledge of quantitative comparisons between joint tissues can give extensive insight into how to better tissue engineer the TMJ disc.

Introduction

Cartilage, tendon, and the temporomandibular joint (TMJ) disc are, for the most part, avascular, aneural, and alymphatic. This makes them ideal candidates for tissue engineering, since a final engineered construct would not need to account for these systems. To engineer a tissue, it is essential to understand the biological and mechanical requirements of that tissue through proper characterization of the original, healthy, adult tissue. These characteristics are essential to establish appropriate success criteria for tissue engineering. While these requirements are reasonably well understood for tissues like hyaline cartilage and tendon, the TMJ disc has only recently undergone extensive characterization. With an appropriate grasp of the disc's properties, we can understand how it compares to other tissues and begin attempts to engineer it.

Perhaps one of the best approaches for TMJ disc tissue engineering, which is largely still in its infancy, is to consider other cartilaginous tissues that have been more widely studied. However, one must keep in mind that the properties of the TMJ disc are drastically different from hyaline articular cartilage and also very different from other fibrocartilages, like the knee meniscus. It is, therefore, potentially advantageous for TMJ disc researchers to consider the necessary characteristics for engineering fibrous tissues, such as tendon or ligament. This review considers both articular cartilage and tendon properties in relation to the TMJ disc. Researchers attempting to engineer a complex tissue like the TMJ disc should benefit from previous engineering work done with other similar tissues. Through a detailed understanding and comparison of various tissues' properties, one can hypothesize on appropriate ways to engineer a tissue through a combination of previous approaches. Examples of this are included in the final discussion of this article.
Motivation for tissue engineering

In addition to cartilage, tendon, and the TMJ disc being ideal for tissue engineering, there is a great demand for replacing these tissues. These tissues are frequently injured or become diseased causing pain and/or difficulty during joint movement. Disease and injury are common in these tissues at all ages, and while they are not life threatening, limited mobility in joints can greatly decrease a patient's quality of life. Patients with joint diseases or injuries are often unable to work or participate in sports and various hobbies without pain.

The most common motivation for tissue engineering articular cartilage is the prevalence of osteoarthritis. In the United States, 9% of the population aged 30 and older has osteoarthritis of either the hip or the knee. Inherent to this problem is cartilage's inability to repair over any extended period of time. This means that most patients are treated for pain and forced to live with unhealthy tissue. Progression of these diseases may eventually lead to a total knee or hip replacement, but both procedures involve invasive surgeries, and complications can occur from these prostheses. Additionally, artificial joints have a limited lifetime of about 10-20 years, making them less than ideal for a population that is living beyond the implant's useful life.

A lack of options for patients with TMJ disorders is also common. Based on epidemiological studies from various populations, it has been reported that 12-57% of the population exhibits patient reported symptoms, and 28-88% have physical signs of a TMJ disorder. In 1999, the U.S. Food and Drug Administration estimated there were 10 million TMJ disorder patients in the United States alone. Additionally, displacement of the TMJ disc is seen in 70% of patients with disorders. Methods for treatment of TMJ disorders, described here briefly, are reviewed in more detail by Wong et al. and Dimitroulis. Proper treatment of disorders is not agreed upon, but usually involves pain medication and possibly physical therapy. In serious TMJ disorders where surgery is considered, there is even less agreement about surgical procedures. Attempts at repositioning and/or reshaping the TMJ disc aim at restoring the disc's native position and function. This surgery in minimally invasion, but the disc lacks the ability to easily repair on its
own, thus these attempts have limitations. When the disc becomes very diseased and becomes a hindrance to jaw movement, patients may opt for a disectomy; however, the absence of a disc may lead to degradation of the mandibular cartilage and even the underlying bone. Various attempts have been made to replace the disc after removal, but synthetic replacements have been very unsuccessful lacking durability and resulting in an immune response. The final option for TMJ surgery is total joint replacement.

Unlike cartilaginous tissues like articular cartilage and the TMJ disc, fibrous tissues do have a limited ability to repair. However, the repair mechanisms are such that the new tissue may be inferior to the original. Jozsa and Kannus give an extensive description of various types of common and rare tendon conditions; these range from sports overuse and repetitive movements in a work environment to drug-induced complications and gunshot injuries. The injuries and diseases described suggest that much of the population may experience some type of tendon complication in a lifetime. When an injured or diseased tendon requires surgical intervention, tissue engineering could become a potential option.

**General tissue characteristics**

**Articular cartilage**

Hyaline articular cartilage is found on the surface of long bones in articulating joints. It is extremely important in the lubrication and load distribution in the joint. The tissue is 60-85% water and cells comprise less than 5% of the mature cartilage volume. Figure 1a shows a schematic of a cartilage section. Articular cartilage is entirely comprised of chondrocytes. Cells in the superficial zone are somewhat flatter than the cells in the middle and deep zone and are aligned in a direction parallel to the surface. Middle cells are more randomly distributed, and deep zone chondrocytes align to some degree with each other in columnar fashion perpendicular to the articulating surface.
**TMJ disc**

The TMJ disc is located superiorly to the condyle of the mandible. Its likely functions are to lubricate the surfaces of motion, absorb shock, stabilize the joint, and support loading of this ginglymo-diarthrodial joint. The posterior and anterior bands of the disc are connected to the mandible via fibrous attachment tissue. The TMJ disc contains both chondrocyte-like cells and fibroblasts. The quantity of fibroblasts exceeds the chondrocyte-like cells in all regions of the disc. Overall, there are approximately 70% fibroblasts and 30% chondrocyte-like cells observed in the porcine TMJ disc.\(^{15}\) Regionally, this ratio of fibroblasts to chondrocyte-like cells increases in the bands and decreases in the intermediate zone.\(^{16}\) The disc is 70% water; it contains a very limited blood supply, mostly in the anterior band.\(^{15,16}\)

**Tendon**

Tendon transmits the force of contraction of a muscle to bone. There are junctions (myotendinous junction or osteotendinous junction) on either end of the tendon's primary tensile region. The junctions, as well as the outer regions of the tendon, have some blood supply, lymphatic drainage, and nerve endings, but the central region of tendon is relatively alymphatic, aneural, and avascular.\(^{13}\) Tendon is around 80% water, and cells comprise 20% of the tissue volume.\(^{17}\) The cells of tendon, termed tenocytes, are entirely fibroblastic in nature. They are not metabolically inert, as was once thought; their primary function in adult tendon is maintenance of the extracellular matrix.\(^{13}\)

**Comparison**

Considering only cellular characteristics, it is already clear that the TMJ disc exhibits cartilage-like and tendon-like characteristics, and is therefore appropriately termed fibrocartilage. It is interesting to note that both tendon and the disc have a limited blood supply. Tendon has some
ability to heal or form scar tissue; similarly the TMJ disc may have some ability to heal or form scar tissue if displacements and injuries are identified early. The ability to heal and form scar tissue may be directly related to the metabolic activity of the cells. The activity of tenocytes, which is greater than was once expected, suggests that TMJ disc cells may also be more metabolically active than chondrocytes. These cellular characteristics could aid attempts to implant all or part of a tissue engineered TMJ disc.

**Animal models**

The use of animal models in medical research is arguably one of the most difficult factors for translating laboratory research into a clinical setting. However, it is necessary to choose a model for its similarities to human as well as its feasibility. The caprine model is used frequently for articular cartilage and tendon research while the porcine model is agreed to be an appropriate model for TMJ research. Complete biochemical and mechanical characterization for these tissues have not been examined in a single species. For this reason, this review contains data from many animal types, which makes quantitative comparison of the tissue properties difficult. While this factor must be kept in mind when comparing the properties, the fundamental differences between these three tissues are still apparent.

**Extracellular Matrix**

**Collagen**

Collagen is the most abundant protein by mass in mammals and arguably the most important extracellular matrix component in biomechanical tissues. It is composed primarily of proline and glycine. Fibrillar collagens, including types I, II, III, V, and XI, have three α-chains that wind around one another to form a helical fiber. Type V is generally associated with type I, and type XI
is associated with type II. While fiber-forming collagen is found in non-load bearing tissues like skin and nerve sheath, tightly packing collagen fibers provide tensile strength in tissues like tendon and ligament. A non-uniform or bimodal distribution of fiber diameters allows for tight, lateral packing. Crimping of collagen fibers also allows for tissue extensibility. Non-fibrillar collagens have functions other than directly supporting mechanical function that include anchoring connective tissue or forming a network like the basal lamina (type IV).

Articular cartilage

The primary collagen type found in hyaline cartilage is type II (Table 1); it is 60% of the dry weight of the tissue. Muir et al. characterized the collagen fibers of human articular cartilage. There are small fibers in all layers, which have a diameter of less than 10 nm. In the superficial layer there are larger fibers that are approximately 34 nm in diameter. The middle zone's fibers are larger yet: between 70 and 100 nm. The deep zone has the largest fibers, which reach 140 nm. The collagen fibers are oriented parallel to the surface in the superficial zone, mostly randomly in the middle zone, and radially in the deep zone. Types I, VI, IX, X, and XI are also present in the extracellular matrix. Type V was concluded to be absent in one study, but more recent work has found it in the extracellular matrix (ECM), pericellular matrix (PCM), and cellular region of rabbit articular cartilage. Type X, while present, is generally considered indicative of cartilage ossification or hypertrophic cartilage.

TMJ disc

Collagen type I is the main component of the TMJ disc, comprising 85% of the dry weight of the tissue. In humans, the collagen forms bundles that are oriented in a ring-like structure around the outer bands of the disc. The anterior band also has a thin layer of fibers on the inferior and superior surfaces, which are oriented in the anteroposterior direction and transition with the predominately mediolaterally oriented fibers of the anterior band. The middle portion of the disc has layers of fibers oriented in numerous directions. Table 1 summarizes the types of collagen
seen in the TMJ disc for various species. Type II is generally seen in trace amounts, although it was not found in primates. Type III has been found in rabbits and humans but not bovine or primates. Gage et al. have researched type III in the attachments of human TMJ discs and found it primarily in discs from patients with TMJ disorders. Researchers have also detected types VI, IX, and XII in the disc. While not much work has looked at the collagen fiber diameter, Detamore et al. found a diameter of 18 ± 9 μm with a range of 2.9 to 37.4 μm in the porcine model.

Tendon

As reviewed elsewhere, tendon is 60-85% collagen by dry weight. There is generally more collagen in tendons that experience strictly tension, such as the Achilles or patellar tendon, than tendons which also experience some compression, like the supraspinatus tendon. Human, Achilles tendon is nearly 100% type I collagen, but other types, including II, III, IV, V, VI, are found in very small quantities. (Table 1). The most prominent of these is type III. Type III may comprise up to 5% of the total collagen in certain types of tendon. Ligament, another fibroblastic tissue similar to tendon, has even more type III collagen (9-12%). Fibrillar collagen is generally oriented longitudinally to support the great tensile stress imparted on the tissue; however, fibers may also form transversely depending on the tissue’s particular function. Evanko and Vogel measured the fiber diameter of adult bovine tendon and found a bimodal distribution. This is consistent with tendon’s tensile nature. The diameter range of these fibrils was 110 nm to 300 nm with a mean of 158 ± 68 nm.

Comparison

Figure 2 shows the percent by dry weight of each of the collagen types in hyaline cartilage, the TMJ disc, and tendon. Based on collagen content, the TMJ disc is clearly more similar to a fibroblastic tissue like tendon with both tissues having a very large content of collagen I. Articular cartilage has a lower overall percent of collagen, and that collagen is almost entirely a different
variety: type II. However, in terms of collagen fiber diameter, the TMJ disc is similar to that of the superficial zone of articular cartilage. Table 1 shows the various collagen types present in each of the three tissues. It appears that type III collagen may be important in both the TMJ disc and tendon, but not articular cartilage. The presence of type V collagen in tendon and type XI collagen in cartilage is consistent with the understanding that those types are associated with types I and II, respectively. It is interesting to note that no researchers have looked into type V collagen in the TMJ disc.

Elastin and other non-collagenous ECM proteins

Elastin is another biomechanically important protein. It aids in tissue recovery after non-failure loading primarily in tension, and since elastin is not as stiff as collagen, it exhibits larger strains during loading.48 Both the cross-linking of tropoelastin molecules and hydrophobic regions of elastin contribute to its ability to recoil. Other notable proteins include fibronectin and laminin, which bind to cell surface receptors. Since fibronectin connects integrins to collagen or heparin, it is important in mechanotransduction, by allowing the transmission of mechanical signals from the environment to the cell. Laminin is an essential glycoprotein of the basal lamina that binds integrins to proteoglycans, such as perlecan or nidogen. Cartilage oligomeric matrix protein (COMP) is another protein of interest in joint tissues. The function of COMP is not entirely understood; however, after its discovery and naming, COMP has been found in other tissues including tendon, ligament, and the knee meniscus.49 It is hypothesized to perform some function in tissue genesis.50,51

Articular cartilage

To our knowledge, elastin has not been detected in articular cartilage.32,52 This fact is understood by considering a relative lack of tensile stress experienced by articular cartilage. The proteoglycan aggrecan (discussed later) imparts much of the compressive stiffness that is necessary for the cartilage’s viscoelastic function under compression. Fibronectin is present in
normal cartilage but increases greatly during a degenerative state.\textsuperscript{53,54} Laminin is present in human adult articular chondrocytes' pericellular matrix but only in the upper layers and not the deep zone.\textsuperscript{56} COMP is seen throughout cartilage ECM and around the chondrocytes in the bovine model.\textsuperscript{50}

\textbf{TMJ disc}

Elastin is present in all regions of the TMJ disc and its posterior attachments.\textsuperscript{16,39,56,57} In the primate disc, elastin is aligned with collagen fibers in the middle zone.\textsuperscript{56} Minarelli and Liberti\textsuperscript{39} found elastin fibers parallel to the collagen fibers in the intermediate zone, anterior, and posterior bands of the human disc. Additionally, numerous elastic fibers were seen in immature discs that decreased in adult discs. Immunostaining of the porcine disc shows more numerous elastin fibers in the posterior band.\textsuperscript{16} Fibronectin immunostained moderate to intense in the primate TMJ disc, but no laminin was detected.\textsuperscript{37} To our knowledge no one has studied the presence of COMP in the TMJ disc.

\textbf{Tendon}

Elastin comprises about 2\% of the dry weight of tendon.\textsuperscript{43} Fibronectin and laminin are not seen in the normal connective tissue. However, they are both found in the myotendineal junction and vascular walls.\textsuperscript{58} Fibronectin also forms on a tear or ruptured tendon.\textsuperscript{58} Despite its name, COMP was also found in numerous species' tendons including human\textsuperscript{59}, equine\textsuperscript{51}, and canine\textsuperscript{49}, but not bovine.\textsuperscript{50}

\textbf{Comparison}

The presence of elastin in the TMJ disc and tendon and its absence in cartilage, again suggests that the TMJ disc is more like tendon than cartilage. It is an essential protein for these tissues as both tissues function under tensile elastic strain and recovery. This is in contrast to cartilage,
which primarily resists compressive stress. The presence of COMP in cartilage and most tendon indicates it may be present in the TMJ disc, although no research has confirmed this. Studying the presence of the COMP gene or protein in the TMJ disc could improve the understanding of the phenotype of TMJ disc cells.

Glycosaminoglycans

Glycosaminoglycans (GAGs) are polysaccharides having an amino sugar and a uronic acid as the repeating disaccharide unit. An important characteristic of these molecules is their negative charge. The types of GAGs that this paper will investigate are hyaluronan (HA), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), and heparin or heparan sulfate (HS). The distinction between these groups comes from the specific sugars that exist in their structure.

Articular cartilage

Articular cartilage is 10% GAG by dry weight. HA is not found in immature tissue, but is 7% of the total GAG in mature cows and 5% of total in mature dogs. CS is the most abundant GAG in articular cartilage. In terms of total GAG in human articular cartilage, Mankin and Lippiello report the percentage of chondroitin-6-sulfate (C-6-S) and chondroitin-4-sulfate (C-4-S) as 42.5±6.1% and 5.7±2.5%, respectively. However, these numbers are likely somewhat overestimated as the researchers calculated the percentages without considering HA. This is also true of their reported KS percentages, which state KS as 51.7±4.5% of total GAG. Tomiosso et al. did not detect any DS or HS in their investigation of ostrich cartilage.

TMJ disc

Total GAG per dry weight of TMJ disc tissue is around 5%. HA was reported between 2.8% and 10% of the total GAG. As with articular cartilage, CS is also the most abundant of all GAGs in the TMJ disc at 70-80% of total GAG and is more abundant in the medial region of
the anterior band.\textsuperscript{16} CS from bovine tissue can further be divided quantitatively into 75\% C-6-S and 21\% C-4-S with the remaining found to be unsulfated.\textsuperscript{35} DS comprises 15-25\% of the total GAG\textsuperscript{35,65,67}, while the KS content ranges from trace amounts\textsuperscript{16,65,67} up 2\%.\textsuperscript{35} HS was measured as 3.8\% in the anterior portion and 4.8\% in the posterior band of the human disc.\textsuperscript{64}

**Tendon**

A typical tendon (i.e., bicep) has 0.1-0.3\% GAG by dry weight.\textsuperscript{68,69} A fibrocartilaginous tendon like the supraspinatus tendon of the rotator cuff 1.23\% GAG in humans.\textsuperscript{69} Large quantities of HA are found in some regions on the surface of the flexor tendon of the bovine model, but HA is present in only small quantities in the central region.\textsuperscript{46} HA accounts for around 2\% of the total GAG.\textsuperscript{70} For the bicep tendon, CS is only 6\% of the total GAG, but in the supraspinatus tendon CS is 56\% of the total GAG. This vast difference reverses for DS and KS. DS is 67\% of the GAG in the bicep tendon and 20\% of the GAG for the supraspinatus tendon. KS is 33\% of the total GAG in the bicep tendon, but only 3\% of the GAG in the supraspinatus tendon.\textsuperscript{69} HS is found only in the myotendinous junction and not in the tensile region of human tendon.\textsuperscript{13}

**Comparison**

Total GAG for each of the tissues is shown in Fig. 3. The tendon data are for the typical bicep tendon and not a fibrocartilaginous tendon. Here we quantitatively see the TMJ disc falling in the center of the GAG spectrum between cartilage and tendon. Table 2 illustrates the types and amounts of GAGs in each tissue according to each researcher.

**Proteoglycans**

Proteoglycans (PGs) are glycoproteins that bind to glycosaminoglycans. The structure and function of PGs vary greatly, but they all have at least one core protein bound to a link protein bound to at least one GAG. Proteoglycans can be very large (like aggrecan at 10-30 MDa) or
very small (like decorin at 70 kDa). When GAGs aggregate into a large PG, their negative charge makes them very hydrophilic; therefore, they can serve to hydrate and lubricate the tissue. Other functions of PGs include aiding in the alignment and size of collagen fibrils. There are many PGs that have been characterized and still more to be discovered. This paper will focus on just a few based on the tissue-specific interest.

**Articular cartilage**

In cartilage, PGs make up 4-7% of the wet weight, of which 50-85% are large-aggregating PGs.¹⁴ Large-aggregating PGs include aggrecan and versican, which may bind to an HA backbone. Aggrecan is a fundamental component of cartilage composition, while versican is mostly seen in cardiovascular tissues. Aggrecan is either a CSPG or KSPG. In cartilage, the size and hydrophilic nature of aggrecan allows for its compressive resistance (as mentioned earlier) and lubrication of the joint. Decorin and biglycan are also found in human articular cartilage, although biglycan is seen mostly in the PCM.⁷¹ Decorin and biglycan are small leucine-rich, dermatan sulfate PGs that have only one or two GAG chains attached to their core. Decorin binds to collagen and controls collagen's fiber size and lateral packing capacity. Cartilage may contain the KSPG fibromodulin based on work done with ostrich articular cartilage.⁶³ Fibromodulin is another small leucine-rich PG, which is important in collagen fiber orientation.

**TMJ disc**

Aggrecan is present in the TMJ disc and comprises 31.2% of the total PGs.⁷² Mizoguchi et al.⁷³ observed aggrecan mostly in the outer bands. Biglycan and decorin are present at 39.5% and 29.3% of total PGs, respectively.⁷² Mizoguchi et al.⁷³ also found biglycan throughout the murine TMJ disc but mostly in the posterior band. Decorin is significantly more abundant in the intermediate zones than the bands, and immunostains most strongly in the disc attachments.¹⁶,⁷³
Tendon

While many more types of PGs have been characterized in tendon, most are associated with the endothelial walls of the capillaries. PGs that are present and relevant to tendon's biomechanical function are decorin, biglycan, and aggrecan.\textsuperscript{13,43,46,68,69,74} Tendon may also contain fibromodulin.\textsuperscript{43,68} Vogel and Peters\textsuperscript{46} found decorin to be strongly present in bovine tendon while biglycan is only located around the cells and on the surface of flexor tendon tissue. Berenson et al.\textsuperscript{68} concluded from their study on various types of human tendon that the amount of PGs and GAGs depends on the mechanical load required of the tissue. In compressed tendon (like in the human rotator cuff where it wraps around the bone), large aggregating PGs are found, whereas strictly tensile tendons contain mostly small PGs like decorin.

Comparison

Following up on Berenson et al.'s\textsuperscript{68} conclusion regarding the content and type of PGs in various tendons, one can speculate about the PG-function relationships in other biomechanical tissues, such as the TMJ disc and cartilage. Because of aggrecan's ability to resist compression, it is immensely important to tissues like cartilage or regions of other tissues that must sustain compressive loading. It is probable that mechanotransduction of various types of cells in response to compression will lead to aggrecan production. Similarly, decorin and biglycan, which are important in tensile tissues, may be produced when cells experience a certain amount of tensile strain.

Mechanical properties

When engineering the tissues considered within this review, mechanical properties are equally important to the biological properties of the engineered tissue. If constructs cannot withstand the mechanical load necessary for native tissue function, then they are of no significant use in clinical applications. While these tissues can experience many types of stresses, including shear and
plowing, the properties most relevant to the initial functional validation of an engineered construct are tensile and compressive.

**Tensile properties**

**Articular cartilage**

Mow *et al.*\(^{14}\) reviewed the elastic modulus of cartilage and stated it is 13.2 MPa, noting that this value varies greatly depending on the joint origin or region within a specific joint, orientation of testing, and cartilaginous zone tested. Akizuki *et al.*\(^{75}\) determined that in most zones the low weight bearing area had a higher tensile modulus than a high weight bearing area. Huang *et al.*\(^{76}\) tested both the femoral head and glenohumeral cartilage from humans and found the elastic modulus of the femoral head to be significantly greater than that of glenohumeral cartilage at 4.23 and 2.24 MPa, respectively. These researchers also concluded the superficial zone of cartilage is significantly stiffer than the middle zone in femoral head cartilage. Additionally, they found significantly higher moduli from samples taken parallel to the cartilage split lines than those perpendicular to the split lines. Roth and Mow\(^{77}\) studied the failure stress of cartilage; it failed in the range of 1-25 MPa with a lateral strain range of 0.03-0.7 and longitudinal strain range of 0.06-2.5.

**TMJ disc**

Beatty *et al.*\(^{76}\) determined that the tensile modulus of the porcine TMJ disc in the mediolateral direction was 3.2 MPa and the ultimate tensile strength (UTS) was 1.6 MPa. In the anteroposterior direction, the modulus was 76.4 MPa and UTS was 37.4 MPa. The values for the moduli increased after the disc was impulsively loaded.\(^{78}\) Detamore and Athanasiou\(^{79}\) noted the same differences between the properties of the two directions. They found the modulus of the porcine disc in the mediolateral direction was 9.48 MPa in the anterior band, 0.58 MPa in the intermediate zone, and 23.4 MPa in the posterior band. The posterior band was significantly
stiffer than the anterior band, and both bands were significantly stiffer than the intermediate zone. When the modulus was measured in the anteroposterior direction, only the central region was significantly larger than the lateral region at 18.5 and 10.6 MPa, respectively; the medial region was 14.3 MPa. The UTS of the porcine disc in the mediolateral and anteroposterior directions were approximately 2.02 and 2.62 MPa, respectively, when averaged between regions. Tanne et al.\textsuperscript{80} also tested various regions of the canine disc anteroposteriorly, and found that the middle region had a significantly higher modulus than the lateral band. The modulus of the medial region fell between these two values but was not significant from either one. Tanaka et al.\textsuperscript{81} tested the human disc in only the anteroposterior directions and found an instantaneous elastic modulus of 44.0 ± 9.1 MPa at a 0-2\% strain and 95.7 ± 17.7 MPa at a 2-4\% strain. They also observed a relaxed modulus of 29.9 ± 7.5 MPa at the lower strain, which did not change significantly with the higher strain.

**Tendon**

Tendon is usually tested along the direction of its loading. General convention is to test the entire tendon, and while the TMJ disc and cartilage researchers can only test a section of the tissue, it is still of interest to compare them to the tendon data. In fact, whole tendon may be less stiff than a smaller section of tissue.\textsuperscript{82} Tensile tests of whole human patellar tendon yield moduli of 504 and 660 MPa for humans of different ages.\textsuperscript{83} Rabbit patellar tendon moduli were reported between 955 and 1855 MPa.\textsuperscript{84} Human supraspinatus tendon is less stiff than patellar tendon having an average modulus of 86 MPa.\textsuperscript{85}

**Comparison**

Figure 4 shows the average mechanical properties of each tissue. The TMJ data in the graph were averaged from the relaxed moduli in the anteroposterior direction. It is once again apparent that the TMJ disc, being both fibrous and cartilaginous in nature, exhibits tensile properties that fall in between those of articular cartilage and tendon. Table 3 illustrates, in more detail, tensile
characteristics of each tissue. Comparing just the relaxed moduli of cartilage and the TMJ disc, it is clear that the TMJ disc has a higher relaxed modulus, particularly when compared to that of bovine cartilage. This may be indicative of a tensile load experienced during mastication. Comparing just the elastic moduli of the TMJ disc and tendon shows that tendon has a higher modulus than the disc, again indicative of tendon's more intense in vivo tensile loading. However, the ranges of elastic moduli are not very different from one another.

Compressive properties

Articular cartilage

Articular cartilage, like all soft tissues, is viscoelastic. Its compressive function makes these properties extremely important when attempting to engineer cartilage. Modeling of cartilage using the biphasic theory gives us an idea of its viscoelastic properties. Athanasiou et al. performed biphasic theory-based testing on cartilage from numerous species under creep indentation and found an average aggregate modulus of 0.85 MPa, a Poisson's ratio of 0.22, and permeability of $2.88 \times 10^{-15}$ m$^4$/Ns. Other researchers found similar results with their animal models. Aggregate modulus is lowest at the superficial layer and increases significantly with deeper layers of cartilage.

TMJ disc

The biphasic theory has also been employed to characterize the porcine TMJ disc. The average aggregate modulus was found to be 20.1 kPa, Poisson's ratio was 0.45, and permeability was $24.1 \times 10^{-15}$ m$^4$/Ns. Another group measured the compressive properties of the canine TMJ disc with unconfined compression and determined an elastic modulus from a linearly elastic model. The moduli were 30.9 and 15.8 MPa at 0 and 2 minutes (where the disc is allowed to relax during that time), respectively. Allen and Athanasiou measured the regional compressive properties of the porcine TMJ disc under unconfined compression, stress relaxation conditions.
Instantaneous moduli and relaxation moduli ranged from 90-3870 kPa and 16.9-74.6 kPa, increasing with applied strain. The coefficient of viscosity was between 1.3 and 13.8 MPa*s. The two methods used on the porcine disc give reasonably similar stiffness results, but the testing on the canine disc yielded stiffness results orders of magnitude higher. Comparing these to elastic, compressive moduli of human discs (211 kPa and 514 kPa, depending on the strain rate) suggests that the porcine model may be an appropriate model for human tissue engineering.\textsuperscript{94}

\textbf{Tendon}

It is not surprising that relatively little research has been done regarding the compressive properties of tendon, since compression occurs rarely in most tendinous tissues. It should be noted, however, that there are parts of tendons like the supraspinatus tendon that do experience compression. Lee \textit{et al.}\textsuperscript{95} performed indentation on the supraspinatus tendon from humans but still used a substantially different method than those presented previously. This testing was probably based on confined compression, though this aspect was not taken into consideration when analyzing the results. This group later compared these results to a finite element model and concluded an initial compressive modulus of 0.024 to 0.09 MPa that increased with increasing compressive stress.\textsuperscript{96} It is unlikely that other types of tendon, which perform a purely tensile function, would have as large a modulus.

\textbf{Comparison}

Table 4 shows the various compressive moduli for each tissue. Again, it is difficult to compare these results because of different testing modalities used, but we can look at articular cartilage and tendon individually and compare them to the TMJ disc. Comparing the aggregate moduli of cartilage and the TMJ disc from biphasic modeling shows the disc has a substantially lower modulus than cartilage. This is not surprising based on the tissues' functions. Looking at the elastic moduli of supraspinatus tendon and the TMJ disc from indentation testing, we see that the values are close suggesting that they may be similar tissues and are appropriately characterized
as fibrocartilaginous. However, we must recall that the data for tendon were from a specific tendon, which experiences compression. The TMJ disc and supraspinatus tendon experience a combination of tension and compression, but this is not true of most tendinous tissue, which experiences tension almost exclusively.

**Tissue engineering**

Performing a search in the United States National Library of Medicine's National Center for Biotechnology Information PubMed database gives revealing information regarding tissue engineering studies on these joint tissues. Figure 6 shows the number of hits for original articles for “TMJ disc,” “articular cartilage,” and “tendon” with “tissue engineering.” Clearly, the TMJ disc and even tendon have received relatively little attention compared to cartilage. It is difficult to imagine that an area like the TMJ disc has been largely ignored in tissue engineering despite an incredible number of people affected by TMJ disorders. Tissue engineering research in the area of mechanical tissues focuses on cell source, scaffold selection, mechanical stimulation, and biochemical environment optimization.

