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Interspecies Characterization and Tissue Engineering
of the Temporomandibular Joint Disc

by

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ABSTRACT

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By Kerem N. Kalpakci

Disorders of the temporomandibular joint (TMJ) are widespread, afflicting millions of people. The majority of these cases involve displacement or injury to the TMJ disc. Current treatments do not fully address severe cases of TMJ dysfunction; therefore, efforts to engineer functional tissues for repair or replacement are warranted. While previous studies have laid the groundwork for these efforts, significant challenges remain, including (1) identification of appropriate animal models, (2) development of methodologies for *in vitro* TMJ tissue engineering, and (3) refinement of tissue culture procedures for clinically relevant cells sources. This thesis contributes to overcoming these challenges by (1) exploring topographical and interspecies variation in functional properties of the TMJ disc, (2) developing an *in vitro* tissue engineering strategy capable of recapitulating native tissue characteristics, and (3) enhancing protocols for chondrogenesis of dermis-derived cells.

The first aim of this thesis characterized the biomechanical and biochemical properties of human TMJ disc in relation to several animal models. Significant regional and interspecies variations were indentified, though certain characteristics were observed across all species. While the human disc displayed properties distinct from the other species, the pig was the most similar and was therefore identified as the most appropriate animal model. The second aim applied these findings as design criteria in the development of an *in vitro* tissue engineering strategy. Scaffoldless constructs derived
from co-cultures of chondrocytes and fibrochondrocytes were enhanced through optimization of growth factor and serum supplementation, such that they recapitulated many characteristics of native TMJ cartilage. Finally, the third aim refined the differentiation process for chondroinduction of dermis-derived cells. Using an optimized, low-cost surface coating, chondrogenesis was significantly enhanced through incorporation of hypoxia during culture. These experiments address several aspects of fibrocartilage tissue engineering and represent a significant step towards *in vivo* application of these technologies.
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INTRODUCTION

The overall objectives of this thesis were 1) to characterize the temporomandibular joint (TMJ) disc and 2) to develop a strategy for in vitro tissue engineering using a clinically favorable cell source. As presented in Chapter 3, technologies for regeneration of TMJ fibrocartilage remain immature and significant challenges must be overcome before clinical treatments are brought forth. By providing a comparison of the human disc to potential animal models, and by enhancing strategies for in vitro chondrogenesis of dermis-derived cells, this thesis represents a significant step toward that goal.

The objectives of the characterization work were to compare the regional biochemical and biomechanical properties of the human disc to several animal models, and to establish an appropriate animal model for future studies. Functional properties elucidated from this work were applied as design criteria to develop a tissue engineering strategy using primary chondrocytes and fibrochondrocytes. Finally, the expansion and differentiation protocols for dermis cell chondrogenesis were refined to advance these technologies toward treatments for tissue regeneration. Work toward these objectives was conducted under the following governing hypothesis: a strategy for TMJ fibrocartilage regeneration using a clinically favorable cell source could be developed from design criteria established via characterization of native tissue. This was investigated under the following specific aims:

1) To compare the functional properties of the human TMJ disc to those of four animal models. This aim was accomplished via assessment of biomechanical and biochemical properties of discs from human, goat, cow, pig, and rabbit
In order to develop a scaffoldless approach for engineering TMJ fibrocartilage, it was hypothesized that an appropriate animal model, which approximates the native characteristics of the human TMJ disc, could be identified. This aim was accomplished by modulating the type and manner of application of anabolic media supplements to co-cultures of primary chondrocytes and fibrochondrocytes. It was hypothesized that a tissue culture regimen capable of engineering constructs with functional properties approaching those of native TMJ fibrocartilage could be identified.

To refine \textit{in vitro} culture methodologies to enhance chondrogenesis of dermis cells, it was hypothesized that systematic refinement of cell culture protocols would enhance chondrogenesis of dermis cells.

The following chapters provide a basis for the topics explored herein and give detailed descriptions of the experiments that achieve the above aims. Chapter 1 is a review of current knowledge of the biomechanics, cell content, and extracellular matrix of the TMJ disc. This review sets the stage for the characterization study described in Chapter 2. In Chapter 3, the current literature on TMJ disc tissue engineering is reviewed, providing a context for the scaffoldless tissue engineering strategy developed in Chapter
4. Finally, Chapters 5 and 6 outline a series of experiments that refine and enhance an approach for chondrogenesis using dermis-derived cells.

Specific aim 1 is addressed in Chapter 2, which describes an interspecies characterization study of the functional properties of the TMJ disc. This study tested the hypotheses that the pig disc would be the most similar to the human, and that aspects of regional variation would be consistent among the species. Human disc explants were characterized in comparison to those from porcine, bovine, caprine, and leporine sources. The biomechanical aspect examined tensile and compressive properties under instantaneous and equilibrium conditions, while the biochemical section investigated collagen and glycosaminoglycan content and topographical distribution. Connections between biomechanical functionality and underlying matrix content and organization were explored, and inferences were made regarding the similarities and differences among the species.

Specific aim 2 is addressed in Chapter 4, where the effects of TGF-β1, IGF-1, and serum application on scaffoldless co-cultures of fibrochondrocytes and chondrocytes were explored. It was hypothesized that fibrocartilages displaying heterogeneous function and matrix composition would result from different co-culture combinations and growth factor serum treatments, and that modulating the type and manner of application of anabolic agents would result in near recapitulation of native tissue material properties and matrix content. This study was carried out in two phases; the first examined growth factor application, in the presence or absence of serum, on fibrocartilage constructs derived from three cell ratios, and the second tested intermittent and continuous application of the
most effective treatments. Constructs were assessed for tensile and compressive properties, biochemical content, and cellularity.

Specific aim 3 is addressed in Chapters 5 and 6, which describe studies that explore methodologies for chondrogenesis of dermis-derived cells. In Chapter 5, a series of refinements, aimed at reducing cost, improving homogeneity, and increasing cell yield over existing cell culture protocols, are described. Chondroitin sulfate was investigated as a substrate and a new method for creating coatings was studied. In addition, the effect of passaging on cell morphology, clonogenicity, proliferation, and matrix production were examined. The specific hypotheses explored in this chapter were that nodule formation could be induced by chondroitin sulfate coated surfaces, and that passaging would affect dermis cell morphology, clonogenicity, and chondrogenesis.

Chapter 6 explores the effects of reduced oxygen tension on dermis cell chondrogenesis. Based on positive outcomes with mesenchymal stem cells, and guided by native cartilage physiology, it was hypothesized that exposure to hypoxia during expansion and/or differentiation would enhance chondrogenesis of dermis cells. To test this hypothesis, cells were exposed to hypoxia during expansion, and cell proliferation and clonogenicity were assessed. Subsequently, these cells were differentiated on the optimized substrate from Chapter 5 in hypoxia and normoxia, and chondrogenesis was assessed through biochemical and histological analyses.

The thesis closes with the Conclusions chapter, which contains a discussion of its salient results and cumulative findings. The impact of this work and directions for future study are described.
CHAPTER 1: Fibrocartilage of the TMJ disc *

Introduction

The temporomandibular joint (TMJ) disc (also intra-articular disc or TMJ meniscus) is a unique structure that allows for normal jaw movement and concomitant functions, including the ability to eat and talk. The disc is often mistakenly assumed to be functionally and structurally equivalent to the better-characterized hyaline articular cartilage that covers the end surfaces of long bones and the fibrocartilaginous menisci of the knee joint. In the following sections, similarities and differences among these tissues will be highlighted to provide the tissue engineer a complete set of design criteria, which will be valuable when working to create a successful engineered disc. Particular attention will be paid to connections linking mechanical function and behavior to underlying disc structure.

Anatomy: Structure and Attachments

The articulating disc of the human TMJ is a biconcave, elliptical, fibrocartilaginous tissue situated between the mandibular condyle and the glenoid fossa. Because of its position, it effectively divides the joint into superior and inferior spaces, as noted in Chapter 1. From a superior view (Fig. 1.1), it appears as an ellipse, longer in the mediolateral direction than the anteroposterior direction, and measuring approximately 19 by 13 mm in humans. The disc can be roughly divided into three topographical zones: the posterior band, the intermediate zone, and the anterior band. When viewed in a sagittal section (Fig. 1.1), it is clear that the anterior and posterior bands of the disc are much thicker than the intermediate zone. The posterior band is the thickest region, measuring approximately 4 mm. The anterior band is slightly thinner than the posterior, and the intermediate zone is the thinnest region, approximately 1 mm. This shape imparts some important functional characteristics to the disc. The intermediate zone fills the void space between the two joint surfaces when the jaw is occluded, separating and protecting these two incongruent surfaces (Fig. 1.2a). During mastication, the intermediate zone imparts some flexibility to the disc, allowing smooth and coordinated movement amid the complex rotational and translatory action present within the joint (Fig. 1.2b). On the other hand, the thicker peripheral bands help to maintain disc positioning and structure, thereby preventing internal derangement.

The TMJ disc is attached along its entire periphery to both the condyle and the temporal bone through a complex network of fibrous connective tissues that form a synovial capsule that envelops the joint (Figs. 1.3 and 1.4). The rear of the disc blends with a loose network of vascular, fibro-elastic tissue called the bilaminar zone, which is
attached to the posterior wall of the glenoid fossa superiorly, and the base of the condyle inferiorly. The anterior end of the disc is attached to the articular eminence of the temporal bone and the anterior horn of the condyle at a depression called the pterygoid fovea. On the medial and lateral sides, the disc merges with the capsule and in turn attaches to the medial and lateral sides of the condylar neck. Generally speaking, the superior surface of the disc translates with respect to the glenoid fossa. In contrast, the inferior surface of the disc, closely matched to the round contours of the condylar head, experiences mainly rotational movements.

Biochemical Content

Aside from the water content, which constitutes between 66 and 80% of the disc’s weight [1], the primary extracellular matrix (ECM) component of the TMJ disc is collagen type I. This marks an important distinction between the fibrocartilage of the disc and hyaline articular cartilage, which is primarily collagen type II, and also the fibrocartilage of the knee meniscus, which contains substantial type I and II components. In addition to collagen, the other major ECM components in the disc are elastic fibers, glycosaminoglycans, and proteoglycans. These components, and their regional distribution and organization, are covered in detail in the following subsections.

Collagen Composition and Distribution

A dense network of collagen fibers defines the architecture of the TMJ disc. This ubiquitous protein, present in nearly all connective tissues, is responsible for the majority of the disc’s mechanical behaviors. Overall, collagen constitutes approximately 30% of
the wet weight [2], 83-96% of the dry weight [3, 4] and 55% of the total volume [5]. Regional variation in collagen concentration is contradictory. Mills et al. [6] reported higher concentrations in the posterior and anterior bands than in the center of the rat disc, but later Almarza et al. [4] reported the opposite in porcine discs. However, in both cases, the reported differences were small. As mentioned previously, the overwhelming majority of collagen in the disc is type I, although the presence of other types has been reported. Collagen type II, the primary fibrilar collagen present in hyaline cartilage, has been found localized around disc cells using immunohistochemistry [6] and later detected in digested discs with Western blot analyses [7]. Detamore et al. [8] observed only faint collagen type II staining in the posterior and anterior bands of the porcine disc via immunohistochemical staining, while large clusters of collagen II were found separating dense bands of collagen type I throughout the intermediate zone. Type III collagen is a fibrilar cartilage most notably associated with skin, blood vessels, and granulation tissue, and some research suggests at least trace amounts are present in the TMJ disc at early developmental stages [9] and in newly formed tissue [10]. However, neither Landesberg et al. [7] nor Gage et al. [2] detected collagen type III in bovine or human discs, respectively.

Non-fibrilar collagen types VI, IX, and XII have also been identified in small quantities in bovine and rabbit discs [7, 11]. Collagen Type VI is known to be present in larger quantities in immature articular cartilage and also localized in the pericellular matrix of mature chondrocytes. Collagen types IX and XII form molecular bridges between adjacent type II and type I fibers, respectively, and there is some evidence that they also bind GAG chains of small dermatan sulfate proteoglycans. In light of these
findings, more research into the origins of the various collagens and their potential role in
development and function in the disc is warranted. For simplicity, however, the collagen
of the disc is mostly type I with type II in relatively small amounts found in the
intermediate zone. Researchers should make use of this distinction when evaluating the
similarities between tissue engineered constructs and the native disc.

_Collagen Organization_

The orientation of collagen contributes greatly to a tissue’s mechanical behavior, and
scanning electron microscopy (SEM) studies have revealed considerable information
about the organization of collagen in the disc. In a study of the TMJ disc of rhesus
monkeys, Taguchi _et al._ [12] described a thinly woven network of collagen fibers
superficially on the disc, anteroposteriorly oriented fibers deeper in the intermediate
zone, and dense fibers around the periphery encircling the central region. In another SEM
study, Minarelli _et al._ [13] examined healthy human discs from fetal to mature adult
donors, noting that collagen was arranged in compact bundles. In the posterior region,
collagen formed thick bundles arranged in a ring which continued around the periphery
of the disc and into the anterior region where they blended with bundles of
anteroposteriorly and obliquely oriented fibers. Detamore _et al._ [8] corroborated several
of these findings in a more recent study of the porcine disc, noting a ring of collagen
fibers around the periphery, with some branching in the posterior band and considerable
branching in the anterior band. Mills _et al._ [6] used polarized light to examine the
organization of collagen in primate discs, noting the presence of two distinct regions. In
the posterior and anterior bands, fibers were primarily oriented mediolaterally with
additional fibers traveling obliquely through the center of these zones. In the intermediate band, the fibers were oriented sagittally with a noticeably greater density and crimping than in the outer bands. Fibers arched to form connections in the areas between these zones. Scapino et al. [14] examined coronal sections of a human disc with a polarized light microscope and discovered thick vertical bundles in the anterior and posterior bands. Many fibers branched diagonally joining the horizontal fibers of the intermediate zone, while some fibers were seen spanning the entire thickness before turning transversely at the surfaces.