**Articular cartilage**

Clearly, there is too much research in articular cartilage tissue engineering to cover in this review; so only some recent and promising developments are mentioned. Developments in cell source primarily come from research in mesenchymal stem cells. Numerous groups are considering differentiated progenitor cells as a viable cell source. Several interesting improvements have also occurred with scaffold choice research. Chang et al. used a gelatin/chondroitin-6-sulfate/hyaluronan tri-copolymer scaffold to replace full thickness defects in a porcine model. In our group, Hu and Athanasiou have developed a scaffoldless method for *in vitro* cartilage tissue engineering, which results in tissue with clinically-relevant dimensions and 1/3 the mechanical properties of native tissue.
TMJ disc

Tissue engineering of the TMJ disc is still in its infancy. The research so far has focused on culture techniques, scaffold choice, and mechanical stimulation. Initially, researchers used cells from the cartilage of the mandible and generally looked for collagen type II production as their success criterion.\textsuperscript{106,106} Another group seeded the cells on polyglycolic acid (PGA) and polylactic acid fibers.\textsuperscript{107} Springer \textit{et al.}\textsuperscript{108} studied cells from TMJ cartilage on various scaffolds: polyamide, expanded polytetrafluoroethylene, PGA, and bone mineral blocks. In these studies mechanical properties and GAG content of the constructs were not considered. In our group, it was found that seeding cells in a spinner flask with the highest concentration of cells and 25 µg/mL of ascorbic acid lead to better results than other techniques.\textsuperscript{109-111} Furthermore, the use of a rotating wall bioreactor was not found to be beneficial for tissue engineering the TMJ disc.\textsuperscript{112} When examining various growth factors, the greatest success in promoting collagen synthesis was found with insulin-like growth factor and transforming growth factor-β1, while platelet derived growth factor and basic fibroblast growth factor increased GAG production.\textsuperscript{113-115} Almarza and Athanasiou\textsuperscript{116} studied intermittent and constant hydrostatic pressure on PGA constructs seeded with TMJ disc cells. They found that constant stimulation improved collagen production in the constructs.

Tendon

Similar to cartilage, there is extensive research in many areas of tendon tissue engineering; so this article will focus on some recent and interesting tissue engineering attempts. Cell source is also of great interest in this area and numerous researchers are looking at various progenitor cells to differentiate into fibroblasts.\textsuperscript{117-122} Many researchers use collagen gels as their tissue engineering scaffold.\textsuperscript{119-121} However, recent attempts have found success with polydioxanone scaffolds\textsuperscript{123}, alginate-based chitosan hybrid polymer\textsuperscript{124}, and chitosan-based hyaluronan hybrid scaffolds.\textsuperscript{117} Fang and Yang\textsuperscript{125} studied the culture of tendon cells on human hair, carbon fibers, PGA, human hair and PGA, and carbon fibers and PGA. They found the best mechanical
properties with the human hair followed by the carbon fibers. PGA did not display acceptable mechanical strength. Calve et al.\textsuperscript{128} have also developed a method for scaffoldless tissue engineering of tendon with a 60\% formation success rate and mechanical properties similar to other tissue engineered tendon constructs. Hankemeier et al.\textsuperscript{122} found an increase in cell proliferation, collagen type I, collagen type III, and alpha-smooth muscle actin expression from a low-dose (3\textmu g/mL) of fibroblast growth factor-2 on bone marrow cells. Altman et al.\textsuperscript{121} used simultaneous tension and torsion on progenitor cell seeded collagen gels to upregulate collagen I, collagen II and tenascin-C for ligament tissue engineering.

\textbf{Discussion}

The tissue characteristics compared in this review clearly illustrate the extent to which the TMJ disc is both chondrocytic and fibroblastic. The cell population of the TMJ disc is mixed, containing both chondrocyte-like cells and fibroblast-like cells. The disc has some vascularity and limited healing potential, which is also true for tendon but not for articular cartilage, which is avascular and has no ability to heal. The TMJ disc’s ECM is somewhat more fibroblastic than chondrocytic; the total collagen content of the TMJ disc and tendon are very similar in both quantity and collagen type. Articular cartilage has less collagen, and it is a different type (type II not I). Both tendon and the TMJ disc contain elastin and similar GAGs and PGs. However, the overall GAG content of the disc is between that of tendon and articular cartilage. In terms of mechanical properties, the TMJ disc is again in between fibroblastic and chondrocytic tissue. While cartilage is primarily a compressive tissue and tendon is a tensile tissue, the TMJ disc has mechanical integrity for both compression and tension.

It is evident that \textit{in vivo} mechanical forces affect the biochemical and mechanical properties of biomechanical tissues. Based on the characteristics presented here and in previous publications, Fig. 7 illustrates the spectrum of structure-function characteristics of joint tissues in terms of
mechanical properties and ECM. Articular cartilage is on the left and represents the tissues with the highest compressive properties, also experiencing the most compression during loading. It has the most GAG and collagen type II, but lowest overall collagen by dry weight. Tensile tendons like Achilles or biceps are on the right side of the spectrum, representing the tissues that experience the most tension in vivo and can withstand the most tensile stress. These tissues also contain the most collagen I and least amount of GAG.

It is with this spectrum in mind that research should continue in the area of tissue engineering of joint tissues. Researchers must not ignore what has been accomplished in tissues similar to their tissue of interest. This is particularly true for tissue engineering the TMJ disc. While little research has been conducted in this area beyond culturing techniques, research in that field could receive a jump-start by considering tissues similar to it, particularly the tendons that experience compression, such as the supraspinatus tendon of the rotator cuff. Furthermore, tissue engineering approaches for the TMJ disc may benefit from considering methods between those that have been successful for tendon and those for articular cartilage. For example, tendon tissue engineering has used tension bioreactors to improve construct properties, while cartilage tissue engineering has applied compression to improve the constructs’ properties. Combining these approaches into a tension/compression bioreactor—applying a central compressive stress as well as a hoop stress, which has compressive and tensile stress components may lead to a very successful TMJ disc tissue engineered construct. Cell source is another important area of future research for TMJ disc tissue engineering. As discussed previously, researchers in both cartilage and tendon tissue engineering have recently examined progenitor cells in their approaches. This may be a more clinically relevant cell source for TMJ disc tissue engineering as well. Differentiation methods from tendon and cartilage tissue engineering may be combined to provide an ideal fibrochondrocyte-like phenotype for TMJ disc tissue engineering. Additionally, a more appropriate cell source may come from a co-culture of fibrous and cartilaginous cells. These are just a few examples of how researchers of complex tissues like the TMJ disc may benefit from the previous work of more fundamental tissues to create a functional tissue engineered construct.
<table>
<thead>
<tr>
<th>Tissue</th>
<th>Reference</th>
<th>Species</th>
<th>Chief collagen type</th>
<th>Other present types</th>
<th>Types not found</th>
</tr>
</thead>
<tbody>
<tr>
<td>Articular Cartilage</td>
<td>Mow et al. 14 ᵃ</td>
<td>Human</td>
<td>II</td>
<td>VI, IX, XI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Naumann et al. 32</td>
<td>Rabbit</td>
<td>II</td>
<td>I, V, VI, X</td>
<td></td>
</tr>
<tr>
<td>TMJ disc</td>
<td>Landesberg et al. 40</td>
<td>Bovine</td>
<td></td>
<td>II, IX, XII</td>
<td>III, X</td>
</tr>
<tr>
<td></td>
<td>Minarelli et al. 39</td>
<td>Human</td>
<td>I</td>
<td></td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Ali &amp; Sharawy 38</td>
<td>Rabbit</td>
<td>I</td>
<td>III, VI, IX</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Detamore et al. 16</td>
<td>Porcine</td>
<td>I</td>
<td></td>
<td>II</td>
</tr>
<tr>
<td>Tendon</td>
<td>Amiel et al. 42</td>
<td>Rabbit</td>
<td>I</td>
<td></td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Hanson &amp; Bentley 127</td>
<td>Human</td>
<td>I</td>
<td></td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>Jozsa &amp; Kannus 13 ᵃ</td>
<td>Human</td>
<td>I</td>
<td>II, III, IV, V</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vogel &amp; Peters 48</td>
<td>Bovine</td>
<td>I</td>
<td></td>
<td>VI</td>
</tr>
</tbody>
</table>

ᵃ symbol indicates that reference is a review.
Table 1-2: Quantitative GAG data for various animal models used in articular cartilage, TMJ disc, and tendon characterization

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Reference</th>
<th>Species</th>
<th>Total GAG (% of dry weight)</th>
<th>HA (% of total GAG)</th>
<th>CS (% of total GAG)</th>
<th>DS (% of total GAG)</th>
<th>KS (% of total GAG)</th>
<th>HS (% of total GAG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Articular Cartilage</td>
<td>McDevitt, ** 47, 48</td>
<td>multiple</td>
<td>10</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Manicourt &amp; Pita</td>
<td>canine</td>
<td>N/A</td>
<td>5</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Murata &amp; Bjelle40</td>
<td>bovine</td>
<td>N/A</td>
<td>6.76^3</td>
<td>9.51^3</td>
<td>N/A</td>
<td>10.3^3</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Mankin &amp; Lippillo56</td>
<td>human</td>
<td>15</td>
<td>N/A</td>
<td>48.1</td>
<td>N/A</td>
<td>51.7</td>
<td>N/A</td>
</tr>
<tr>
<td>TMJ disc</td>
<td>Kobayashi165</td>
<td>Human</td>
<td>N/A</td>
<td>5.6</td>
<td>69.9</td>
<td>24.5</td>
<td>trace</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Kobayashi165</td>
<td>Porcine</td>
<td>N/A</td>
<td>2.8</td>
<td>5.8</td>
<td>91.4</td>
<td>trace</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Axelsson et al.164</td>
<td>human</td>
<td>5.8^3</td>
<td>10</td>
<td>84.7^3</td>
<td>trace</td>
<td>4.3</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Nakano &amp; Scott57</td>
<td>Bovine</td>
<td>7^2</td>
<td>5.3^9</td>
<td>70.6^9</td>
<td>15.2^9</td>
<td>8.8^9</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Nakano &amp; Scott55</td>
<td>Bovine</td>
<td>5</td>
<td>5</td>
<td>79</td>
<td>14</td>
<td>2</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Detamore et al.16</td>
<td>Porcine</td>
<td>5.3</td>
<td>trace</td>
<td>74</td>
<td>16.8^9</td>
<td>9</td>
<td>N/A</td>
</tr>
<tr>
<td>Tendon</td>
<td>Riley et al.69</td>
<td>Human</td>
<td>1.23</td>
<td>9.3</td>
<td>56</td>
<td>20</td>
<td>3</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Riley et al.69</td>
<td>Human</td>
<td>0.12</td>
<td>10.8</td>
<td>7</td>
<td>29</td>
<td>3</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Berenson et al.88</td>
<td>Human</td>
<td>0.22^3</td>
<td>44^4</td>
<td>56^5</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Berenson et al.88</td>
<td>Bovine</td>
<td>0.178^9</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Jozsa &amp; Kannus13, 10</td>
<td>Human</td>
<td>0.2</td>
<td>6</td>
<td>34</td>
<td>60</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

\* symbol indicates that reference is a review.

* from wet weight

\$ averaged from various regions

# Amount of DSPGI (decorin), not total DS

BT-Bicep tendon

RC-rotator cuff tendons
### Table 1-3: Tensile moduli for articular cartilage, the TMJ disc, and tendon

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Reference</th>
<th>Species</th>
<th>Tensile Modulus (MPa)</th>
<th>Direction</th>
<th>Relaxed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Articular Cartilage</td>
<td>Akizuki et al.(^5)</td>
<td>Human</td>
<td>1-21</td>
<td>Par.</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Huang et al.(^6)</td>
<td>Human</td>
<td>2-37</td>
<td>Par.</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Woo et al.(^6)</td>
<td>Bovine</td>
<td>2-2.6</td>
<td>Par.</td>
<td>Yes</td>
</tr>
<tr>
<td>TMJ disc</td>
<td>Beatty et al.(^7)</td>
<td>Porcine</td>
<td>76.4</td>
<td>AP</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.2</td>
<td>ML</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Detamore and Athanasiou(^7)</td>
<td>Porcine</td>
<td>11-19</td>
<td>AP</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Tanne et al.(^8)</td>
<td>Canine</td>
<td>40-101</td>
<td>AP</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Tanaka et al.(^8)</td>
<td>Human</td>
<td>19-73</td>
<td>AP</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>27-118</td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>Tendon</td>
<td>Johnson et al.(^9)</td>
<td>Human PT</td>
<td>504-660</td>
<td>Par.</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Danto &amp; Woo(^4)</td>
<td>Lapine PT</td>
<td>955-1855</td>
<td>Par.</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Itoi et al.(^5)</td>
<td>Human ST</td>
<td>40-180</td>
<td>Par.</td>
<td>No</td>
</tr>
</tbody>
</table>

Par.-parallel to collagen alignment; AP- anteroposterior, ML- mediolateral

Yes indicates stress relaxation

No means the modulus is taken from the slope of stress-strain curve

PT-Patellar tendon

ST-supraspinatus tendon
Table 1-4: Compressive moduli for articular cartilage, the TMJ disc, and tendon

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Reference</th>
<th>Species</th>
<th>Compressive Modulus (MPa)</th>
<th>Method</th>
<th>Relaxed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Articular Cartilage</td>
<td>Athanasiou et al.</td>
<td>Multiple</td>
<td>0.3-1.4</td>
<td>I,B</td>
<td>Yes, creep</td>
</tr>
<tr>
<td></td>
<td>Athanasiou et al. [87]</td>
<td>Multiple</td>
<td>0.5-1</td>
<td>I,B</td>
<td>Yes, creep</td>
</tr>
<tr>
<td></td>
<td>Schinagl et al.</td>
<td>Bovine</td>
<td>0.08-1.4</td>
<td>C</td>
<td>Yes, SR</td>
</tr>
<tr>
<td></td>
<td>Naumann et al.</td>
<td>Lapine</td>
<td>0.5</td>
<td>I,B</td>
<td>Yes, creep</td>
</tr>
<tr>
<td></td>
<td>Huang et al.</td>
<td>Human</td>
<td>0.1-0.8</td>
<td>C</td>
<td>Yes, SR</td>
</tr>
<tr>
<td>TMJ disc</td>
<td>Kim et al.</td>
<td>Porcine</td>
<td>0.016-0.029</td>
<td>I,B</td>
<td>Yes, creep</td>
</tr>
<tr>
<td></td>
<td>Tanaka et al.</td>
<td>Canine</td>
<td>31</td>
<td>U</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Chin et al.</td>
<td>Human</td>
<td>0.211-0.514</td>
<td>U</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Allen &amp; Athanasiou</td>
<td>Porcine</td>
<td>0.08-3.9</td>
<td>U</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.017-0.075</td>
<td></td>
<td>Yes-SR</td>
</tr>
<tr>
<td>Tendon</td>
<td>Zobitz et al.</td>
<td>Human ST</td>
<td>0.024-0.09</td>
<td>I</td>
<td>No</td>
</tr>
</tbody>
</table>

I-indentation, B-Biphasic, C-confined, U-unconfined, SR-stress relaxation

ST-supraspinatus tendon
**Figure 1-1: Schematic diagrams and histology for articular cartilage, the TMJ disc, and tendon**

Figure 1 Schematic diagrams representing tissue sections of a) articular cartilage, b) the TMJ disc, and c) tendon and hematoxylin and eosin stained tissue sections of d) bovine articular cartilage, e) a porcine TMJ disc, and f) bovine tendon. Dotted lines in the schematics suggest the various zone distinctions in the tissue, which possess different characteristics. In articular cartilage, these zones are the superficial middle and deep zone. The superficial zone has flatter cells that align, like the collagen fibers, with the articulating surface. Cells in the middle and deep zone are rounder. Deep zone cells form columns perpendicular to the surface, while middle cells are more randomly oriented. In the TMJ disc, the dotted line represents a transition between the intermediate zone and the bands of the disc. Cells in the bands align circumferentially along with the collagen fibers, while the intermediate zone cells and collagen fibers are more randomly aligned. The tendon tissue schematic shows only the central region, which would experience tension in the horizontal direction. The collagen fibers and fibroblasts align parallel to that force.
Figure 1-2: Collagen quantities for articular cartilage, the TMJ disc, and Achilles tendon

![Graph showing collagen percentages for different tissues.]

Figure 2: Collagen quantities for articular cartilage, the TMJ disc, and Achilles tendon. Data for this graph was used from McDevitt, Nakano and Scott, and Amiel et al. for cartilage, the TMJ disc, and tendon, respectively.
Figure 1-3: Total GAG percent by dry weight for articular cartilage, the TMJ disc, and bicep tendon.

Figure 3 Total GAG percent by dry weight for articular cartilage, the TMJ disc, and bicep tendon. These data were averaged from the literature.
Figure 1-4: Tensile modulus for articular cartilage, the TMJ disc, and tendon

![Graph showing tensile modulus for articular cartilage, the TMJ disc, and tendon.](image)

**Figure 4** Tensile modulus for articular cartilage, the TMJ disc, and tendon. Cartilage data is relaxed modulus and averaged from Akizuki et al.\(^76\) and Huang et al.\(^78\) TMJ disc data is averaged relaxed modulus in the anteroposterior direction from Beatty et al.\(^78\) and Detamore and Athanasiou.\(^79\) Tendon data is the elastic modulus averaged from Johnson et al.,\(^83\) Danto and Woo,\(^84\) and Itoi et al.\(^85\)
Figure 1-5: Tissue engineering attempts for articular cartilage, the TMJ disc, and tendon

Figure 5 Number of original articles from searching Pubmed database for “cartilage tissue engineering,” “tendon tissue engineering,” and “TMJ disc tissue engineering.”
Figure 1-6: Spectrum of structure-function characteristics of various soft joint tissues

Figure 6 Spectrum of structure-function characteristics of various soft joint tissues. Abbreviations not defined previously are articular cartilage (AC), knee meniscus (KM), rotator cuff tendon (RCT), ligament (L), and strictly tensile tendon (T). The top arrow shows the range of ECM characteristics, specifically percent collagen and percent GAG by dry weight. The bottom spectrum gives tensile and compressive moduli for each tissue. The tensile moduli are from stress relaxation data except for tendon and ligament data, and the compressive moduli are from unconfined indentation data using biphasic modeling. Articular cartilage is placed farthest left, representing the most compressive with the highest GAG content tissue. Farthest right are the most tensile and highest collagen content tissues. Ligament ECM data were taken from Amiel et al.,44 ligament and tendon tensile data were averaged from Danto and Woo,64 and knee meniscus data were taken from Almarza and Athanasiou.4 Other tissue data were referenced previously in this review. Data are shown to scale with the exception of tendon and ligament in the tensile modulus spectrum.
Chapter 2: Is tissue engineering of the TMJ disc a feasible process?

Abstract

Temporomandibular joint (TMJ) disorders are common and difficult to remedy. Tissue engineering is one alternative that seeks to improve TMJ surgical treatment options. Tissue engineering aims to replace diseased or injured tissue with biologically engineered constructs. These constructs should reproduce native function and limit an immune response. To achieve tissue engineering success, it is important to first understand the tissue’s cellular, biochemical, and mechanical properties in order to create validation and design criteria. Reviewed herein are the known properties of the TMJ disc and initial attempts toward TMJ disc tissue engineering. Important aspects of tissue engineering are scaffold selection, cell source, biochemical factors, and mechanical stimuli.

Motivation

The temporomandibular joint (TMJ), or jaw joint, is used throughout normal everyday functions such as eating or talking. Thus, disease or injury of this joint greatly decreases a patient's quality of life. Common activities become difficult and painful for patients with a TMJ disorder (TMD). The prevalence of TMJ dysfunction is surprisingly high; based on various epidemiological studies, 28-88% of the population exhibit some physical sign or symptom of a TMJ dysfunction.\textsuperscript{7}

Around one-fifth of patients exhibiting symptoms seek medical treatment for TMDs.\textsuperscript{129} In the United States, there is an estimated 10 million TMD patients,\textsuperscript{6} around 70% of patients seeking treatment exhibit a displaced TMJ disc.\textsuperscript{9} Figure 1 illustrates the five stages of TMJ disc internal derangement as described by Wilkes;\textsuperscript{18} the patient population from this study had an average age of 31 years and a female to male ratio of 7:1, common characteristics of the TMD patient population.

In addition to joint pain, TMD symptoms include headaches, earaches, jaw clicking, limited jaw opening, and jaw lock.\textsuperscript{7,9,130} Unfortunately, TMD symptoms offer little aid in understanding the cause of TMDs. Numerous treatment options for TMD patients exist, but standard approaches and treatments are rarely agreed upon, even among experts. TMJ treatments and surgical approaches are presented in greater detail in reviews by Wong \textit{et al.}\textsuperscript{10} and Dimitroulis.\textsuperscript{11} Briefly, non-surgical options are the first treatment modality and include pain medication and physical therapy. Minimally invasive surgery, like arthrocentesis or arthroplasty, may be attempted in dysfunctional joints with limited tissue degradation; these procedures aim to reduce inflammation or repair the disc/attachments. When the disc is beyond repair, it may be removed (discectomy). Post-discectomy the joint may be left empty or replaced with autologous tissue. Synthetic discs are no longer implanted due to extensive wear and immune response.\textsuperscript{12} In the most extreme cases of degeneration, patients may opt for total joint replacement. Unfortunately, many TMDs are progressive, leading to extensive joint remodeling. Treatments primarily focus on the
reduction of pain. This leaves the field of TMJ research primed for tissue engineering alternatives that have the potential to reduce pain and restore total function.

**Disc characteristics**

The TMJ disc is located between the mandibular condyle and fossa-eminence of the temporal bone (Figure 2). The joint is enclosed in a synovial capsule; the synovium serves to nourish and lubricate the joint.\(^{131}\) The TMJ is a ginglymo-diarthrodial joint, meaning it exhibits both hinge-like and rotational motions. During normal movements, the disc translates anteriorly during jaw opening and posteriorly during closing. The presence of the disc's fibrous attachments is important to joint motions, but their exact mechanical function and location is heavily debated. The disc is believed to aid in joint lubrication as well as load distribution, jaw stabilization, and shock absorption.

The TMJ disc is divided into three regions: anterior band, posterior band, and intermediate zone (Figure 2). The posterior band is thicker than the anterior band; both bands are significantly thicker than the intermediate zone.\(^{132}\) The disc is generally divided into these three regions for characterization purposes. The bilaminar zone, a fourth element of the disc, exists between the posterior band and the posterior attachments, but generally, is not considered part of the disc. This region possesses some vasculature and is difficult to discern from the posterior attachment tissue.

While the disc is cartilaginous, it is very different from hyaline articular cartilage or even the knee meniscus.\(^4\) A healthy TMJ disc is primarily avascular, although some vasculature can be found near the attachment regions. It is well hydrated, containing 70% water.\(^{15}\) Similar to the knee meniscus, the TMJ disc exhibits a mixed population of cell types. In the porcine disc, there are approximately 70% fibroblast-like cells and 30% chondrocyte-like cells.\(^{15}\) The percent of
chondrocyte-like cells increases in the intermediate zone and decreases in the bands. This cell population is indicative of the disc's proper characterization as fibrocartilage.

The extracellular matrix (ECM) of the disc is essential to tissue function and important to thoroughly understand before attempting to engineer a construct. The TMJ disc is primarily collagen, and the collagen of the TMJ disc is nearly all collagen type I. Collagen type I makes up the majority of the disc's dry weight, approximately 85%. Trace amounts of types II, III, VI, IX, and XII can be found in various animal models. The fibers of the disc are primarily oriented circumferentially around the outer regions of the disc. In the intermediate zone, fibers are more random but possess a primarily anteroposterior alignment. Collagen fibers in the porcine disc have an average diameter of 18 ± 9 μm with a range of 2.9 to 37.4 μm. Parallel to the collagen fibers are elastin fibers, which are found in all regions.

Glycosaminoglycans (GAGs) and proteoglycans (PGs) are also important components of tissue ECM. The TMJ disc contains approximately 5% GAGs on a dry weight basis. Chondroitin sulfate is the most prevalent GAG in the disc, comprising 70-80% of the total GAG content. Aggrecan is an example of a chondroitin sulfate PG that is present in the disc and is important in hydration, lubrication, and compressive strength. Dermatan sulfate is the next most abundant GAG in the disc, making up 15-25% of total GAG content. Dermatan sulfate PGs include decorin and biglycan, which are important in controlling the collagen fiber lateral packing ability and diameter size. Hyaluronic acid, which binds non-convenantly to aggrecan, has been found in the range of 2.8-10% of the total GAG content. Heparan sulfate was found as 4.3% of total GAG content in the human disc. Keratan sulfate GAGs are generally considered a trace component of the TMJ disc but have been measured up to 2% of the total GAGs.

Mechanical properties of the TMJ disc are important to understand since engineered constructs must support the necessary load imparted on the native tissue. The tensile elastic modulus of the porcine TMJ disc is higher in the anteroposterior direction than the mediolateral direction at 76.4
MPa and 3.2 MPa, respectively. In the mediolateral direction, Detamore and Athanasiou found significant differences between the posterior band, anterior band, and intermediate zone with relaxation moduli of 23.4 MPa, 9.5 MPa, and 0.58 MPa, respectively. In the anteroposterior direction, the stiffest region was the central section followed by the medial section and then lateral section.

Several methods have proved useful in modeling the compressive properties of the TMJ disc. An elastic, compressive modulus for human discs was observed in the range of 211 kPa to 514 kPa, dependent on the strain rate. The biphasic theory has been employed frequently since its conception to illustrate a tissue's viscoelastic characteristics. Biphasic modeling of the porcine TMJ disc yielded properties of 20.1 kPa for the aggregate modulus, 0.45 for the Poisson's ratio, and $24.1 \times 10^{-16}$ m$^3$/Ns for the permeability. Most recently, unconfined compression, stress relaxation tests were performed to give the surface-regional instantaneous and relaxation moduli of the porcine disc. These values were found to be strain dependent, ranging from 90-3870 kPa (instantaneous modulus) and from 16.9-74.6 kPa (relaxation modulus) for 10%-30% strain, respectively. The coefficient of viscosity was also strain dependent, ranging from 1.3-13.8 MPa·s.

Shear properties of the TMJ disc have recently received due attention. Tanaka et al. found a storage modulus between 0.78-2.0 MPa depending on the compressive strain and percent shear. A loss modulus near 0.4 MPa and loss tangent ranging from 0.2-0.25 MPa was observed.

**Tissue engineering**

Tissue engineering is a potential option for the future treatment of diseased or injured discs. The general approach to tissue engineering involves selection of a cell source, seeding these cells on an appropriate scaffold, and applying external stimuli to encourage ECM production and
organization. These external stimuli may be grouped into two general categories: biochemical and mechanical. Tissue engineering approaches may commence *ex vivo* or *in vivo* and may exclude one or more of the aforementioned factors (cells, scaffold, and stimuli). For example, skin therapies have been successful using acellular collagen scaffolds. However, all tissue engineering therapies aim to replace the native tissue characteristics through tissue remodeling or regeneration. TMJ tissue engineering has focused on the combination of scaffolds, cells, and stimuli *in vitro* as illustrated in figure 3.

**Scaffolds**

Scaffolds, an important part of a construct's initial mechanical integrity, provide surface area for cell attachment. The earliest tissue engineering study used a porous collagen scaffold; after two weeks the construct appeared similar to the disc in gross morphology and cell shape. Later, researchers attempting to create a replacement for the TMJ disc used fibers of polyglycolic acid (PGA) and polylactid acid (PLA) and concluded that both scaffold materials were able to support cell attachment, matrix production, and retain testable mechanical properties after 12 weeks. Another study compared PGA, polyamide filaments, expanded polytetrafluoroethylene (ePTFE) filaments, and bone blocks. While all these scaffolds supported cell attachment and a small amount of collagen production, they were unable to form neotissue after 4 or 8 weeks. Tissue engineering studies in our lab have primarily used PGA non-woven meshes. While PGA supports cell attachment and matrix production, it degrades very rapidly, leaving constructs with limited mechanical integrity after only a few weeks. PLA non-woven mesh, however, has shown promise in retaining tensile and compressive integrity over a similar time scale.

Some researchers have investigated novel materials for TMJ disc engineering that would allow custom-shaped scaffolds to be implanted through minimally invasive surgery. Acrylated collagen type I scaffolds were successfully photopolymerized through a layer of rat skin; in this study, viability of osteoblasts in a photopolymerized poly(ethylene oxide) dimethacrylate was demonstrated, suggesting this process could be accomplished with other cell types. However,
corresponding data for TMJ disc cells encapsulated in alginate showed a drastic decrease in cell numbers at 4 and 8 weeks of culture with no ECM production at any time point, suggesting TMJ disc cells may not survive an encapsulated environment.\textsuperscript{109}

\textit{Cell source}

The cell source for a tissue engineering study is tremendously important, but limited research has been conducted in TMJ disc engineering studies. The most commonly used cells for these experiments are derived from the TMJ disc\textsuperscript{106,108-111,113-116} or articular cartilage.\textsuperscript{105,107,108} A major hurdle to overcome in tissue engineering is that tissue engineering generally requires a large cell population to create a construct. While passaged cells may seem appealing, chondrocytes have been found to de-differentiate to a more fibroblastic phenotype after only a couple of passages.\textsuperscript{1} Additionally, TMJ disc cells showed a decreased expression for ECM proteins with the exception of decorin and biglycan due to passage (Figure 4).\textsuperscript{2} Thus, for the future of TMJ disc engineering, a cell source that can yield a large population of TMJ disc cells, or a population of cells that rapidly fill a scaffold, must be identified.

As mentioned previously, after a discectomy, surgeons may replace the disc with some type of autologous tissue, such as skin, auricular cartilage, dura mater, temporalis muscle, or temporalis fascia.\textsuperscript{105} Any of these tissues may serve as potential cell sources for the TMJ disc, but one of the most appealing in terms of clinical feasibility and patient comfort is dermis. Adult dermal fibroblasts have been shown to produce matrix indicative of a chondrocytic phenotype when seeded on aggrecan-coated plates (Figure 4).\textsuperscript{3}

\textit{Biochemical factors}

Growth factors are commonly used in tissue engineering studies. Four studies have demonstrated the potential of growth factors for TMJ disc tissue engineering. This potential was first observed using transforming growth factor-\(\beta_1\) (TGF-\(\beta_1\)) and prostaglandin \(E_2\) (PGE\(_2\)) on
bovine TMJ disc cells in monolayer. TGF-β1 increased cell proliferation by 250%, while PGE₂ had no significant effect.\(^{40}\) Also in monolayer, the effects of platelet derived growth factor (PDGF), insulin like growth factor (IGF) and basic fibroblast growth factor (bFGF) on porcine TMJ disc cells demonstrated that lower concentrations of these growth factors favored biosynthesis, while higher concentrations favored proliferation.\(^{114}\) The most beneficial growth factors were IGF-I and bFGF, which both showed significant increases in collagen synthesis and cell proliferation. The effects of IGF-I, bFGF and TGF-β₁ on porcine TMJ disc cells in PGA scaffolds showed increased collagen production when exposed to low concentrations of IGF-I and TGF-β₁,\(^{115}\) but no other significant differences between the experimental groups existed. In the end, IGF-I was recommended for future tissue engineering studies due to low cost and beneficial collagen production. Of course, the native tissue is exposed to a variety of growth factors; so, it is possible growth factor combinations will be more beneficial than any single factor. IGF-I, bFGF, and TGF-β₁ have been investigated in combinations of two to determine if synergistic effects exist.\(^{113}\) All constructs exposed to growth factor combinations improved in structural integrity compared to a no growth factor control, but no combination was statistically significant in terms of biochemical or mechanical properties. While synergistic effects were not observed, improved overall cellularity of the constructs was noted when both growth factors were used at a high concentration.

Although growth factors have received the most attention, positive biochemical stimulation is also likely to come from culture conditions and cellular interactions as well. An ascorbic acid concentration of 25 \(\mu\text{g/mL}\) has been shown to produce constructs with higher total collagen content and higher aggregate modulus relative to concentrations of 0 \(\mu\text{g/mL}\) or 50 \(\mu\text{g/mL}\).\(^{111}\) This was likely associated with improved seeding observed for the constructs cultured in 25 \(\mu\text{g/mL}\) of ascorbic acid. Initial cell seeding is another important consideration in any tissue engineering construct due to cell-to-cell interactions and signaling. Almarza and Athanasiou\(^{119}\) showed that PGA scaffolds seeded at saturation increased cellularity and ECM content relative to scaffolds seeded below saturation.
Mechanical stimulation

The native TMJ disc undergoes significant loading, which is often broken down into compression, tension, and shear components.\textsuperscript{136} While cells proliferate and produce ECM in static culture, mechanical stimuli may be required to produce an optimal tissue engineered construct. A variety of mechanical stimuli may be beneficial including compression, tension, hydrostatic pressure, and fluid shear stress. Darling and Athanasiou\textsuperscript{137} have published an extensive review of the mechanical bioreactors that have been used in engineering cartilaginous tissues.

Three recent studies have investigated the effects of mechanical stimulation on TMJ disc constructs. A low-shear fluid environment by means of a rotating wall bioreactor created constructs with dense matrix and cell composition;\textsuperscript{112} however, when the biochemical content of these constructs was compared to those grown in static culture, no clear benefit of the bioreactor was observed. When disc cells were exposed to hydrostatic pressure in monolayer or PGA scaffolds, constant hydrostatic pressure at 10 MPa increased collagen production compared to static culture.\textsuperscript{116} In contrast, intermittent hydrostatic pressure from 0 to 10 MPa at 1 Hz frequency was detrimental to the constructs, producing less collagen and GAGs than unloaded controls. These results were consistent in both two and three-dimensional culture. In another recent study, dynamic tensile strain significantly reduced interleukin-1β induced up regulation of matrix metalloproteinase.\textsuperscript{138} This may have implications on future tissue engineering studies since MMPs play an important role in ECM degradation and remodeling.

Future directions for TMJ disc tissue engineering

While TMJ disc tissue engineering is in its infancy, other musculoskeletal tissues have been studied to a greater extent. These tissues include articular cartilage, bone, and tendon. TMJ disc tissue engineering should build on not only past TMJ research but also successes in these other tissues, while keeping in mind the disc's structural and functional differences.
The issue of scaffold certainly requires further investigation. Scaffolds that degrade too quickly are unable to provide the necessary mechanical integrity; thus, future research may focus on polymers with longer degradation times or that encourage rapid ECM production. Alternatively, using natural polymers like collagen may be effective since cells would simply remodel existing matrix instead of forming a new collagen network, thereby decreasing the time until the scaffold reaches a functional state. A third option is a scaffoldless or self-assembling process. Such approaches have been examined in both tendon and articular cartilage.\textsuperscript{104,120} While these methods require refinement to increase mechanical strength, data suggest these approaches may offer a new direction in soft tissue engineering. Furthermore, by eliminating the scaffold material within an engineering construct, concerns over mechanical integrity and cell toxicity due to the scaffold degradation process are diminished.

An optimal cell source is necessary for tissue engineering to be realized. To date, no such source has been identified that is likely to be clinically sound. However, research in other musculoskeletal tissues like cartilage, tendon, and bone has explored the possibility of using mesenchymal stem cells for tissue engineering.\textsuperscript{98-102,117-122,139} Using progenitor cells may also be desirable for the TMJ disc, since bone marrow or adipose tissue could potentially yield a large population of autologous, pluripotent cells. Alternatively, research on other potential cell sources, such as embryonic stem cells and dermis-derived fibroblasts, continues to demonstrate promise.

The inclusion of biochemical signaling will be an integral part of producing a TMJ disc tissue engineering construct. Significant work has been performed in both two- and three-dimensional cultures to determine optimal growth factor signaling for TMJ disc engineering. Recent work showed the growth factors IGF-I and TGF-β; used alone produced increases in collagen production.\textsuperscript{116} This provides a basis for growth factor selection in future TMJ disc tissue engineering studies. Beyond growth factors, the media used for culturing should also be further investigated. Ascorbic acid concentration has influenced the outcome of engineered constructs;\textsuperscript{111} thus, other media supplements may need further optimization as well. Cell-to-cell
interactions are important, and seeding the cells in scaffolds at saturation was shown to produce constructs with significant increases in ECM production.\textsuperscript{110} This is clearly vital for fabrication of an optimal TMJ disc construct.

Cartilage is a mechanical tissue; thus, mechanical stimulation should be expected for regeneration of any cartilaginous tissue. The most successful mechanical stimulation used to date for the TMJ disc has been constant hydrostatic pressure.\textsuperscript{116} Hydrostatic pressure should certainly be pursued further, because there are likely to be other beneficial loading regimens. Tension has shown promise in monolayer culture and should be pursued for future three-dimensional tissue engineering studies.\textsuperscript{138} Success in engineering the knee meniscus has been seen using direct compression; these results may apply to the TMJ disc due to the fibrocartilaginous nature of both tissues.\textsuperscript{140} Additionally, perfusion increased cellularity and ECM production in articular chondrocytes and may hold the same potential for the TMJ disc.\textsuperscript{141} Perfusion may also create larger constructs due to increased nutrient circulation.

In conclusion, while the field of TMJ disc engineering remains young, significant progress has been achieved. With this progress have come new, challenging questions and a wealth of knowledge on the disc’s characteristics. Related research may begin to merge with TMJ disc engineering due to the increased knowledge of TMJ disc design criteria. Tissue engineered TMJ constructs may now be validated with the increased fund of information on the tissue’s native characteristics. With these tools at hand, TMJ research will continue to rapidly progress to, hopefully, a viable tissue engineering implant.

\textbf{Figure 2-1: The stages of TMJ internal derangement}
**Figure 1** The stages of TMJ internal derangement as described by Wilkes. Schematics describe the progression of TMJ internal derangement; these schematics were created based upon radiologic findings described by Wilkes. In early stages, clinical symptoms are limited (no significant pain or mechanical symptoms); however, a slight anterior displacement of the disc can be observed. As the derangement progresses towards the intermediate stage, a few episodes of pain along with occasional joint tenderness, headaches, and mechanical problems are reported. Here, the disc displacement is slightly more forwards and the posterior edge thickens. At the intermediate stage, pain intensifies along with other clinical symptoms; anterior displacement of the disc is significant and coupled with disc prolapse. As the disorder progresses toward late stages, chronic pain develops; disc displacements are severe and hard tissue remodeling ensues. In late stages, joint scraping and difficulty in function are evident. The disc may be out of position, degenerated, or perforated. Hard tissue remodeling is severe; the joint is essentially arthritic.

*Figure 2-2: Joint anatomy and disc regions*
Figure 2 The TMJ disc is located between the mandibular condyle and fossa-eminence of the temporal bone. The disc is fibrocartilaginous and has a biconcave shape in both sagittal and coronal views. Thickness variations are evident in the sagittal view, where the thick posterior and anterior bands differ significantly from the intermediate zone. In the coronal view, thickness variations are less pronounced; however, the medial and lateral extents of the disc are slightly thicker than the intermediate zone.
**Figure 2-3: A tissue engineering paradigm: history of TMJ disc engineering**

**Cell Source**
- Tissue Engineering
- Detamore (2004, 2005a)
- Bean (2005)
- Shoulder Cartilage: Puelacher (1994)
- Mandibular Condyle: Girdler (1998)
- Fossa-Eminence: Springer (2001)

**Scaffolds**
- Collagen I: Thomas (1991)
- Poshusta (2001)
- PGA: Puelacher (1994)
- Polyamide: Springer (2001)
- ePTFE: Springer (2001)
- Bone Blocks: Springer (2001)

**Related Studies**
- Articular Cartilage: Darling (2005)
- TMJ Disc Cells: Allen (Submitted)
- Stem Cells: Many

**Biochemical Signals**
- TGF-β1: Landesberg (1996)
- Detamore (2005b)
- Almarza (2005b)
- PGE2: Landesberg (1996)
- IGF-1: Detamore (2004, 2005b)
- Almarza (2005b)
- bFGF: Detamore (2004, 2005b)
- Almarza (2005b)
- Ascorbic Acid: Bean (2005)
- Cell-to-Cell Contact: Almarza (2005a)

**Biomechanical Signals**
- TMJ Disc
- Low Shear Fluid: Detamore (2005a)
- Hydrostatic Pressure: Almarza (2005)

**Relative Tissues**
- Tension: Deschner (2005)
- Direct Compression/Tension: AufderHeide (2005)
- Perfusion: Davission (2002)

**Figure 3** Tissue engineering, generally, is conducted by combining cells and signals on an appropriate scaffolding material. This approach has been the standard thus far in TMJ disc engineering. References to significant studies of scaffolding, signals, and cell source for the TMJ disc are placed within the classic paradigm figure. Clearly, TMJ disc engineering is very young; however, it is apparent that the field is rapidly expanding.
Figure 4 In our laboratory, we have investigated the relationship between chondrocytes, fibrochondrocytes, and dermal fibroblasts. First, chondrocytes progressively dedifferentiate as a function of monolayer culture. As these cells are passaged, they become more fibroblastic in nature, characterized by a loss of chondrocytic ECM gene expression and a gain in fibroblastic expression. Fibrochondrocytes follow a similar loss in gene expression; however, fibroblastic gene expression is also lost as a function of passage. However, it may be possible to regain these losses by seeding passaged cells over proteins; dermal fibroblasts have demonstrated a chondrocytic response when seeded over specific extracellular matrix proteins.
Chapter 3: Improving culture conditions for TMJ disc
tissue engineering*

Abstract

Background: The temporomandibular joint (TMJ) is extremely important for activities like eating and talking, which can become painful and difficult for patients with TMJ dysfunction. Tissue engineering is a potential alternative to current surgical interventions through replacement of diseased or injured tissue with a functional construct. Since research with TMJ disc cells began relatively recently, optimal culturing conditions must be determined. Methods: Metabolic additives, L-glutamine, L-alanyl-L-glutamine, sodium pyruvate, and insulin, were examined for their effects on TMJ disc cells in monolayer. Effects of L-proline were examined in 3-D culture at concentrations of 0, 25, and 100 mg/L. Results: The combination of L-glutamine, sodium pyruvate, and insulin improved cell proliferation rates without affecting collagen production or gene expression. No differences were observed in mechanical properties of the engineered constructs; however, collagen and glycosaminoglycan quantities normalized to cell number decreased at the highest concentration of L-proline. Conclusion: This work identified supplements for 2-D monolayer expansion. Other supplements or culture conditions still need to be investigated for 3-D tissue production. This work improves upon porcine TMJ disc cell culturing conditions, taking us closer to being able to engineer the TMJ disc.

Introduction

The temporomandibular joint (TMJ) is crucial to everyday function, allowing smooth motion of the mandible in all directions. When problems occur, TMJ patients may experience symptoms like joint pain, jaw clicking, headaches, earaches, limited jaw opening, and jaw lock.\cite{7,8,130} It is estimated that 28-88\% of the population exhibit one or more physical symptoms for TMJ dysfunction based on various epidemiological studies.\cite{7} The patient population is unusual, exhibiting an average age of 31 years and a female to male ratio of 7:1.\cite{18} In patients where the disorder is severe, discs can become deformed and/or perforated, and the cartilage of the joint can become degraded and arthritic.\cite{18} In these cases, treatment options are limited, rarely agreed upon, and have varied success rates. The inability to talk or eat without pain places heavy physical and emotional burdens on the patient. Of the patients that pursue treatment for a TMJ disorder, around 70\% have a displaced disc in the joint.\cite{9} Thus, when surgical intervention is required, tissue engineering of the disc presents a potential solution for the future of TMJ replacement.

Tissue engineering aims to replace diseased or injured tissue with a functional replacement. By creating a living, healthy tissue alternative, problems associated with synthetic tissues and a lack of viable donated tissue can be circumvented. Producing a biochemically robust and mechanically functioning tissue requires a strong understanding of the characteristics of the disc and its cells. These characterization data can then be applied to a tissue engineering approach that involves the expansion of cells in passage, seeding these cells onto a scaffold, and applying external stimuli \textit{in vitro} to allow for production of proper extracellular matrix (ECM) and development of mechanical strength before implantation (see Fig. 1). Mechanical and biochemical properties for the adult porcine TMJ disc have now been well characterized thereby establishing suitable success criteria for tissue engineering.\cite{15,16,79} There is, however, a weaker understanding of the TMJ disc cells and the factors needed to stimulate their proliferation and ECM production. Proliferation is of particular importance for the two-dimensional (2-D), monolayer
cell expansion phase of the approach, and ECM production is essential during the three-dimensional (3-D) tissue formation phase. Previous work with TMJ disc cells observed an inverse relationship between proliferation and matrix synthesis, and so separate optimizations of these two phases and culture conditions (2-D and 3-D) may be necessary.\textsuperscript{114} Previous work has determined that culturing conditions can have profound effects on porcine TMJ disc cells.\textsuperscript{109-111} The porcine TMJ model is agreed to be the closest to human.\textsuperscript{25} This work looks at several important aspects of the culturing conditions to examine their effects, first on cell proliferation and then on matrix production, in two separate studies.

In a relatively acellular tissue like the TMJ disc, large amounts of cells are rarely available for use in tissue engineering. Insulin has been shown previously to improve cell proliferation in meniscal fibrochondrocytes,\textsuperscript{142} and both sodium pyruvate (NaPyr) and insulin are important in cell metabolism. Also, GlutaMAX\textsuperscript{TM}, the dipeptide L-alanyl-L-glutamine, provides a more stable alternative to L-glutamine, which degrades to result in a loss of the additive and an accumulation of the toxic degradation product. This alteration seems beneficial, but some cell types may have more difficulty metabolizing the dipeptide. The objective of the work presented here is to find the combination of the additives above that will be most beneficial in inducing rapid cell proliferation in TMJ disc cells in monolayer.

Medium components also affect ECM production in 3-D. For the present study, the effects of various concentrations of L-proline will be examined in 3-D culture. Proline, an important component of the collagen molecule, is a common supplement for chondrocytes\textsuperscript{143-145} and has been added to tenocyte culture as well.\textsuperscript{146} If collagen production in tissue engineered constructs can be increased, the mechanical integrity of the construct is likely to increase as well. Thus, the hypothesis of this study is that L-proline will increase collagen synthesis. Metabolic media additives or the addition of L-proline could greatly enhance the final properties of a tissue engineered construct, taking us closer to being able to engineer the TMJ disc.
Materials and methods

Cell isolation

Porcine TMJ discs were obtained from a local abattoir as follows: TMJ joints were removed en bloc within 24 hours of death and transferred into a sterile environment before breaking into the joint capsule. Discs were removed, minced, and digested for 24 hours in 0.1% collagenase (Worthington, Lakewood, NJ) in base media (see monolayer cell culture) to liberate the cells, which were either seeded in monolayer or frozen in freezing media [base media with DMSO, 20% fetal bovine serum (FBS), 1% Penicillin-Streptomycin-Amphotericin B (PSF), and 1% non-essential amino acids (NEAA)] at −80°C, until ready for use in 3-D cell culture.

Monolayer cell culture

Base culture medium in the monolayer study was either high glucose Dulbecco’s modified Eagle medium (DMEM) with GlutaMAX (Gibco, Carlsbad, California) or DMEM with L-glutamine and 4.5 g/L glucose (Biowhittaker, Walkersville, MS). Both were supplemented with 10% FBS (Gemini Bio-Products, Woodland, CA), 1% PSF (Cambrex, Walkersville, MD), 1% NEAA (Life Technologies, Carlsbad, CA), and 1% L-ascorbic acid (Sigma, St. Louis, MO). Primary cells from 6 different animals were cultured in 6-well tissue culture-treated plates in a standard incubator at 37°C and 5% CO₂ until they were 70-90% confluent. At this confluence, cells were passaged with 1x trypsin-EDTA (Gibco, Carlsbad, California). One well in each group was left unpassaged for hydroxyproline samples. Of the passaged cells, 100,000-500,000 were collected for quantitative real-time polymerase chain reactions (qRT-PCR), and the remaining cells were seeded again at 30% confluence. Passaging continued until passage 3. During culture there were six media treatments (see table 1). Each type of glutamine was tested either with sodium pyruvate, without sodium pyruvate, or with the combination of sodium pyruvate and insulin.
Division rate calculation

To assess cell proliferation, equation 1 was used to calculate the cells’ division rates.

\[ d = \log_2 \left( \frac{N_f}{N_i} \right) \frac{\text{t}}{t} \]

<Eq. 1>

Equation 1 describes the division rate of cells \( d \) where \( N_i \) is the initial number of cells (at seeding), \( N_f \) is the final number of cells (at passage), and \( t \) is the time in seconds (to passage). This value was calculated at each passage from cell counts measured via trypan blue exclusion and counted with a hemocytometer. While this equation does not take into account cell death and is therefore an underestimate of the actual division rate, it provides a simple metric of cell expansion based on cell counts.

Gene expression

Before plating for passage 0 (P0) and after the first passage (P1), 100,000-500,000 cells were collected and suspended in 1 mL of TriZol reagent (Invitrogen) for RNA isolation. The RNA was extracted from the solution with chloroform and precipitated with isopropanol. The RNA was pelleted, washed with 75% ethanol, and resuspended in 40 \( \mu \)L RNAase-free water. The RNA concentration was assessed using a spectrophotometer (Nanodrop, Wilmington, DE).

Stratagene’s StrataScript\textsuperscript{TM} First Strand Synthesis System protocol was used for reverse transcription. Briefly, 200 ng of RNA was diluted in 17.7 \( \mu \)L of RNAse-free water. Random hexamers were annealed to the RNA by incubating samples with primers, buffer, and dNTPs at 65°C for 5 min then cooled to room temperature. Reverse transcriptase and RNase block were added to the solution and incubated at 42°C for 60 min followed by incubation at 70°C for 15 min to terminate the reaction.
qRT-PCR was performed for collagen type I, type II, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with a Rotor-gene 3000 real-time PCR machine (Corbett Research). These genes were previously optimized for simultaneous detection and measurement. Primer-probe sequences are available in Upton et al.\textsuperscript{147} for collagen or in Darling et al.\textsuperscript{148} for GAPDH. Gene-specific primer-probe sets, HotStar Taq (Qiagen), buffer (Qiagen, 1x), MgCl\textsubscript{2} (Qiagen, 3.5 mM), dNTPs (Promega, 0.2 mM), and RNase-free water were used as the PCR mix. The PCR reaction began with a 15 min denaturing step at 95°C followed by 50 cycles of 15 s at 95°C and 30 s at 60°C. Fluorescence measurements were taken every cycle at 60°C to provide a quantitative, real-time analysis of the PCR reaction for the specific genes mentioned above.

The efficiency ($E_{\text{GOI}}$) of the PCR reactions was determined through a series of standard sample dilutions (3x, 9x, 27x, and 81x). Equation 2 shows the calculation for the abundance value for a gene of interest ($A_{\text{GOI}}$) where $C_{\text{GOI}}$ is the take-off cycle determined via the comparative quantification package available in the Roto-gene software.

$$A_{\text{GOI}} = \frac{1}{(1 + E_{\text{GOI}})^{C_{\text{GOI}}}} \quad \text{<Eq. 2>}
$$

3-D cell culture

Base culture medium for the 3-D study (selected from the monolayer analysis) was DMEM with L-glutamine and 4.5 g/L glucose (Biowhittaker) supplemented with 10% FBS, 1% PSF, 1% NEAA, 1% L-ascorbic acid (Sigma), and 1 μL/mL of insulin (Sigma). Primary TMJ disc cells from four porcine heads were defrosted and seeded with a modified spinner flask method as described previously.\textsuperscript{109} Primary cells were thawed and seeded on poly-L-lactic acid (PLLA) scaffolds (Biomedical Structures, Warwick, RI) that were 5 mm in diameter and 2 mm thick. Scaffolds were strung on a stainless steel wire and hung in 250 mL of media in one of three spinner flasks. The spinner flasks contained either 0 mg/L, 25 mg/L, or 100 mg/L of L-proline. Seventy-five million cells per mL scaffold volume were spun with a stir bar in a standard tissue culture incubator for 3 days. After an additional 4 days of seeding without spinning to allow for proper cell attachment,
the scaffolds were removed and assessed using histological, biochemical and mechanical assays. This was designated as the “0 wk” time point. Scaffolds for later time points were then placed into shallow 5 mm diameter agarose wells. Cell culture with the three L-proline concentrations continued for another 6 weeks with half of the medium changed daily. At wk 3, samples were assessed using histology and biochemistry. At wk 6, all assessments from wk 0 were performed again.

**Histology**

In the 3-D study, histological characterization was performed at each of three time points. Samples were covered in Histo prep™ (Fisher), frozen in liquid nitrogen, and cut into 14 μm sections. Since PLLA samples frequently wash off in aqueous solutions, adhesive slides (Instrumedics, St. Louis, MO) were used. Slides were fixed in formalin and stained with hematoxylin and eosin for cell distribution, picrosirius red for collagen, and safranin O/fast green for proteoglycans. Slides for immunohistochemistry (IHC) were fixed in acetone and exposed to primary mouse antibodies for either collagen type I (Accurate Chemical and Scientific, Westbury, NY) or type II (Chemicon, Temecula, CA). Secondary detection was accomplished with a Vectastain ABC kit (Burlingame, CA) as described previously.¹⁶

**Biochemistry**

Five scaffolds were acquired after 0, 3, and 6 wks of static culture to assess the concentration of DNA, glycosaminoglycans (GAGs), and collagen per scaffold. Scaffolds were lyophilized for 48 hrs, and then digested using 125 μg/ml papain (Sigma) in 50mM phosphate buffer (pH=6.5) containing 2 mM N-acetyl cysteine (Sigma) and EDTA at 60°C for 18 hrs.¹⁴⁹ DNA quantification was accomplished with picogreen analysis by comparison to a calf thymus DNA standard. Conversion of DNA to cell number (7.7 pg DNA/cell) was used to determine cellular content per scaffold.¹⁵⁰ Samples were tested for total GAG content via a dimethylmethylene blue colorimetric assay kit, with standards provided by the manufacturer (Accurate Chemical and Scientific Corp.,
Westbury, NY). The hydroxyproline assay for total collagen content was accomplished by first hydrolyzing samples in 4N NaOH at 121°C for 1h. Samples were then neutralized and placed in a buffer. Hydroxyproline content was colorimetrically quantified via a modified chloramine T assay and standardized to bovine collagen.  

*Mechanical testing*

Construct tensile and compressive properties were obtained on an Instron 5565 (Norwood, MA) using a 50 N load cell on four samples per experimental group. Relevant data that were collected are force, displacement, and time. Figure 2 shows an example of the data obtained from compressive (Fig. 2a) and tensile (Fig. 2b) testing.

Unconfined compression testing under stress relaxation conditions was performed to determine the viscoelastic properties of the samples as described previously. Briefly, the Instron used a height detection protocol, which accounts for the buoyancy of the compression platen in the phosphate buffered saline, to determine the precise height of the sample. Ten, 20, and 30% strains were then applied to the samples, with the resulting stress allowed to equilibrate. Compression testing was performed on whole 5 mm constructs; the diameter was unaltered during culture and was used to calculate the area of the sample surface.

Data were then fit with a curve-fitting tool in Matlab (The Math Works). Equation 3 shows the general case used in curve fitting, where $\sigma(t)$ is the stress at time $t$, $E_r$ is the relaxation modulus, $z$ is the height of the specimen at a give strain, $u$ is displacement, $\tau_r$ is the constant stress time constant, $\tau_o$ is the constant strain time constant, and $t_i$ is the time at which a single stress relaxation curve begins. $\tau_r$ and $\tau_o$ can be used to determine the coefficient of viscosity ($\mu$) and instantaneous modulus ($E_i$), which along with $E_r$, are considered important properties of a viscoelastic material.
\[ \sigma(t) = \sum_{i=1}^{n} \left\{ \frac{3}{2} \frac{E_r(u_i - u_{i-1})}{z} \left[ 1 + \left( \frac{r_\sigma}{r_e} - 1 \right) e^\frac{-(r-r_i)}{r_e} \right] \right\} \]  

<Eq. 3>

For tensile testing, 2mm of the construct was removed using a cutting template to give a rectangular cross section. One-third of the hydrated sample on either end was clamped to prevent slippage. The gauge length was measured with a digital micrometer. A constant strain rate (0.01 mm/sec) was applied under tension until the sample failed. Force and displacement data were then converted into stress and strain, by dividing by the cross sectional area and original gauge length, respectively. The elastic modulus was calculated from the initial slope of this new curve. Yield stress was found at the stress where the curve began to deviate from the straight line, and ultimate tensile stress (UTS) was determined at the highest stress endured by the sample.

**Statistical analysis**

Data from the monolayer study were analyzed by a 4-way analysis of variance (ANOVA) with factors of Glutamine (monomer or dimer), additive (none, sodium pyruvate, or sodium pyruvate + insulin), and passage (0-3). The animal (1, 2, 3, 4, and 5) was also used as a block since previous studies have found significant differences in results among various animals; these repeated measures resulted in a total of 5 samples per experimental group. This sample number carried through all the data analysis performed in the monolayer phase. These factors and levels are shown in table 2. From this statistical model, effects on division rate, collagen I gene expression, and total collagen protein were elucidated. When the F-test showed a significant difference \((p < 0.05)\), a Tukey’s HSD post hoc test was performed to compare within main factor effects.
The 3-D study was analyzed with a 2-way ANOVA. Factors were time and L-proline concentration (see table 3). This statistical model was applied to data from biochemical (n=5) and mechanical testing (n=4), as well as wet and dry weights (n=5). Again, a Tukey’s HSD was performed when factors were found to be significant (p < 0.05).

Data were verified for ANOVA assumptions including normality. When data did not meet the normality criterion, they were transformed based on a Box-Cox analysis transformation. In all cases, this transformation resulted in normal data sets.

Results

Monolayer phase

Figure 3 shows the results of division rate calculations. Passage significantly decreased the division rate from passages 1 to 2 and 2 to 3 compared to passage 0 to 1. Cells exposed to the additive combination of sodium pyruvate and insulin showed a significantly higher division rate than without the presence of both of those additives (‘no additive’ in Fig. 3a). The use of L-glutamine was significantly better than using its dimer Glutamax (Fig. 3b).

Total collagen was in the range of 4-10 μg per sample (see Fig. 4). These data did not demonstrate significant differences for the use of any particular additive. Passage showed a detrimental effect for collagen production, which, as an overall factor, significantly decreased at passage 3.

Gene expression for collagen I did not change due to any additive. GAPDH significantly decreased from P0 to P1, but was not significant among the various media supplements. Collagen II expression was rarely seen among all treatments and, when present, was only at the lower limits of detection. Among the various treatments, division rate data identified the optimal
combination for cell proliferation of TMJ disc cells to be basal DMEM with L-glutamine, sodium pyruvate, and insulin. Based on these results and the observation that this medium was not detrimental to collagen expression and synthesis, this medium was selected for use in the 3-D phase.

3-D phase

No gross morphological differences among treatments were observed in the 3-D, L-proline study. Dry weights of constructs showed no significant changes with either time or L-proline concentration. Wet weight significantly decreased with time. Cell numbers for the L-proline study exhibited a non-normal distribution and were transformed by raising the cell data to the −1.5 power, in accordance with the Box-Cox procedure. The transformed data were then used to determine significance of the factors. These data showed a decrease in cell number with each increasing time point (Fig. 5). Additionally, the 25 mg/L L-proline treatment was significantly lower than the other two treatments (0 and 100 mg/L) with \( p=0.012 \).

Collagen per construct ranged from 8-30 \( \mu \text{g} \) and increased significantly at wk 3 from wk 0, but neither was different from wk 6 data. This is the same result as that seen for the transformed data \((\text{collagen/wet weight})^1\), based on the Box-Cox transformation used due to the data lacking a normal distribution. Collagen per dry weight and collagen per cell both illustrated significantly higher results at wks 3 and 6 than at wk 0. Collagen per cell also showed interesting results in terms of the concentration of L-proline (Fig. 6). The 100 mg/L concentration of L-proline resulted in a significantly lower collagen per cell than either 0 mg/L or 25 mg/L.