Consistent findings from these studies and others [15-18] can be summarized as follows: The fibers display a characteristic crimping pattern throughout with a reported mean periodicity between 8 and 23 μm [2, 12, 17, 19, 20]. Thick bands of fibers are assembled in a ring-like structure around the periphery of the disc. In the anterior and posterior bands, the fibers run mediolaterally (Fig. 1.5). Conversely, in the medial and lateral regions the fibers align anteroposteriorly. Fibers in the intermediate zone align prominently in the anteroposterior direction, though mediolateral and oblique fibers are present in smaller numbers. In the boundaries between these regions, transitional fibers are found bridging the discordant groupings. Covering the upper and lower surfaces of the disc is a thin layer of undulating fibers with foveae of varying diameter. Taken as a whole, these observations suggest certain aspects of functionality in the disc. The undulating collagen fibers may impart relative compliance to the disc at low strains, thereby allowing it to conform more readily to the incongruencies of the joint surfaces. The anteroposteriorly-oriented fibers suggest tensile loading occurs mostly in this direction. The thick peripheral bands probably help to maintain the shape of the disc.
during loading and also help to restore the initial shape upon removal of loads. This collagen ring is reminiscent of the radial bands of collagen in the knee meniscus. There, the bands constrain the lateral displacement during compressive loading through development of tensile hoop stresses. The analogous fibers in the disc may function in a similar way, constraining lateral deformation during compressive loading [17]. Lastly, the thin foveae present on the disc surfaces allow for storage and diffusion of synovial fluid, effectively reducing friction.

Elastin

The presence of elastin in the TMJ disc has been described in many studies [6, 8, 21-26]. Elastic fibers are found throughout the disc in small numbers making up between 1-2% of the tissue mass [24]. In contrast to elastin found in the ear or nose, which forms large bundles, the elastin in the disc exists as intermittent fibers with a relatively small diameter of approximately 0.5 \( \mu \text{m} \) [21]. Individual elastin fibers are predominantly found running parallel to collagen and in the interstices between bands of collagen, and cross-link to form branched networks with adjacent elastin fibers. There may be a higher degree of branching and multidirectionality in the posterior band than in the intermediate zone, where elastin is predominantly oriented in an anteroposterior direction [25]. The peripheral bands contain considerably more elastin than the intermediate zone [8, 24-26], and the superior surface contains more than the inferior surface [26].

In the human disc, 69% of the elastin is found in the anterior band, compared with 26% in the posterior band, and 5% in the intermediate zone [26]. Conversely, the porcine disc contains more elastic fibers in the posterior band than other regions [8, 25].
bilaminar zone and the anterior attachments are significantly more elastin-dense than the disc [23, 26].

Elastin is highly compliant and extensible across a broad elastic region. When considering the dense network of relatively stiff collagen bundles, the sparse network of extensible elastic fibers certainly does not contribute much to the mechanical stiffness or strength of the disc [17]. More likely, elastin aids in restoring the original form after removal of loads [6, 21, 22, 25].

Proteoglycans and Glycosaminoglycans

Proteoglycans are ECM molecules that consist of a core protein and at least one branched glycosaminoglycan (GAG) assembly. GAGs are highly negatively charged molecules that, through attractive interactions with water, resist fluid flow, thereby increasing a tissue’s compressive integrity. The large proteoglycans bind hundreds of GAG chains and form aggregates by attaching to hyaluronan via link protein (Fig. 1.6). These assemblies interweave with the collagen network, becoming effectively immobilized in the ECM due to their size and highly branched structure. Smaller proteoglycans (Fig. 1.7), which express collagen binding domains, are believed to regulate fibrillar aggregation, packing density of fibrils [27], and regulate growth factor bioactivity [28].

Much variation exists in the literature for GAG concentration in the disc. Reports have suggested concentrations as high as 10% [29] and as low as 0.5% [4] of total dry weight. Taking into account the whole body of literature, a reasonable approximation would be around 5% of the dry weight [3, 4, 8, 30]. This value represents roughly 10-20% of the GAG concentration typically found in articular cartilage, though it is close to
that of the knee meniscus [31]. As with GAG concentration, there is little agreement in the literature regarding the regional distribution of GAG in the disc due to varying test methods and different animal models. Almarza et al. [4] found higher GAG content in the medial regions of porcine discs relative to the lateral and central portions, with the posterior band yielding fewer GAGs than central and anterior portions. Detamore et al. [8] had similar findings using porcine discs regarding the distribution of GAGs, though the total content varied between these two studies. Kopp [32] and Nakano and Scott [33] recorded higher GAG content in the central region relative to the periphery in human and bovine discs, respectively. However, Mills et al. [6] found higher concentration of GAGs in the anterior and posterior bands relative to the intermediate zone in the primate TMJ disc. It is possible that the GAG distribution in the disc is heterogeneous and highly variable across these animal models, though a larger study examining an array of animal models using a standardized testing protocol is needed to decisively characterize the distribution of GAGs and proteoglycans in the disc.

The most abundant GAG found in the disc is chondroitin sulfate [3, 8, 29, 30]. The combined total of chondroitin-4 and chondroitin-6-sulfate represents approximately 74-79% of the GAG content in the disc [3, 8, 30]. The majority of these chains are attached to aggrecan [3], the large aggregating proteoglycan found in abundance in hyaline articular cartilage. Keratan sulfate chains, which associate with aggrecan in smaller numbers, constitute approximately 2-9% of the GAG content in the disc [8, 30]. The non-sulfated GAG hyaluronan, which forms the backbone of the aggrecan molecule, is also present in low concentration in the disc constituting between 0.05-10% of the total GAG content [8, 30, 34]. Detamore et al. [8] found a higher concentration of chondroitin-
6-sulfate in the intermediate and anterior bands than in the posterior band, and found more chondroitin-4-sulfate in the intermediate zone than in the anterior and posterior bands of porcine discs. These results were consistent with an earlier report by Nakano and Scott [33], which showed the concentration of chondroitin sulfate to be 10 times higher in the center relative to the periphery of the bovine disc. Detamore et al. [8] also examined mediolateral variation of chondroitin sulfate concentration, reporting higher values in the medial regions relative to the lateral regions.

In contrast to the chondroitin sulfate proteoglycans (aggrecan, versican), the dermatan sulfate proteoglycans decorin and biglycan are small (~100 kDa) and only bind one or two GAG chains [35]. The biological roles of these proteoglycans remain ill-defined, though regulation of collagen fibrillogenesis is likely a key function. Indeed, the absence of these molecules in knockout mice results in increased variability in collagen fibril size, shape, and aggregation in skin, tendon, and bone, ultimately leading to more brittle tissues [36]. Other purported roles include growth factor modulation and sequestration, and regulation of cell growth [37]. Detamore et al. [8] reported 14.4 and 2.5 times higher dermatan sulfate concentrations in the intermediate zone of porcine discs relative to the posterior and anterior bands, respectively, although no mediolateral differences were found. Scott et al. [38] examined decorin and biglycan individually using gel-electrophoresis and reported roughly equivalent concentrations of biglycan in the central and peripheral regions of bovine discs, but found a higher concentration of decorin in the peripheral tissue relative to the central region. They also noted longer dermatan sulfate chains in the central tissue relative to the outer tissue. Both of these
studies reported that dermatan sulfate constitutes about 15% of the total GAG content in the disc [8, 30].

Biomechanical Properties

As discussed in Section 1.6, the TMJ disc experiences tensile, compressive, and shear forces \textit{in situ}. The number of studies devoted to mechanical characterization of the native disc has increased dramatically over the last two decades. This section provides a broad overview of these studies, with particular emphasis on relating biomechanical properties and behavior to the underlying disc structure.

\textit{Tensile Properties}

Upon initial loading, from 0% to \(\sim 5\%\) strain, the disc appears to be more compliant than at higher strains. This initial behavior, known as the toe region, has been attributed to the straightening of crimped collagen bundles, and may be correlated with the normal physiological range of motion [19, 20]. After this initial toe region, the disc deforms linearly up to the yield point. Tensile properties are higher when tested parallel to the primary direction of collagen orientation since collagen fibers only resist deformation along their long axis. This is reflected in a number of studies that tested the intermediate zone in the mediolateral direction and found lower stiffness and strength relative to other regions [39-42]. Teng \textit{et al.} [42] tested three regions in the canine TMJ disc in the mediolateral direction. The anterior and posterior bands exhibited higher ultimate tensile strength (47 ± 17 and 70 ± 31 MPa) than the intermediate zone (14.7 ± 5.9 MPa), and also yielded a higher elastic modulus (both \(\sim 30\) MPa) than the intermediate zone (18.4
MPa). Beatty et al. [40] tested samples from the central region of the porcine disc in the mediolateral and anteroposterior directions and similarly found the disc to be much softer mediolaterally (7.35 ± 0.23 MPa) than anteroposteriorly (33.8 ± 1.3 MPa).

Detamore et al. [39] tested porcine discs in three directions in both mediolateral and anteroposterior directions under incremental stress relaxation and obtained data for strength, failure energy, and peak and relaxed moduli. In the mediolateral direction, the intermediate zone had the lowest relaxed modulus (0.58 ± 0.39 MPa), followed by the anterior (9.5 ± 3.3 MPa) and posterior (23.4 ± 6.5 MPa) bands. In the anteroposterior direction, the medial and central regions were stiffer than the lateral region, with elastic moduli of 14.3 ± 3.7, 18.5 ± 4.9, and 10.6 ± 3.0 MPa, respectively. The regional peak strengths of the disc ranged from 0.58 MPa to 7.38 MPa, with the weakest being the intermediate zone tested mediolaterally.

Tanaka et al. [43-45] have performed multiple studies of the TMJ disc in the anteroposterior direction. In one study [43], bovine discs from three age ranges (young adult, adult, mature adult) were divided into three regions (medial, central, lateral) and loaded at either 1.0 MPa or 1.5 MPa for 20 minutes. Values for the instantaneous modulus were highest in the medial region and lowest in the central region for all three age groups, though the range of values across all groups was relatively small (20.2 - 25.9 MPa). In another study [44], the central and medial regions of human discs from donors ranging from 22-67 years were tested, with values reported for instantaneous modulus varying from 27.1 - 65.2 MPa. Lastly, Tanaka et al. [45] tested healthy human TMJ discs and compared them to those from patients suffering from internal derangement under tensile stress relaxation. They reported a relaxed elastic modulus of 59.2 ± 4.2 MPa for
deranged discs compared to $60.5 \pm 9.3$ MPa for healthy tissue, and instantaneous moduli of $96 \pm 17$ MPa for deranged and $96 \pm 19$ MPa for normal discs.

**Compressive Properties**

Reported values for the compressive modulus of the disc are highly variable, ranging from 16 kPa to 60 MPa [46, 47]. This wide variation can be attributed to interspecies differences, testing method, material model, preconditioning, sample preparation, and environmental conditions. Therefore, the values obtained from these studies should be taken with caution, and more credence should be handed to the regional variation described within an individual work. As a corollary, future characterizations should be performed using uniform testing criteria similar to those set forth by the ASTM for materials testing, or by adapting techniques used for testing other musculoskeletal soft tissues such as tendons or ligaments.

Despite inconsistencies in the literature regarding its compressive properties, it is generally accepted that the disc is likely 10-1000 times softer under compression than tension, with a compressive elastic modulus between 100 kPa and 10 MPa. This difference is stark when comparing to articular cartilage, which displays less of a disparity between moduli under tension and compression [31]. Proteoglycans (aggrecan) are known to increase compressive stiffness by increasing local hydrostatic pressure and impeding fluid flow through the matrix. GAGs play a large role in hyaline articular cartilage mechanics as they compose 15-25% of the tissue’s dry weight [48]. As discussed previously, GAGs are relatively sparse in the disc, suggesting its compressive stiffness is dependent on some other structural characteristic. Some important
observations were made by Allen et al. [49] after examining the disc’s compressive behavior during step-wise stress relaxation tests. First, the relaxation time constants obtained for the disc are substantially shorter than those observed for other cartilaginous tissues (a trait shared by mandibular condylar cartilage as well [50], as noted in Chapter 3). Second, the modulus increases steadily as a function of increasing strain (also observed with mandibular condylar cartilage [50]). The authors attributed short relaxation times to the lack of fluid impedance at low strain levels due to the relative scarcity of GAGs in the tissue. Strain dependence suggests that as the tissue is compressed, collagen fibers become more closely packed together, reducing the effective void space and increasing resistance to fluid flow. Beek et al. [47] hypothesized another mechanism by which collagen might mediate compressive behavior. In their study, whole human discs were dynamically compressed in three regions in the anteroposterior direction, yielding 2 - 3 times higher compressive moduli in the intermediate zone relative to the anterior and posterior bands. To explain these results, the authors proposed a mechanism in which collagen directs fluid flow anteroposteriorly in the intermediate zone during compression up to the bands, where the fluid is obstructed by the mediolaterally oriented collagen fibers. If indeed the compressive properties of the disc are primarily mediated by the underlying collagen structure, then tensile and compressive behaviors are inextricably linked. It is important that future characterizations analyze the interplay of compressive and tensile forces to test these hypotheses and provide a more accurate description of disc behavior in situ.

Three studies by Athanasiou’s group noted significant compliance in the lateral side of the intermediate zone relative to other regions [46, 49, 51]. First, Kim et al. [46]
performed creep indentation tests on the superior surface of the disc and modeled the data using linear biphasic theory. The lateral side of the intermediate zone yielded a significantly lower aggregate modulus (16.3 ± 2.1 kPa) than the medial side (29 ± 12 kPa). Allen and Athanasiou [49, 51] published two studies that tested the surface-regional compressive properties of the disc under step-wise stress relaxation and modeled the data using viscoelastic theory. In both studies, samples from the lateral region demonstrated less mechanical integrity relative to other regions. Along with finite element analyses, which have identified the presence of large forces in the lateral aspects of the disc, these studies provide additional rationalization as to why perforations are preferentially located on the lateral side of the disc [52].

Shear and Frictional Properties

The disc is subjected to shearing and frictional forces as a result of translatory and rotational movements during disc loading. The degree of shearing and abrasion in the disc is dependent on multiple factors, including surface roughness and joint lubrication. Lubricating characteristics in the joint are primarily attributed to the presence of synovial fluid, which derives its rheological properties from the unsulfated GAG hyaluronan [53]. In patients suffering from osteoarthrosis, hyaluronan concentration and molecular weight is seen to decrease. Surface roughness can be measured directly using optical profilometry or atomic force microscopy, though no such tests have been performed on the TMJ disc to date. However, several studies have examined the frictional properties of the TMJ disc [54-56]. In tests on porcine explants, the frictional coefficient of the disc was determined to be approximately 0.015 - 0.025, increasing with magnitude and time
of loading [55]. Another study of porcine discs demonstrated that tractional forces increase with increasing strain and velocity [56].