GAG per construct, GAG per wet weight, GAG per dry weight, and GAG per cell all resulted in significantly higher values at wks 3 and 6 than at wk 0. GAG per construct and GAG per dry weight also showed that the 100 mg/L concentration resulted in significantly lower values than 0 mg/L L-proline, while values obtained with 25 mg/L were not significantly different from either. 100
mg/L L-proline resulted in significantly lower GAG per cell compared to the other two concentrations (see Fig. 7). In terms of dry weight, GAG comprised 0.03-3% of the total.

The quantitative ECM data were supported by histology, which showed somewhat more staining with the 0 mg/L of L-proline treatment for cells and collagen than other treatments. The control group also exhibited more intense picrosirius red staining as time increased. No safranin-O staining was seen for any concentration of L-proline at any time point. Both cells and collagen were concentrated more heavily around the periphery of the PLLA construct than in the center (Fig. 8). The PLLA was still seen at the end of the culture period. IHC staining for collagen type I was very similar to that seen for the total collagen with picrosirius red staining. IHC showed no staining for collagen type II.

Tensile testing resulted in no significant changes for yield stress, UTS, or tensile modulus for either factor: time or concentration. The yield stress range was 100-600 kPa. UTS range was 139-950 kPa, and tensile modulus range was 246-1120 kPa.

Compressive properties were also not significant between experimental groups. At 10% strain $\varepsilon_R$, $E_i$, and $\mu$ values were in the ranges of 1.5-22 kPa, 2.5-23 kPa, and 1.8-44 MPa·s, respectively. For 20% strain these ranges were 1.5-16 kPa, 2.5-20 kPa, and 8-104 MPa·s, respectively. The coefficient of viscosity for both 10% and 20% strain demonstrated a significant increase at wk 6 compared to wk 0.

**Discussion**

Tissue engineering of the TMJ disc is an important goal toward improving surgical options for sufferers of TMJ disorders. Unfortunately, this is a complex and extensive task that requires examination of numerous aspects of the tissue engineering approach including cells, scaffold
choice, and external stimuli needed to create a functional tissue. Relatively little work has been conducted toward tissue engineering the TMJ disc, and these aspects have not yet been fully optimized. Previous work optimized the concentration of ascorbic acid in the media thereby improving cellularity in TMJ disc cell-seeded scaffolds and increasing collagen content. Our own experience has also found that TMJ disc cells have higher ECM production in Dulbecco's modified Eagle Medium (DMEM) when compared to Ham's F-12 nutrient mixture or to the combination of DMEM-F12 (unpublished data). The studies presented here give further insights into the culturing of TMJ disc fibrochondrocytes for monolayer expansion and 3-D tissue formation, offering a necessary base for future TMJ disc or other fibrocartilage tissue engineering studies.

The results of examining metabolic regulators in DMEM media on TMJ disc cells showed that using the combination of L-glutamine, sodium pyruvate, and insulin provides a significant improvement in the division rates of the cells. The improvement of cell division with L-glutamine over its dimer suggests that the porcine TMJ disc cells appear to be unable to metabolize the dimer as readily, and the byproducts produced from the L-glutamine degradation are not detrimental to the cells. Though proliferation is generally observed with the tradeoff of lowered ECM production, no changes were seen in collagen production or gene expression. Passaging the cells caused a significant decrease in division rate after passage 1 and a significant decrease in total collagen production after passage 2. In the 3-D phase of this work, neither compressive nor tensile mechanical properties of the TMJ disc cell-seeded scaffolds changed with variable amounts of L-proline added to the media. The ECM content normalized per cell decreased with the high concentration of L-proline and was largest with no L-proline. This did not support the hypothesis of this phase, as L-proline did not result in increased collagen synthesis.

Passaging the TMJ disc cells in monolayer had a negative effect on both cell proliferation rate and ECM production. The decrease in cell proliferation rate with passage contrasts what our group has observed previously for chondrocytes, which proliferate more quickly after passage 2.
(unpublished data). However, decreases in gene expression of important ECM proteins with increasing passage have been seen previously for articular chondrocytes, meniscal fibrochondrocytes, and TMJ disc fibrochondrocytes. Cell proliferation rate significantly decreased between P1 and P2 compared to the passage between P0 and P1, but collagen production did not decrease significantly until beyond P2. Based on these results, it would be reasonable to passage these cells to P2 without large consequences to the tissue engineering approach.

Large numbers of cells, which produce organized ECM, are essential to tissue engineer a biochemically and mechanically functional tissue. Pyruvate is an important component of the glycolysis pathway—providing energy for cell function. Insulin is a hormone that can encourage protein synthesis and carbohydrate utilization. Both these additives together were able to improve the cell proliferation rates in the porcine TMJ disc cells without significantly affecting the collagen production or gene expression. Since it is often argued that cell proliferation and cell production are inversely related, it is encouraging that the additives were able to increase cell proliferation rates without changing the collagen production. The stability in collagen protein production and gene expression suggests that these additives should not be detrimental in the 3-D study, where the goal is ECM production. It is for this reason that these additives were not reassessed in the 3-D phase.

Surprisingly, L-proline was not beneficial, and sometimes even detrimental, to TMJ disc fibrochondrocyte ECM production. Even though L-proline is commonly used in articular chondrocyte culture and proline is a large component of collagen, when normalized to the number of cells both collagen and GAG production significantly decreased at the high concentration (100 mg/L) of L-proline. It is difficult to speculate about the reasons for these counterintuitive results. It is possible that such a high concentration could result in down regulation of these ECM genes or an increase in matrix metalloproteinase (MMP) production leading to the overall decrease in
measured matrix. MMPs are an important regulator of collagen and GAG breakdown and measuring the activity of MMPs in these cells is an interesting area of future research.

The deficiency of substantial amounts of ECM production in the 3-D constructs contributed to the relatively weak mechanical properties, particularly in tension. The lack of significant differences in tensile or compressive properties between experimental groups or time points suggests that the scaffold was the main contributor to the constructs' integrity. Still, the tensile properties obtained were much lower than those exhibited in the native disc. While properties vary greatly depending on the region of the disc tested and the testing method, Beatty et al.\textsuperscript{78} used a similar method on porcine disc tissue as was used in this study and observed a tensile modulus of 76.4 MPa and a UTS of 37.4 MPa in the anteroposterior direction of the disc. The tensile properties observed in this study were orders of magnitude lower. Compressive testing of native porcine discs performed with the same methods used in this study yielded an averaged relaxed modulus of 4.5 kPa, an average instantaneous modulus of 20 kPa, and an average coefficient of viscosity of 3.6 MPa\textperiodcentered s at 10% strain.\textsuperscript{93} At 20% strain, these values were 6.5 kPa, 170 kPa, and 11.1 MPa\textperiodcentered s, respectively. The compressive properties obtained for the tissue engineered constructs are reasonably close to the tissue values, but are largely due to the presence of the polymer scaffold and not ECM created by the cells.

Histological analysis of the tissue engineered constructs indicated that cells and ECM were located primarily on the surface of the construct. While the scaffold was very porous, allowing for cell migration into the scaffold, only limited migration was actually observed. Large groups of cells attached to one another rather than the scaffold suggesting that PLLA may not be an optimal scaffold for TMJ disc tissue engineering. Previous work with polyglycolic acid scaffolds demonstrated that the degradation of polyglycolic acid was too rapid for proper tissue formation, resulting in constructs that were not robust enough to be tested mechanically.\textsuperscript{109,112-114,118,134} Exploration into other scaffolds or scaffoldless approaches is necessary for TMJ disc tissue engineering success.
Cells used in these studies were obtained from hogs that are considered skeletally mature. This suggests the TMJ disc cells, like other cartilaginous cells, have likely developed to the point where their need for ECM production is largely complete. The function of cells in adult cartilaginous tissues is primarily to maintain the matrix that was produced during skeletal growth, although work specifically regarding TMJ disc development is extremely limited. However, both collagen per cells and GAG per cells did show significant increases at wks 3 and 6 over wk 0, suggesting that these cells do retain some ability for ECM production even though they are from adult tissue. However, both collagen and GAG were well below native tissue values. Since these ECM components function to provide mechanical integrity to the tissue, low collagen and GAG values explain the low tensile and compressive properties seen in the constructs.

Overall, there is still a strong need for further improvement to TMJ disc tissue engineering approaches. Both biological and mechanical properties of the tissue engineered constructs are still not close enough to native tissue values to function in vivo. The inability of L-proline to significantly improve the ECM production suggests the need for further research in 3-D for the purpose of functional tissue engineering. Increasing ECM production is essential for both the tissue’s biological and mechanical role in the jaw. However, improvements with cell proliferation rates were seen here, suggesting the ability to obtain more cells quickly, thereby decreasing time and cost of the cell expansion phase in this tissue engineering approach.

In addition to the additives examined in this study, there are several factors that should be investigated more extensively to improve on the tissue engineered constructs. First, further biological stimuli including growth factors should be examined further. Growth factors including insulin-like growth factor,\textsuperscript{113,115,152,154} platelet-derived growth factor,\textsuperscript{114,155} basic fibroblast growth factor,\textsuperscript{113,115} and transforming growth factor-\(\beta\) (TGF-\(\beta\))\textsuperscript{113,115,152,154} have been examined previously on TMJ disc cells. These studies all exhibited some beneficial effects on proliferation, ECM production, or both. Perhaps the most profound effects in 3-D studies were seen with application
of TGF-β1, but construct properties were still well below native tissue values, suggesting TMJ disc cells may require stimuli other than soluble factors.\textsuperscript{115,154} Mechanical stimulation may be necessary to promote ECM production in 3-D culture of TMJ disc cells. To date, only three studies have applied mechanical stimulation to TMJ disc cells. Detamore \textit{et al.}\textsuperscript{112} found that using a rotating wall bioreactor was detrimental to TMJ disc constructs compared to a non-loaded control. In contrast, Almarza \textit{et al.}\textsuperscript{116} observed an increase in collagen production when constant hydrostatic pressure was applied. As this stimulus may be promising, further optimization of the stimuli parameters such as magnitude, frequency, and duty cycles need to be conducted to further improve the construct properties. Another promising mechanical stimulus is cyclic tension, which was applied to TMJ disc cells, resulting in the downregulation of various MMPs.\textsuperscript{136} Other types of mechanical stimulation like direct compression or a combination of stimuli may also hold the key to successful TMJ disc tissue engineering and should be examined.

Lastly, it is important to reflect on the proliferative and productive capacity of the cell type used in this study. The enormous deterioration in quality of life resulting from TMJ disc maladies compels for rapid progress toward therapeutic solutions. Thus, in parallel to the advancements being made in understanding the behavior of TMJ disc cells, the need to consider alternative cell sources is evident. Stem cells, which have the ability to proliferate extensively and to differentiate to produce numerous types of tissues, may hold promise for TMJ disc tissue engineering.\textsuperscript{98-102,117-122,138} Additionally, adult cells that are considered “terminally differentiated” may also hold promise in forming other, similar cell types. One example of this is dermal fibroblasts, which have been induced to make collagen type II, indicative of a cartilage phenotype.\textsuperscript{3} Dermal fibroblasts have been shown to function in a tissue engineered tendon replacement as well.\textsuperscript{156} Alternative cell sources for cartilaginous tissue engineering, specifically for the TMJ disc, are an important area for future research.

This work improved on the monolayer expansion phase of the tissue engineering approach for the TMJ disc by increasing the proliferation rates without compromising ECM production. Future
research is needed in refining the 3-D culturing techniques to create a functional tissue engineered construct. Biochemical stimuli, like growth factors or other biological regulators, or mechanical stimuli may drastically affect the ECM production of these cells. Alternatively, other cell types, such as stem cells or phenotypically-flexible adult cells, may provide a more feasible and functional replacement. By directing future tissue engineering research in these areas we may one day be able to provide sufferers of TMJ disorders with a more desirable approach to TMJ disc replacement.
### Table 3-1: Experimental groups for monolayer study

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<th>L-Glutamine</th>
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<td>D</td>
</tr>
<tr>
<td>With NaPyr</td>
<td>B</td>
<td>E</td>
</tr>
<tr>
<td>With NaPyr and insulin</td>
<td>C</td>
<td>F</td>
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NaPyr = Sodium pyruvate
Table 3-2: Statistical design for monolayer study

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<tr>
<td></td>
<td>Glutamax</td>
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<tr>
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<tr>
<td></td>
<td>with NaPyr</td>
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<tr>
<td></td>
<td>with NaPyr and insulin</td>
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<tr>
<td>Passage</td>
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<td>Animal</td>
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NaPyr = Sodium pyruvate
Table 3-I: Statistical design for 3-D study

<table>
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<td>100 mg/L</td>
</tr>
<tr>
<td>Time after seeding</td>
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<td>3 wks</td>
</tr>
<tr>
<td></td>
<td>6 wks</td>
</tr>
</tbody>
</table>
Figure 3-1: Tissue engineering paradigm

Figure 1 This schematic shows a common approach to tissue engineering, which involves adding passaged cells to a scaffold in vitro and applying external stimuli to create a functional replacement tissue.
Figure 2 Graph "a" shows the data collected during a typical stress relaxation experiment. Fitting these curves to equation 3 yields the relevant viscoelastic properties in compression. Graph "b" shows the stress-strain data from tensile testing that were used to determine tensile properties.
Figure 3 Data are shown as the mean ± SD; (additives and passage) factors separated by different symbols († and ‡, or * and ** respectively) are statistically significant (p < 0.05). Graph “a” illustrates the significantly higher division rate for the combination of additives over none, when other factors (except passage) remain unchanged. Graph “b” shows division rates are also higher for L-glutamine over Glutamax™, when other factors are held constant.
Figure 3-4: Total collagen results from monolayer study

Figure 4 This graph shows μg of total collagen for each of the various media types (lettered according to table 1). Data are shown as the mean ± SD. Statistical analysis of the passage factor indicate that passage 3 has significantly less collagen production than earlier passages as indicated with different symbols (*) and **) ($p < 0.05$).
Figure 3-5: Results of picogreen assay from L-proline study

![Graph showing cell numbers over time for different L-proline concentrations.]

Figure 5 Cell number decreased significantly at each time point. The 25mg/L of L-proline group also exhibited significantly less cells compared to the other two concentrations. Significance levels were determined based on a Box-Cox transformation of cells raised to the $-1.5$ power, but data is plotted here untransformed for clarity. Groups separated by different symbols († and ‡ for time factor, or * and ** for concentration factor) are considered significantly different ($p < 0.05$) and are shown as mean ± SD.
**Figure 3-6: Collagen results normalized per million cells from L-proline study**

![Graph showing collagen results](image)

**Figure 6** Total collagen per million cells from hydroxyproline assay are shown as mean ± SD. Factors separated by different symbols († and ‡ for time, or * and ** for concentration) are considered significantly different (p < 0.05). 100 mg/L L-proline resulted in significantly lower collagen per cell than the other two concentrations. Significantly higher collagen was seen at 3 and 6 wks compared to wk 0.
Figure 3-7: GAG results normalized per million cells from L-proline study

Figure 7 Results based on DMMB assay calculated for total GAG content per million cells is presented. Data separated by different symbols († and ‡ for time factor, or * and ** for concentration factor) are considered significantly different ($p < 0.05$) and are shown as mean ± SD. As with the collagen data, 100 mg/L L-proline concentration resulted in significantly lower ECM production, and later time points had significantly higher ECM.
**Figure 3-8: Histology of L-proline PLLA constructs at week 6 time-point**

Figure 8 Frames a-c illustrate representative samples of the picrosirius red staining for collagen; d-e show hematoxylin and eosin staining. Micrographs “a” and “d” are 0 mg/L of L-proline, “b” and “e” are 25 mg/L of L-proline, and “c” and “f” are 100 mg/L of L-proline. Cells and collagen were seen sparsely throughout the constructs and were primarily located on the edge of the constructs. The edge of the constructs in each frame is indicated by the black arrows, which also shows the localization of the cells or collagen. Scale bar = 0.1 mm.
Chapter 4: Exploring alternative cell sources for TMJ
disc tissue engineering

Abstract

This study looks at a variety of possible cell sources that could be utilized in a tissue engineering approach for the TMJ disc. Articular chondrocytes, costal chondrocytes, dermal fibroblasts, mixtures of dermal fibroblasts with both types of chondrocytes, passaged costal chondrocytes, and bone marrow derived mesenchymal stem cells were examined in a scaffoldless approach for extracellular matrix (ECM) production relevant to the TMJ disc using biochemical assays and histology. Sulfated glycosaminoglycans and total collagen were highest for the passaged and the primary costal chondrocytes compared to the other groups. Constructs containing fibroblasts or stem cells contracted, forming smaller constructs over 4 wks in culture. The size and ECM data suggest the potential of costal chondrocytes to function in an engineered TMJ disc replacement.
Introduction

Despite the prevalence of TMJ dysfunction, which exist in 28-88% of the population, treatment options remain inadequate.\(^7\) Surgical options are limited and frequently involve the use of implant materials that prevent proper tissue healing or regeneration and subsequent success of the surgery.\(^10,11\) Tissue engineering seeks to create a potential alternative to these materials by creating a functional, biological substitute. The engineered tissue could be implemented into various components of a diseased or damaged TMJ. Approximately 70% of TMJ patients have a displaced disc, which may lead to degeneration of the joint; current work seeks to replace this tissue.\(^9\) The temporomandibular joint (TMJ) disc is a fibrocartilaginous tissue that is essential to everyday function. The anatomy of this joint is shown in Fig. 1.

In spite of the numerous attempts to tissue engineer the TMJ disc with primary cells, construct properties remain greatly inferior to those of native tissue. Additionally, cells from the TMJ disc are scarce and likely abnormal in a patient interested in an engineered disc replacement, suggesting that the use of autologous TMJ disc cells is not feasible. The need for a more clinically relevant and functional cell source is evident. This study examines a variety of cell sources for their biochemical potential to function in a TMJ disc replacement.

Mesenchymal stem cells (MSCs) have recently gained tremendous attention in the field of tissue engineering, particularly for their potential use in musculoskeletal tissues.\(^86,99,101,102,117-119,121,122,139\) Their multipotency, self-renewing ability, and ease of procurement suggest that they are an ideal candidate for TMJ disc tissue engineering. MSCs are an alternative to embryonic stem cells, which are also self-renewing and pluripotent but can potentially form teratomas, are difficult and expensive to grow, and are ethically controversial.

Another potential adult cell source is dermal fibroblasts (DFs). While these cells are fibroblastic in nature, producing primarily collagen type I, they have recently been shown to express
chondrocytic genes and produce chondrocytic proteins like collagen II and glycosaminoglycans (GAGs) under certain conditions. Their intrinsic fibroblastic characteristics coupled with their chondrogenic potential suggests the ability of DFs to function as a fibrochondrocytic cell source.

Other types of cartilage may also provide a possible cell source for TMJ disc tissue engineering. Hyaline articular chondrocytes (ACs) have been studied in the greatest detail, even though the tissue does not repair itself, nor is it very abundant. Nevertheless, ACs are still examined as a possible cell source for cartilage engineering. Similarly, there have been several studies that have examined costal chondrocytes (CCs) as a potential cell source for articular, auricular, nasal, or tracheal cartilage tissue engineering, but none for the purposes of fibrocartilage. This is surprising when one considers the characteristics of these tissues (fibrocartilage and costal cartilage). Fibrocartilage contains collagens type I and II, elastin, and GAGs and functions under both tensile and compressive loads. Costal cartilage also has these components suggesting that its cells could be used for fibrocartilage tissue engineering. The tensile strength in healthy, human, costal cartilage was measured as 4.30-5.31 MPa. Another study measured the properties of young, human, costal cartilage using a slower strain rate and found a tensile strength of 2.27 MPa and a compressive strength of 8.29 MPa. Collagen type II and type I are present at a ratio of around 5:1, which increases with age in humans. There is a large quantity of GAGs: primarily chondroitin sulfate and keratan sulfate. CCs also produce elastin. A schematic of the tissue engineering approach using costal chondrocytes is shown in Fig. 2. Here, the harvested cells are dissociated, assembled into a construct, and implanted as a TMJ disc replacement.

This study assesses the feasibility of numerous cell types for their use in TMJ disc tissue engineering. In addition to constructs of MSCs, DFs, ACs, primary CCs, and passaged CCs, 50:50 co-cultures of the two types of cartilage with the dermal fibroblasts are examined. The hypothesis of this study is that there exists a clinically-relevant cell source that produces
constructs of a reasonable size and similar extracellular matrix (ECM) to that seen in the native TMJ disc.

Materials and methods

Cell isolation

Cells were obtained from an 8 month old, female goat within 4 hrs of death using methods described elsewhere: AC, CC, DF, MSC. ACs, CCs, and DFs were frozen until ready for use. MSCs were cultured on tissue culture treated plastic in minimum essential medium α with L-glutamine (Invitrogen, Carlsbad, CA) with 20% fetal bovine serum (FBS) (Atlanta Biologicals), 1% Penicillin-Streptomycin (PSF) (Invitrogen), 1% non-essential amino acids (NEAA) (Life Technologies, Carlsbad, CA). Some of the CCs were also expanded in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Carlsbad, California) with 10% FBS, 1% PSF, 1% NEAA, and 25 μg/mL L-ascorbic acid. All cells were cultured in a standard incubator at 37°C and 5% CO2. At 70-90% confluence, cells were passaged with 1X trypsin-EDTA (Gibco). The passaged CC group was composed of cells at passage 3.

Construct culture

Constructs were formed by a modified method from Hu and Athanasiou. Each group was seeded with 2 million cells into 3 mm 2% agarose wells. Co-cultures of CCs or ACs with DFs (abbreviated CC/DF and AC/DF, respectively) were at a ratio of 1:1. All constructs were cultured in the wells for 2 wks before being transferred into agarose coated well plates. Media changes occurred everyday with a chemically defined medium, shown previously to enhance the chondrocytic phenotype: DMEM with 1% PSF, 1% NEAA, 1% insulin-transferrin-selenium+ premix (BD Biosciences, San Jose, CA), 0.1 μM dexamethasone, 40 μg/mL L-proline (EMD
Chemicals, Gibbstown, NJ), 50 μg/mL ascorbate 2-phosphate (Sigma), and 100 μg/mL sodium pyruvate (Fisher). Passaged CCs or MSCs were seeded 2 wks after the other groups.

Histology

Constructs were examined at 2 and 4 wks with histological staining and immunohistochemistry (IHC) as described elsewhere.180 Briefly, constructs were sectioned in a cryotome stained for collagen with picrosirius red, for GAG with safranin O/fast green, and for cells with hematoxylin and eosin. Collagen types I and II were detected with appropriate antibodies and DAB visualization.

Biochemistry

Four samples were taken at 2 and 4 wks for biochemical analysis. Sample wet and dry weights were taken before and after a 2 day lyophilization. The dry samples were digested overnight in 125 μg/mL papain (Sigma) at 60°C. Digested samples were stored at −20°C prior to assaying. A hydroxyproline assay was performed to determine the total collagen content with a modified method described previously.181 GAG content was measured with a dimethylmethylene blue Blyscan kit according to the manufacturer’s protocol (Biocolor, Newtownabbey, Ireland). DNA content was determined with a PicoGreen® dsDNA reagent (Molecular probes), and cell numbers were calculated using a conversion factor of 7.7 pg DNA/cell as determined previously.181

Statistics

Data were analyzed with a 2-way analysis of variance. Cell type and time were the two factors, having seven and two levels, respectively. A Tukey’s post hoc test was run to elicit differences between the levels, when the main effects test indicated significance (p < 0.05).
Results

Morphology and histology

Figure 3 shows the morphological and histological differences between the constructs at 4 wks. ACs, CCs, and CC P3s formed constructs about 3 mm in diameter. They appeared mostly cylindrical in shape, but the CC P3 constructs did bulge somewhat in the center, indicative of a fluid-filled central region. AC/DF and CC/DF constructs were also primarily cylindrical, but they contracted to diameters around 1.5 to 2 mm. DF and MSC constructs contracted to spherical shapes, approximately 1 mm in diameter. Staining with safranin-O was only evident in CC, CC/DF and CC P3 constructs, although only in the center of the constructs and not on the outermost edge. Picrosirius red staining was apparent in all constructs and was mostly uniform throughout. Many constructs exhibited denser picrosirius red staining on the outer periphery, which was also apparent on some of the IHC micrographs. Collagen I was seen on CC, DF, CC/DF, CC P3, and MSC constructs, but mostly on the edge of the DF and MSC constructs. Collagen type II staining was present in all but the DF and MSC constructs. Hematoxylin also stained densely around the edge of the constructs, otherwise the cell distribution was mostly uniform.

Wet and dry weight data are shown in Table 1. CC P3 constructs had the largest wet weight, followed by the CC constructs. DF constructs had the smallest wet weight of any of the groups. Similarly, CC and CC P3 constructs had statistically larger dry weights than any other group. Weight significantly increased from 2 wks to 4 wks.

Biochemistry

Figure 4a shows quantitative cell content. Cell number was greatest for the CC P3 group, which was significantly larger than any group except the AC group. The cell number of the AC constructs was significantly greater than those of the CC/DF and MSC constructs. The CC P3 constructs had statistically greater collagen content than any other group, producing more than
twice the closest group at 4 wks (Fig. 4b). GAG content was also greatest for CC P3s, nearly
double the primary CC group GAG content, and the primary CC constructs had greater GAG than
the remaining groups, producing greater than 10 times the GAG of the AC group (Fig. 4c). All
biochemical quantities assayed showed an increase from 2 wks to 4 wks.

Discussion

This study examines a large variety of cell sources in a scaffoldless approach in an effort to find a
more synthetic cell type for TMJ disc tissue engineering. Articular chondrocytes, costal
chondrocytes, dermal fibroblasts, mixtures of both types of chondrocytes with fibroblasts,
passed costal chondrocytes, and bone marrow-derived stem cells were examined for their
ability to synthesize ECM relevant to the TMJ disc. Costal chondrocytes consistently produced
the most GAG and collagen, and they produced the largest tissue volume. Both primary and
passed CCs exceeded the other groups in terms of physical size and ECM production, but the
passed CC group was consistently the best.

While this scaffoldless tissue engineering approach has shown success using young, bovine
articular chondrocytes for the purposes of cartilage tissue engineering, this is the first study to
explore the potential of this approach in TMJ disc tissue engineering. Previous TMJ disc
tissue engineering studies used scaffolds which may inhibit intercellular communication, degrade
prior to the adequate production of matrix, and/or induce an inflammatory response from
degradation byproducts. Previous TMJ disc tissue engineering studies primarily used TMJ disc
fibrochondrocytes as the cell source. These cells produced constructs with limited matrix,
insufficient mechanical properties, and poor cell retention upon three-dimensional
seeding. Additionally, healthy TMJ disc cells are limited in a patient desiring a TMJ
disc replacement, and these cells have limited expansion capabilities in vitro due to their
deciduation, which occurs with passage and expansion. This is also the first study to examine a variety of cell types with this scaffoldless approach.

Creating constructs that maintain their size throughout the culture process is necessary for clinical translatable. The AC, CC, and CC P3 samples exhibited the largest constructs, which did not contract during culture, suggesting they are the most relevant for tissue replacement. Changes in the culturing conditions may reduce the contraction of MSCs and DFs. Alternatively, manipulating the agarose mold may elicit benefits from construct contraction, for example, by causing changes in ECM organization. However, these approaches have yet to create constructs of clinically-relevant sizes.

Biochemical data for both GAG and collagen was highest for the passaged CC group followed by the primary CC group. CC, CC/DF, and CC P3 also look promising because they stained positive for both collagen types I and II. While mechanical data were not obtained in this study, one can postulate about the mechanical properties from the ECM content. GAG contributes to compressive stiffness, suggesting that CC and CC P3 constructs would be most apt at functioning under a compressive load. Larger amounts of collagen were produced by CC P3 samples, indicative of their potential for higher tensile properties than other groups.

Despite the success of other investigators in using MSCs to tissue engineer other mesenchymal tissues, MSCs performed consistency poorly, relative to the other groups studied here. A similar media formulation and high-density culture as used here has shown success in cartilage formation with MSCs, but perhaps additional growth factors would be needed to promote a more chondrocytic phenotype. Other external stimuli may also be necessary to bring about greater ECM production from these cells.