Large shearing forces are believed to cause deformation and damage in cartilage and fibrocartilage [57-59], and the TMJ disc is believed to experience some degree of shear loading based on finite element analysis [60, 61]. Nevertheless, only a handful of studies have been published that examined the shear properties of the disc [62-64]. First, Lai et al. [62] examined regional variation in shear properties of the human TMJ disc using an axiotorsional device. Cylinders were tested from three regions along the mediolateral axis, yielding a shear modulus of around 1.0 MPa for the central region and 1.75 MPa for the medial and lateral regions. Tanaka et al. [64] exposed porcine discs to 0.5% shear strain across a range of loading frequencies (0.1 - 100 Hz) in the mediolateral and anteroposterior directions. The dynamic shear moduli increased non-linearly with increasing frequency. Storage and loss moduli were about 1.5 times greater in the anteroposterior direction relative to the mediolateral direction across the range of frequencies. Tanaka et al. [63] repeated the same procedure in another study but this time varied the compressive strain (5% - 15%) and shear strain (0.5% - 1.5%). Dynamic shear moduli increased with increasing compressive strain, which the authors attributed to a decrease in porosity with increased loading. This result would seem to corroborate the strain dependence of compressive properties [49, 51]. Interestingly, shear moduli decreased with increasing shear strain. The explanation for this behavior is less clear. The authors speculated that water and proteoglycans in the disc may display non-Newtonian shear-softening behavior similar to synovial fluid.
Overall, the frictional and shear properties of the disc remain relatively unknown. It is reasonable to assume that unphysiological shear loading may have a degradative effect, similar to what is seen in other cartilages [58, 59, 65], necessitating the execution of more characterization work. It is likely that disc traction forces increase with the onset of osteoarthrosis as a result of synovial fluid degradation and increased surface roughness. These increased forces could then cause greater shearing in the disc, possibly leading to derangement and disease [66]. An understanding of shear and frictional forces in joint disease processes will no doubt be necessary to avoid premature deterioration of a tissue engineered TMJ disc.

**Cell Types**

The TMJ disc contains a heterogeneous collection of morphologically variable cells [67]. Some cells are flattened and spindle-shaped much like the tenocytes found in tendons, while others appear rounded, surrounded by a distinct pericellular matrix similar to what is seen in hyaline articular cartilage (Fig. 1.8). When taken as a whole population, the TMJ disc cells may be appropriately referred to as *fibrochondrocytes*.

Detamore *et al.* [68] studied the regional distribution of cells in the porcine disc using histology and transmission electron microscopy, yielding an overall density of $681 \pm 197$ cells/mm$^2$, 70% ± 11% of which appeared fibroblast-like based on morphology. The intermediate and posterior bands were significantly more cellularized than the anterior band, and the central region of the intermediate zone had approximately 10% fewer cells than the lateral and medial regions. The anterior and posterior bands contained a higher percentage of fibroblasts than the intermediate zone, and the superior
and middle layers had higher levels of fibroblast-like cells than the inferior surface. In contrast to these findings, Milam et al. [69] found mostly rounded, chondrocyte-like cells surrounded by lacunae in primate TMJ discs. Mills et al. [6] also reported the presence of rounded, chondrocyte-like cells in the interstices between collagen bundles in primate TMJ discs. Cells were typically found in groups of three to six cells, and were generally smaller and less rounded at the surfaces and close to the peripheral attachments.

Berkovitz and Pacy [70, 71] examined TMJ disc cell anatomy in two studies. The first study [70] explored age-related differences in rats and marmosets, and reported the presence of a microfilamentous pericellular matrix surrounding the cells. This matrix was structurally different from the pericellular matrix of hyaline articular chondrocytes, as it was lacking a pericellular capsule separating the cells from the ECM. Perhaps the most interesting finding was a transition in cell morphology from a fibrous cell to a more chondrocyte-like morphology with advancing age. In the second study [71], the authors examined mature adult human discs removed due to TMJ dysfunction, though the investigation only focused on areas of tissue that appeared to be the least affected by disease processes. The cells in this study were oval-shaped, contained only moderate amounts of intracellular organelles, and were characterized by a large volume of filamentous material that filled the cytoplasm. Cells lacked a distinct pericellular matrix similar to those seen in the previous study [70], and most cells were found closely associated with the collagen fiber network. Cell appearance in this study would suggest the human disc at advanced age is more fibrous than fibrocartilaginous. However, the authors correctly note that it would be inappropriate to assume equivalence between apparently non-affected regions of a diseased disc and a wholly undamaged tissue.
In vitro examinations of the metabolic processes of isolated TMJ disc cells give clues to their phenotype and in situ functional roles [6, 7, 72, 73]. Mills et al. [72] cultured rabbit cells from both the disc and its fibrous attachments. Cells isolated from the disc maintained a polygonal morphology and synthesized cartilage-like proteoglycans, while cells derived from the attachments were spindle shaped and produced fibroblast-like proteoglycans. A later study by Mills et al. [6] confirmed these reports, as cells from primate discs maintained a polygonal shape during in vitro culture. Landesberg et al. [7] analyzed cell proliferation, collagen synthesis, proteoglycan synthesis, and RNA in cultured bovine disc cells. Disc cells proliferated very rapidly in culture, much faster than chondrocytes. Cells synthesized collagen as 12% of total protein produced, similar to what is seen for cultured articular chondrocytes. Collagen type II RNA was found, though at low levels relative to control mandibular condyle cells. Kapila et al. [73] cultured leporine disc cells and assessed the conditioned media for proteinases using polyacrylamide gels followed by western blots. They noted the presence of several matrix metalloproteinases (MMPs), including gelatinase, procollagenase and prostromelysin. They also isolated two proteinases inhibitors, most likely TIMP and TIMP-2, using reverse zymography. These findings suggest disc cells contribute to ECM remodeling though secretion of MMPs, and additionally, they express an MMP profile more reflective of a synovial fibroblast than a chondrocyte.

In summary, there is no single description of the phenotype of a TMJ disc cell. Instead, the cells should be viewed as a heterogeneous distribution expressing characteristics that fit somewhere along the phenotypic spectrum between a fibroblast
and a chondrocyte. Therefore, the term fibrochondrocyte most accurately describes the heterogeneous population of TMJ disc cells [7].

Age-Associated Changes in the Disc

The mechanical strength and stiffness of many connective tissues, such as articular cartilage, skin, and bone, increase into adulthood, then gradually decrease with advancing age [74-76]. However, several studies indicate that the mechanical integrity of the TMJ disc is sustained or continues to increase past the point of skeletal maturation [43, 44, 62, 77, 78]. Tanaka et al. [43, 77, 78] examined the tensile, compressive, and dynamic viscoelastic properties of discs from young-adult (3 year-old), adult (7 year-old), and mature-adult (10 year-old) cattle. Under creep tension [77], mature-adult discs were around 10% stiffer than adult discs and also maintained the least residual strain after unloading. For compressive stress relaxation [78], the instantaneous (17 MPa) and relaxed moduli (4 MPa) were similar between adult and mature-adult groups. Under dynamic (cyclical) compressive loads applied at 1 Hz [43], the storage modulus of young-adult discs (0.69 MPa) was significantly smaller than that of adult (1.21 MPa) and mature-adult (1.44 MPa) discs. In addition, the loss modulus for the mature-adult group (0.23 MPa) was significantly larger than the loss moduli of the younger groups. In a study of discs from human donors, Tanaka et al. [44] reported a significant correlation ($p < 0.01$) between aging (range 22 - 67) and tensile modulus (27.1 - 65.2 MPa). Finally, Lai et al. [62] found the shear modulus of human discs increased significantly ($p < 0.01$) with increasing age (range 36 - 76).
Age-related changes in mechanical properties are accompanied by, and can be largely attributed to, changes in ECM composition and organization. Nakano and Scott [33] quantified the biochemical composition of bovine discs, separated into inner and peripheral regions, from prenatal development through maturation. In both regions, collagen content increased rapidly during prenatal development, then plateaued thereafter, while water concentration decreased steadily, though not significantly, from fetus to adult. Most notably, there was a dramatic increase in chondroitin sulfate (13-fold) and keratan sulfate (1600-fold) concentration from immature to mature adult in the inner tissue, though the outer tissue remained relatively constant in this regard. The authors hypothesized that increased GAG concentration was an adaptive response to cyclic compressive loading, which occurs predominantly in the central region of this disc. Indeed, it has been shown that direct compression of disc explants leads to increased production of chondroitin sulfate in vitro [79], that cyclic compression increases production of GAGs in articular cartilage in vitro [80, 81], and that moderate exercise stimulates GAG production in vivo [82]. A higher concentration of GAGs would reduce the mechanical load-carriage requirement of the solid matrix by increasing interstitial fluid pressure, thus allowing more energy dissipation through viscous effects.

Collagen microarchitecture also undergoes significant changes during maturation and aging. Ahn et al. [83] examined collagen fibril size and arrangement in the intermediate zone of rat discs aged 1 day to 1 year using quantitative analysis of scanning electron micrographs. From birth up to 2 weeks, individual fibrils were 45 ± 3 nm in diameter. At 4 weeks, smaller and larger fibrils had deposited, which doubled the standard deviation, though the average size remained the same. By 8 weeks, the average
fibril diameter increased to 58 ± 19 nm, with a similar value shown for the 1 year samples. A broad distribution of sizes could clearly be seen in the electron micrographs at 1 year, and the packing density was much higher than in younger tissue, where uniformly-sized fibrils were separated by regular void spaces. A broadening distribution of fibril diameters in collagen bundles during development is typical of all collagenous tissues, and tensile load-bearing tissues such as tendon also show an increasing fibril diameter during postnatal development [84, 85]. Larger fibril diameter is correlated with increased tissue stiffness and a reduction in extensibility, as evidenced by a shortened toe region [85]. In the same way that increased deposition of GAGs is a response to cyclic compressive loading, an increase in disc fibril diameter is likely a response to cyclic tensile stresses during mastication.

Greater levels of calcium in the disc are correlated with advancing age [74]. Jibiki et al. [86] showed calcifications in 37% of discs obtained from cadaveric donors of ages 47 to 107 using x-ray radiography and electron microscopy. In this study, calcium deposits were associated with lesions predominantly located in the posterior band. The majority of these deposits had structures characteristic of an endochondral ossification process, including lamellae, thick collagen fibers, Haversian canals, and lacunae. Previous studies found similar features in discs from patients with TMD [87, 88]. Though calcifications were related to arthropathy or mechanical injury in this study, it is possible that calcification is inherent to the aging process.

In summary, the composition and structure of disc ECM changes in response to mechanical stresses during the aging process, and these changes are manifested in increased mechanical stiffness and strength. Collagen concentration increases and fibrils
widen and become more densely packed, leading to increased tensile modulus and strength. Increased calcification may also contribute to increased mechanical properties. GAG concentration in the compressively loaded intermediate zone rises with age, leading to higher compressive and viscous properties. Considerable evidence suggests this adaptation process continues past the point of skeletal maturity.
Figure 1.1: Schematic representation of the regions of the human disc
The disc is shown in superior (top) and sagittal (bottom) views. The disc has a biconcave shape, characterized by thicker band regions on the posterior and anterior ends separated by a thinner intermediate zone. The approximate dimensions of an adult TMJ disc are given.
Figure 1.2: Location of the disc relative to the joint surfaces
The disc is positioned between the temporal bone and the mandibular condyle. The illustrations show the location of the disc a) with jaw occlusion and b) with translation and forward rotation of the mandible.
Figure 1.3: Superior view of the disc showing peripheral attachments
The disc is attached to the mandible and the temporal bone via fibrous attachments along its periphery.
Figure 1.4: The disc in situ with attachments
These illustrations show sagittal views of the disc a) without the joint capsule, revealing the bilaminar zone and anterior attachments, and b) with the joint capsule and the lateral ligament shown.
Figure 1.5: The disc with arrows representing the predominant collagen orientation
Though collagen fiber orientation is heterogeneous throughout the disc, the predominant orientation is directed anteroposteriorly in the intermediate zone and mediolaterally in the band regions.
Figure 1.6: Aggrecan, a large aggregating proteoglycan

(a) Aggrecan monomer showing two chondroitin sulfate-rich domains (CS1, CS2), a keratin sulfate rich region (KS), three globular protein domains (G1, G2, G3), and link protein (LP).

(b) Several aggrecan monomers binding to hyaluronan to create a large, immobile structure.
Figure 1.7: Decorin, a small leucine-rich proteoglycan
a) Decorin molecule showing its leucine-rich domain and a single attached dermatan sulfate molecule. b) Decorin associating with a collagen bundle.
Figure 1.8: Transmission electron micrographs of TMJ disc cells
The disc contains a heterogeneous cell population of a) spindle-shaped cells and b) rounded cells. From Detamore et al. [68]
CHAPTER 2: An Interspecies Comparison of the Temporomandibular Joint Disc *

Abstract

The temporomandibular joint (TMJ) disc plays a critical role in normal function of the joint, and many disorders of the TMJ are a result of disc dysfunction. Previous quantitative TMJ characterization studies examined either the human or a specific animal model, but no single study has compared different species in the belief that differences in joint morphology, function, and diet of the subject would be reflected in the composition and mechanical behavior of the disc. In this study, we examined topographical biochemical (collagen, glycosaminoglycan and DNA content) and biomechanical (tensile and compressive) properties of the human TMJ disc, and also discs from the cow, goat, pig, and rabbit. Regional and interspecies variations were identified in all parameters measured, and certain disc characteristics were observed across all species, such as a weak intermediate zone under mediolateral tension. While human discs possessed properties distinct from the other species, pig discs were the most similar to the human, suggesting that the pig may be a suitable animal model for foundational studies in TMJ bioengineering efforts.

Introduction

The temporomandibular joint (TMJ) disc has several important physiological functions, most notably the dissipation and distribution of masticatory loads [89]. Disorders of the TMJ are widespread, likely afflicting between 5% and 15% of the adult population [90], and approximately 70% of these are associated with displacement of the disc [91]. In its normal relationship, the disc is positioned between the mandibular condyle and glenoid fossa of the temporal bone, separating the joint into superior and inferior spaces. However, displacement leading to internal derangement can occur, resulting in uncoordinated movements of the disc relative to the joint surfaces [92]. Previous attempts to replace the disc with alloplastic devices have failed, resulting in further joint degradation [93, 94]. Therefore, studies aimed at tissue engineering the TMJ, and especially the disc, are warranted [95, 96].

To establish design criteria for tissue engineering efforts, thorough characterization of native TMJ disc tissue is necessary. A general characterization of the biochemical makeup of the disc can be elucidated from previous examinations. Collagen constitutes approximately 30% of the wet weight [2], of which the majority is collagen type I. Total collagen concentration of the porcine disc is highest in the center relative to the lateral region [4]. The total concentration of glycosaminoglycans (GAGs) in the TMJ disc is between 0.6 to 10% of the dry weight [97]. Studies of pig and cow discs indicate that the greatest GAG content is located in the center relative to the periphery [4, 33], but the opposite trend has been seen in primate discs [6]. The cells of the TMJ disc reflect a heterogeneous fibrochondrocyte cell population consisting of both fibroblast and chondrocyte-like cells. Regionally, the greatest DNA concentration and cell number has
been seen in the medial portion of the pig disc [4], and the anterior and posterior bands of the primate disc [6].

Previous biomechanical examinations have highlighted distinct anisotropic and heterogeneous properties. Tensile strength and stiffness correlate with local collagen orientation, with greater values present in the central region when tested in the anteroposterior direction relative to the mediolateral direction [39, 40, 98]. Compressive properties vary topographically; the relaxation modulus of the medial region is highest while the posterior and anterior bands appear to support the highest instantaneous loads [46, 49, 51, 99]. Overall, the disc is 10-1000 times softer under compression than it is under tension [89].

While these studies have vastly increased the collective knowledge of TMJ disc physiology, no single study has compared biomechanical and biochemical characteristics of the human disc to an animal model. Furthermore, there are no comprehensive studies examining topographical variations or orientation-dependent characteristics. The porcine disc has been identified as the model most appropriate for comparison to the human based on morphological similarities such as disc size and shape, anatomical similarities of the respective joints, similar masticatory patterns, and the fact that pigs are omnivorous [100-103]. Other species examined include cows [78, 104], dogs [98, 105], goats [106], rabbits [17, 107], rats [108], and sheep [109]. In an effort to better understand the similarities and differences between some of these models, and to gain a quantitative evaluation of their properties, this study compares the regional biochemical and biomechanical properties of the human, cow, goat, pig and rabbit discs.
Materials and Methods

Specimen Procurement

Tissue specimens were procured from skeletally mature sources over a period of several months. Goat, pig, rabbit, and cow heads with intact joint capsules were obtained from local abattoirs within hours of slaughter. Human TMJ discs were dissected from seven female cadaver donors of age 63-84 years, mean age 73. TMJ discs were carefully dissected from their attachments and verified to be grossly normal. Discs were washed in 0.01 M phosphate-buffered saline (PBS), then wrapped in PBS soaked gauze and frozen at -20°C until testing.

Biochemical Analysis

For quantitative biochemistry six (rabbit, goat, pig) or four (human and cow) left discs from each species were thawed in PBS at room temperature for 4h, and then sectioned into five pieces as depicted in Fig. 2.1a. All specimens were blotted to remove excess moisture, weighed to obtain a wet weight, then lyophilized for 48 h. Digestion of the samples was completed in 1.5 mL of 125 mg/mL papain (Sigma, St. Louis, MO) solution overnight at 60°C. The DNA content of the samples was measured by reaction of DNA with Picrogreen reagent (Invitrogen, Carlsbad, CA). The total amount of sulfated GAG was measured using a dimethylmethylene blue colorimetric assay kit (Biocolor, Newtownabbey, UK). The total collagen content was determined using a hydroxyproline assay, as described previously [4].

Histology

For topographical histology, a right disc from each species was divided into five regions as shown in Fig. 2.1a. Disc samples were snap frozen in tissue freezing medium (Triangle
Biomedical, Durham, NC) and cryo-sectioned at 12 \textmu m in the anterior-posterior direction. Qualitative analysis of sulfated GAG was conducted using safranin-O/fast green staining.

**Tensile Sample Preparation and Testing Procedure**

Discs from each species were thawed and cut into three regions in either an anteroposterior or mediolateral direction as described in Fig. 2.1b. Regions were sectioned in a cryotome to a uniform thickness between 300 and 600 \textmu m. These sections were taken from the middle zone after removing the superior surface. Rectangular specimens, one mm wide, were then removed. Tests were conducted on a materials testing machine (Instron 5565, Canton, MA, USA). A 0.02 N tare load was applied to the samples followed by preconditioning with 15 cycles of 5% strain at a rate of 10 mm/min. After preconditioning, step strains were applied at 5% increments beginning with 10% and up to 40%, with 10 min between steps for stress relaxation. If the sample did not fail during application of these strains, the sample was pulled at a constant rate of 0.1 mm/s until failure. Data were retained only from samples that failed away from the grips. Peak and relaxed moduli were obtained by constructing stress vs. strain plots through points of peak and relaxed stresses at each step strain [39].

**Compression Sample Preparation and Testing Procedure**

Cylindrical tissue plugs were taken from five regions of each disc as shown in Fig. 2.1a, and sectioned such that the superior and inferior surfaces were parallel using a cryotome. The final sample thicknesses ranged from 0.8-4 mm. Unconfined compression testing was performed on the Instron. A 0.02 N tare load was applied to the sample, followed by preconditioning with 5% strain for 15 cycles. During the test, 10% step strains were
applied from 10% up to 30% strain, with 10 min intervals between steps for stress relaxation. Values for instantaneous modulus, relaxation modulus, and coefficient of viscosity were obtained by fitting data to a viscoelastic solution for a Kelvin solid developed previously [51].

Results

Gross Morphology

Gross morphology and measured dimensions of discs from all five species are presented in Figs. 1c-d. The pig was the only species that was not significantly different from the human in both mediolateral and anteroposterior dimensions.

Biochemical Analysis

Quantitative biochemical results and two-factor ANOVA analysis is shown in Fig. 2.2. The mean collagen content normalized to wet weight (ww) was found to vary between 16.5% and 30.1% for all species and regions tested. TMJ discs from the cow showed significantly greater collagen per wet weight than all other species except the pig (Fig. 2.2a). Human discs were not statistically different in collagen concentration from either the pig or rabbit discs. The posterior band displayed significantly greater collagen concentration than the lateral region of the intermediate zone, but there were no other statistical differences among the disc regions.

The mean DNA/ww varied between 0.0055% and 0.0358%, showing stark differences in both region and species. Human discs contained significantly less DNA/ww than all of the other species, but there were no other interspecies variations (Fig. 2.2b). Regionally, the anterior and posterior bands contained significantly more
DNA than all three regions of the intermediate zone. DNA content is proportional to cell number, so these variations can also be described in terms of differences in cellular density.

The mean sulfated GAG/ww content was found to vary between 0.273% and 0.936% for all samples tested. Cow discs contained significantly more GAG/ww than all species except the goat (Fig. 2.2c). The GAG concentration of the human TMJ discs was significantly different from all other species, with its value in-between the rabbit and pig discs. While all three regions of the intermediate zone possessed significantly more GAG than the anterior band, only the medial portion of the intermediate zone contained more GAG than the posterior band.

**Histology**

Histological staining supports the quantitative biochemical results. Positive safranin-O staining for sulfated GAGs is clearly visible in all samples except the anterior band of the human disc and all regions of the pig disc (Fig. 2.3).

**Tension**

Values for tensile properties are shown in Table 1A and results of the two-way ANOVA are presented in Table 2.1b. Analysis of intraspecies topographical variation demonstrated significance for all parameters. Topographically, the ML C samples exhibited weaker and softer behavior than all other groups, though no other regional variation was found. With regard to interspecies variation, human tissue was significantly stiffer and stronger than all other species, while rabbit tissue was softer than all other groups. Strength values ranged from 0.2 MPa for bovine ML C to 13.8 MPa for the human AP C tissue. The human AP C group also displayed the highest peak and relaxed
moduli of 51.7 MPa and 34.4 MPa, compared with the bovine ML C values of 0.2 MPa and 0.1 MPa, respectively.

Compression

Values for compressive assessments are shown in Table 2.2a and results of the two-way ANOVA are shown in Table 2.2b. All parameters increased with increasing strain. The interspecies analysis showed that the bovine, leporine, and caprine tissue had the highest relaxation and instantaneous moduli, with the exception of the goat relaxed modulus at 30% strain. Porcine tissue had the lowest moduli overall, and was similar to human tissue at 20% and 30% strain for both moduli. Topographically, the bands yielded higher instantaneous and relaxed moduli relative to the intermediate zone samples, with the exception of the relaxation modulus of the IZC region, which was similar to the ABC group. The highest relaxed modulus of 199 kPa was obtained from the bovine PBC samples, while the highest instantaneous modulus of 6.55 MPa was noted from the goat ABC group. The softest tissue was from the lateral region of the pig, with relaxation and instantaneous moduli under 10% strain of 12.4 kPa and 16.0 kPa respectively. The band regions of the goat and cow displayed the highest viscosity coefficients, with values between 35 and 40 MPa \cdot s at 30% strain.

Discussion

While previous studies of the TMJ disc investigated regional variation in biochemical and biomechanical properties, this study is the first to examine these properties across several species. Furthermore, this is the first study to quantify both biochemical and biomechanical properties concurrently. The advantage of this study’s approach is that
animal models could be compared to the human disc using the same testing methods in a consistent environment, mitigating variability associated with comparisons made across different studies. Due to the contradictory nature of prior studies, the results of this investigation do not agree with all prior work, but they are consistent with previous studies performed in our group using the pig model [4, 8, 39, 49, 51]. The interspecies characterization data collected here will provide valuable design parameters for tissue engineers seeking to recapitulate the intrinsic properties of the disc in vitro, and for those looking to study functional replacements in vivo.

Prior studies have indicated that structure function relationships exist within the porcine TMJ disc [97], but now a comprehensive comparison can be made, not only within a single species, but also across species. Sulfated GAG content is frequently related to compressive stiffness, and indeed in this study, the species with the greatest GAG content (cow) also had the highest compressive moduli. In contrast, regional variations in GAG content showed no relationship with compressive properties. Instead, the region with the highest total collagen content (PBC) also possessed the highest compressive properties, as seen previously for the pig disc [49]. Although collagen is generally thought to mediate tensile properties, no correlations with total collagen content were observed in this study. As seen previously, tensile properties of the disc depend more on the orientation of collagen rather than total content [39]. These data emphasize that future tissue engineering studies will have to account not only for biochemical content, but also organization to produce heterogeneous mechanical properties similar to native tissue.
Collecting a significant amount of interspecies data within a single study allows correlation of disc properties to the greater functional requirements of each animal. It is apparent that some properties of the disc vary with the specific anatomy or diet of each species. For example, the herbivores (cow, goat, and rabbit), whose joint motion is primarily translatory [110], had greater GAG content and compressive properties across all disc regions compared to the omnivores (human and pig), whose motion is both rotatory and translatory. On the other hand, some regional variations were constant across species, indicating that certain portions of the disc may have similar functional requirements within all species tested. For example, the intermediate zone under mediolateral tension was always weaker than other regions and the DNA content of the intermediate zone was consistently lower than the bands. While it is clear that the disc from each species is unique, it does appear that some properties of the disc transcend species.

A major goal of this study was to quantify the differences and similarities among animal models, and specifically, how these relate to their appropriateness as analogs of the human disc. Comparisons to the human disc provide valuable information though the advanced age of available specimens may result in stiffer tissues [78] with lower cellularity [111]. Taking into account all of the parameters tested in this study, it can be argued that the pig disc is more similar to the human than the other species since the pig had the fewest differences overall (dimensions, collagen and GAG content, compressive properties). Therefore, the results of this study point to the pig as the most appropriate animal model and support prior anatomical studies.
Table 2.1: Tensile values and assessment of statistical variation

A) Values for peak modulus, relaxed modulus, and strength under unilateral tension accompanied by analysis of intraspecies topographic variation. Data are represented as mean ± S.D. Statistically significant intraspecies topographic variation, as determined using a one-way ANOVA and Tukey’s HSD post-hoc test with α = 0.05, is represented by superscript letters where applicable. B) Interspecies and topographic variation of tensile properties analyzed using a two-way ANOVA and Tukey’s HSD post-hoc test with α = 0.05. Values not connected by same letter are significantly different. Between four and six samples were examined for each topographical location in each species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sample</th>
<th>Strength (MPa)</th>
<th>Peak modulus (MPa)</th>
<th>Relaxed modulus (MPa)</th>
</tr>
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<tbody>
<tr>
<td>Human</td>
<td>AP C</td>
<td>$A^{13.8 \pm 2.8}$</td>
<td>$A^{51.7 \pm 7.7}$</td>
<td>$A^{34.4 \pm 12.2}$</td>
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<tr>
<td></td>
<td>AP M</td>
<td>$AB^{12.8 \pm 3.3}$</td>
<td>$A^{37.2 \pm 4.1}$</td>
<td>$AB^{30.0 \pm 3.8}$</td>
</tr>
<tr>
<td></td>
<td>MLA</td>
<td>$B^{6.7 \pm 4.3}$</td>
<td>$B^{25.5 \pm 13.2}$</td>
<td>$AB^{18.5 \pm 10.0}$</td>
</tr>
<tr>
<td></td>
<td>ML C</td>
<td>$C^{4.2 \pm 1.5}$</td>
<td>$C^{9.5 \pm 1.7}$</td>
<td>$B^{6.1 \pm 1.9}$</td>
</tr>
<tr>
<td></td>
<td>ML P</td>
<td>$BC^{6.7 \pm 3.9}$</td>
<td>$BC^{21.2 \pm 12.1}$</td>
<td>$B^{17.0 \pm 9.6}$</td>
</tr>
<tr>
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<td>$A^{24.0 \pm 6.9}$</td>
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<td>$AB^{11.4 \pm 6.3}$</td>
</tr>
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<tr>
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<td>$A^{28.5 \pm 8.2}$</td>
<td>$A^{19.6 \pm 6.3}$</td>
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<td>$A^{13.0 \pm 4.9}$</td>
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<tr>
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<td>AP C</td>
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<tr>
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<td>AP M</td>
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<td>$A^{16.7 \pm 5.9}$</td>
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<td>$B^{5.0 \pm 2.3}$</td>
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<td>ML P</td>
<td>$AB^{6.7 \pm 3.9}$</td>
<td>$A^{21.2 \pm 12.1}$</td>
<td>$A^{17.0 \pm 9.6}$</td>
</tr>
<tr>
<td>Rabbit</td>
<td>AP L</td>
<td>$AB^{2.9 \pm 1.2}$</td>
<td>$A^{11.5 \pm 6.2}$</td>
<td>$AB^{6.8 \pm 3.8}$</td>
</tr>
<tr>
<td></td>
<td>AP C</td>
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<td>$AB^{7.0 \pm 2.4}$</td>
<td>$AB^{4.5 \pm 1.8}$</td>
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<tr>
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<tr>
<td></td>
<td>MLA</td>
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<td>$AB^{6.6 \pm 1.5}$</td>
<td>$AB^{4.4 \pm 1.0}$</td>
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<td>ML C</td>
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<tr>
<td></td>
<td>ML P</td>
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<td>$AB^{7.3 \pm 3.4}$</td>
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b) Species Strength Peak Relaxed Region Strength Peak Relaxed