Since costal chondrocytes, dermal fibroblasts, and MSCs represent more clinically-feasible cell sources than TMJ disc cells or articular chondrocytes, further studies on these cell types are
needed. The costal cartilage samples are of particular interest for future research due to their ECM content being most similar to a native TMJ disc. Future work will certainly need to examine the mechanical nature of these constructs, because of the importance of load bearing in the TMJ disc. Nevertheless, these results imply an exciting possibility for a new cell source in TMJ disc tissue engineering. In fact, the passaged costal chondrocyte results are quite promising as they suggest that, in the future, a small costal cartilage biopsy could allow the engineering of a new TMJ disc. If, in addition, we could use skin cells in a co-culture to limit the biopsy amount, then this opens a new avenue toward treating the vexing and intractable problem of TMJ disorders.
### Table 4-1: Wet and dry weights of all groups at both time points

<table>
<thead>
<tr>
<th>Wet weight (mg)</th>
<th>2 wk</th>
<th>4 wk*</th>
<th>Dry weight (mg)</th>
<th>2 wk</th>
<th>4 wk*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC^B</td>
<td>5.6 ± 0.56</td>
<td>9.4 ± 1.7</td>
<td>CC^A</td>
<td>0.52 ± 0.093</td>
<td>1.3 ± 0.41</td>
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<tr>
<td>AC^C</td>
<td>2.0 ± 0.42</td>
<td>4.3 ± 0.85</td>
<td>AC^B</td>
<td>0.17 ± 0.12</td>
<td>0.45 ± 0.14</td>
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<tr>
<td>DF^D</td>
<td>0.88 ± 0.26</td>
<td>0.63 ± 0.33</td>
<td>DF^B</td>
<td>0.24 ± 0.055</td>
<td>0.25 ± 0.014</td>
</tr>
<tr>
<td>AC/DF^C</td>
<td>2.0 ± 0.68</td>
<td>2.5 ± 1.5</td>
<td>AC/DF^B</td>
<td>0.19 ± 0.15</td>
<td>0.45 ± 0.15</td>
</tr>
<tr>
<td>CC/DF^C</td>
<td>1.7 ± 0.63</td>
<td>2.8 ± 0.60</td>
<td>CC/DF^B</td>
<td>0.24 ± 0.16</td>
<td>0.41 ± 0.16</td>
</tr>
<tr>
<td>CC P3^A</td>
<td>12 ± 0.96</td>
<td>18 ± 2.0</td>
<td>CC P3^A</td>
<td>1.0 ± 0.054</td>
<td>2.41 ± 0.12</td>
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<td>MSC^C</td>
<td>0.90 ± 0.24</td>
<td>1.2 ± 0.17</td>
<td>MSC^B</td>
<td>0.063 ± 0.051</td>
<td>0.42 ± 0.16</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD. Groups separated by different letters are statistically significant (p < 0.05). Both wet and dry weights were significantly larger at the later time point as indicated by the *. The CC P3 group had the highest weights followed by the primary CCs. The rest were significantly lower with the DF group having a statistically lower wet weight than any other group.
Figure 4-1: Anatomy of the Temporomandibular Joint

Figure 1 The TMJ disc is shown in red, positioned between the condyle of the mandible and the articular eminence. This cross-section shows the thicker bands of the disc, which circumnavigate the central portion or intermediate zone.
Figure 4-2: Schematic diagram for tissue engineering the TMJ disc with costal chondrocytes

Figure 2 Schematic diagram showing a potential approach for tissue engineering the TMJ disc, using costal chondrocytes. First, costal cartilage is scraped from the patient. The tissue is digested in vitro to give a single cell suspension. These cells are then cultured in a three-dimensional, scaffoldless approach. Once a functional tissue is engineered in vitro the construct would be implanted into the TMJ as a disc replacement.
Figure 4-3: Morphology and histology of the various constructs at 4 wks

<table>
<thead>
<tr>
<th></th>
<th>AC</th>
<th>CC</th>
<th>DF</th>
<th>AC/DF</th>
<th>CC/DF</th>
<th>CCP3</th>
<th>MSC</th>
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<tr>
<td>Saf-O</td>
<td></td>
<td></td>
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<tr>
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<tr>
<td>Col I</td>
<td></td>
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<tr>
<td>Col II</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

**Figure 3** Abbreviations on the left side indicate the type of staining for each row, and the abbreviations at the top indicate the cell type for each column. The scale bar is located in the upper left of the histology micrographs. Morphologically, AC, CC, and CC P3 constructs were mostly cylindrical, with diameters around 3 mm. The other constructs contracted, with CC/DF and AC/DF constructs having diameters between 1.5 and 2 mm. DF and MSC constructs contracted to mostly spherical shapes with diameters about 1 mm. Safranin-O staining was apparent for CC, AC, and CC P3 constructs. Picosirius red staining was seen for all constructs, and was frequently more intense around the outer periphery of the construct, as seen in the AC, DF, CC/DF, CC P3, and MSC groups. Collagen type I was seen for all constructs except the AC and AC/DF constructs, and collagen type II was seen for all constructs except the DF and MSC constructs.
**Figure 4-4: Quantification of biochemical content**

**Figure 4** Data are shown as mean ± SD. Groups separated by different letters in the figure legend are statistically significant ($p < 0.05$). All metrics were significantly larger at 4 wks as indicated by the *.

For cells, the CC P3 had greater quantities than all groups except the AC group, which was significantly greater than the CC/DF or MSC groups. Collagen per construct and GAG per construct were significantly greatest for the CC P3 constructs. The CC group also had greater GAG quantities than all except the CC P3 group.
Chapter 5: Clinically-relevant cell sources for TMJ disc engineering

Abstract

Tissue engineering of the temporomandibular joint (TMJ) disc aims to provide patients with TMJ disorders an option to replace diseased tissue with autologous, functional tissue. This study examined clinically-relevant cell sources by comparing costal chondrocytes, dermal fibroblasts, a mixture of the two, and TMJ disc cells in a scaffoldless tissue engineering approach. It was hypothesized that all constructs would produce matrix relevant to the TMJ disc, but the mixture constructs were expected to appear most like the TMJ disc constructs. Costal chondrocyte and mixture constructs were morphologically and biochemically superior to the TMJ disc and dermal fibroblast constructs, and their compressive properties were not significantly different. Costal chondrocyte constructs produced almost 40 times more collagen and 800 times more glycosaminoglycans than TMJ constructs. This study demonstrates the ability of costal chondrocytes to produce extracellular matrix that may function in a TMJ disc replacement.

Introduction

There are approximately 10 million patients in the United States that suffer from TMJ disorders.\textsuperscript{188} In severely diseased joints, surgical options are met with varying amounts of success, as reviewed elsewhere.\textsuperscript{10,11} Efforts to engineer the TMJ disc may create a viable alternative to current treatment options.

While previous work using TMJ disc cells characterized their \textit{in vitro} behavior for the purpose of tissue engineering, a clinical solution will likely not involve these cells. This conclusion was reached following the experience of our laboratory and others, demonstrating the difficulty in culturing these cells, failure of constructs to achieve sufficient morphology, and poor mechanical strength of the engineered tissue.\textsuperscript{105,108,110,115,116,152,183}

A clinically-feasible cell source should be abundant, healthy, and leave little donor site morbidity. Selection of an alternative source must also consider the functionality of the cells. Previous characterization data guide this selection; the TMJ disc has properties of both fibrous tissue and cartilage, indicating the need for a fibrochondrocytic cell source.\textsuperscript{4} Specifically, the cells should produce tissue containing collagen type I, type II, and glycosaminoglycans and should support both tensile and compressive loads. Dermal fibroblasts exhibit chondrogenic potential despite being inherently fibrogenic in nature.\textsuperscript{3,157} Costal cartilage contains both collagen types II and I (in a ratio of 5:1) and glycosaminoglycans suggesting its potential to function as a fibrocartilage replacement.\textsuperscript{172} Indeed, costochondral grafts are already used in mandibular reconstruction.\textsuperscript{189} In addition to their functional potential, costochondral cartilage and dermal fibroblasts are easily harvested and more abundant than TMJ disc cells, making them a more clinically-feasible source of cells for tissue engineering.

Constructs of costal chondrocytes, dermal fibroblasts, TMJ disc cells, and a 50/50 costal chondrocyte/dermal fibroblast mixture were examined in this study. Based on known tissue
characteristics, it was hypothesized that all cell types would produce extracellular matrix similar to the TMJ disc cell construct. However, the costal chondrocyte/dermal fibroblast mixture was expected to produce the most TMJ disc cell-like construct. Specifically, mechanical and biochemical properties that are most similar to the TMJ would be observed via glycosaminoglycan and collagen II production by the costal chondrocytes and collagen I production by the dermal fibroblasts.

**Materials and methods**

**Cell isolations**

Cells were isolated from three skeletally-mature, Spanish, female goats. TMJ disc cells were isolated as previously described and cultured until 70-90% confluent. They were passaged with trypsin-EDTA (Gibco, Carlsbad, CA) until passage 2. Culture medium was Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine and 4.5g/L glucose (Biowhittaker, Walkersville, MS), 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA), 1% Penicillin-Streptomycin-Fungizone (Cambrex, Walkersville, MD), 1% non-essential amino acids (Gibco), 25μL/mL L-ascorbic acid (Sigma, St. Louis, MO), and 1μL/mL insulin (Sigma).

Skin was cut into 1cm² squares, digested in 0.5% dispase (Gibco) at 4°C overnight, and then epidermis and adipose layers were removed. The remaining dermis was placed in 0.05% type II collagenase (Worthington, Lakewood, NJ). After 24hrs, samples were passed through a 70μm cell strainer, yielding a single-cell suspension, which was plated and fed with DMEM containing Glutamax, 10% fetal bovine serum, 1% Penicillin-Streptomycin-Fungizone, and 1% non-essential amino acids. When confluent, cultures were exposed to 0.5% dispase for 30min to remove keratinocytes. The purified dermis cells were allowed to expand, and passage 2 cells were used.
Cartilage was scraped from non-floating ribs, minced into cubes of approximately 1mm³, and digested overnight with 0.2% collagenase in DMEM. After isolation, cells were frozen in DMEM with 10% dimethyl sulfoxide, 20% fetal bovine serum, 1% Penicillin-Streptomycin-Fungizone, and 1% non-essential amino acids to allow for concurrent seeding of costal cartilage constructs with the other groups.

**Construct culture**

Constructs were formed using a scaffoldless method described previously.¹⁰⁴ Two million cells (TMJ, costal chondrocytes, dermal fibroblast or a 50:50 costal chondrocyte: dermal fibroblast mix) were seeded in 3mm wells formed with 2% agarose (Fisher Scientific). Media changes occurred everyday using DMEM with 1% Penicillin-Streptomycin-Fungizone, 1% NEAA, 1% insulin-transferrin-selenium+ premix (BD Biosciences, San Jose, CA), 0.1μM dexamethasone, 40μg/mL L-proline (EMD Chemicals, Gibbstown, NJ), 50μg/mL ascorbate 2-phosphate (Sigma), and 100μg/mL sodium pyruvate (Fisher). After 2wks, constructs were transferred to agarose-coated plates. Samples were removed for biochemistry and histology at 3wks and 6wks. Additionally, mechanical testing was performed at 6wks.

**Histology**

Samples were frozen in HistoPrep™ (Fisher), and 14μm sections were prepared. Slides were stained with picrosirius red for collagen or safranin-O/fast green for glycosaminoglycans. Immunohistochemistry slides were stained for collagen types I and II, as described previously.¹⁶

**Biochemistry**

Four samples per group were lyophilized for 2days and digested at 4°C with constant agitation for 7days with 125μg/mL papain (Sigma) digest, followed by 2days of 1mg/mL elastase (Sigma) digestion. Samples were stored at −20°C.
Cell numbers were determined using PicoGreen® (Molecular probes) with a conversion factor of 7.7pg DNA/cell.\textsuperscript{181} Total collagen was measured using a modified hydroxyproline assay.\textsuperscript{151} Sulfated glycosaminoglycans were quantified with a dimethylmethylen blue Blyscan kit (Biocolor, Newtownabbey, Ireland). Type I collagen was quantified with an indirect ELISA, described previously.\textsuperscript{177}

**Mechanical testing**

At least five samples per group were tested in tension and compression. Tensile testing was performed on an Instron 5565 (Norwood, MA) to determine ultimate tensile strength and elastic modulus. Samples were cut into a dog bone shape using a scalpel blade and biopsy punch and tested at 10% strain rate/min until failure.

Specimens were tested in unconfined compression with an indentation apparatus.\textsuperscript{190} Each sample was tare-loaded with 0.00196mN until equilibrium was reached (deformation less than 10\textsuperscript{-6}mm/s) or loading time reached 10min. A step load of 0.00686N was then applied until equilibrium or 1hr elapsed. Creep data were analyzed with Matlab's (The Math Works, Inc) curve fitting tool using the viscoelastic model.\textsuperscript{191}

**Statistics**

Data were analyzed with a 2-way analysis of variance where time and cell type were factors with two and four levels, respectively. When an F-test indicated significance ($p < 0.05$), a Tukey's post hoc test was performed.
Results

Morphology and histology

Morphologically, dermal fibroblast and TMJ disc constructs contracted, yielding mostly spherical constructs measuring about 1mm in diameter. Mixture constructs contracted to a diameter just less than 2mm and maintained a more cylindrical shape. Costal chondrocyte constructs expanded to a diameter and height just over 3mm (Fig.1 and Table 1).

TMJ and dermal fibroblast groups did not stain with safranin-O (Fig.1 m-p). All groups stained positive for collagen (Fig.1 i-l). The costal chondrocyte group stained intensely for glycosaminoglycans throughout the construct, whereas the costal chondrocyte/dermal fibroblast group stained only around the periphery of the construct. Immunohistochemistry demonstrated positive collagen I staining for all groups. Only groups which contained costal chondrocytes, however, stained positive for collagen II (Fig.2).

Biochemistry

Costal chondrocyte constructs had significantly more cells than any other group, and costal chondrocyte/dermal fibroblast constructs had significantly more than dermal fibroblast or TMJ constructs (Fig.3a). Cell numbers were not significantly affected by time. The initial seeding was 2 million cells/construct, which only the costal chondrocyte constructs maintained.

Costal chondrocyte constructs had significantly more total collagen than any other group (Fig.3b). Collagen type I was normalized to the amount in the TMJ constructs, and there was significantly more collagen I for costal chondrocyte and costal chondrocyte/dermal fibroblast over the other constructs (Fig.3c). Both total collagen and collagen I increased significantly at wk6 from wk3.

Glycosaminoglycan per construct also increased significantly at the later time point. For costal chondrocyte constructs it was orders of magnitudes higher than the other groups with values at 3
and 6wks of 640±17μg and 1700±94μg, respectively. TMJ constructs made 1.7±0.6μg at 3wks and 1.1±0.4μg at 6wks. At 3wks the dermal fibroblast constructs contained 2.6±1.0μg and 1.1±0.3μg at 6wks. The co-culture constructs produced 1.8±0.7μg and 2.0±0.8μg at 3 and 6wks, respectively. Despite cell quantity changes, matrix normalization to cell number resulted in similar trends to the per construct normalization.

**Mechanical properties**

There were no statistical differences between any of the measured compressive properties. For all three tensile properties, the TMJ constructs were significantly higher (Table 2).

**Discussion**

Due to the prevalence of TMJ disorders and limited treatment options, it is essential to examine possible alternatives to current surgical techniques, such as engineering a replacement disc. Using TMJ disc cells in this approach has numerous drawbacks, such as a limited population of healthy cells and donor site morbidity. The current study examines the potential of dermal fibroblasts and costal chondrocytes as alternative cell sources for fibrocartilage tissue engineering. The results show significant increases in extracellular matrix produced by costal chondrocytes from that produced by dermal fibroblasts or TMJ disc cells, while co-culture of costal chondrocytes and dermal fibroblasts made extracellular matrix in quantities between either individual cell type. These distinctions were also apparent in construct size and weight, where costal chondrocyte constructs were significantly larger. The hypothesis that a costal chondrocyte/dermal fibroblast co-culture would produce constructs most similar to those produced by the TMJ cells was not supported by this work. Instead, dermal fibroblast constructs were most like TMJ constructs. Costal chondrocyte/dermal fibroblasts trended toward improved biochemical content in addition to improved morphology over TMJ cells and dermal fibroblasts.
However, costal chondrocyte constructs exceeded expectations by producing significantly more glycosaminoglycans, total collagen, and collagen type I than any other construct.

The extracellular matrix results obtained with the TMJ disc cells are representative of previous studies, which suggest that these cells do not exhibit a robust synthetic ability. In contrast, costal chondrocyte constructs demonstrate that cells derived from this source are highly productive relative to the others tested. The most collagen produced previously with TMJ disc cells was approximately 60μg total collagen per construct—made with over 6 million cells. At the same time point (6wks), the costal chondrocyte constructs produced over 450μg of total collagen with an initial seeding density of 2 million cells. Additionally, the costal chondrocyte constructs yielded almost 100 times more glycosaminoglycans than previous TMJ disc cell constructs.

The 200-300% increase in collagen I of costal chondrocyte constructs over TMJ constructs further illustrates the productive capacity of the costal chondrocytes. Since rib cartilage contains both collagen types I and II, and skin contains primarily type I, the dermal fibroblast constructs were expected to produce the most collagen type I followed by costal chondrocyte/dermal fibroblast constructs, and, finally, costal chondrocyte constructs. However, like total collagen, dermal fibroblast constructs contained the least collagen I, and no statistical difference was seen between the costal chondrocyte and costal chondrocyte/dermal fibroblast group. This reinforces the total extracellular matrix data that suggest costal chondrocytes alone may be a viable cell source for functional tissue engineering of the TMJ disc.

While extracellular matrix data indicate that costal chondrocytes are most likely to succeed in fibrocartilage tissue engineering, mechanical data did not correspond to extracellular matrix changes. Generally, an increase in glycosaminoglycans increases compressive resilience, while more collagen improves tensile strength. In this experiment, the TMJ disc cell constructs had significantly higher tensile properties. This could be due to tighter cell packing or better organization of the extracellular matrix. However, even the largest of any of the constructs' tensile
properties were still orders of magnitude below the native values for the TMJ disc.\textsuperscript{76} Despite the lower tensile strength, the high quantities of extracellular matrix suggest that with the proper stimuli (biochemical or mechanical) the costal chondrocytes can produce a more mechanically robust construct, perhaps through better organization of the collagen fibers. Mechanical stimuli are particularly well-suited for altering organizational changes, as seen with a variety of tissue engineering studies, and will be an important area of future research for TMJ disc tissue engineering.\textsuperscript{140,192-194}

While the scaffoldless approach used in this study has clear advantages, like avoiding immune responses due to biomaterials, many of the TMJ disc cells and dermal fibroblasts were not retained at even the first time point. Low cell retention was also seen previously with TMJ disc cells on scaffolds.\textsuperscript{110} At both time points, only one-eighth of the original cells were measured for the TMJ and dermal fibroblast constructs, and one-fourth remained in the costal chondrocyte/dermal fibroblast constructs. Dermal fibroblast and TMJ constructs also contracted significantly from the initial well diameter, while the costal chondrocyte constructs retained their initial size or grew slightly. A reduction in size makes it more difficult to engineer a replacement tissue with functional dimensions. Considering the retention of cells, most overall extracellular matrix production, and ability to create a replacement tissue with clinically-relevant dimensions, the costal chondrocytes appear to be the most likely cell source candidate, of those studied here, for TMJ disc replacement, particularly with this scaffoldless method.

Finally, the costal chondrocyte constructs offer several other advantages as a cell source for TMJ disc reconstruction. Large quantities of costal cartilage can be obtained from almost any patient with a minimally-invasive harvest technique producing limited morbidity and complications.\textsuperscript{195} While costal cartilage is a relatively acellular tissue,\textsuperscript{196} the protocol could be optimized to limit the amount of needed tissue, for example, by expanding the cells before construct formation. However, patients requiring a tissue engineered disc would not have sufficient quantity of nonpathological tissue to provide an adequate number of TMJ disc cells, even with passaging,
particularly considering the size reduction discussed previously.\textsuperscript{15} While using costal cartilage without \textit{in vitro} manipulation is appealing, previous work reveals complications, like tissue overgrowth\textsuperscript{197,198} and undesirable calcification. These concerns can be addressed by controlling the \textit{in vitro} environment used in a tissue engineering approach. By influencing growth conditions and applied stimuli, constructs can be engineered to produce the appropriate dimensions, mechanical properties, and biochemical properties. After examining the integrative capacities of engineered neotissue at different maturities, grafts may be more readily integrated with the native joint.

Although costal chondrocytes are clearly superior in this experiment as a highly productive and feasible cell source for tissue engineering, the simplicity of dermal fibroblast harvest warrants its continued examination. Further optimization is also needed to improve the costal chondrocyte constructs' mechanical properties. With the application of external stimuli, like growth factors or mechanical forces, scaffoldless costal chondrocyte constructs may produce sufficient quantities of organized matrix to function as a TMJ disc replacement and serve as a feasible alternative for patients.
Table 5-1: Quantitative size data

<table>
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<tr>
<th></th>
<th>Diameter wk3 (mm)</th>
<th>Diameter wk6 (mm)</th>
<th>Volume wk3 (mm$^3$)</th>
<th>Volume wk6 (mm$^3$)</th>
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<tbody>
<tr>
<td>TMJ</td>
<td>0.970±0.037$^{a}$</td>
<td>0.863±0.109$^{c}$</td>
<td>0.493±0.135$^{a}$</td>
<td>0.357±0.158$^{a}$</td>
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<td>costal chondrocyte</td>
<td>3.05±0.088$^{a}$</td>
<td>3.12±0.088$^{a}$</td>
<td>19.5±1.95$^{a}$</td>
<td>26.4±1.77$^{a}$</td>
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<td>costal chondrocyte/dermal fibroblast</td>
<td>1.61±0.165$^{b}$</td>
<td>1.73±0.132$^{a}$</td>
<td>2.38±0.861$^{b}$</td>
<td>3.54±0.564$^{b}$</td>
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<tr>
<td>dermal fibroblast</td>
<td>1.03±0.110$^{c}$</td>
<td>0.955±0.090$^{c}$</td>
<td>0.578±0.174$^{cc}$</td>
<td>0.525±0.132$^{cc}$</td>
</tr>
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</table>

Table 1 Quantitative size data (mean ± standard deviation) of all groups. Groups separated by different letters are considered significantly different ($p < 0.05$). Volumes were significantly greater at wk6 than at wk3 as indicated by the *. Time was not a significant factor for diameter. Costal chondrocytes groups were consistently the largest, followed by the costal chondrocyte/dermal fibroblast mixture.
### Table 5-2: Mechanical properties

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<td>Relaxed modulus (kPa)</td>
<td>Instantaneous modulus (kPa)</td>
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<tr>
<td>TMJ</td>
<td>41.2 ± 9.37</td>
<td>179 ± 40.3</td>
</tr>
<tr>
<td>CC</td>
<td>36.4 ± 25.0</td>
<td>851 ± 1470</td>
</tr>
<tr>
<td>CC/DF</td>
<td>23.3 ± 9.05</td>
<td>77.1 ± 36.3</td>
</tr>
<tr>
<td>DF</td>
<td>41.9 ± 11.1</td>
<td>135 ± 58.0</td>
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</tbody>
</table>

**Table 2** Mechanical properties (mean ± standard deviation) of all groups at wk6. TMJ disc cell constructs were significantly stronger in tension than any other group as indicated by the * (p < 0.05). No other statistical differences were observed.
Figure 5-1: Gross morphology and histology at wk6

Figure 1 Gross morphology and histology at wk6 for tissue engineered constructs with various cell sources: TMJ (a column), dermal fibroblast (b column), costal chondrocyte/dermal fibroblast (c column), and costal chondrocyte (d column). a-h illustrate top and side views of the constructs. Spaces on the ruler below the images are 1mm. These clearly show the extensive contraction that occurred in TMJ and dermal fibroblast constructs, while costal chondrocyte/dermal fibroblast had substantial but less contraction. Staining with picrosirius red (i-l) and safranin-O/fast green (m-p) are also shown here. Of particular interest is the glycosaminoglycan staining, which is most apparent in costal chondrocyte construct and around the edge of the costal chondrocyte/dermal fibroblast construct. Dermal fibroblast and TMJ disc cell constructs did not stain with safranin-O. Scale bar = 0.1mm.
**Figure 5-2: Immunohistochemistry staining for constructs**

![Immunohistochemistry staining for constructs](image)

**Figure 2** Immunohistochemistry staining for constructs made from TMJ disc cells (a, f), dermal fibroblasts (b, g), costal chondrocyte/dermal fibroblast co-culture (c, h), and costal chondrocytes (d, i). a-e show the collagen I staining which is present in all constructs. (e is the positive control: knee meniscus.) f-j were stained for collagen type II. (j is the positive control, articular cartilage, for collagen II.) While collagen type II staining is not as intense as the collagen I it is clearly apparent in CC/DF and CC constructs. DF and TMJ disc cell constructs did not stain for collagen type II. Staining for both collagen types was mostly uniform throughout the constructs. Scale bar = 1mm.
Figure 5-3: Biochemical results for all groups

Figure 3 Biochemical quantities for cells (a), collagen per construct (b), and percent increase in collagen type I (c). All data are shown as mean + standard deviation with a sample size equal to four for all groups. Groups separated by different letters are considered significantly different (p<0.05). Costal chondrocyte constructs have more cells (determined by picogreen quantification) and total collagen (measured with hydroxyproline assay) than any other group. Time was a significant factor in total collagen with larger values at wk6. Costal chondrocyte/dermal fibroblast constructs had significantly more cells than dermal fibroblast or TMJ constructs and had a nearly significant increase in collagen over dermal fibroblast and TMJ constructs. Graph c shows the percent increase in collagen I from the TMJ control at the respective time point. Costal chondrocyte and costal chondrocyte/dermal fibroblast constructs had significantly more collagen type I.
Chapter 6: Overcoming limitations in tissue engineering the temporomandibular joint with articular and costal chondrocytes

Abstract

Objective: This study examines the tissue engineering potential of passaged (P3) and primary (P0) articular chondrocytes (ACs) and costal chondrocytes (CCs) from skeletally-mature goats for use in the temporomandibular joint (TMJ). Design: These four cell types were assembled into scaffoldless tissue engineered constructs and cultured for 4 wks. The constructs were then tested for cell, collagen, and glycosaminoglycan (GAG) content with biochemical assays, and collagen types I and II with enzyme-linked immunosorbent assays. Constructs were also tested under tension and compression to determine biomechanical properties. Results: Both primary and passaged CC constructs had greater GAG/wet weight than AC constructs. Primary AC constructs had significantly less total collagen and contained no collagen type I. AC P3 constructs had the largest collagen I/collagen II ratio, which was also greater in passaged CC constructs relative to primary groups. Primary AC constructs were not mechanically testable, while passaged AC and CC constructs had significantly greater tensile properties than primary CC constructs. Conclusions: Primary CCs are considerably better than primary ACs and have potential use in tissue engineering when larger quantities of collagen type II are desired. The poor performance of the ACs, in this study, which contradicts the results seen with previous studies using immature bovine ACs, may thus be attributed to the animals’ maturity. However, CC P3 cells appear particularly well-suited for tissue engineering fibrocartilage of the TMJ due to the high quantity of collagen and GAG, and tensile and compressive mechanical properties.

Introduction

Tissue engineering presents a potential solution to the complex problem of temporomandibular joint (TMJ) disorders. Current approaches to treating TMJ disorders include pain medication and physical therapy, but surgical approaches are often necessary when the disorder becomes severe or the patient has extensive trauma. These treatment options, described in greater detail elsewhere, are not always universally accepted.\textsuperscript{10,11} Reconstruction of the joint requires the use of non-biologic materials, which can restore some function to the joint, but lack of integration of the materials with the soft tissues prevents perfect functional restoration. In some situations, the disc is removed, which may temporarily alleviate pain but will frequently result in degeneration of the joint after time. A possible long-term, non-immunogenic solution for severe TMJ problems is the creation of autogenic, functional tissues. Regeneration of various tissue types within the joint may be necessary; however, this work focuses on the creation of cartilaginous tissues found in the joint: fibrocartilage of the disc and articular cartilage of the mandibular condyle and temporal bone.

Creating these tissues requires a solid understanding of the structural and functional characteristics of the tissues. The properties of the TMJ disc and articular cartilage are distinct from one another and other tissues, as reviewed elsewhere.\textsuperscript{4,169} Some key distinctions include the primary type of collagen present in the tissues and the mechanical function of the tissues. While articular cartilage mainly supports compressive loading, the TMJ disc has an important additional tensile role. Articular cartilage contains nearly 100% collagen type II, while the TMJ disc is almost 100% collagen type I. However, other tissues fall between these, containing significant quantities of both collagens type I and II, including the mandibular condylar cartilage\textsuperscript{169,200} and the knee meniscus.\textsuperscript{201,202} As with collagen content, mechanical properties follow a similar spectrum. The TMJ disc has a relatively low aggregate modulus—around 20 kPa for the porcine disc with indentation testing,\textsuperscript{91} while articular cartilage has a modulus over 1 MPa.\textsuperscript{88} In tension, the TMJ disc elastic modulus ranges between 1-100 MPa, depending on the species and direction tested, while articular cartilage has a modulus less than 20 MPa.\textsuperscript{4} Both
contain a substantial quantity of glycosaminoglycans (GAGs). These characteristics must be considered when evaluating potential replacements for the tissues.

Previous attempts to tissue engineer the TMJ disc have frequently used TMJ disc cells.\textsuperscript{110,112,162,163} Despite numerous attempts, these cells have yet to approach the quantitative biochemical content or mechanical strength necessary to function in a tissue replacement. Additionally, these cells are difficult to obtain and very limited in quantity, which is not likely remedied through \textit{in vitro} cell expansion and passaging.\textsuperscript{2,184} Recent work with costal chondrocytes (CCs), however, suggests their potential in TMJ disc tissue engineering both in functionality and clinical translatability.\textsuperscript{178} CCs have been shown to produce substantial, relevant matrix—dramatically more than seen previously with TMJ disc cells. Collagen/wet weight at 3 wks after culture was approximately 1\% while GAG/wet weight was 3\%.\textsuperscript{178} These values are below the collagen seen in native tissues [20\% collagen/wet weight for cartilage\textsuperscript{14} and 30\% collagen/wet weight for the TMJ disc\textsuperscript{42}] but near to those seen for GAG content in the native tissues [4-7\% GAG/wet weight for cartilage\textsuperscript{14} and 2\% GAG/wet weight for the TMJ disc\textsuperscript{72}]. Constructs made from these CCs were mechanically testable and manipulatable with surgical tools, which was not true of previous TMJ disc cell constructs.\textsuperscript{109,113,178} The improvements in translatability include the surgeons' familiarity harvesting costal cartilage, limited complications of this surgery, and abundance of healthy tissue.\textsuperscript{189,195}

While tissue engineering of the TMJ disc is still in its infancy, articular cartilage regeneration has been explored considerably longer. The initial paradigm for tissue engineering used cells seeded on a scaffold, but previous work with articular chondrocytes (ACs) has shown the ability of these cells to form neotissue without a scaffold and with properties approaching those of native tissue.\textsuperscript{104,203,204} The importance of the cell in this approach is apparent, and while functional tissue has been created with young, calf chondrocytes, the usefulness of this approach with adult ACs remains uncertain. Finding a functional, skeletally-mature cell source would surge forward the clinical translatability of this approach.
Skeletally-mature CCs and young ACs have shown previous functional potential and do not require cells from the TMJ, suggesting their clinical usefulness to patients in need of TMJ surgery, whose TMJ cells may be phenotypically or functionally abnormal. However, the quantity of chondrocytes needed is quite substantial, and so there is considerable interest in expanding and passaging these cells. Previous work on both these cell types shows dedifferentiation of the cells from a chondrocyte-like to a more fibroblast-like phenotype.\textsuperscript{164,177} While this response is often considered an undesirable result of the cell passaging process, in the case of fibrocartilage tissue engineering, it may be beneficial. The shift from collagen type II to collagen type I and decrease in GAGs yields a fibrochondrocyte-like cell type. These chondrocytes, phenotypically altered by passaging, may function most effectively in a tissue engineered construct for the purposes of fibrocartilage replacement.

This study examines ACs and CCs at both passage 0 (P0) and passage 3 (P3) in a scaffoldless tissue engineering approach. The hypotheses of this study are twofold: 1) CCs will produce constructs that are equal to or better than AC constructs in biological and biomechanical properties. 2) P3 constructs will have characteristics that are more amenable to fibrocartilage tissue engineering, while P0 constructs will be better suited for cartilage tissue engineering; specifically, P3 constructs will have greater collagen I than collagen II and visa versa for P0 constructs. P3 constructs will have greater tensile properties, while P0 constructs will have greater compressive properties.

\textbf{Materials and Methods}

\textit{Cell isolation}

Tissue was obtained from three skeletally-mature, Spanish, female goats from a local abattoir within 4hrs postmortem. Costal cartilage was scraped from the non-floating ribs, and articular
cartilage was taken from the distal femur. Both cartilages were minced and digested overnight in 0.2% type II collagenase (Worthington, Lakewood, NJ) in Dulbecco's modified Eagle medium (DMEM) (Gibco, Carlsbad, California) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA), 1% Penicillin-Streptomycin-Amphotericin B (PSF), 1% non-essential amino acids (NEAA) (Life Technologies, Carlsbad, CA), and 25 μg/mL L-ascorbic acid (Sigma, St. Louis, MO). Some cells were expanded on tissue culture treated plastic, while the remaining primary cells were frozen in liquid nitrogen in DMEM with DMSO and an additional 10% FBS. Cells were passaged at 70-90% confluence with trypsin-EDTA (Gibco) until passage 3.

**Construct culture**

Constructs were formed from passage 3 and primary ACs and CCs by a modified method, described previously. Two million cells were seeded into 5mm agarose molds. After 2 wks, constructs were removed and transferred into agarose-coated tissue culture treated plates. Chondrogenic medium consisting of DMEM supplemented with 1% PSF, 1% NEAA, 1% insulin-transferrin-selenium+ premix (BD Biosciences, San Jose, CA), 0.1 μM dexamethasone, 40 μg/mL L-proline (EMD Chemicals, Gibbstown, NJ), 50 μg/mL ascorbate 2-phosphate (Sigma), and 100 μg/mL sodium pyruvate (Fisher) was changed everyday. Constructs were examined at 4 wks using the various assays described below.

**Histology**

Samples were frozen in Histoprep™ Frozen Tissue Embedding Media (Fisher) and cut to 14 μm sections in a cryotome. Sections were fixed in formalin and stained with picrosirius red for collagen, safranin-O/fast green for GAGs and von Kossa stain for calcification using standard histological protocols. Immunohistochemistry (IHC) was also used to examine the localization of collagen types I and II, using methods described previously.
Biochemistry

Six samples per group were used to determine the biochemical content. Samples were weighed and dried to determine wet and dry weights. Dry samples were then digested at 4°C with constant mechanical agitation in 125 μg/mL papain (Sigma) for 7 days followed by 1mg/mL elastase (Sigma) digestion for 2 days. Cell number was determined by quantifying DNA with a PicoGreen® dsDNA reagent (Molecular probes) and converting with a factor of 7.7 pg DNA/cell, as determined previously. Collagen was quantified with a modified colorimetric hydroxyproline assay, described previously. In brief, samples were hydrolyzed with NaOH. The reaction was then neutralized with HCl. Chloramine T and Ehrlich's reagents were added and incubated at 60°C. Sulfated GAGs were measured with a dimethylmethylen blue (DMMB) Blyscan kit, according to the manufacturer's protocol (Biocolor, Newtownabbey, Ireland). Enzyme-linked immunosorbent assays (ELISA) were also performed to quantify the amounts of collagen type I and type II. Type I collagen was quantified using an indirect ELISA, described previously. Briefly, digested samples and standards were incubated overnight on high-affinity plates at 4°C. A primary mouse antibody (Accurate Chemical) was added for 2hrs, followed by a HRP-conjugated secondary antibody (Chemicon). Tetramethyl benzidine (Chemicon) was used for visualization. A Chondrex (Redmond, WA) collagen detection kit was used for collagen type II quantification, according to the manufacturer's protocol.

Mechanical testing

Six samples per group were tested for tensile and compressive properties. Tensile testing was performed on an Instron 5565 (Norwood, MA) with samples glued to a paper frame. This allowed for a defined gauge length and proper securing of the samples to the Instron. After cutting the paper, samples were loaded at 1% strain/sec until failure. Ultimate tensile strength (UTS) and elastic modulus (E) were determined from the stress/strain curves. Compressive properties were determined with creep indentation testing. Samples were cut through the diameter to make a level testing surface and put in saline solution. A tare load of 0.00196 N was applied until
equilibrium was reached (deformation less than $10^{-5}$ mm/s) or 10 min elapsed. Afterwards, a step load of 0.00686 N was added until equilibrium was again reached or 1 hr passed. The creep load was then removed, and the sample equilibrated once again. These data were used to determine aggregate modulus, Poisson's ratio, and permeability.

**Statistics**

Data were analyzed with a one-way analysis of variance. When an F-test indicated significance ($p < 0.05$) a Tukey's *post hoc* test was performed to determine differences among the groups.

**Results**

**Morphology and histology**

Weight and diameter data for the constructs are shown in Table 1, and gross morphology is illustrated in Fig. 1. Wet weight was significantly greatest for CC P0 constructs. AC P0 constructs had greater wet weights than AC P3 and CC P3 constructs. Both P0 groups had the greatest percent water, followed by CC P3 and then AC P3, which were statistically different. AC P0 constructs had the greatest diameter, around 5.5 mm. The CC P0 constructs had diameters around 4.5 mm, which were significantly greater than either of the P3 groups. CC P3 constructs had significantly greater diameters than the AC P3 constructs, measuring about 3 mm and 2 mm, respectively. CC P0 constructs remained cylindrical in shape, while CC P3 and AC P3 formed more spherical shapes. AC P0 constructs were of a hydrated, gelatinous consistency, which did not hold a solid shape, but rather relaxed to the position seen in Fig. 1a,e. Histological micrographs are also shown in Fig. 1. Safranin O staining (Fig. 1i-l) was positive for both CC groups but only lightly stained in the AC P0 group and not stained in the AC P3 group. The picrosirius red stain (Fig. 1m-p) was positive for all groups, but particularly intense on the outer
few micrometers of the constructs. Collagen I (Fig. 1q-t) was positive for all groups except AC P0.
Collagen II (Fig. 1u-x) was positive for all groups, although AC P3 stained only faintly.

**Biochemistry**

Cell numbers at 4 wks were all around 1.5 million (Fig. 2a). The number of CC P0 was significantly greater than the other groups, and the AC P3 number was significantly greater than the CC P3 number. The CC P3 group, however, had the greatest GAG per wet weight (Fig. 2b). CC P0 constructs also had significantly greater GAG per wet weight than either passage of AC constructs. Collagen per wet weight (Fig. 3c) was significantly greater in CC P3, AC P3, and CC P0 than AC P3 constructs. The collagen type I ELISA did not detect any collagen I in the AC P0 samples. In contrast, AC P3 constructs had 86 ± 5 times the collagen I relative to the collagen II content measured with the ELISAs. The type I to type II ratios for CC P3 and CC P0 were 0.87 ± 0.3 and 0.05 ± 0.009, respectively, suggesting the prevalence of collagen type II in the primary constructs.

**Mechanical properties**

As was explained in the morphology description of the AC P0 constructs, this group was not solid and unable to withstand mechanical loading; therefore, these constructs were not testable in either tension or compression. Tensile elastic modulus (Fig. 3a) and UTS (Fig. 3b) were significantly greater for passage 3 groups than primary. Aggregate modulus was not significantly different for any of the testable groups. Poisson's ratio was also not significant and ranged from 0 to 0.39. CC P3 constructs had a significantly greater permeability than AC P3 or CC P0 groups with results of $5.46 \times 10^{-13} \pm 4.32 \times 10^{-13}$, $1.52 \times 10^{-13} \pm 1.02 \times 10^{-13}$, and $5.20 \times 10^{-14} \pm 1.71 \times 10^{-14}$, respectively.
Discussion

This work examined the biochemical and mechanical properties of scaffoldless, tissue engineered constructs created from passaged and primary costal chondrocytes and articular chondrocytes. The constructs made from primary cells were generally larger, had less mechanical integrity, and contained less ECM per wet weight. The AC P0 constructs, in particular, had significantly less collagen, and were not mechanically testable in either tension or compression. While smaller and rounded in shape, the P3 groups had significantly greater tensile strength, and the CC P3 constructs had the most GAG/wet weight. ELISA and IHC analyses demonstrated the lack of collagen type I in AC P0 constructs and limited quantity of collagen type II in AC P3 constructs.

These results supported the hypotheses of this study. The first, that CCs would produce constructs equal to or better than AC constructs, was particularly apparent in the primary constructs. CC P0 constructs were better than AC P0 constructs for cell quantity, overall size, mechanical properties, collagen/wet weight, and GAG/wet weight. CC P3 constructs also had significantly greater size and GAG/wet weight than AC P3 constructs, but only trended higher in aggregate modulus and collagen/wet weight. In regard to the second hypothesis, which suggested that P3 constructs would be more like fibrocartilage and the P0 constructs more like articular cartilage, there are both significant and trending results in support of this. AC P3 constructs had an infinitely higher collagen type I/collagen type II ratio than AC P0 constructs. This ratio was also greater for CC P3 constructs compared to CC P0 constructs but not significantly different. Additionally, the P3 constructs had significantly greater tensile properties than the P0 constructs, but compressive properties were not significantly different.

The results seen for the primary costal chondrocytes were similar to those seen previously. While the previous study used similar methods, samples were tested biochemically at 3 and 6 wks. The quantitative results of this 4 wk study fell between the previous values for both hydroxyproline and DMMB assays. Tensile results were 3-5 times lower in this study than those seen previously at 6 wks, but the previous study used smaller agarose molds with the same
number of cells to form the constructs, resulting in smaller constructs. This change in construct size may alter the organization of the ECM and therefore the mechanical properties. In addition, tensile properties of CC constructs have been seen to increase over time (data not shown).

In contrast, the primary articular chondrocyte constructs produced in this study are quite different from those created previously. Previous work showed substantially higher GAG, total collagen, and collagen type II content for the constructs. While the constructs produced here were gelatinous, difficult to handle, and not mechanically testable, previous constructs have produced aggregate moduli around 200 kPa, elastic modulus approaching 1.4 MPa, and UTS near 350 kPa. While these mechanical properties were seen on constructs created with 5.5 million cells, even when previous work used 2 million cells (as was used here), an aggregate modulus of 74 kPa was obtained. The primary change in the present study from previous ones, which may account for these differences, is the source of the chondrocytes. Both studies obtained the ACs with similar methods from the distal femur, but previous work used ACs from 1 wk old, male calves, while this study used cells from skeletally mature, female goats. While the sex or species may account for the differences, the more likely explanation is the age difference. There is considerable work that demonstrates age-related changes in ECM structure, which may relate to the changes in mechanical properties seen with these different-aged cells. Previous work also showed decreases in collagen type II, aggrecan core protein, and Sox 9 gene expression of cultured, mature chondrocytes compared to immature chondrocytes. This suggests that the immature cells have a greater productive capacity for the relevant ECM than mature chondrocytes.

Despite the somewhat disappointing results for primary chondrocytes relative to previous results, the passaged ACs and CCs showed considerable improvements in their constructs' biochemical and mechanical properties. While both cell types have been shown to dedifferentiate with passage, this effect appears to have benefits, particularly for this current use of the cells.
Passaged AC constructs, unlike their primary counterparts, were mechanically testable in both tension and compression and contained 11 times more total collagen/wet weight. AC P3 constructs also had almost 2.5 times more GAG/wet weight than AC P0 constructs, although the differences were not significant. Passaged CC constructs had almost 3 times more GAG/wet weight, 16 times greater elastic modulus, and 7 times greater UTS than primary constructs. The aggregate modulus was not significantly different between the CC P3 and CC P0 constructs.

While these passaged constructs were dramatically improved from P0 constructs, the characteristics of the tissues of interest for tissue engineering must be considered. As reviewed previously, articular cartilage contains primarily collagen type II, while the TMJ disc contains primarily collagen type I. Additionally, articular cartilage is most important under compressive loading, while fibrocartilage is also important under tensile loading. In this study, only the AC P0 constructs did not contain any collagen type I, while the AC P3 cells produced the lowest amount of collagen II, particularly relative to the large quantity of collagen type I produced in this group. CC constructs, however, had measurable quantities of both types of collagen. CC P0 constructs had around 20 times more collagen II than collagen I, while CC P3 had less than 1.5 times the collagen II/collagen I ratio. These data, combined with the CC constructs’ mechanical properties, suggest the potential usefulness of primary CCs for cartilage tissue engineering and passaged CCs for fibrocartilage tissue engineering.

Comparing the data to native tissue, the GAG content was similar to both articular cartilage and the TMJ disc, as was seen previously. The GAG/wet weight was approximately 2% for CC P0 constructs and 5% for CC P3 constructs. This suggests the potential of either of these cell types to function for a variety of soft tissues in the TMJ. The collagen/wet weight was, again, lower than native tissue with the CC P0 group having 0.5% collagen/wet weight and the CC P3 constructs having 0.7% collagen/wet weight. Mechanical properties were also still many times lower than native. The most dramatic difference between these two groups, which relates their potential to function in either cartilage or fibrocartilage is the collagen II/collagen I ratios, as discussed
previously. While improvements in collagen content and mechanical properties must be made before such a construct could be implanted, these cells exhibit considerable potential for functionality.

While the passaged ACs were, for most metrics, statistically equivalent to passaged CC constructs, CC constructs had 8 times more GAG/wet weight. The CC constructs were also significantly larger in size. This makes the translation from bench top to bedside more feasible. Considering the accessibility of this cell source, limited surgical complications, and abundance of costal cartilage tissue, this cell source appears feasible for future use. While considerable aspects of this work should be explored in greater detail (i.e., passage number, expansion conditions, temporal changes in culture), these data are promising. The affirmation of the hypotheses, combined with the significant results seen here, suggest that passaged CCs should be considered as a possible cell source for engineering TMJ fibrocartilage tissues.
Table 6-1: Quantitative size data for all groups

<table>
<thead>
<tr>
<th></th>
<th>Wet weight (mg)</th>
<th>% water</th>
<th>Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC P0</td>
<td>20.40 ± 5.74 B</td>
<td>94.79 ± 0.58 A</td>
<td>5.41 ± 0.62 A</td>
</tr>
<tr>
<td>AC P3</td>
<td>3.91 ± 0.08 C</td>
<td>82.95 ± 0.04 C</td>
<td>2.07 ± 0.04 D</td>
</tr>
<tr>
<td>CC P0</td>
<td>59.76 ± 10.36 A</td>
<td>93.26 ± 0.86 A</td>
<td>4.52 ± 0.32 B</td>
</tr>
<tr>
<td>CC P3</td>
<td>9.66 ± 2.40 C</td>
<td>84.88 ± 0.19 B</td>
<td>3.12 ± 0.31 C</td>
</tr>
</tbody>
</table>

Table 1 Data are shown as mean ± standard deviation. Groups separated by different letters are considered significantly different (p < 0.05). CC P0 constructs were significantly heavier in wet weight than any other group. AC P0 constructs had greater wet weights than both types of P3 constructs. Primary groups had the greatest percent water, and the CC P3 constructs also had more water than AC P3 constructs. All diameters were statistically different from one another with AC P0 being the greatest, followed by CC P0, CC P3, and AC P3.
Figure 6-1: Morphology and Histology from 4 wks

AC P0  AC P3  CC P0  CC P3

Figure 1 Representative constructs from each group at 4 wks. Safranin-O/fast green staining (i-l), picrosirius red staining (m-p), collagen type I IHC (q-t), and collagen type I IHC (u-x) illustrate the localization of the ECM. AC P0 had limited safranin-O staining throughout the construct, and AC P3 showed none. CC P0 and CC P3 had dense staining throughout the construct, although CC P3 did not stain the very outer edge of the construct. All constructs stained positively and uniformly for picrosirius red. AC P0 did not stain for collagen type I, while collagen type II staining was seen throughout the construct. AC P3 stained positively for collagen I, though only a little for collagen type II. CC constructs from both passages stained positive for collagen types I and II throughout the constructs. Scale bar = 0.1 mm.
Figure 6-2: Quantitative biochemical data for all groups

![Bar charts showing data for different groups at passage 0 and 3.](chart_images)

Figure 2 Data are shown as mean ± standard deviation. Groups separated by different letters are considered significantly different ($p < 0.05$). While some statistical differences are observed between the groups for the number of cells (Fig. 2a), the groups aggregate around 1.5 million cells. Dramatic differences were seen in the GAG per wet weight (WW) (Fig. 2b) with CC P3 and CC P0 being significantly more concentrated than either of the AC groups. CC P3 also had significantly more GAG per wet weight than CC P0. AC P0 was significantly lower than the other groups for collagen per wet weight (Fig. 2c).
**Figure 6-3: Mechanical data for all groups**

**Figure 3** Data are shown as mean ± standard deviation. Groups separated by different letters are considered significantly different (p < 0.05). AC P0 groups were not testable in tension or compression. For the tensile properties, UTS and E, CC groups were significantly better than AC P3. No statistical differences were seen between the groups for aggregate modulus (Fig. 3c).
Chapter 7: Passaged costal chondrocytes provide a viable cell source for temporomandibular joint tissue engineering

Abstract

Costal cartilage is commonly harvested for various types of facial reconstructive surgery. The ability of costal chondrocytes (CCs) to produce relevant extracellular matrix, including glycosaminoglycans (GAGs), collagen type I, and collagen type II, makes them an appealing cell source for fibrocartilage tissue engineering. In order to obtain enough cells for tissue engineering, however, cell expansion will likely be necessary. This study examined CCs at passages 0, 1, 3, and 5, as well as temporomandibular (TMJ) disc cells, in a scaffoldless tissue engineering approach. TMJ disc constructs had over twice the collagen content of any other group, as well as the largest tensile properties; however, the substantial contraction of the constructs and limited cell numbers make it a non-feasible cell source for tissue engineering. In general, statistical differences in mechanical properties or total collagen content of the various CC groups were not observed; however, significantly more GAG was produced in the passaged CCs than the primary CCs. More collagen type II was also observed in some of the passaged cell groups than in passage 0. These results suggest not only feasibility but potential superiority of passaged CCs over primary CCs, which may lead to a functional engineered fibrocartilage tissue.

Introduction

The temporomandibular joint (TMJ) has the essential function of allowing fluid jaw movement. Degeneration or injury of this joint leads to pain during everyday activities like eating or talking, which can become physically and emotionally painful. In the United States alone, there are over 10 million patients with TMJ disorders.\textsuperscript{186} There are various treatment options depending on the level of degeneration as reviewed elsewhere.\textsuperscript{10,11} In severe cases, treatment options have limited success, and there is no consensus as to a standard method of treatment.

When TMJ disorders are severe or traumatic injury occurs in the TMJ, total joint reconstruction may be necessary. A widely accepted approach for replacing the mandible of the jaw is to use a rib and costal cartilage graft.\textsuperscript{189,212-218} Costal cartilage grafts are also used in ear, nose, and other craniofacial reconstructions.\textsuperscript{212} By using autologous tissue, complications with patient rejection are eliminated. The costal cartilage can be harvested easily, and donor site morbidity is largely eliminated with current surgical techniques.\textsuperscript{195} A common problem with this approach in the jaw is overgrowth of the costal cartilage in the TMJ, which can require further surgery.\textsuperscript{175,197,198,219} The tissue overgrowth suggests that using the costal cartilage directly may not be appropriate for reconstructing a soft tissue, like the TMJ disc; however, creation of a tissue engineering construct \textit{in vitro} with costal chondrocytes may yield a completely functional tissue without undesirable complications. Ease of use and practicality in a clinical setting motivates the exploration of costal chondrocytes (CCs) in a tissue engineering approach.

Indeed, CCs have been used in tissue engineering for various cartilage applications: articular,\textsuperscript{158,160,183,220,221} tracheal,\textsuperscript{164,166} elastic,\textsuperscript{159,162,154,165} and, most recently, fibrocartilage.\textsuperscript{178} Previous work suggests that CCs are quite productive and could provide a more clinically feasible cell source for TMJ disc tissue engineering.\textsuperscript{178} However, the acellular nature of cartilage limits the amount of viable cells available from a piece of tissue.\textsuperscript{196} For this reason, cell expansion and passaging appeals to tissue engineers as a way to obtain large numbers of cells, which are
frequently needed for a tissue engineering approach. Previous work has shown that CCs have the ability to expand up to passage 5, at which point they appear to lose their proliferative ability, showing little expansion beyond passage 5.\textsuperscript{161} Passaging, however, causes chondrocyte dedifferentiation to a more fibrochondrocyte-like cell type; just a single passage is capable of causing a significant drop in collagen type II and glycosaminoglycan (GAG) production.\textsuperscript{1,164} It is encouraging to note, however, that the resultant dedifferentiation may prove beneficial for the purposes of tissue engineering fibrocartilages, like the TMJ disc, which contain less collagen type II, more collagen type I, and less GAG than articular cartilage.

This study examines the use of CCs at passage 0, 1, 3, and 5 and TMJ disc cells in a scaffoldless tissue engineering approach. While TMJ disc cells are not a feasible option for tissue engineering as they are extremely limited in cell number, difficult to harvest, and likely diseased in any patient interested in a TMJ replacement, they will serve as a control in this experiment. It is hypothesized that increasing passages decreases the cartilaginous proteins while increasing the fibrocartilaginous proteins in the constructs.

**Materials and Methods**

**Cell isolation**

Both cell types were taken from three skeletally mature goats, which were obtained from a local abattoir immediately after death. TMJ disc cells were removed and isolated, as described previously.\textsuperscript{163} These cells were expanded in Dulbecco's modified Eagle medium (DMEM) with L-glutamine and 4.5 g/L glucose (Biowhittaker, Walkersville, MS) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA), 1% Penicillin-Streptomycin-Amphotericin B (PSF), 1% non-essential amino acids (NEAA) (Life Technologies, Carlsbad, CA), 25 μg/mL L-ascorbic acid (Sigma, St. Louis, MO), and 1 μg/mL insulin (Sigma). At 70-90%
confluence, TMJ disc cells were passaged with 1X trypsin-EDTA (Gibco, Carlsbad, California) and used in this experiment at passage 2.

Costal cartilage tissue was scraped from non-floating ribs, minced, and digested with 0.2% type II collagenase (Worthington, Lakewood, NJ) for 18 hrs. A portion of the primary cells (P0s) were used for construct formation and the remainder were plated on tissue culture treated plastic and cultured as described above. CCs were expanded in DMEM (Gibco) with 10% FBS, 1% PSF, 1% NEAA, and 25 μg/mL L-ascorbic acid. CCs at passage 1 (P1), passage 3 (P3), and passage 5 (P5) were collected to form tissue engineered constructs. All cells were cultured in a standard incubator at 37°C and 5% CO2.

Construct culture

Constructs were created via a modified method used by Hu and Athanasiou. Briefly, each sample group of cells was seeded into 3 mm, 2% agarose wells to form self-assembled constructs, containing 2x10⁶ cells each. These constructs were cultured in the wells for 2 wks before being transferred into agarose coated 6-well plates. Partial media changes of DMEM with 1% PSF, 1% NEAA, 1% ITS+ premix (BD Biosciences, San Jose, CA), 0.1 μM dexamethasone, 40 μg/mL L-proline (EMD Chemicals, Gibbstown, NJ), 50 μg/mL ascorbate 2-phosphate (Sigma), and 100 μg/mL sodium pyruvate (Fisher) occurred everyday.

Histology

Two constructs were removed at both time points (3 and 6 wks), frozen in HistoPrep™ Frozen Tissue Embedding Media (Fisher), cut to 14 μm sections in a cryotome, and put on glass slides. Slides were placed on a 30°C warm plate overnight and formalin-fixed for histology. Sections were stained with picrosirius red for collagen, safranin O/fast green for GAGs, and hematoxylin and eosin for cell visualization. Immunohistochemistry (IHC) for collagen types I and II was also performed on these samples. IHC slides were stored at -80°C, fixed in acetone, and stained with
a Biogenex i6000 autostainer (San Ramon, CA). Samples were washed in phosphate buffer saline solution with Tween before every step except the primary antibody. Slides were blocked with 3% H₂O₂ in methanol and Vectastain protein block (Vector Laboratories, Burlingame, CA). Primary antibodies were incubated with the samples for 1 hr. Antibodies for collagen type I were mouse monoclonal (Accurate Chemical and Scientific, Westbury, NY), and rabbit polyclonal antibodies were used for collagen type II (Chemicon, Temecula, CA). Secondary antibodies for the appropriate species were provided by the Vectastain ABC kit. Staining was visualized with a DAB substrate kit (Vector Laboratories). IHC slides were counter stained with Harris's hematoxylin (Fisher).

Biochemistry

Six samples were taken at 3 and 6 wks for biochemical analysis. Samples were weighed before and after a 2 day lyophilization step to determine both wet and dry weights of the constructs. Once dried, samples were digested in 125 μg/mL papain (Sigma) in 50 mM phosphate buffer (pH=6.5) containing 2 mM N-acetyl-cysteine (Sigma) and EDTA for 7 days followed by 2 days of 1 mg/mL elastase (Sigma) digestion. The entire digest occurred at 4°C with constant mechanical agitation.

DNA was quantified with a PicoGreen® dsDNA reagent (Molecular probes), and cell numbers were calculated using a conversion factor of 7.7 pg DNA/cell, as determined previously.¹⁸¹ Total collagen was measured with a modified colorimetric hydroxyproline assay, described previously.¹⁵¹ Briefly, samples were hydrolyzed with NaOH and neutralized with HCl. Chloramine T and Ehrlich's solutions were added and incubated at 60°C. Sulfated GAGs were quantified with a dimethylmethylen blue Blyscan kit, according to the manufacturer's protocol (Biocolor, Newtownabbey, Ireland).
ELISA

Collagen type I was measured with an indirect ELISA, as described previously. Briefly, the sample digestes were incubated on high-affinity plates for 18 hrs at 4°C. Samples were then exposed to a primary mouse antibody (Accurate Chemical) followed by a HRP-conjugated secondary antibody (Chemicon). Samples were visualized by incubating wells with tetramethyl benzidine (Chemicon) and quantified by comparing them to an ELISA grade collagen I standard. Collagen type II was quantified with a Chondrex (Redmond, WA) collagen detection kit, according to the manufacturer's protocol.

Mechanical testing

Mechanical testing was performed on at least 6 samples at the 6 wk time point. Compressive properties were determined by conducting creep tests under unconfined compression. Costal chondrocyte samples were cut in half through the diameter of the samples to create an even testing surface. The initial heights of all the samples were measured with digital calipers. Samples were then placed in saline solution and positioned under the platen so that the sample surface and platen were parallel. Each specimen was loaded with a tare weight of 0.002 N until equilibrium was reached (deformation less than 10^-6 mm/s) or 10 minutes elapsed. A step load was then applied to the sample with a creep test weight of 0.007 N until equilibrium was again reached or the sample crepeed for 1 hr. The load was then removed, and the sample was again allowed to equilibrate to the specimen's recovery height. Creep data were then analyzed with the curve fitting tool in Matlab (The Math Works, Inc) using the viscoelastic model described previously. Equation 1 describes the behavior of the viscoelastic solid where u_x is the deformation, σ is the applied stress, E_w is the relaxed modulus, z is the creep distance, τ_σ is the stress relaxation time constant, τ_γ is the creep time constant, and h(t) is the step function. Fitting the data gives solutions for the relaxed modulus and time constants, from which the viscosity and instantaneous modulus (E_0) can be calculated.
\[ u_z(r, z(r, o), t) = \frac{2\sigma}{3E_\infty} z(r, 0) \left[ 1 + \left( \frac{\tau_z}{\tau_o} - 1 \right) e^{-t/\tau_o} \right] h(t) \]  

<Eq. 1>

An Instron 5565 (Norwood, MA) with a 50 N load cell was used for tensile testing. Samples were cut into a dog bone shape and measured with digital calipers across the smallest cross sectional area. Samples were secured with cyanoacrylate glue on a paper frame with a standard gauge length. The paper was attached to the Instron grips before cutting it—leaving only the sample to be tested. Samples were tested at a 10% strain per minute until failure. Displacement and load data were collected and converted into stress and strain. Ultimate tensile strength (UTS) and elastic modulus (E) were calculated for each data set.

Statistics

When applicable, data were analyzed for statistical significance with a 2-way analysis of variance (ANOVA). Factors of cell type and time had five and two levels, respectively. When a main effects test indicated significance (\( p < 0.05 \)), a Tukey's post hoc test was used to determine differences among the levels. This statistical model was used for ELISA and biochemical data. Mechanical data were analyzed with a 1-way ANOVA, where cell type was the only factor.

Results

Morphology and histology

Quantitative size data are shown in Table 1 and illustrated in the first two rows of Fig. 1. TMJ and P5 constructs contracted early in culture resulting in statistically smaller diameters than the other constructs. TMJ constructs were also statistically smaller in both diameter and volume than P5 constructs. Contraction in the TMJ constructs resulted in a primarily spherical shape. Constructs composed of passaged CCs formed rounder shapes compared to the P0 constructs, which were
cylindrical in appearance. CC constructs grew over time resulting in a significant difference in diameters between 3 wks and 6 wks.