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<th>Species</th>
<th>Strength</th>
<th>Peak</th>
<th>Relaxed</th>
<th>Region</th>
<th>Strength</th>
<th>Peak</th>
<th>Relaxed</th>
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<td>A</td>
<td>AP L</td>
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<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Pig</td>
<td>BC</td>
<td>B</td>
<td>B</td>
<td>AP C</td>
<td>A</td>
<td>A</td>
<td>A</td>
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<tr>
<td>Goat</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td>MLA</td>
<td>A</td>
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<td>C</td>
<td>C</td>
<td>C</td>
<td>ML P</td>
<td>A</td>
<td>A</td>
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Table 2.2: Compressive values and assessment of statistical variation

A) Values for relaxation modulus, instantaneous modulus, and coefficient of viscosity for stress relaxation tests under unconfined compression. Data are represented as mean ± S.D. Statistically significant interspecies topographic variation, as determined using a one-way ANOVA and Tukey’s HSD post-hoc test with α = 0.05, is represented by superscript letters where applicable. B) Interspecies and topographic variation of compressive properties analyzed using a two-way ANOVA and Tukey’s HSD post-hoc test with α = 0.05 where applicable. Values not connected by same letter are significantly different. Between four and six samples were tested for each topographical location and species.

### A) Relaxation modulus (kPa)

<table>
<thead>
<tr>
<th>Species</th>
<th>Region</th>
<th>Strain 10%</th>
<th>Strain 20%</th>
<th>Strain 30%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>PBC</td>
<td>60.9 ± 28.1</td>
<td>71.9 ± 44.9</td>
<td>102.3 ± 72.1</td>
</tr>
<tr>
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<td>ABC</td>
<td>31.4 ± 15.8</td>
<td>43.4 ± 23.7</td>
<td>60.7 ± 34.6</td>
</tr>
<tr>
<td></td>
<td>IZM</td>
<td>32.6 ± 19.5</td>
<td>17.8 ± 7.8</td>
<td>27.9 ± 10.9</td>
</tr>
<tr>
<td></td>
<td>IZC</td>
<td>24.3 ± 11.0</td>
<td>34.8 ± 14.4</td>
<td>54.4 ± 17.2</td>
</tr>
<tr>
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<td>IZL</td>
<td>15.8 ± 16.3</td>
<td>19.0 ± 22.2</td>
<td>30.5 ± 36.9</td>
</tr>
<tr>
<td>Pig</td>
<td>PBC</td>
<td>24.3 ± 7.4</td>
<td>570.9 ± 19.1</td>
<td>112.5 ± 31.4</td>
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<td>ABC</td>
<td>17.1 ± 9</td>
<td>30.4 ± 5.8</td>
<td>64.3 ± 14.7</td>
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<tr>
<td></td>
<td>IZM</td>
<td>14.3 ± 5.9</td>
<td>23.2 ± 13.4</td>
<td>42.3 ± 24.3</td>
</tr>
<tr>
<td></td>
<td>IZC</td>
<td>15.0 ± 3.1</td>
<td>26.4 ± 6.4</td>
<td>82.0 ± 16.5</td>
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<tr>
<td></td>
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<td>21.6 ± 10.9</td>
<td>63.8 ± 17.3</td>
</tr>
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<td>91.1 ± 29.4</td>
<td>126.3 ± 28.5</td>
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<td>ABC</td>
<td>44.5 ± 10.1</td>
<td>83.8 ± 7.2</td>
<td>117.6 ± 13.1</td>
</tr>
<tr>
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<td>IZM</td>
<td>22.2 ± 9.0</td>
<td>42.7 ± 15.2</td>
<td>85.7 ± 20.0</td>
</tr>
<tr>
<td></td>
<td>IZC</td>
<td>34.3 ± 4.7</td>
<td>53.8 ± 6.8</td>
<td>82.0 ± 16.9</td>
</tr>
<tr>
<td></td>
<td>IZL</td>
<td>23.7 ± 18</td>
<td>33.2 ± 5.7</td>
<td>52.0 ± 9.1</td>
</tr>
<tr>
<td>Cow</td>
<td>PBC</td>
<td>70.2 ± 18.1</td>
<td>119.5 ± 23.7</td>
<td>196.8 ± 35.6</td>
</tr>
<tr>
<td></td>
<td>ABC</td>
<td>67.2 ± 18.3</td>
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<td>166.5 ± 97.3</td>
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<td>85.7 ± 32.5</td>
</tr>
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### B) Instantaneous modulus (kPa)

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<tr>
<th>Species</th>
<th>Strain 10%</th>
<th>Strain 20%</th>
<th>Strain 30%</th>
</tr>
</thead>
<tbody>
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<td>Human</td>
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<td>2823 ± 2157</td>
<td>4800 ± 3597</td>
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<tr>
<td>ABC</td>
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<td>1783 ± 768</td>
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</tr>
<tr>
<td>IZM</td>
<td>216 ± 114</td>
<td>908 ± 666</td>
<td>2402 ± 1368</td>
</tr>
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<td>IZC</td>
<td>226 ± 170</td>
<td>774 ± 527</td>
<td>2085 ± 662</td>
</tr>
<tr>
<td>IZL</td>
<td>57 ± 17</td>
<td>287 ± 78</td>
<td>1116 ± 153</td>
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<td>Pig</td>
<td>121 ± 66</td>
<td>2287 ± 1307</td>
<td>3310 ± 1551</td>
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<td>ABC</td>
<td>64 ± 182</td>
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<td>4039 ± 178</td>
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<tr>
<td>IZM</td>
<td>32 ± 32</td>
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<td>IZC</td>
<td>20 ± 2</td>
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<td>IZL</td>
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<tr>
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<td>11/14 ± 5.6</td>
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<td>2603 ± 1656</td>
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<tr>
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### Coefficient of viscosity (MPa s)

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<th>Strain 20%</th>
<th>Strain 30%</th>
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### Region

<table>
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<td>IZL</td>
<td>A</td>
<td>A</td>
<td>AB</td>
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<tr>
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<td>AB</td>
<td>A</td>
<td>B</td>
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</tbody>
</table>

47
Figure 2.1: Description of the regions tested and gross morphology of the discs
A) Regions of the TMJ disc used for biochemical, histological, and compression testing: posterior band central (PBC), intermediate zone (IZ) medial (IZM), IZ central (IZC), IZ lateral (IZL), and anterior band central (ABC). B) Regions of the TMJ disc used for tensile testing: mediolateral (ML) posterior (ML P), ML central (ML C), ML anterior (ML A), anteroposterior (AP) medial (AP M), AP central (AP C), AP lateral (AP L). C) Scaled figure showing the gross morphology of TMJ discs collected from the five different species tested. D) Dimensions of discs collected from each species measured in the mediolateral (M-L) and anteroposterior (A-P) direction. Data are presented as mean ± S.D. A one-way ANOVA was conducted on the data from each direction and animals not connected by the same letter are statistically different from one another. The pig was the only animal with dimensions not significantly different from the human in both the A-P and M-L directions.
Figure 2.2: Biochemical analysis of collagen, DNA, and GAG
A-C) show an interspecies comparison of the quantitative biochemical content of the TMJ disc. Data were normalized to wet weight and are presented as mean ± S.D. A two-way ANOVA is presented with the factors of species and region. Samples not connected by the same letter are statistically different from each other. A) Total collagen content of human samples was not statistically different from pig or rabbit discs. B) Human discs contained significantly less DNA content than the other species, likely due to age. C) Sulfated GAG content of human samples fell in between the pig and rabbit samples.
Figure 2.3: Histological analysis of GAG content
Safranin-O/fast green staining of sections from the TMJ disc. Positive Safranin-O staining (red to purple color) is clear in all samples except human ABC and all regions of the pig disc. Images were taken on a with a 20X objective.
CHAPTER 3: Tissue Engineering of the TMJ Disc *

Introduction

In the most common tissue engineering approach, cells are seeded on an appropriate scaffold and then cultured in an environment that promotes production of native ECM tissue, leading to tissue-specific biomechanical characteristics. The initial choice of a particular cell, scaffold, or exogenous stimulation regimen is based on characterization data, native tissue development, or engineering studies of other tissues. As attempts are made, the techniques become refined to where a construct ready for in vivo implantation and testing is produced. Whereas these aspects have become increasingly well defined for articular cartilage and bone tissue engineering, significant questions regarding cell source, scaffold choice, and stimulation regimen still remain for the TMJ disc.

From 1991-2001, four articles were published that suggested it would be possible to engineer a cartilaginous tissue in the shape of a TMJ disc [102, 112-114]. Since that time, numerous studies have considered scaffolding options, biomechanical stimulation regimens, growth factor strategies, and alternative cell sources toward the goal of recapitulating the biochemical and biomechanical properties of the native disc in tissue engineered constructs. These studies will be introduced in the following section to provide researchers a base from which to devise a well-informed in vitro tissue engineering strategy. Following that section, there will be sections on scaffold selection, biochemical factors, and bioreactors that draw on insights garnered from tissue engineering studies of the TMJ disc and other cartilaginous tissues.

Previous Tissue Engineering Efforts

Thomas et al. [112] published the first *in vitro* TMJ disc tissue engineering study in 1991. In this study, second passage leporine disc cells were suspended with unpolymerized collagen type I and allowed to polymerize after injection in a porous collagen matrix. The constructs underwent a significant change in size over two weeks, decreasing from 16 to 12 mm in diameter. Cells cultured in collagen scaffolds were more chondrocytic than monolayer controls, exhibiting a rounded morphology and staining positively for proteoglycans. Three years later, Puelacher et al. [113] created human disc-shaped constructs by seeding primary bovine articular chondrocytes on pre-shaped polylactic acid/polyglycolic acid (PLA/PGA) scaffolds. After 1 week of *in vitro* culture, constructs were implanted subcutaneously in nude mice and cultured *in vivo* for 12 weeks. Histological staining of constructs revealed the presence of GAGs and collagen II, and most significantly, the constructs retained their shape after the culture period.

Girdler [114] created disc-shaped constructs from mandibular cartilage cells of marmosets. The author indicated that chondroprogenitor cells were isolated but did not specify the zone from which these cells were procured, or provided verification that they were, in fact, chondrocyte progenitors. The isolated cells were expanded in monolayer for 3 weeks, maintaining a polygonal morphology throughout the culture period. After trypsinization, cells were suspended in an unpolymerized solution of type I collagen and fibrinogen, which was then seeded into collagen type I sponges and polymerized using thrombin in sodium chloride and sodium citrate. Constructs were cultured for 14 days, then semi-quantitative biochemical assessments for proteoglycans and collagen types I and II were performed. These constructs stained positively for collagen type II and
proteoglycans, and round-shaped cells were embedded within a dense matrix. Despite the thorough histological matrix assessment, the mechanical integrity of these constructs was not assessed.

Springer *et al.* [102] sought to create a durable replacement disc by culturing disc and articular eminence cells from humans and pigs on non-absorbent scaffolds, including expanded polytetrafluoroethylene (ePTFE) monofilaments, PGA monofilaments, polyamide monofilaments, and bone mineral blocks. The idea was that these scaffolds would provide a stress-absorbing framework within a tissue engineered construct. Isolated cells were expanded for one passage, and then seeded in scaffolds for 2 hours before medium containing 10% serum was added. Constructs were cultured for 2, 4, or 8 weeks in the same medium, then evaluated using electron microscopy and gel electrophoresis. Cells from human and pig sources showed no differences in monolayer culture or on scaffolding material. Cells became confluent on scaffolds within 4 weeks, assuming either a spherical or fusiform shape. SDS-PAGE revealed the presence of collagen type II, suggesting the cells were of a chondrocytic phenotype. Polyamide and ePTFE scaffolds did not degrade over the culture period, though PGA fibers showed fissures within 4 weeks and were mechanically unstable at 8 weeks. One large concern for the future applicability of this study is the biocompatibility of the polyamide and ePTFE scaffolds. Though these materials did not degrade in this study, some degradation could occur after continued exposure to the mechanical forces present in the joint, leading to formation of wear particles and subsequent foreign-body responses.