Figure 1 illustrates the histological and gross morphological differences between the different cell types at 6 wks. In terms of staining localization and intensity, little difference was observed between 3 and 6 wks. TMJ constructs contracted into dense spheres of cells with little ECM, as seen by the H&E staining. These constructs did not stain for GAGs, but did stain for collagen throughout. P0 constructs stained for collagen, GAG, and cells throughout the construct in a mostly uniform manner. P1, P3, and P5 constructs stained positive for GAG and collagen, but staining was only seen in an outer ring of the construct. Cells for these constructs were localized to the outer ring. While a few cells were located in the center, trypan blue staining of the fluid from this inner region indicated the cells were dead.

IHC staining is shown in Fig. 2. Collagen type I was seen throughout all constructs, but was denser on the outer surface and in the center of the passaged constructs. All but the TMJ constructs stained positive for collagen II.

**Biochemistry**

The number of cells (Fig. 3a) was significantly higher in the P3, P0, and P5 groups with P1 being significantly lower and the TMJ group the lowest. For most groups, cells tended to increase (p = 0.05) with increasing time. Quantitative sulfated GAG content normalized by construct wet weight is compared in Fig. 3b. GAG significantly increased from 3 to 6 wks, approximately doubling in all groups except the TMJ. The GAG content of the TMJ constructs were significantly lower than any other group, and P0 constructs contained significantly lower GAG than any of the passaged groups. P5 constructs also contained significantly less GAG than P1 or P3 constructs. Total collagen from the hydroxyproline assay normalized by wet weight (Fig. 4) was the greatest in the TMJ constructs, with no other statistical differences.
ELISA

Collagen I and II quantities are also illustrated in Fig. 4. As with total collagen, TMJ constructs produced the most collagen I per weight wet (p < 0.05). No collagen II was produced in the TMJ constructs at 3 wks, which supports the IHC results, and only trace amounts (at the limits of detection) were measured at 6 wks. P1 constructs had the most (significantly more than P0, P3, or TMJ constructs) collagen type II per wet weight, and P5 constructs had significantly more than P3 or TMJ constructs.

Mechanical properties

Mechanical properties are listed in Table 2. In compression, TMJ constructs had a significantly larger $E_o$ than P0, P1, and P3 constructs and a significantly larger viscosity than all other groups. The P5 group had a significantly higher $E_\infty$ than P3. In tension, the TMJ constructs had a significantly larger $E$ than any other group and a significantly larger UTS than all the groups except P1.

Discussion

With the limited treatment options available for patients with TMJ disorders, tissue engineering seeks to create a functional TMJ disc replacement from a patient's own cells. Previous work has shown the potential functionality of costal chondrocytes in a scaffoldless, fibrocartilage tissue engineering approach.\textsuperscript{178} Costal cartilage is used frequently in various reconstructive surgeries, because it is an abundant source of cartilage that is easy to obtain.\textsuperscript{189,212-216,218,222} However, complications that have been seen when using the whole tissue for jaw replacement suggest that using the cells with an in vitro tissue engineering approach may provide greater control for creating a functional TMJ disc replacement. To achieve the desired cell numbers from this hypocellular tissue, cell passaging may be necessary and may provide a more functional construct for the purposes of engineering the TMJ disc. This study examined the biochemical and mechanical characteristics of constructs from P0, P1, P3, and P5 CCs, in addition to TMJ disc
cells. The data presented here show that passaged CCs have the greatest capacity for creating a functional fibrocartilage tissue replacement.

The hypothesis that increasing passage would decrease the chondrocytic proteins while increasing the fibrochondrocytic proteins proved partially true. Higher passages showed a significant decrease in collagen type II with P3 containing less collagen II per wet weight than P1 constructs. However, the higher passages did not increase collagen type I quantities. GAG per wet weight was significantly decreased in P5 from other passage constructs but was also significantly less in P0 constructs over the passaged constructs. It was originally expected that higher passage constructs would most closely resemble TMJ disc cell constructs, but this was largely not the case. The TMJ constructs had the smallest diameter, highest percentage of collagen I and total collagen, and lowest amounts of GAG and cells. While P5 constructs also had the smallest average diameter of the CC groups, P0 constructs had the closest GAG quantity, P1 had the closest cell quantity, and P3 had the closest collagen II quantities to the TMJ constructs. None of the CC constructs approached the total collagen or collagen I quantities seen in the TMJ disc constructs.

The overall appearance of the constructs created in this study suggests that CCs are a promising cell source for tissue engineering. TMJ constructs contracted to a small sphere with a diameter one-third the size of the other groups; this contraction event is undesirable, because the decrease in diameter and tissue volume makes it more difficult to create a tissue replacement of clinically relevant dimensions. These results were also seen with fibrocartilage from the knee meniscus. Alternatively, CC constructs grew in diameter over time (although P5 constructs contracted in diameter initially). The time-dependent growth was particularly prominent in the P3 constructs, which exhibited a 7% increase in diameter from 3 wks to 6 wks.

Dead cells were noted only in the center of passaged constructs. Previous research has shown that cartilage dedifferentiates with passage, and it is possible there are multiple cell
subpopulations that form with slightly different phenotypes.\textsuperscript{1,164} When these cells are seeded into a three-dimensional construct, the subpopulations may aggregate into distinct regions through their surface receptors.\textsuperscript{224} Additionally or alternatively, the outer core of cells may limit nutrient diffusion into the center or waste transport removal causing the inner population of cells to die. Experimentation with passaged CCs following this study suggests that the "cyst" forms within the first 48 hrs after seeding, but using a lower cell seeding density (cells per area) may eliminate this phenomenon (unpublished data). Altering the media composition or delivery, for example, by adding growth factors or using a perfusion bioreactor, may also prevent cell death in the core. Eliminating this unique structural characteristic may improve the cellular communication and/or overall biochemical and mechanical properties of the constructs such that passaged cells may create more functional constructs.

Biochemical assessment showed that passaged CC constructs produced almost twice as much GAG and equivalent amounts of collagen type I and total collagen to primary CCs. This ECM production is critical to the functionality of a tissue engineered construct. Collagen type II was also greater for passaged constructs with the exception of passage 3; however, this collagen type is uncharacteristic of a TMJ disc and may be detrimental to its functionality.\textsuperscript{16}

The total collagen measured with hydroxyproline was not fully accounted for by the collagen types I and II ELISAs. This is likely due to other ECM molecules that would be detected with the hydroxyproline assay including other types of collagen and elastin. CCs have been shown to produce elastin,\textsuperscript{164} collagen type III,\textsuperscript{225} and collagen type X.\textsuperscript{226} Additional collagen types could also be present like types IX and XII, which are associated with types II and I, respectively.

CC constructs were able to retain the initial cell seeding density of 2x10\textsuperscript{6} cells. These groups had between 1.5 x10\textsuperscript{5} to 2 x10\textsuperscript{6} cells per construct, while TMJ constructs lost 7/8 of this initial seeding density within the first 3 wks of culture. These cells either did not initially assemble into the construct, or the cells died and sloughed off within the first 3 wks. Also, the cell number did not
increase over time in this group, suggesting a lack of proliferation, or proliferation was equilibrated with cell death. In any case, the loss of cells again suggests that even more cells would be needed to produce a TMJ disc cell construct of relevant dimensions. This illustrates once again the lack of feasibility in using TMJ disc cells in this tissue engineering approach.

Mechanical properties can frequently be linked to ECM content. While the GAG content of the constructs was not well correlated to the trend in compressive properties, the significant differences between the groups for UTS and E almost exactly correspond to the differences in total collagen and collagen type I. TMJ constructs had 2-10 times greater tensile properties than the other groups and about a 5 times greater total collagen content. Unfortunately, the TMJ disc cell constructs, in addition to being made of a non-feasible cell source and contracting significantly, still lack the mechanical integrity to function in vivo. Even the best mechanical data are still 4-84 times (depending on the direction tested) less than the native TMJ disc in tension\textsuperscript{78} and 2-8 times less than the native tissue in compression.\textsuperscript{93}

Overall, this study illustrates the potential for passaged CCs in tissue engineering and also suggests the need for future work with these cells. Despite the larger tensile properties, collagen content, and in some cases compressive properties, TMJ disc cells are not a feasible option for tissue engineering. They are difficult to harvest, likely diseased in a patient considering a TMJ disc replacement, and extremely limited in number. Additionally, the cell number in the constructs decreased from initial seeding and contracted to become prohibitively small. On the other hand, CCs can be easily obtained through a minimally invasive procedure and produce constructs of reasonable size with relevant ECM. Furthermore, while there was not a clear trend in the effects of passaging on CCs, the passaged CCs consistently outperformed primary CCs. Passaging increases time between tissue harvest and implantation but yields more cells, which may be essential to create a construct of relevant size. Mechanical properties and collagen content also need to be improved, and employing some of the strategies mentioned previously, like adding growth factors or a bioreactor, will be important future work. Incorporating these strategies could
improve this tissue engineering approach such that it becomes a feasible option for sufferers of TMJ disorders.
Table 7-1: Dimensions and weights of constructs at 6 wks

<table>
<thead>
<tr>
<th></th>
<th>Diameter (mm)</th>
<th>Thickness (mm)</th>
<th>Wet weight (mg)</th>
<th>Dry weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMJ</td>
<td>0.837 ± 0.119&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.893 ± 0.211&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.218 ± 0.0608&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.0617 ± 0.0204&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>P0</td>
<td>3.08 ± 0.224&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.64 ± 0.936&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.9 ± 1.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.38 ± 0.245&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P1</td>
<td>3.35 ± 0.0727&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.32 ± 0.133&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.8 ± 1.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.67 ± 0.151&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P3</td>
<td>3.35 ± 0.127&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.76 ± 0.176&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.1 ± 2.60&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.829 ± 0.391&lt;sup&gt;es&lt;/sup&gt;</td>
</tr>
<tr>
<td>P5</td>
<td>2.85 ± 0.0841&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.51 ± 0.0952&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.0 ± 0.904&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.81 ± 0.0974&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 1 Data are shown as mean ± SD. Groups separated by different letters are statistically significant (p < 0.05). TMJ constructs were significantly smaller by all metrics when compared to the other groups. P5s were significantly smaller than other CCs in diameter and dry weight. P1 and P3 constructs had the largest diameters. P0 constructs had the largest wet weight and a significantly greater dry weight than the P1 group.
Table 7-2: Mechanical properties at 6 wks

<table>
<thead>
<tr>
<th></th>
<th>Compressive Properties</th>
<th>Tensile Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E&lt;sub&gt;-&lt;/sub&gt; (kPa)</td>
<td>E&lt;sub&gt;o&lt;/sub&gt; (kPa)</td>
</tr>
<tr>
<td>TMJ</td>
<td>44.5±12.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>190±58.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P0</td>
<td>32.2±24.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>93.9±72.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P1</td>
<td>28.3±10.3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>76.8±32.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P3</td>
<td>23.1±6.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>55.0±14.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P5</td>
<td>50.0±10.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>117±22.1&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 2 Data are shown as mean ± SD. Groups separated by different letters are statistically significant (p < 0.05). In compression, statistical differences were observed in E<sub>o</sub> and viscosity with TMJ being significantly larger than all except P5s and all groups, respectively. The P5 group had a significantly larger E<sub>-</sub> than the P3 group. In tension, the UTS for TMJ constructs was significantly larger than P0, P3, and P5 constructs. TMJ constructs also had a significantly larger elastic modulus than all other constructs.
Figure 7-1: Morphology and histology at 6 wks

<table>
<thead>
<tr>
<th>TMJ</th>
<th>P0</th>
<th>P1</th>
<th>P3</th>
<th>P5</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>b</td>
<td>c</td>
<td>d</td>
<td>e</td>
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<tr>
<td>f</td>
<td>g</td>
<td>h</td>
<td>i</td>
<td>j</td>
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<tr>
<td>u</td>
<td>v</td>
<td>w</td>
<td>x</td>
<td>y</td>
</tr>
</tbody>
</table>

Figure 1 Images a-j show gross morphology of each construct from the top (a-e) and side (f-j) at 6 wks. Spaces below the constructs are 1mm. H&E (k-o), safranin O/fast green for GAG (p-t), and picrosirius red for collagen (u-y) staining are also shown for each group. The scale bar in the bottom left is 1mm. TMJ constructs contracted into small spheres, primarily consisting of cells, as indicated by the H&E staining. They did not stain for GAG and stained uniformly for collagen. P0 constructs were tall cylinders while passaged constructs also rounded into spheres. P0 constructs stained mostly uniform for collagen, GAG, and cells. Passaged cells, however, formed fluid-filled spheres that did not stain for cells, GAG, or collagen. Only an outer ring of tissue stained for cells and ECM.
Figure 7-2: IHC staining for collagen types I and II

Figure 2 IHC staining for collagen type I (a-f) and type II (g-l) for all constructs at 6 wks. Positive controls are seen in frames f and l. Collagen I was seen throughout TMJ and P0 constructs. Passaged constructs showed the most intense collagen I staining around the outside of the constructs and within the fluid-filled center. Collagen type II was seen throughout all the tissue-like areas of the CC constructs, but was not seen in TMJ constructs. Scale bar = 1mm.
Figure 3 Cell (a) and GAG per wet weight (b) quantities for all groups (mean ± SD). Groups separated by different letters are considered significantly different ($p < 0.05$). TMJ constructs were significantly lower for both cell and GAG content from all other groups. P1 had significantly lower cell numbers than other CC constructs. P0 had the lowest GAG content of all the CC constructs, and P5 had significantly lower GAG quantities than P1 constructs. Time was a significant factor for GAG with 6 wks being greater than 3 wks as was very apparent in CC constructs.
Figure 4 Collagen content normalized by wet weight (mean ± SD) for all groups at 3 and 6 wks. Gray and black bars show the stacked quantities of collagen type I and collagen type II, respectively, from the ELISAs. The remaining white bars were the quantities measured with the hydroxyproline assay, which were unaccounted for by the ELISAs. All results indicated a significant increase in the time factor from 3 to 6 wks. The TMJ group had the most (p < 0.05) collagen type I and total collagen, as indicated by the asterisk. Collagen II results (statistics not represented in the figure) were greatest in P1 constructs, which had significantly more collagen type II per wet weight than P0, P3, or TMJ constructs, followed by P5 constructs, which were also significantly greater than P3 or TMJ constructs.
Chapter 8: Hydrostatic pressure effects on costal cartilage constructs

Abstract

This study examined passaged costal chondrocytes under intermittent (1 Hz) and static (0 Hz) 10 MPa hydrostatic pressure for the purposes of improving tissue engineered constructs for fibrocartilage engineering. Hydrostatic pressure regimens were applied from days 7-10, and constructs were evaluated immediately after stimulation (10 days) and after 21 days. Constructs were assayed for collagen content (total, type I, and type II), glycosaminoglycan (GAG) quantity, cell number, tensile properties, and compressive properties. Cell number, GAG, collagen, and ultimate tensile strength increased significantly from 10 days to 21 days. Few differences were seen among the treatment groups and controls, suggesting the applied stimuli may not be effective for these cells, or the regimens used were not optimal.
Introduction

Costal chondrocytes (CCs) have shown promise in fibrocartilage tissue engineering.\textsuperscript{178,180,227} Costal cartilage can easily be harvested with a minimally-invasive surgical technique that has relatively negligible complications.\textsuperscript{195} Using a scaffoldless approach, CCs have produced a more functional fibrocartilaginous extracellular matrix (ECM) than other cell sources, including dermal fibroblasts, bone marrow-derived stem cells, temporomandibular joint (TMJ) disc cells, articular chondrocytes (ACs), and various co-cultures.\textsuperscript{178,227} Additionally, they have sufficient expansion potential and abundance to make them clinically translatable.\textsuperscript{180,227} Despite these exciting initial results, more work must be done to improve the biomechanical properties of the tissue engineered constructs so that they can be fully functional within the joint.

Mechanical stimuli are one method of improving the properties of tissue engineered constructs, particularly those intended for use in a mechanically functional tissue. As reviewed elsewhere, tissue engineering of musculoskeletal tissues has benefited from tensile, compressive, shear, and hydrostatic pressure bioreactors.\textsuperscript{223} Our laboratory and others have seen success with hydrostatic pressure for stimulating constructs made from a variety of cell sources: articular ACs,\textsuperscript{229-232} meniscal fibrochondrocytes,\textsuperscript{233,234} TMJ disc fibrochondrocytes,\textsuperscript{116} and tendon fibroblasts.\textsuperscript{235} In three-dimensional culture, TMJ disc cell constructs contained more glycosaminoglycans (GAGs) and collagen with static (0 Hz) hydrostatic pressure; less cells, collagen, and GAG were seen with the application of intermittent pressure at 1 Hz.\textsuperscript{116} Similar results were seen with meniscal fibrochondrocytes seeded on scaffolds. The collagen per scaffold and GAG per scaffold was significantly higher for the 10 MPa application at 0 Hz than for 10 MPa at 0.1 or 1 Hz and controls.\textsuperscript{233} Alternatively, AC constructs were most often improved with intermittent application.\textsuperscript{229-231} ACs in monolayer increased collagen II and aggrecan expression with 10 MPa intermittent hydrostatic pressure at 1Hz. These changes were not seen when static pressure was applied.\textsuperscript{231} Similar application of intermittent hydrostatic pressure on three-dimensional tissue engineered AC constructs resulted in significant increases in collagen and
GAG production.\textsuperscript{229} Passaged ACs on scaffolds also increased GAG and collagen production with hydrostatic pressure application. The increase in collagen was only significant with a higher pressure application (6.86 MPa versus 3.44 MPa).\textsuperscript{232} However, the effects of any of these stimuli on CCs, either passaged or primary in either monolayer or three-dimensional constructs, are unknown. The objective of this study was to apply intermittent (1 Hz) and static (0 Hz) hydrostatic pressure at 10 MPa to scaffoldless, costal chondrocyte tissue engineered constructs and observe the biochemical, mechanical, morphological, and histological changes.

**Materials and Methods**

*Cell isolation*

Costal cartilage was obtained from three skeletally-mature, Spanish, female goats within 4 hrs postmortem. Tissue was scraped aseptically from the non-floating ribs, minced, and digested for 18 hrs in 0.2\% type II collagenase (Worthington, Lakewood, NJ) in Dulbecco’s modified eagle’s medium (DMEM) (Gibco, Carlsbad, CA) with Penicillin-Streptomycin-Amphotericin B (PSF) (Cambrex, Walkersville, MD), 1\% non essential amino acids (Gibco), and 25 µL/mL L-ascorbic acid (Sigma, St. Louis, MO). Isolated cells were then expanded and passaged at about 90\% confluence with trypsin-EDTA (Gibco) until passage 3.

*Construct culture*

Two million cells were seeded into 5 mm agarose wells, based on a method described previously.\textsuperscript{104,182} Constructs were fed DMEM with 1\% PSF, 1\% NEAA, 1\% insulin-transferrin-selenium+ premix (BD Biosciences, San Jose, CA), 0.1 µM dexamethasone, 40 µg/mL L-proline (EMD Chemicals, Gibbstown, NJ), 50 µg/mL ascorbate 2-phosphate (Sigma), and 100 µg/mL sodium pyruvate (Fisher). After 1wk constructs were transferred into agarose-coated plates, and mechanical stimulation began. Hydrostatic pressure was applied with an apparatus, described previously.\textsuperscript{116,229} Three groups of constructs were heat sealed in non-permeable pouches (Kapak,
Minneapolis, MN) with 30 mL of media. One group, non-treated control (NTC) was not put in a bag. One group, bagging control (BC), was bagged, but not stimulated with pressure. The static pressure (SP) group was stimulated with 10 MPa of continuous hydrostatic pressure and the intermittent pressure (IP) group was stimulated with 10 MPa of pressure at 1 Hz. These stimuli were applied for 1 hr/day for 4 days.

**Histology**

Samples were taken from each group at 10 days and 21 days for histological examination. Constructs were frozen in HistoPrep™ (Fisher) and cut into 14 μm thick sections. Slides were fixed in formalin and stained with safranin-O/fast green for GAGs or picrosirius red for collagen.

**Biochemistry**

Six constructs per group were removed at each time point and weighted before and after a two-day lyophilization step. Dried constructs were digested with constant mechanical agitation for seven days in 125 μg/mL papain (Sigma) solution containing N-acetyl-cysteine (Sigma) and EDTA at 4°C. Elastase (Sigma) at 1mg/mL was added for another 2 days of digestion. Digest solutions were tested for sulfated GAGs with a dimethylmethylen blue Blyscan kit (Biocolor, Newtownabbey, Ireland) according to the manufacturer's protocol. Cell number was determined with a conversion factor of 7.7 pg DNA/cell from DNA quantification with PicoGreen® (Molecular probes, Carlsbad, CA) reagent by comparing to calf thymus DNA (Sigma). Collagen content was measured with a modified colorimetric hydroxyproline assay. Enzyme-linked immunosorbent assays (ELISAs) for collagen types I and II were also performed, as described previously.
Mechanical testing

At least six samples per groups were tested at each time point for tensile and compressive mechanical properties. For tensile testing, constructs were cut into a dog bone shape and glued to a paper frame to give a standardized gauge length. Samples were tested on a 5565 Instron (Norwood, MA) with a 1% strain/sec until failure; the elastic modulus (E) and ultimate tensile strength (UTS) of the constructs were calculated from the resulting stress-strain data.

Constructs were cut in half through the circumference and tested with a creep indentation apparatus to determine compressive properties. An indentation tip of 1 mm was used to apply a tare load of 0.002 N and a creep load of 0.007 N. Loading occurred until the sample equilibrated (defined as deformation less than $10^{-6}$ mm/s) or 10 min passed (tare load) and 1 hr passed (creep load). The step load was removed and recovery distance measured. Data were analyzed with a semi-analytic, semi-numeric model to determine the biphasic properties: aggregate modulus, permeability, and Poisson's ratio.

Statistics

Biochemical and mechanical data were analyzed with a two-way analysis of variance, using factors of time and pressure stimulation having two and four levels, respectively. When an F-test indicated significance of a factor ($p < 0.05$), a Tukey's post hoc test was performed to determine differences within the factor.

Results

Morphology and histology

Constructs were primarily cylindrical in shape, and there were no changes in shape over time or among the treatment groups. There were no significant differences in construct diameter among
the treatment groups, with a range of 2.72 - 3.47 mm. Wet and dry weights of the constructs are shown in Table 1. Both increased significantly with time.

Histology micrographs from 21 days are shown in Fig. 1. No substantial differences were noted among the groups. Constructs stained uniformly throughout for both picrosirius red and safranin-O, except the outer edge which did not stain for safranin-O and stained more intensely for picrosirius red. While not shown, constructs from 10 days also stained in a similar fashion but were less intense in color.

Biochemistry

Results from biochemical assays and ELISAs are illustrated in Fig. 2. Cells and ECM all increased significantly from 10 days to 21 days. The only difference among the groups was seen in cell number/construct, for which IP was greater than SP; however, this was not a dramatic difference and was only apparent at the later time point. When the quantity of collagen type I was normalized to the amount of collagen type II (Fig. 2d), the ratio decreased significantly over time. While both quantities increased individually with time, the increase in collagen II relative to collagen I was more dramatic.

Mechanical properties

Mechanical data are shown in Fig. 3. There were no differences in tensile properties among the groups, but a temporal increase was noted, which was significant for UTS. No significant differences were observed among the groups for any of the compressive properties, but permeability decreased significantly with time.
Discussion

To the best of our knowledge, this study was the first to examine the effects of hydrostatic pressure on costal chondrocytes. Passaged CCs in scaffoldless, three-dimensional constructs were examined under 10 MPa intermittent (1 Hz) and static (0 Hz) hydrostatic pressure. Constructs were examined immediately after stimulation (10 days) and after 3 wks of total culture. Few differences were seen for the gross morphology, ECM content, or mechanical properties among the groups. The time factor, however, did result in significant increases in cell number, GAG, collagen, and UTS.

Unlike in prior studies, neither intermittent nor static pressure resulted in enhanced ECM or better mechanical integrity.\cite{116,229-231,233} One possible explanation for this is that passaged CCs are not sensitive to hydrostatic pressure. Since a precise mechanism for the effects of hydrostatic pressure has not been determined, it is difficult to speculate why these cells would not be sensitive. Alternatively, the regimen applied in this study may not have been effective for the CCs. The 10 MPa at 0 Hz or 1 Hz stimuli were chosen based on previous work with ACs, meniscal fibrochondrocytes, and TMJ fibrochondrocytes, but these may not be the most appropriate regimens for stimulating these cells. Assessing different application times, durations, magnitudes, and/or frequencies may result in a beneficial or detrimental outcome.

Time was a consistently significant factor in this study, with increases seen for several biochemical and mechanical properties. Collagen/construct quadrupled, and GAG/construct more than quadrupled. Cell quantity also increased, and UTS increased 2-5 times with the later time point. While both quantities increased individually with time, the increase in collagen II relative to collagen I was more dramatic. This may be due to the chondrogenic medium, which was employed in the construct culture and has been shown previously to promote a chondrocytic phenotype.\cite{98,233} Although no major differences resulted from the selected treatments, the intermittent pressure group had a significantly higher cell number than the static pressure group, apparent at the later time point. This suggests that intermittent pressure may encourage
proliferation, while static pressure decreases it, but the differences were not very dramatic and have not been seen in previous studies.\textsuperscript{237-239}

While examining alternative application times, pressures, frequencies, and durations may be important, the use of other mechanical stimuli may be of greater interest. Considering the important tensile component of fibrocartilage, employing a tensile bioreactor to stimulate these constructs may lead to particularly exciting results. Work with tendon and ligament tissue engineering has shown that tensile stimuli can promote collagen alignment, collagen gene expression, and tensile properties.\textsuperscript{121,240} These promising results for fibrous tissue engineering may also translate well into improved properties of fibrocartilage tissue engineered constructs with costal chondrocytes.
Table 8-1: Wet and dry weights of the constructs

<table>
<thead>
<tr>
<th></th>
<th>Day 10</th>
<th></th>
<th>Day 21*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wet weight (mg)</td>
<td>Dry weight (mg)</td>
<td>Wet weight (mg)</td>
<td>Dry weight (mg)</td>
</tr>
<tr>
<td>NTC</td>
<td>6.5 ± 0.78</td>
<td>0.79 ± 0.11</td>
<td>12 ± 0.53</td>
<td>1.5 ± 0.071</td>
</tr>
<tr>
<td>BC</td>
<td>7.27 ± 0.60</td>
<td>0.85 ± 0.13</td>
<td>11 ± 1.0</td>
<td>1.4 ± 0.058</td>
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<tr>
<td>SP</td>
<td>6.9 ± 1.5</td>
<td>0.73 ± 0.11</td>
<td>12 ± 0.59</td>
<td>1.5 ± 0.096</td>
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<tr>
<td>IP</td>
<td>7.4 ± 0.88</td>
<td>0.77 ± 0.060</td>
<td>12 ± 0.71</td>
<td>1.5 ± 0.065</td>
</tr>
</tbody>
</table>

Table 1 Wet and dry weight of all groups at 10 days and 21 days. Data are shown and mean ± standard deviation. Weights at 21 days were significantly greater than 10 days, as indicated by the *. 
Figure 8-1: Histology images at 21 days

<table>
<thead>
<tr>
<th>NTC</th>
<th>SP</th>
<th>BC</th>
<th>IP</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
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<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
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</tbody>
</table>

**Figure 1** Histological analysis of all the groups at 21 days. Picrosirius red staining was particularly prominent on the outer edge of the constructs and otherwise uniform throughout the neotissue. Safranin-O staining was positive for all constructs and seen throughout the constructs except on the outermost edge. Scale bar = 0.1 mm
Figure 8-2: Biochemical data

Figure 2 Data are shown as mean ± standard deviation. Groups separated by different letters are considered significantly different. Graph a) shows the results of the picogreen assay. Cells were significantly greater at 21 days and IP was greater than CP. No treatment differences were seen for any of the other assays, but collagen/construct (b) and GAG/construct (c) were also significantly greater at 21 days than at 10 days. The ratio of collagen type I to collagen type II (d) was significantly greater at 10 days than at 21 days.
Figure 3 Data are shown and mean ± standard deviation. No statistical differences were observed for the elastic modulus (a) or aggregate modulus (c), but for UTS (b) values were significantly greater at 21 days than 10 days.
Chapter 9: Growth factor effects on costal chondrocytes for tissue engineering fibrocartilage

Abstract

Tissue engineered fibrocartilage could become a feasible option for replacing tissues like the knee meniscus or temporomandibular joint disc. This study employed five growth factors insulin-like growth factor-I, transforming growth factor-β1, epidermal growth factor, platelet-derived growth factor-BB, and basic fibroblast growth factor in a scaffoldless approach with costal chondrocytes, attempting to improve biochemical and mechanical properties of engineered constructs. Samples were quantitatively assessed for total collagen, glycosaminoglycans, collagen type I, collagen type II, cells, compressive properties, and tensile properties at two time points. Most treated constructs had lower biomechanical and biochemical properties than the no growth factor control, suggesting a detrimental effect, but the IGF treatment tended to improve the constructs. Additionally, the 6wk time point was consistently better than 3wks, with total collagen, glycosaminoglycans, and aggregate modulus doubling during this time. Further optimization of the time in culture and exogenous stimuli will be important in making a more functional replacement tissue.

Introduction

Tissue engineering seeks to create functional replacement tissue and often employs an autogenic cell source to avoid issues with immune rejection. Identifying an appropriate cell source can be particularly difficult, since cells from the tissue of interest are often scarce and/or already diseased. This is especially true for cartilage and fibrocartilage tissues. Fibrocartilage, like that seen in the knee meniscus or temporomandibular joint (TMJ) disc, is frequently injured or diseased and largely lacks the ability to repair itself.\textsuperscript{241,242} Unlike healthy, hyaline articular cartilage, fibrocartilage also contains collagen type I, and while cartilage functions primarily in compression, fibrocartilage has an important tensile component to its mechanical role.\textsuperscript{4} The biochemical and mechanical demands of this unique category of tissues require a highly productive cell type.