From 2004-2008, Athanasiou *et al.* [115-127] published 13 articles refining the use of porcine TMJ disc cells for tissue engineering. These studies optimized monolayer
culture conditions, scaffold selection, seeding density and technique, growth factor regimens, and biomechanical stimulation regimens. The first scaffold used was alginate, though it was unsuccessful due to a marked decrease in cell number and a lack of ECM production [116]. PGA nonwoven meshes were seeded successfully using spinner-flasks, and constructs produced significantly higher levels of collagen type I relative to PGA scaffolds seeded using other methods [116]. Cell seeding density was then optimized, noting that initial seeding should not exceed 75 million cells/mL of scaffold volume [118]. Later, a poly-L-lactic-acid (PLLA) scaffold was tested in an effort to limit construct contraction, hypothesizing that its slower degradation rate would allow for significant matrix deposition and thereby increase the mechanical integrity of constructs [127]. At 4 weeks, PGA constructs had contracted to roughly 5% of their original volume, while PLLA constructs retained their initial dimensions.

Several growth factors were tested for their effects on disc cells in monolayer and in scaffolds. First, the growth factors IGF-I, PDGF, and bFGF were examined at concentrations of 10 and 100 ng/mL for their effects on cell proliferation and matrix production after 14 days of monolayer culture [115]. All growth factors increased the proliferation rate relative to controls. PDGF and bFGF induced a more than two-fold increase in GAG production relative to controls, and IGF-I and bFGF increased production of collagen. In general, higher growth factor concentrations enhanced cell proliferation while the lower concentrations increased biosynthesis. Next, IGF-I, bFGF, and TGF-β1 were added at two concentrations during 3-D culture [117]. All growth factor groups had improved mechanical and structural integrity relative to controls, and IGF-I and TGF-β1 were shown to increase collagen synthesis. Combinations of these
three growth factors were then assessed [119]. All combinations improved cellularity and mechanical integrity, though no single combination stood out as particularly beneficial. Finally, IGF-I, TGF-β1, and TGF-β3 were tested on PLLA-seeded constructs [127]. Constructs treated with TGF-β1 had double the number of cells and GAG content and 15 times the collagen content of those treated with IGF-I. This finding was particularly interesting, considering these two growth factors behaved similarly when tested on PGA-seeded constructs [117]. This dramatic difference could be a result of changes in the local pH due to degradation rates, or as a result of the dynamic cell density changes due to PGA contraction.

Almarza and Athanasiou [121] examined the effects of cyclic and constant hydrostatic pressure on disc cells. A 10 MPa amplitude treatment was applied for 4 hours at 0 and 1 Hz to cells in monolayer to test for gene expression of cartilaginous matrix components. Cyclic application resulted in increased collagen type II and decreased collagen type I, aggrecan, and decorin expression relative to controls, while constant application increased collagen type I and decreased aggrecan expression. Next, these treatments were applied intermittently over 1 week to measure matrix production in 3-D constructs. Constant application increased collagen production (24.5 µg/construct) relative to controls (18.2 µg/construct) and groups receiving cyclic treatment (7.5 µg/construct). Detamore and Athanasiou [120] tested the effectiveness of a rotating wall bioreactor, which had been shown to enhance biosynthesis and improve matrix homogeneity in articular chondrocyte-seeded constructs. The results of this study did not support its future use with these cells, as statically cultured constructs generally outperformed the rotating groups.
The stability of the TMJ disc cell phenotype during *in vitro* culture was examined in three studies [123, 124, 126]. Gene expression did not vary depending on the region of the disc from which cells were isolated, though cells were found to rapidly down-regulate aggrecan, collagen type I, and collagen type II expression with passage [123]. Expansion of cells would be necessary to obtain enough cells for creation of a full-sized autologous construct; therefore attempts were made to recover the lost phenotype. Cells passaged up to five times were exposed to the growth factors IGF-I, TGF-β1, and TGF-β3 for 24 hours in monolayer or pellet culture and analyzed using real time RT-PCR [124]. The growth factors did not have a positive effect on cells cultured using either method, and pellet culture had a negative effect on expression relative to monolayer. Recovery of phenotype was then attempted through culture on ECM coated surfaces [126]. Passage 0, 1, and 2 cells were plated on surfaces coated with aggrecan, collagen type I, collagen type II, or decorin, though no treatment had a significantly positive effect.

More recently, a series of studies were published that suggested that costal cartilage could serve as an autologous cell source for disc tissue engineering [128-131]. This tissue would be particularly appealing as many craniofacial surgeons have experience replacing the mandible with a rib graft. In one study [130], scaffoldless constructs (see *scaffolds* section below) were created from primary and passaged goat costal chondrocytes (CCs), and compared to control TMJ disc cells. Cellularity and GAG content of primary and passaged CCs constructs were nearly an order of magnitude higher than disc-cell constructs after 6 weeks of culture, and most importantly, CC constructs retained their size and shape throughout the culture period (~ 3 mm dia.), while disc-cell constructs contracted severely (0.84 mm dia.). Immunostaining revealed
the presence of collagen types I and II throughout primary CC constructs, though constructs from passaged cells only stained around the periphery and in the middle where a large, fluid-filled core had formed. This sphere did not form in primary CC or disc-cell constructs. Not surprisingly, similar results were observed when mandibular condylar cartilage cells were compared directly to hyaline cartilage cells from the ankle [132, 133], with hyaline cartilage cells outperforming the TMJ cells.

Scaffolds

The purpose of a scaffold is to provide a vehicle upon which cells may grow and deposit matrix. A suitable scaffold must be biocompatible, sterilizable, and biodegradable. It must be sufficiently porous and allow unrestricted cell growth and diffusion of nutrients through large, interconnected pores [134]. The physical properties of the scaffold should support matrix deposition, and the degradation profile should allow newly deposited matrix to gradually assume mechanical loads. Additionally, there should be a method for which the size and shape of the scaffold can be modified to fit the specific dimensions of the target tissue. It may also be advantageous for a scaffold to direct cellular processes though the release of growth factors, or by providing mechanical feedback through cell-substrate interactions.

Synthetic scaffolds are highly versatile. The shape and size can be modified to fit specific applications. Important characteristics, such as porosity, mechanical properties, degradation rate, and hydrophilicity, can be tuned by modifying production procedures or by creation of custom copolymers. PLA and PGA are two widely used biodegradable and biocompatible scaffolding materials. In addition to their use in previous TMJ disc tissue
engineering studies, they have also been tested for regeneration of the knee meniscus [135-137] and intervertebral disc [138-140]. These polymers are hydrolyzed into lactic acid and glycolic acid, which are then metabolized further by the body and excreted [141]. A primary difference between these two scaffolds is the degradation rate. Initial PLA degradation products are larger and do not diffuse as readily as PGA products, and therefore PLA degrades more slowly [127]. The previous work with PGA demonstrated that its degradation rate was far too fast at its given formulation and structure for use with TMJ disc cells, however PLA/PGA copolymers or higher molecular weight polymers could show promise for future work. Polyurethane materials such as polycaprolactone (PCL) have been explored for engineered menisci [142, 143] and mandibular condyles [144] due to their slow degradation rate and high mechanical properties. Foams made from these materials and copolymers of PCL and PLA can be formed to any size or shape; porosity and degradation rate can be adjusted by modifying molecular weight/intrinsic viscosity, composition, and fabrication method. Because of the existence of literature based on the use of polyurethane polymers for meniscus and mandibular condyle tissue engineering, studies examining their possible use for TMJ disc tissue engineering are warranted.

The most extensively studied scaffolding material for cartilage tissue engineering is collagen [145]. Type I collagen is most commonly used due to its abundance. The collagen can be used either intact or after proteolytic digestion, allowing for formation of gels. Gels have shown promise for repair of cartilage defects functioning as delivery systems for injectable cell suspensions [146], though this approach would not work for the TMJ disc. A more realistic approach for the disc would involve the use of
prefabricated collagen sponges [147]. These scaffolds are highly porous (>95%) and have pore diameters greater than 120 μm. A possible limitation for collagen and other natural scaffolds, such as hyaluronan and alginate, is their inherently low rigidity [148]. Constructs may not retain their shape in light of past experiences with PGA.

Another possible approach involves the use of decellularized tissues, such as periosteal tissue [149], perichondrial tissue [150], and small intestine submucosa [151] to serve as scaffolds. Of course, the porosity and shape of these tissues cannot be varied to recreate the specific morphology of the disc. Lumpkins et al. [152] proposed using a decellularized porcine disc as a xenogenic scaffold, comparable to approaches seen with other tissues such as heart valves [153]. In this study, discs decellularized with sodium dodecyl sulfate (SDS) maintained their size and shape, and displayed similar mechanical energy dissipation characteristics similar to the native disc. This study was published very recently, and the future utility of these scaffolds is unknown, as these constructs have yet to be tested with cells.

Though the byproducts of polymer degradation are non-toxic, they are acidic in the case of aliphatic polyesters, such as PGA and PLA and their copolymers, and cause a drop in pH in the local environment. This acidity could potentially have deleterious effects on cells. In addition, scaffolds may shield cells from stresses imparted from bioreactors or from the in vivo environment, thereby preventing mechanotransductive events. Therefore, efforts have been made to devise a method for scaffoldless tissue engineering [154]. One such process, termed the self-assembly process, involves seeding cells at a high density in non-adherent agarose wells, and has been used to create tissue engineered articular cartilage constructs with clinically relevant dimensions (15 mm dia.
and 1 mm thick), and aggregate modulus approaching 1/3 that of native tissue [154]. This technique has also been used to fabricate semilunar knee menisci using co-cultures of articular chondrocytes and meniscal fibrochondrocytes [155]. By using an appropriate mold shape, along with cells that can survive in a scaffold-free environment, self assembly could prove to be a successful approach for engineering the TMJ disc.

Bioactive Agents

The effects of growth factors on TMJ disc cells and disc-cell seeded constructs have been studied previously. The specific growth factors examined include TGF-β1, TGF-β3, PDGF, bFGF, and IGF-1. Salient results are presented in Table 4.1. Perhaps the most important finding from these studies is that higher concentrations of growth factors tend to increase cell proliferation, while lower concentrations enhance biosynthesis [156]. Therefore, a good approach might be to expose constructs to high levels of growth factors upon seeding to increase cellularity, and then gradually reduce the concentration to encourage ECM deposition.

Biochemical agents other than growth factors may have utility for TMJ disc tissue engineering. Natoli et al. [157] found that the GAG-depleting agent chondroitinase-ABC can be used to increase tensile properties in scaffoldless tissue engineered cartilage constructs. This process involves temporarily removing GAGs to allow for enhanced organization and alignment of the collagen network. Also, the pro-inflammatory cytokine interleukin-1 (IL-1) has been shown to encourage migration of meniscal fibrochondrocytes [158]. Future tissue engineering studies should examine the use of
catabolic treatments for matrix modification and cell signaling, in addition to the more widely studied anabolic factors.

**Mechanical Stimulation and Bioreactors**

Because of their inherent avascularity, cartilaginous tissues require mechanical loading to exchange nutrients and waste products. In addition, biomechanical stimuli may be essential for cell survival and matrix synthesis, as unused cartilage atrophies [159]. The TMJ disc is exposed to direct forces imparted through contact with the condyle and fossa, and hydrostatic loading from fluid pressurization in the joint capsule. As discussed in Chapter 2, a lifetime of exposure to forces leads to changes in matrix structure and mechanical properties. Tissue engineers seek to recreate these forces *in vitro* by using devices which simulate the *in situ* mechanical environment.

The only mechanical stimulation that has been experimentally applied to TMJ disc cells is hydrostatic pressure, when Almarza and Athanasiou [121] demonstrated that static loading at 10 MPa was most beneficial to biosynthesis while cyclic loading was found to be detrimental. This finding was surprising since the disc is assumed to be subjected to cyclic hydrostatic loading *in situ*. Hydrostatic pressure is transmitted through the fluid surrounding tissue. Because water is nearly incompressible at physiological pressures, this loading does not cause an initial change in tissue volume. Instead, the increased fluid pressure upsets the balance between negatively charged GAGs and the water molecules in the matrix, forcing a gradual release of fluid to the synovium. This explains the effect hydrostatic pressure can have on nutrient diffusion, and may help explain why static pressure is beneficial to tissue engineered constructs [160]. Research
suggests that receptors on the surface of cartilage cells, namely the NA/K, Na/K/2Cl, and Na/H pumps, change conformation and thereby alter intracellular ion concentrations in response to hydrostatic pressure [161, 162]. This change may stimulate signal transduction cascades, causing upregulation of ECM related genes, and enhancing matrix production.

In contrast to hydrostatic pressure, direct stimulation physically compresses the tissue. This type of loading occurs in the TMJ disc during chewing or clenching. Clenching forces would limit small molecule diffusion, and many studies testing static loading on articular cartilage constructs have reported adverse effects [163-166]. However, dynamic stimulation has shown beneficial effects in cartilage tissue engineering [163, 164, 167]. Mauck et al. [167] reported a 45% increase in GAG and a 37% increase in collagen deposition in response to 10% applied at 1 Hz, and Buschmann et al. [164] reported similar results using 3% strain at 1 Hz. A primary reason cited for the positive effects of this treatment is that mass transfer is enhanced relative to static culture. Compression moves fluid out of the tissue, while unloading allows fluid to be drawn back in to the tissue, thus a complete media change is accomplished through repeated cycles. Additionally, hydrostatic pressure builds up in constructs in response to dynamic loading, and shear stresses develop in response to moving fluids. Although cyclic direct compression was beneficial in cartilage tissue engineering, it actually had a deleterious effect with mandibular condylar cartilage cells [168] (see Chapter 5). Given the similarity of behavior observed with condylar cartilage cells and TMJ disc cells, it is possible that cyclic direct compression may not be efficacious for TMJ disc cells. In the event that this approach is attempted for TMJ disc cells, or with any cell source for a TMJ
application, it is important to make sure that constructs are sufficiently resilient to handle this type of loading, as high amplitudes and frequencies could easily be detrimental to tissue formation. Such studies would need to optimize the frequency, amplitude, duration, and duty cycle of loading.

Bioreactors are meant to enhance the exchange of nutrients and wastes within constructs. Diffusion limitations in static culture can prevent cells in the center of constructs from having access to fresh media leading to death or inviability. As more biomimetic constructs with greater thicknesses and matrix densities are created, this issue will become more important. In addition, it could be desirable to have closer control over the temporal exposure of cytokines than would be allowed in a system which is limited by the rate of diffusion. Lastly, some bioreactors are able to provide a continuous culture environment. Such a setup would significantly reduce the number of manipulations performed by researchers during culture, greatly reducing construct variability and limiting chances for contamination.

A rotating-wall is an example of a bioreactor. In this system, a vertically oriented culture dish is rotated to create fluid-flow and impart a low-level shear to suspended cells or to constructs [169]. Results with its use for cartilage constructs have been positive, showing significant increases in GAG production, collagen deposition, and increases in equilibrium modulus relative to static controls [170-172]. This bioreactor is the only one that has been used for culture of TMJ disc cells, though it was found to have mixed effects [120]. The authors speculated that the shearing fluid washed portions of the scaffold loose along with attached cells and matrix. The results of this study illustrated the need to temper the level of mechanical perturbation at early stages so not to damage
immature constructs, for example by preceding bioreactor culture by a period of static culture. This study aside, a low-shear environment provided by the rotating-wall could be beneficial to early stage constructs by enhancing nutrient exchange, so long as the scaffold and cells can withstand shear forces.
Table 3.1: Effects of growth factors on TMJ disc cells

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Concentrations</th>
<th>Cell source</th>
<th>Notable effects</th>
<th>Ref.</th>
</tr>
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<tr>
<td></td>
<td><strong>In monolayer</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TGF-β1</td>
<td>0.03 - 3 ng/mL</td>
<td>Cow</td>
<td>250% increase in cell number</td>
<td>[7]</td>
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<tr>
<td>bFGF</td>
<td>3 ng/mL</td>
<td>Cow</td>
<td>Upregulation of Erk1/2 and p38</td>
<td>[156]</td>
</tr>
<tr>
<td></td>
<td>10, 100 ng/mL</td>
<td>Pig</td>
<td>96% increase in cell number</td>
<td>[115]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>280% increase in GAG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>420% increase in collagen</td>
<td></td>
</tr>
<tr>
<td>IGF-1</td>
<td>10, 100 ng/mL</td>
<td>Pig</td>
<td>49% increase in cell number</td>
<td>[115]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>270% increase in GAG</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>450% increase in collagen</td>
<td></td>
</tr>
<tr>
<td>PDGF</td>
<td>20 ng/mL</td>
<td>Cow</td>
<td>Upregulation of Erk1/2 and p38</td>
<td>[156]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pig</td>
<td>41% increase in cell number</td>
<td>[115]</td>
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<td></td>
<td><strong>In 3-D culture</strong></td>
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</tr>
<tr>
<td>TGF-β3</td>
<td>5, 30 ng/mL</td>
<td>Pig</td>
<td>110% increase in collagen</td>
<td>[117]</td>
</tr>
<tr>
<td></td>
<td>5 ng/mL</td>
<td>Pig</td>
<td>Maintained cellularity</td>
<td>[127]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10-fold increase in collagen</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>340% increase in GAG</td>
<td></td>
</tr>
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<td>Pig</td>
<td>128% increase in collagen</td>
<td>[117]</td>
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<td>Pig</td>
<td>300% increase in collagen</td>
<td>[117]</td>
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<tr>
<td>TGF-β3</td>
<td>5 ng/mL</td>
<td>Pig</td>
<td>Maintained cellularity</td>
<td>[127]</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>300% increase in collagen</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>80% increase in GAG</td>
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<td>Combinations of:</td>
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<td>Pig</td>
<td>All growth factor combinations enhanced construct cellularity and viability relative to controls.</td>
<td>[119]</td>
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<td>TGF-β1</td>
<td>10, 100 ng/mL</td>
<td>Pig</td>
<td></td>
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<td>IGF-1</td>
<td>10, 100 ng/mL</td>
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<tr>
<td>bFGF</td>
<td>10, 100 ng/mL</td>
<td>Pig</td>
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CHAPTER 4: Assessment of Growth Factor Treatment on Fibrochondrocyte and Chondrocyte Co-Cultures for TMJ Fibrocartilage Engineering *

Abstract

The effects of TGF-β1 and IGF-1 were examined on three ratios of scaffoldless co-cultures of fibrochondrocytes (FC) and articular chondrocytes (AC) in a two-phase sequential study. In the first phase, growth factors were applied alone and in combination, in the presence or absence of serum, and the best overall treatment was applied at intermittent dosing in the second phase. The objective was to identify co-culture ratios and anabolic treatments that result in constructs with properties approaching those of native TMJ fibrocartilage. Modulating these parameters resulted in constructs with GAG/WW (12.2%), instantaneous compressive moduli (790 kPa), relaxed compressive moduli (120 kPa), and Young’s moduli (1.87 MPa) that overlap with native TMJ disc values. On FC/AC co-cultures, TGF-β1 treatment increased collagen concentration ~20%, compressive stiffness ~130%, and Young’s modulus ~170% relative to no growth factor controls. IGF-1 did not appear to have an effect on functional properties alone or in combination with other agents. Serum supplementation, though generally detrimental to functional properties, was identified as a powerful mediator of FC construct morphology. Finally, both intermittent and continuous TGF-β1 treatment showed positive effects, though continuous treatment resulted in greater enhancement of construct functional

properties. This work proposes a strategy for the development of constructs for replacement or repair of TMJ fibrocartilage, and its future application will be realized through translation of these findings to clinically viable cell sources.
Introduction

Disorders of the temporomandibular joint (TMJ) affect millions in the United States [173, 174]. Many cases of temporomandibular dysfunction (TMD) are associated with malformations or displacements of the TMJ disc, a fibrocartilaginous tissue that assists in normal function of the joint, and/or injuries to the fibrous cartilage that covers the surface of the mandibular condyle [175, 176]. Sufferers experience pain and difficulty performing routine activities such as eating and talking. The majority of cases are best managed through conservative treatments and pain mitigation, though the most severe cases of joint degeneration require surgical intervention [177]. While current surgical methods, such as arthrocentesis, arthroscopic repositioning, discectomy, and joint replacement, can restore some function, these treatments do not fully address severe TMD [96]. Efforts to engineer tissues for repair or replacement are essential to provide the next generation of treatments and an ideal long-term solution [176, 177].

An appropriate cell source for tissue engineering must be abundant, accessible, and functionally appropriate for the target tissue. Primary TMJ disc cells [116, 117, 125, 127] and condylar cartilage cells [178, 179] have been examined for TMJ engineering, however clinical success using these sources is improbable due to cell scarcity and their inability to produce sufficient matrix in vitro. Additionally, the availability of TMJ cells for in vitro studies is limited; therefore the pressing problems of TMD need to be addressed using alternative cell types.

The meniscus, the TMJ disc, and condylar cartilage are comprised of heterogeneous matrices containing predominantly collagen type I with lower levels of collagen type II, as well as glycosaminoglycans (GAGs) [8, 30, 180-182], and exhibit
regional variation and anisotropy in material properties [39, 49, 183-186]. The cells of the knee meniscus are a mixed population of rounded, chondrocyte-like cells and elongated, fibroblast-like cells similar to those found in the disc and condylar cartilage [68, 176]. While knee meniscus cells are not abundant or easily accessible, efforts are underway to differentiate stem cells toward this fibrocartilage phenotype [178, 187-189]. When combined with articular chondrocytes (AC) in a scaffoldless culture system [154], these cells are capable of forming constructs comprised of collagen types I and II, and levels of GAGs similar to native fibrocartilage [155, 190, 191]. Importantly, by modulating the ratio of FCs to ACs, the composition of the resulting matrix could feasibly be tailored to more closely recapitulate the target tissue mechanical properties and ECM. As with fibrochondrocytes, abundant efforts are also underway in differentiating embryonic stem cells [192, 193], bone marrow-derived mesenchymal stem cells [194, 195], adipose-derived stem cells [196, 197] and other cells [198, 199] toward a chondrocyte phenotype. Given the similarities in function, current progress in fibrocartilage tissue engineering, and the rapidly advancing field of stem cell differentiation toward the cartilage and fibrocartilage phenotypes, a co-culture of FCs and ACs is an attractive culture model for TMJ tissue engineering.

In addition to varying co-culture ratios, the use of anabolic agents can modulate matrix synthesis and subsequently affect biomechanical properties. Studies of several growth factors (GF) on fibrochondrocytes in monolayer and 3D culture showed increases in collagen and GAG precursor uptake using TGF-β1 compared to IGF-1, bFGF, and PDGF, though IGF-1 also significantly increased collagen precursor uptake [137, 200]. In addition, previous studies using both IGF-1 and TGF-β1 on fibrochondrocytes led to
significant increases in collagen and GAG synthesis over other GFs [115, 117]. Synergy between IGF-1 and TGF-β1 has been demonstrated in ACs in terms of aggrecan production [201], and recent work suggests that intermittent dosing of GFs may enhance their efficacy relative to continuous treatment, likely the result of reduced receptor desensitization [202, 203]. In addition to GFs, fetal bovine serum (FBS) can modulate the character of fibrocartilage constructs. Though recent work has shown increases in functional properties using a serum-free, chemically defined medium in FC:AC co-cultures [191], the use of FBS in conjunction with IGF-1 and TGF-β1 may be capable of accentuating functional differences between the resulting tissue. For meniscus tissue engineering, previous work in our laboratory has determined serum-free methods using a 50:50 FC:AC co-culture. Whether this can be achieved for other FC:AC ratios has yet to be determined.

The purpose of this study is two-fold. First, a ratio of FCs to ACs must be determined for engineering the TMJ disc and condylar cartilage. Second, a protocol for the use of bioactive factors in culturing these cells must be identified. Specifically, GF type, the contribution of serum to its effectiveness, whether different GFs need to be combined, and whether saturation occurs in their dosing are all variables that must be examined. To achieve these goals, two phases were employed. In the first phase of this study, the effects of TGF-β1, IGF-1, and serum, alone combination, on scaffoldless constructs of MCs and two co-cultures of MCs and ACs, were examined. In the second phase, the best overall treatments, as determined by quantitative assessments of matrix composition and biomechanical integrity, were applied continuously or intermittently. Based on previous experience, it was hypothesized that a spectrum of fibrocartilages
displaying heterogeneous function and matrix composition would result from different co-culture combinations and GF/serum treatments, and that modulating the type and manner of application of anabolic agents would result in near recapitulation of native tissue material properties and matrix content.

Materials and Methods

Chondrocyte and Fibrochondrocyte Isolation and Seeding

Chondrocytes were harvested from the distal femoral condyle, and fibrochondrocytes were harvested from menisci of one-wk-old male calves (Research 87, Boylston, MA) within 36h of slaughter. Tissue was minced and digested overnight in medium containing 0.2% collagenase type II (Worthington, Lakewood, NJ). Medium consisted of DMEM with 4.5 g/L glucose and L-glutamine (Gibco, Grand Island, NY), 1% penicillin/streptomycin/fungizone, 1% non-essential amino acids, 0.4 mM L-proline, 5 mM ascorbate-2-phosphate, and 10 mM sodium pyruvate. Cells from multiple animals were pooled and frozen at -80°C to obtain sufficient numbers for the experiments. Cylindrical agarose molds of 5 mm diameter were prepared as previously described [204]. Frozen ACs and MCs were thawed, resuspended in culture medium, counted, and seeded at 5.5 x 10⁶ cells/well in 150 µl of appropriate culture medium at specified MC:AC ratios. Culture medium was the same as digestion medium with the addition of 1% ITS+ (BD Biosciences, San Jose, CA) and 100 nM dexamethasone. Constructs were given complete media changes of 500 µl on alternating days. After 10 days, constructs were excised from molds and placed in agarose coated 48 well plates and given 500 µl
media changes on alternating days. Constructs were collected after 4 wks for gross measurements and partitioned to perform further analyses.

Phases I and II: GF/Serum Treatments and Intermittent vs. Continuous Treatment

Constructs created from three combinations of FCs to ACs were examined (FC%:AC%): 100:0, 75:25, and 50:50. A full-factorial design was employed to test four levels of GF treatment (10 ng/ml TGF-β1, 5 ng/ml IGF-1, the combination of these, and no GF) and two levels of serum treatment (10% and none). The best overall GF/serum treatment for each culture ratio (that which resulted in the greatest mechanical integrity and matrix content) was carried forward to phase II to examine the effect of intermittent dosing. Intermittent groups were given GFs during the first and third wks, while continuous groups were supplemented throughout the 4 wk culture period.

Compressive and Tensile Biomechanics

Compression samples were prepared by removing a cylindrical portion from the center of the construct using a 2 mm biopsy punch. The sample was submerged in PBS and loaded into an Enduratec 3200 testing machine (Electroforce, Eden Prairie, MN). After determining initial sample thickness by applying a 0.02 N tare load, samples were preconditioned with 15 cycles of 5% strain at 1 Hz. A 10% step strain was applied for 20 min and stress relaxation data were recorded. Viscoelastic material properties were determined by fitting data a Kelvin solid model using Matlab (MathWorks, Natick, MA) [49]. Tensile samples were dog-bone shaped pieces created using a biopsy punch. Samples were glued into paper frames and placed in grips of the Enduratec. Tension was applied at 1% strain/s until failure, and Young’s modulus was determined from the linear region of the stress vs. strain curve.
Quantitative Biochemistry

Portions used for biochemical assessments were weighed, lyophilized, and digested in 1 ml papain at 65°C overnight. Total DNA content was determined in a fluorescence plate reader using Picogreen® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR). Total sulfated GAG content was determined using a dimethylmethylene blue (DMMB) dye-binding assay kit (Biocolor, Newtownabbey, Northern Ireland). Total collagen content was determined with a hydroxyproline assay using Sircol™ standards (Biocolor) [205].

Histology

Histology samples were frozen in tissue embedding medium and sectioned at 14 μm. Sections were fixed in 10% phosphate buffered formalin and stained with safranin-O/fast green to examine GAG distribution, and picrosirius red to examine collagen.

Statistical Analyses

In phase I, a three-factor ANOVA (cell ratio, serum treatment, and GF treatment) was used to analyze quantitative data. For serum treatment, a Student’s t-test was performed post-hoc to determine significance between treatments, and a Tukey’s HSD was used for cell ratio and GF treatment assessments. In phase II, a two-factor ANOVA (cell ratio and GF treatment) was used, with a Student’s t-test or Tukey’s HSD test performed post-hoc for cell ratio and GF treatments, respectively. In both phases, a one-way blocked ANOVA was also performed to determine the best overall treatment for each of the cell ratios tested. Five samples were used for each experimental group, and all data are presented as mean ± S.D.
Results

TGF-β1 in defined medium was determined to be best overall treatment for the 50:50 and 75:25 co-culture ratios and was therefore carried forward to phase II. Since GFs had little discernable effect on the 100:0 groups, this cell ratio was not examined in phase II.