Costal chondrocytes (CCs) appear particularly well suited for the purposes of fibrocartilage tissue engineering due to similarities in their tissue characteristics. Native rib cartilage contains glycosaminoglycans (GAGs), primarily chondroitin sulfate and keratan sulfate,\textsuperscript{174-176} and collagen types I and II in a ratio of 1:5.\textsuperscript{172} Costal cartilage is hypocellular, containing 4-10 cells per 0.22mm\textsuperscript{2} of tissue.\textsuperscript{196}

While CCs have shown potential in cartilage tissue engineering,\textsuperscript{160,163,221} their ability to produce collagen I suggests they may function even more effectively in fibrocartilage tissue engineering. Indeed, CCs have shown potential to produce extracellular matrix (ECM) relevant for tissue engineering the TMJ disc; however, improved collagen content and mechanical properties are necessary before a functional replacement can be achieved.\textsuperscript{178,180}

The growth factors and concentrations were judiciously chosen based on their ability to improve ECM production in CCs or fibrochondrocytes. Transforming growth factor-\(\beta\)1 (TGF), at 1ng/mL was shown to increase proline, thymidine, leucine, and sulphate incorporation in CCs.\textsuperscript{243} At
higher concentrations TGF improved collagen production and mechanical properties in constructs made from TMJ disc fibrochondrocytes.\textsuperscript{115} The combination of fibroblast growth factor (FGF), platelet-derived growth factor-BB (PDGF), and TGF increased proliferation and collagen type I and elastin staining in CCs.\textsuperscript{154} PDGF also increased collagen production in TMJ disc cells.\textsuperscript{155} FGF also increased proline, leucine, and thymidine incorporation in CCs, but decreased the sulfate incorporation.\textsuperscript{243} FGF increased GAGs, collagen, and mechanical properties in TMJ disc cells.\textsuperscript{114,115} Epidermal growth factor (EGF) increased proliferation of growth plate CCs and promoted a more elongated morphology; the effects of proliferation were saturated at 30ng/mL.\textsuperscript{244,245} GAG was increased with insulin-like growth factor-I (IGF) up to 0.5 \textmu g/mL in CCs\textsuperscript{246} and collagen was increased in TMJ disc cells with 100ng/mL of IGF.\textsuperscript{114}

This study attempts to improve biochemical and mechanical properties of tissue engineered constructs using CCs through the addition of growth factors to this \textit{in vitro} approach. IGF, TGF, EGF, PDGF, and FGF were applied continuously to scaffoldless, tissue engineered constructs and examined for changes in ECM quantities and mechanical properties.

\textbf{Materials and Methods}

\textit{Cell preparation}

Costal cartilage tissue was scraped from the non-floating ribs of three skeletally-mature, female goats obtained from a local abattoir. This tissue was minced into cubes approximately 1mm\textsuperscript{3} in size and digested overnight at 37\textdegree C with 0.2\% type II collagenase (Worthington, Lakewood, NJ) in Dulbecco’s modified eagle’s medium (DMEM) (Gibco, Carlsbad, CA) with 10\% fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA), 1\% Penicillin-Streptomycin-Amphotericin B (PSF) (Cambrex, Walkersville, MD), 1\% non essential amino acids (NEAA) (Gibco), and 25\mu L/mL L-ascorbic acid (Sigma, St. Louis, MO). Isolated cells were cultured on tissue culture-treated plastic until 70-90\% confluent. They were then passaged with trypsin-EDTA (Gibco). After
passage 1, cells were frozen in DMEM with 10% DMSO, 20% FBS, 1% PSF, and 1% NEAA. Upon thawing, cells were again cultured in monolayer until passage 3.

Construct preparation

Constructs were formed using a method modified from the self-assembly of articular chondrocytes.\textsuperscript{104} Agarose wells were formed from a mold, and 2M cells were seeded into each 5mm well. After 4hrs, additional construct medium was added: DMEM with 1% PSF, 1% NEAA, 1% ITS+ premix (BD Biosciences, San Jose, CA), 0.1μM dexamethasone, 40μg/mL L-proline (EMD Chemicals, Gibbstown, NJ), 50μg/mL ascorbate 2-phosphate (Sigma), and 100μg/mL sodium pyruvate (Fisher). Five growth factors (in addition to a no treatment control—no GF) were used individually in the construct medium throughout the entire 6wk culture period: TGF (1ng/mL), IGF (100ng/mL), FGF (10ng/mL), EGF (30ng/mL), and PDGF (10ng/mL) (Peprotech, Rocky Hill, NJ). After 1wk, constructs were removed from the wells and cultured for the remaining time in unconfined, agarose-coated, 6-well plates. All groups were studied at 3 and 6wks for gross morphological, histological, biochemical, and mechanical changes.

Histology and immunohistochemistry

At least one construct per group was removed at each time point, frozen in HistoPrep\textsuperscript{TM} (Fisher), and sectioned into 14μm slices. Slides for histology were fixed in formalin and stained with safranin-O/fast green for GAGs or picrosirius red for collagen. Immunohistochemistry (IHC) was used to examine collagen types I and II, as described previously.\textsuperscript{180}

Biochemistry and enzyme-linked immunosorbent assay (ELISA)

Six samples per group were examined at each time point for biochemical content. Samples were weighed before and after lyophilization to determine wet and dry weights. Dry samples were digested under constant mechanical agitation for 7 days at 4°C with a 125μg/mL papain (Sigma)
solution containing N-acetyl-cysteine (Sigma) and EDTA. Elastase (Sigma) at 1mg/mL was added for another 2 days of digestion. Digested samples were stored at -20°C and used for biochemical assays and ELISAs.

DNA was quantified with a PicoGreen® (Molecular probes, Carlsbad, CA) reagent by comparing to calf thymus DNA (Sigma). Cell number was determined with a conversion factor of 7.7pg DNA/cell. Sulfated GAGs were quantified with a Blyscan kit (Biocolor, Newtownabbey, Ireland) according to the manufacturer's protocol. Collagen content was measured with a modified colorimetric hydroxyproline assay. Collagen types I and II were quantified with ELISAs as described previously.

**Mechanical testing**

Five samples were cut in half through the center of the sample, and the remaining piece of neotissue was tested with a creep indentation apparatus to determine compressive properties. An indentation tip of 1mm was used to apply a tare load of 0.002N and a creep load of 0.007N. Loading occurred until the sample equilibrated (defined as deformation less than $10^{-6}$ mm/s) or 10min passed (tare load) and 1hr passed (creep load). The step load was removed and recovery distance measured. Data were analyzed with a semi-analytic, semi-numeric model to determine the biphasic properties: aggregate modulus, permeability, and Poisson's ratio.

At least five samples per group were also tested in tension on a 5565 Instron (Norwood, MA). Constructs were cut in half through the circumference and then cut again to form a dog bone shape so that only a solid piece of neotissue remained. Samples were glued to a paper frame and loaded at 10% strain/min until failure. Elastic modulus (E) and ultimate tensile strength (UTS) were calculated from the stress-strain data.
Statistics

All mechanical and biochemical data were analyzed with a two-way ANOVA. Factors were time and growth factor, which had two and six levels, respectively. An F-test was used to determine if a factor was significant ($p < 0.05$), and a Tukey's post hoc test was used to determine differences between the levels.

Results

Morphology and histology

Morphological differences among the treatment groups at 6wks are illustrated in Fig. 1. Control and IGF constructs retained their initial seeding diameter of 5mm, while other groups contracted into more spherical-shaped constructs. These spheres were actually fluid-filled structures. Staining of the inner fluid with trypan blue showed numerous dead cells (data not shown). At both time points, 100% of the PDGF, FGF, and TGF constructs appeared to have this cyst-like morphology. At 3wks, EGF, IGF, and controls were cyst-like in 80%, 54%, and 64% of the constructs, respectively. At 6wks, 100% of the EGF constructs appeared fluid-filled. The cyst-like IGF constructs also increased to 80% at 6wks, but the control samples remained about the same with 60% forming cyst-like structures; these structures did not stain for any ECM (see Fig. 1). Collagen and GAG staining was seen throughout the outer region of the constructs. The collagen staining was particularly dense on the outermost surface of all the constructs. IHC staining was positive for both collagens type I and II for all constructs and regionally distributed in a similar manner to the picrosirius red stain. The morphology of the cells in the constructs was primarily rounded and chondrocyte-like.
Biochemistry and ELISA

Figure 2 illustrates cell number, GAG content, and total collagen content for all treatments and time points. Data are presented as per construct quantities, but wet and dry weights are shown in Table 1. IGF constructs had the largest wet and dry weights, and no GF constructs had a significantly larger dry weight than TGF, EGF, PDGF, and FGF constructs. The cell number (Fig. 2a) was greatest in IGF constructs, which was significantly more than control, TGF, and PDGF constructs. EGF and FGF groups had significantly more cells than the PDGF group; however, all groups resulted in cell quantities near the initial seeding of 2M cells. The total GAG and collagen in the constructs (Fig. 2b and 2c), on the other hand, showed dramatic differences for the treatment groups. IGF and control groups had the greatest GAG and collagen, significantly more than any other group. TGF constructs contained significantly more GAG than the EGF, PDGF, and FGF constructs and more collagen than PDGF and FGF constructs. Collagen II quantities (Fig. 3a) had the same statistical differences as the GAG quantities, while collagen I content (Fig. 3b) in the EGF group was significantly greater than the control and FGF groups. The ratios of collagen type I to collagen type II are shown in Table 2. EGF constructs had significantly the greatest ratio with EGF and PDGF being significantly greater than TGF, IGF, or control constructs. Time was significant for all biochemical, ELISA, and weight metrics, with the 6wk time point having greater quantities than the 3wk time point.

Mechanical properties

Aggregate moduli (Fig. 4a) of control samples were significantly greater than EGF or FGF samples. Aggregate moduli also significantly increased from 3wks to 6wks. Permeability and Poisson's ratio were not significantly different between the experimental groups with ranges of $3.97 \times 10^{-15}$-$1.87 \times 10^{-13}$ m²/N·s and 0.0108-0.368, respectively. Permeability was significantly greater at 3wks than 6wks. Tensile properties (Fig. 4b and 4c) were highest for the control group. Control constructs had a UTS significantly greater than EGF, PDGF, and FGF constructs. The control group also had a significantly greater elastic modulus than PDGF and FGF groups. The
UTS values of the IGF samples were also significantly higher than the FGF samples. Time was not a significant factor in the tensile properties.

Discussion

This study examined the effects of various growth factors on scaffoldless, costal chondrocyte constructs for the purposes of fibrocartilage tissue engineering. IGF-treated constructs were equal to or better than control for all the metrics examined in this study. The increase in cell number with IGF treatment is consistent with previous work with CCs. The increase in GAG seen previously with CCs or increase in collagen seen previously with TMJ fibrocartilage were not as prominent as expected, however the increasing trend was certainly apparent suggesting the benefits of IGF for fibrocartilage tissue engineering.

TGF, EGF, and PDGF had considerably less GAG, total collagen, and collagen type II, but more collagen type I, demonstrating the promotion of a more fibroblast-like phenotype. Previous work with CCs showed that EGF and FGF treatment led to a more elongated cell morphology in monolayer. This morphology is generally indicative of a cell phenotype which would produce more collagen type I and less collagen type II. While dramatic differences in the cell morphology were not observed and most of the cell appeared rounded in shape, the EGF and FGF groups produced larger ratios of collagen type I to type II than the control group. While previous work supports many of the results seen here, more improvements in ECM content were expected for TGF, FGF, and PDGF. The limited improvements seen with these growth factor treatments are likely due to the base medium used, which had no serum and contained additional additives that have been shown to promote chondrogenesis. The previous cited work added 10% serum to the base medium, which contains small amounts of growth factors. Serum is often considered undesirable in tissue engineering, due to issues with immune response of serum from another species, but it may be necessary to promote growth factor effects. Alternatively, the
presence one or two other growth factors may be sufficient to encourage the effects of a single growth factor. Future work may wish to consider a combination of a small number of growth factors in lieu of adding serum. Other studies may also wish to consider alternative growth factors with the base medium used here. The medium’s chondrogenic ability appeared to decrease with the addition of TGF, EGF, PDGF and FGF, as was seen with the 10-60% decrease in GAG, total collagen, and collagen type II from the control. (IGF treatment was largely unchanged from the group without additional growth factors.) While these results were contrary to expectations, they are partially supported by current work with articular chondrocytes in a scaffoldless approach with serum-free medium, which showed a decrease in GAG with the addition of TGF-β1. While fibrocartilage tissue engineering is still in its early stages, the field of cartilage tissue engineering has been explored in considerably more depth and established this base medium as better than FBS media for cartilage regeneration. However, it is apparent from this study that more work needs to be done, particularly for fibrocartilage tissue engineering, to determine growth factors that can further improve properties from the base medium rather than diminish them.

Regardless of the growth factor used, constructs consistently benefited from a longer time in culture. For all biochemical assays and ELISAs, quantities of ECM and cells increased temporally across all treatment groups. At 6wks, total collagen and GAG increased at least two fold over the values seen at 3wks for all the groups. Aggregate modulus also approximately doubled in most groups. Tensile properties, however, did not have a clear trend with time. Most groups remained the same from 3wks to 6wks; it was not considered a significant factor for either UTS or elastic modulus. This suggests that while collagen quantities are increasing in the constructs, the collagen is likely not being organized or packed appropriately to improve the tensile strength or stiffness. The application of a mechanical stimulus, like tension, could be important to the ECM organization, which would likely improve the mechanical properties. The temporal changes also suggest the need to examine the time factor in greater depth—exploring both longer and shorter time points to elicit an overall trend on the changes that occur with time.
Previous work using passaged chondrocytes in a scaffoldless approach have also observed the fluid-filled structure that formed in many of the constructs from this study. It is well accepted that chondrocytes prefer cell-cell interaction and retain their phenotype better in three-dimensional culture, while attachment to the tissue culture plastic alters their phenotype, promoting a more fibrochondrocytic phenotype. This phenotypic alteration may not occur uniformly, and it is very likely that the cell population after passaging is non-uniform. As discussed previously, in the absence of another surface on which to attach, similar cells attach to one another; this is referred to as the differential adhesion hypothesis. Considering these observations, it is reasonable to infer that the passaged CCs aggregate into distinct cell populations upon construct formation. Within the first 48hrs, formation of the fluid-filled structure becomes apparent, (data not shown). The ensuing death of the inner population of cells may be due to the outer cells forming a barrier for nutrient and/or waste transport. Additionally or alternatively, the inner region may be more amiable to substrate attachment versus cell attachment, causing them to die after three-dimensional seeding. This suggests an interest in determining the characteristics of these populations and purifying them prior to construct seeding. Interestingly, at the end of 6wks, all the growth factors appeared to promote this cyst-like structure either at 3wks or over time (like IGF treatment). This fascinating observation gives insight into the subpopulations of these cells. One could postulate that these growth factors initially encouraged growth of the population of cells that formed in the center. Their subsequent death made the growth factors appear ineffective and even detrimental. If the methods used here were altered so that those cells did not die, the growth factors may have caused beneficial changes in the constructs. Further exploration of ways to eliminate this structure will likely be necessary. Altering the expansion conditions would certainly affect the cell characteristics and may also eliminate the “cyst” structure.

While there is considerable work that still needs to occur to optimize the constructs created here, the approach used is promising for the purposes of fibrocartilage tissue engineering. Comparing the construct properties to those seen in a porcine TMJ disc, it is apparent that compressive
properties are nearly the same, and that GAG content is actually greater than the native tissue. Collagen content was about ten times lower than native, and tensile modulus was 2-40 times lower. The fibroblast-promoting growth factors, like TGF, PDGF, EGF and FGF, were not able to improve these properties from the control, but they were able to alter the ratio of collagen I to collagen II suggesting the potential of the growth factors to augment the construct for various types of fibrocartilages. Additionally, the IGF treated group was better than the other groups for biochemical content and mechanical properties. Future work examining a combination of growth factors, an optimal culture time, purification of the expanded cell population, or improved expansion conditions could further develop these constructs such that they are able to function as native fibrocartilage.
Table 9-1: Wet and dry weights of constructs at both time points

<table>
<thead>
<tr>
<th>Wet weight (mg)</th>
<th>3wk</th>
<th>6wk*</th>
<th>Dry weight (mg)</th>
<th>3wk</th>
<th>6wk*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No GF(^b)</td>
<td>27.3 ± 4.42</td>
<td>36.0 ± 6.05</td>
<td>No GF(^b)</td>
<td>2.97 ± 0.193</td>
<td>4.49 ± 0.366</td>
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<tr>
<td>IGF(^a)</td>
<td>33.9 ± 6.10</td>
<td>46.1 ± 9.80</td>
<td>IGF(^a)</td>
<td>3.33 ± 0.542</td>
<td>6.02 ± 0.272</td>
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<tr>
<td>TGF(^b)</td>
<td>19.8 ± 1.51</td>
<td>28.6 ± 1.07</td>
<td>TGF(^c)</td>
<td>2.22 ± 0.131</td>
<td>3.92 ± 0.378</td>
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<tr>
<td>EGF(^b)</td>
<td>21.1 ± 3.82</td>
<td>35.7 ± 3.25</td>
<td>EGF(^c)</td>
<td>2.00 ± 0.305</td>
<td>3.72 ± 0.379</td>
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<tr>
<td>PDGF(^b)</td>
<td>24.9 ± 19.3</td>
<td>30.2 ± 5.90</td>
<td>PDGF(^c)</td>
<td>1.91 ± 0.422</td>
<td>3.56 ± 0.501</td>
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<tr>
<td>FGF(^b)</td>
<td>22.7 ± 1.72</td>
<td>33.6 ± 3.87</td>
<td>FGF(^c)</td>
<td>2.24 ± 0.224</td>
<td>3.56 ± 0.302</td>
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</table>

Table 1 Data are shown as (mean ± SD). Treatment groups with different letters are statistically significant (\(p < 0.05\)). Weights were significantly larger at 6wks as indicated by the \(*\). IGF constructs had wet and dry weights significantly larger than any other group. No GF constructs also had significantly greater dry weight than the other groups.
Table 9-2: Ratios of collagen types for constructs at both time points

<table>
<thead>
<tr>
<th>Collagen I/Collagen II</th>
<th>3wk*</th>
<th>6wk</th>
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</thead>
<tbody>
<tr>
<td>No GFd</td>
<td>2.33 ± 0.150</td>
<td>2.67 ± 0.206</td>
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<td>IGFd</td>
<td>2.82 ± 0.552</td>
<td>2.61 ± 0.237</td>
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<tr>
<td>TGFEd</td>
<td>3.22 ± 0.265</td>
<td>3.13 ± 0.518</td>
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<td>EGFf</td>
<td>6.35 ± 1.60</td>
<td>3.82 ± 0.180</td>
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<tr>
<td>PDGFab</td>
<td>5.22 ± 0.815</td>
<td>3.42 ± 0.223</td>
</tr>
<tr>
<td>FGFbc</td>
<td>4.73 ± 0.572</td>
<td>3.33 ± 0.580</td>
</tr>
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</table>

Table 2 Ratios of collagen type I to collagen type II at both time points.

Data are shown as (mean ± SD). Treatment groups with different letters are statistically significant (p<0.05). Ratios were significantly larger at 3wks as indicated by the *. EGF and PDGF constructs had significantly higher ratios than IGF, TGF, or no GF treated constructs. The FGF ratio was also significantly greater than IGF and control groups.
Figure 1 Gross morphological images from 6wks are shown in frames a-l. Spaces below the images are 1mm in length. Control and IGF groups retained the initial 5mm diameter, while the other groups contracted and rounded into more spherical morphologies. Frames m-r show picrosirius red staining for the constructs at 6wks. This stain clearly shows the collagen which stained consistently in an outer ring and densely on the very outer edge of the constructs. These images also illustrate the void structure that formed frequently, particularly in the growth factor-treated groups. The scale bar in frame m is 1 mm and applies for all picrosirius red staining micrographs. Frames s-x show safranin-O/fast green staining. Collagen types I and II IHC are seen in frames y-dd and ee- jj, respectively. All samples stained positive for GAGs and collagens types I and II. The scale bar in frame ee is 100 µm and applies to the IHC and safranin-O/fast green stains.
Figure 2 Cell (a), GAG (b), and collagen (c) content at both time points (mean + SD). Treatment groups with different letters are statistically significant ($p < 0.05$). For all the biochemical metrics, the 6wk time point was significantly greater than the 3wk time point as indicated by the *. While some statistical differences in the various growth factors were apparent, cell quantities were around 2M for all groups. GAG/construct and collagen/construct were significantly greatest in the control and IGF constructs. TGF-treated constructs had significantly more GAG content than EGF, PDGF, and FGF constructs and significantly more collagen than PDGF and FGF constructs.
Figure 9-3: Quantities of collagen types I and II of growth factor-treated constructs

Figure 3 Collagen type II (a) and collagen type I (b) quantities for constructs at both time points (mean ± SD). Treatment groups with different letters are statistically significant ($p < 0.05$). The 6wk time point was significantly greater than the 3wk time point in both collagen types as indicated by the *.

Control and IGF samples had significantly more collagen type II than all other groups. TGF samples had significantly more collagen type II than EGF, PDGF, and FGF samples. Collagen type I was significantly greater in EGF constructs than in FGF or no GF constructs.
Figure 9-4: Mechanical data of growth factor-treated constructs

**Figure 4** Aggregate modulus (a), UTS (b), and elastic modulus (c) for all groups at both time points (mean ± SD). Treatment groups with different letters are statistically significant (p < 0.05). The 6wk time point was significantly greater than the 3wk time point as indicated by the * for aggregate modulus. The no GF group had the highest aggregate modulus, which was significantly larger than EGF and FGF groups. The control group also had the highest tensile modulus and UTS—significantly greater than PDGF and FGF. Its UTS was also significantly higher than the EGF group. IGF constructs were significantly greater than FGF constructs for both E and UTS. The TGF group was significantly larger than the FGF group for UTS. Time was not a significant factor for either tensile measurement.
Conclusions

The work presented in this thesis improved upon previous work toward temporomandibular joint (TMJ) disc tissue engineering by optimizing the culturing conditions of TMJ disc cells. It also built upon the relatively well-established tissue engineering fields of articular cartilage and tendon, compared to the TMJ disc. The review seen in chapter 1 highlighted the key aspects of tissue engineering that should be considered. Successes with alternative cell sources and scaffoldless culture methods for articular cartilage and tendon motivated the latter half of the global objective. Thus, this thesis took the previous TMJ disc tissue engineering work in a profound new direction: using new cell sources in a scaffoldless technique to improve the functionality and clinical translatability of the tissue engineering approach. These changes have led to dramatic improvements and suggest exciting directions for future work.

This thesis addressed two global hypotheses: 1) finding optimal culturing conditions for TMJ disc cells in two- and three-dimensional culture will improve the final properties of TMJ disc cell constructs, and 2) there exists a cell source that will create more biomechanically and biochemically functional constructs toward achieving eventual clinical translatability.

The first hypothesis was examined in a two phase study which was able to successfully increase TMJ disc cell proliferation without altering collagen gene expression or protein production in monolayer culture. TMJ disc cells were also seeded on PLLA scaffolds and exposed to varying concentrations of L-proline, but the addition did not improve any biochemical or mechanical properties of the constructs. The need for greater improvements to the three-dimensional constructs in order to produce neotissue approaching native values suggested the necessity for more experimentation with this approach.

The limitations of the TMJ disc cells from the first aim and extensive previous work were apparent. The cells did not produce substantial matrix, nor was the limited matrix organized such
that it could support a sufficient amount of mechanical loading as the polymer scaffold degraded. From a clinical standpoint, the required cell quantity and the difficulty in harvesting TMJ disc cells makes the methods used in aim 1 unappealing. These limitations motivated the second hypothesis. Additionally, the scaffold-based approach was abandoned for a scaffoldless approach that had seen considerable success for cartilage and fibrocartilage tissue engineering.\textsuperscript{104,185}

First, a broad look was taken at a variety of possible cell types that may function in this scaffoldless, tissue engineering approach. Articular chondrocytes (ACs) from the knee, costal chondrocytes (CCs), dermal fibroblasts (DFs), mixtures of dermal fibroblasts with both types of chondrocytes, passaged costal chondrocytes, and bone marrow derived mesenchymal stem cells (MSCs) were examined for their ability to produce glycosaminoglycans (GAGs) and collagen, while retaining their cellular content. The potential clinical translatability of these cell types varies. For example, MSCs would be an ideal candidate due to their self-renewing ability, while ACs would leave tissue morbidity at the harvest site. However, any of these cell types is arguably better than TMJ disc cells for their translatability into a clinical method. In this pilot study, CCs and passaged CCs produced constructs with more GAG and collagen than any other cell type.

CC constructs were examined in more depth and compared to TMJ disc cell constructs, DF constructs, and a mixture of CC/DF constructs. This was the first time that TMJ disc cells were examined with this self-assembly, scaffoldless method. While the TMJ disc cell constructs showed surprisingly good biomechanical and biochemical results, the resulting size of the constructs and the limitations in clinical translatability of the cells suggested that the other cell types should be explored. While DF constructs were also prohibitively small and CC/DF constructs did not produce much matrix, CC constructs were large, mechanically robust, and contained substantial matrix. These constructs were also dramatically improved from previous TMJ disc cell-seeded scaffolds.
Primary CC constructs were also compared to passaged CC constructs and primary and passaged AC constructs for biochemical and biomechanical properties. While previous work with young, bovine ACs showed success,\textsuperscript{104} the results shown here with skeletally-mature, primary ACs were not successful. AC P0 constructs had low biochemical content and were not testable in tension or compression. The difference between these and previous results is likely attributed to the age of the cells. Unlike the AC P0 constructs, CC P0 constructs were mechanically testable and produced GAG and collagen. Passaged constructs, however, were better for tensile properties and biochemical quantities. The dedifferentiation that has been shown previously for passaged chondrocytes, appears beneficial for the purposes of TMJ disc tissue engineering.\textsuperscript{1,184}

The final aim of this thesis was to further examine passaged CCs and improve their properties with external stimuli. Primary CC constructs were compared to P1, P3, and P5 constructs. Passaged constructs produced better compressive properties and more GAG content, but there was not a consistent trend among the various passaged constructs. The CC constructs once again outperformed TMJ disc cell constructs. The ability to use passaged cells further improves the potential clinical translatability of this approach. Since large quantities of cells are required for this scaffoldless method, the ability to passage the cells lessens the quantity of tissue needed from harvest.

Hydrostatic pressure was applied to passaged CC constructs at 10 MPa with both intermittent (1 Hz) and static (0 Hz) stimulation. Comparing the treated groups to bagging and culture controls showed little change among any of the groups, suggesting that CCs were either not responsive to hydrostatic pressure or that an alternative regimen is required to see differences. However, differences between the two time points were profound for many of the construct metrics: GAG, collagen, collagen types I and II, ultimate tensile strength (UTS), and elastic modulus (E).

Growth factors were also applied exogenously to the passaged, CC constructs. Epidermal growth factor (EGF), platelet-derived growth factor-BB (PDGF), and basic fibroblast growth factor (FGF)
showed lower GAG, collagen, collagen type II, UTS, E, and aggregate modulus from the no
growth factor control. However, EGF had significantly more collagen type I than the control.
Transforming growth factor-β1 (TGF) also had less GAG, collagen, collagen type II than controls,
but mechanical properties were not significantly different. Insulin-like growth factor (IGF),
however, had equal or better biochemical and mechanical properties than the control suggesting
its potential to improve these constructs. The limited improvements seen from the other growth
factors may be due to the previously optimized chondrogenic medium that was used in construct
cultures.

This work has contributed substantially to the objective of regenerating the TMJ disc with a
biological replacement. Previous results with TMJ disc cells on scaffolds yielded as high as 35 μg
collagen/construct of collagen\textsuperscript{109,154} and 18 μg GAG/construct.\textsuperscript{111} Using passaged CCs in a
scaffoldless approach, this work was able to obtain 400-450 μg collagen/construct and more than
2 mg GAG/construct. While this still lacked the collagen content compared to native tissue, the
GAG exceeded the native tissue quantity. Biomechanical properties of the constructs were also
below native values, but they too were substantially improved from the previous tissue
engineering results with TMJ disc cells, which were often not testable.\textsuperscript{109} These dramatic
improvements in functionality were also supported by improvements in the translatability of costal
chondrocytes from TMJ disc cells. These conclusions unequivocally support the second
hypothesis of this thesis, that there exists a cell source that will create more biomechanically and
biochemically functional constructs while also improving clinical translatability. Passaged costal
chondrocytes have shown tremendous promise in their applicability toward TMJ disc tissue
engineering.

While this work focused on the TMJ disc, these results have implications for other cartilaginous
tissue engineering applications including the knee meniscus and articular cartilage. The
properties of TMJ disc fibrocartilage are similar to other fibrocartilages, suggesting the potential
for CCs to function in a variety of fibrocartilages for tissue engineering. As was discussed in
chapter 6, costal chondrocytes may also improve current approaches to articular cartilage tissue engineering. While primary ACs were unable to produce high quality neotissue and passaged chondrocyte constructs produced large quantities of collagen type I, which is uncharacteristic of articular cartilage, primary CCs may be beneficial for hyaline cartilage regeneration. These constructs were inferior to constructs produced with young, calf chondrocytes, but using skeletally-mature cartilage tissue may translate more readily into a clinical approach that uses an autologous cell source. Using an autologous cell source that can be easily harvested with little donor site morbidity or surgical complications is highly desirable. This eliminates a potential immune response, and this cell source may decrease the time to shift from bench top to bedside and cost of the procedure.

The work described in this thesis demonstrates the usefulness of costal chondrocytes for tissue engineering and contributes considerably to the field of TMJ disc tissue engineering. It also suggests potential directions to improve on the current properties of the CC construct. Optimizing the expansion conditions and seeding density could improve the collagen production and construct properties. Applying tension during in vitro culture may drastically improve the extracellular matrix organization and mechanical properties of the constructs. By further improving the functional properties of these constructs and generating methods for proper tissue integration, these CC constructs may provide a surgical solution to patients with TMJ disorders.
References


34. Shen, G. The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage. Orthod Craniofac Res. 8:11-7, 2005.


134. Allen, K. D. and K. A. Athanasiou, Comparison of scaffolding biomaterials for TMJ disc tissue engineering, in Biomedical Engineering Society Annual Conference. 2005: Baltimore, MD.


188. NIDCR, TMJ Disorders. 2006.