Gross Characteristics

Construct gross morphology is shown in Figs. 4.1 and 4.2. All constructs were disc-shaped, with the exception of serum treated 100% FC groups, which formed spherical constructs. In phase I, 100% FC groups had smaller diameters and wet weights (WW) than the co-culture groups, as demonstrated in Table 4.1, and 75:25 and 50:50 groups had similar WWs, diameters, and thicknesses for the respective GF and serum treatments. TGF-β1 and combined GF treatment led to 38% and 45% decreases in WW and 16% and 15% decreases in diameter relative to controls, respectively, while IGF-1 treatment did not have a significant effect. The presence of serum in culture medium led to a 12% decrease in WW and an 8% decrease in diameter relative to defined medium. In phase II, 75:25 and 50:50 groups had similar WWs, though 75:25 constructs were 5% smaller in diameter (Table 4.2). Continuous TGF-β1 supplementation caused more contraction and lower WW relative to intermittent and no TGF-β1, and intermittently treated constructs were also smaller and lighter than controls.

Histology

In phase I, collagen staining was more intense in TGF-β1 and GF combination groups relative to IGF-1 and control groups for both co-culture ratios and base media (Fig. 4.3). FC groups showed stronger collagen staining than co-culture groups. All co-culture groups stained for GAG with safranin-O (Fig. 4.4), though TGF-β1 reduced staining in
the presence on serum. In contrast to the co-cultures, only the background stained in 100% FC groups. In phase II, continuous and intermittent groups stained more intensely for collagen and GAG than controls (Figs. 4.5 and 4.6).

Quantitative Biochemistry

The 100% FC constructs maintained more cells than co-culture constructs (Table 4.1), and the presence of serum in base medium coincided with a significant drop in cellularity relative to defined medium co-culture constructs. In phase I, collagen/WW was greatest in chemically defined TGF-β1 and GF combination co-culture groups, with values ranging from 9.9 ± 1.1% to 11.0 ± 0.5%, while IGF-1 and no GF chemically defined co-culture groups ranged from 7.8 ± 0.4% to 9.0 ± 0.5% (Fig. 4.7a). Serum treated constructs yielded significantly less collagen/WW relative to chemically defined constructs, and similar collagen/WW values were obtained for 75:25 and 50:50 groups. Neither serum presence nor GF treatment had an effect on collagen content in 100% FC groups, with values ~5% collagen/WW. In phase II, continuous TGF-β1 constructs had greater collagen/WW than intermittent and no GF groups (Fig. 4.8b). GAG content per WW was greatest in the 50:50 constructs, with values ranging from 10.8 ± 2.4% to 12.2 ± 1.6% for chemically defined groups (Fig. 4.7b), while all 100:0 groups contained less than 1% GAG/WW. Serum treatment resulted in lowered GAG/WW for both co-culture ratios. In phase II, GF treatment did not significantly affect GAG/WW (Fig. 4.8b).

Biomechanics

The serum-treated 100% FC groups were not testable under compression or tension. In phase I, treatment with TGF-β1 in serum-free medium increased the instantaneous compressive modulus two-fold relative to IGF-1 and no GF treatment in both co-culture
groups (Fig. 4.7c). The greatest instantaneous modulus was 785 ± 88 kPa from the 75:25 TGF-β1 treated, chemically defined group. The 100% FC groups were significantly softer, with values ranging between 133 ± 34 and 242 ± 43 kPa. Serum treatment significantly reduced instantaneous and relaxed moduli relative to defined medium, and GF treatment did not significantly affect compressive moduli in the presence of serum. Overall, relaxed moduli trends across GF and serum treatments were similar to instantaneous moduli (Figs. 4.7c and 4.7d). In phase II, continuous and intermittent TGF-β1 treatment increased instantaneous and relaxed moduli relative to control, and continuously treated groups had similar compressive moduli to intermittently treated groups, except for the 75:25 constructs upon instantaneous compression (Figs. 4.8c and 4.8d).

Under tension, TGF-β1 treatment dramatically increased Young’s modulus over IGF-1 treatment and no GF controls (Fig. 4.7e). Young’s modulus values for co-culture groups treated with TGF-β1 in defined medium ranged from 1.49 ± 0.58 to 1.86 ± 0.27 MPa, while IGF-1 and no GF controls had values from 0.56 ± 0.08 to 0.75 ± 0.23 MPa. Overall, serum treatment reduced tensile stiffness 51% overall in the co-culture groups. In phase II, tensile stiffness was not significantly different between continuous and intermittent treatment groups within the 75:25 ($p = 0.080$) or 50:50 ($p = 0.074$) cell ratio groups, though the difference was significant ($p = 0.009$) for the combined groups (Fig. 4.8e).
Discussion

The overall aim of this work was to optimize and employ anabolic treatments to AC/FC co-cultures to develop an *in vitro* culturing regimen for repair or replacement of TMJ fibrocartilages. This study utilized a two-phased approach to elucidate 1) the effects of TGF-β1 and IGF-1 alone and in combination, and in serum-based and serum-free media, on the resulting construct functional properties, and 2) the effects of varying the supplementation regimen for the most effective anabolic treatments. Wide variation in morphological, biochemical, and biomechanical properties was found as a result of GF and serum treatments, and the results support the hypothesis that modulation of anabolic treatments would result in near recapitulation of native tissue functional properties.

Direct comparison of construct properties to native values provides validation of the methodologies explored in this study. Values for instantaneous compressive modulus in this study ranged from 130-790 kPa, while relaxed modulus ranged from 3 – 120 kPa, both of which approach or equal the native values. Under unconfined compression, a regional investigation of the porcine TMJ disc reported instantaneous moduli of 80-420 kPa and relaxed moduli of 17-38 kPa [51]. A similar investigation of porcine mandibular condylar cartilage reported elastic moduli from 826-1526 kPa and equilibrium moduli from 9.6-22.5 kPa [186]. Under tension, values of Young’s modulus from this study ranged from 0.26-1.87 MPa, approaching the lower end of native values, which are between 0.58-31.8 MPa for the TMJ disc [39] and 8.7-29 MPa for condylar cartilage [185]. Collagen/WW of constructs in this study (4.2-11.0%) were low compared with the native TMJ disc (~21%) [4], though GAG/WW values in this study (0.6-12.2%) actually exceeded those in the native disc (0.3-1.6%) [4, 8]. The observations that constructs with
a wide range of functional properties can be obtained by modulating anabolic treatments to varying ratios of FC and AC co-cultures, and that many of these properties overlap those seen in native values, are exciting findings. However, further optimization studies must be explored, especially those aimed at increasing collagen production and directing collagen organization, to recapitulate the higher end tensile values.

In agreement with our hypothesis, TGF-β1 supplementation significantly increased functional properties of co-culture constructs. In serum-free medium, collagen/WW increased 18% and 24% over no GF controls for 75:25 and 50:50 co-cultures, respectively. The effect of TGF-β1 on biomechanical properties was more robust, increasing instantaneous compressive modulus 140% and 120%, and increasing Young’s modulus 130% and 223% for 75:25 and 50:50 co-cultures, respectively. These findings reflect those of previous studies that show enhancement of cartilage construct properties using TGF-β1 [160, 204, 206]. For example, in scaffoldless cultures of articular chondrocytes, 30 ng/ml TGF-β1 increased collagen/WW by 55%, compressive modulus by 73%, and tensile modulus by 58% [204]. In contrast to its effects on FC:AC co-cultures, the effects of TGF-β1 were not as evident on the 100% FC constructs. There were no differences in collagen/WW between GF treatments. While instantaneous compressive modulus was significantly higher than no GF controls ($p = 0.0018$), increases in relaxed modulus ($p = 0.099$) and Young’s modulus ($p = 0.13$) were not significant in TGF-β1 treated groups. These results were consistent with a recent study by Wilson et al. [206] that showed dramatic increases in dynamic compressive modulus in scaffoldless chondrocyte constructs with TGF-β1 supplementation compared with more modest increases in fibrochondrocyte constructs. As a result of its positive effects on
construct functional properties, TGF-β1 treatment was selected for use in phase II on FC:AC co-cultures. The results showed benefits for both intermittent and continuous TGF-β1 treatment, although continuous treatment resulted in greater enhancement of construct functional properties. It is likely that the saturation threshold for TGF-β1 in this system was not met by the 10 ng/ml concentration. Further experimentation with higher concentrations and duty cycles of TGF-β1 treatment could yield further gains in functional properties.

IGF-1 did not appear to have an effect on construct properties in this study. In fact, groups supplemented with IGF-1 were not statistically different from controls in any functional assessment, either in the presence or absence of serum, and in addition, the combination of IGF-1 and TGF-β1 had statistically similar effects on constructs as TGF-β1 treatment alone for all functional assessments. This finding was surprising in light of previous work that shows increases in matrix synthesis in fibrochondrocyte constructs [137], and increases in matrix synthesis and compressive properties in fibrochondrocyte and chondrocyte constructs with IGF-1 treatment [117, 204]. One explanation for this result is the presence of insulin in the base media. Insulin is considered an essential component of serum-free media due to its role in regulating glucose metabolism, though it also acts as a signaling molecule through receptor tyrosine kinases similar to those for IGF-1 [207, 208]. A study by Bohme et al. [209] on chondrocytes in serum-free culture noted similar regulation of cell proliferation and collagen synthesis at identical insulin and IGF-1 concentrations. Additionally, that study noted that insulin continued to induce collagen synthesis after cessation of cell proliferation, while IGF-1 did not. Considering that insulin is supplemented at 6.25 μg/ml in this study, compared with IGF-1
supplementation at 5 ng/ml, the effects of IGF-1 may be overshadowed by those of insulin.

Consistent with previous findings [190, 191], 100% FC constructs contracted greatly from seeded dimensions, and it is therefore likely that a certain percentage of ACs is required to aid in maintenance of shape in cylindrical molds. However, when a nonadherent center post is incorporated into the mold, 100% FC constructs are better able to maintain their shape [155]. It is believed that cell-derived contraction forces, directed towards the center in cylindrical molds, are deflected circumferentially upon contact with the post, causing hoop stresses, which are likely responsible for the resultant circumferential fiber alignment [155]. The observed contraction could therefore be useful for TMJ fibrocartilage engineering assuming an appropriate mold, capable of directing collagen into biomimetic orientations, can be designed for the TMJ.

For the co-cultures, the presence of serum had a detrimental effect shown by a decrease in all functional properties. For co-cultures of FCs and ACs on PLLA scaffolds, serum is necessary for maintenance of cellularity [210], though in this study serum application actually reduced cellularity in the co-cultures by 57%. In addition, the combination of serum and TGF-β1 inhibited GAG production relative to either treatment alone. It is possible that serum application, in addition to the myriad of nutrients and signaling molecules in the defined medium, may have led to overstimulation of the chondrocytes. In the 100% FC groups, normalized matrix production was not affected by serum treatment, but obvious morphological differences were evident. This response suggests a role for serum in future engineering efforts in line with the above argument outlining the utility of contraction. Temporal application of serum could be used to
induce desired morphological changes at appropriate stages in a tissue engineering process.

In conclusion, this study assessed the effects of TGF-β1 and IGF-1, alone and in combination, in the presence or absence of serum, on three ratios of FCs and ACs, leading to construct functional properties approaching those of native TMJ fibrocartilages. Continuous treatment with TGF-β1 in the absence of serum was found to promote the greatest enhancement of ECM synthesis and biomechanical properties in FC/AC co-cultures. Future studies should use this treatment, in combination with mechanical stimulation such as hydrostatic pressure and direct compression, to further enhance collagen synthesis and tensile properties in fibrocartilage constructs. Direct translation of these findings towards the development of treatments for TMD will be realized through application of these strategies to clinically viable cells sources.
Table 4.1: Phase I construct characteristics after 4 wks of culture

Data are represented as mean ± S.D. Letters refer to statistical analyses performed within each cell ratio. Values not connected by same letter are significantly different.

<table>
<thead>
<tr>
<th>Cell ratio</th>
<th>Base medium</th>
<th>GF treatment</th>
<th>WW (mg)</th>
<th>Diameter (mm)</th>
<th>Thickness (mm)</th>
<th>Cells/construct ($10^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:0</td>
<td>Defined</td>
<td>TGF-β1</td>
<td>6.4 ± 0.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.43 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.76 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IGF-1</td>
<td>5.7 ± 0.3&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>3.30 ± 0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.75 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Both</td>
<td>6.9 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.59 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.71 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.9 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>4.5 ± 0.1&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>3.57 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.69 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.2 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>FBS</td>
<td>TGF-β1</td>
<td>3.7 ± 0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.13 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.78 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.7 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IGF-1</td>
<td>5.2 ± 0.6&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.29 ± 0.16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.93 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.2 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>Both</td>
<td>5.3 ± 0.7&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.20 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.00 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>None</td>
<td>5.1 ± 0.1&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>2.28 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.95 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.7 ± 0.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>75:25</td>
<td>Defined</td>
<td>TGF-β1</td>
<td>10.5 ± 0.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.63 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.87 ± 0.05&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.9 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>IGF-1</td>
<td>18.2 ± 2.0&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.41 ± 0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87 ± 0.09&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td></td>
<td>Both</td>
<td>10.0 ± 1.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.50 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.75 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>17.2 ± 1.3&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.85 ± 0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>FBS</td>
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<tr>
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<td>2.6 ± 0.8&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td></td>
<td>Both</td>
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<td>4.65 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.86 ± 0.05&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td>1.01 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0 ± 0.3&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>50:50</td>
<td>Defined</td>
<td>TGF-β1</td>
<td>10.7 ± 0.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.50 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.92 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>IGF-1</td>
<td>19.2 ± 1.0&lt;sup&gt;bc&lt;/sup&gt;</td>
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<td>1.00 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.2 ± 0.8&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>Both</td>
<td>10.4 ± 0.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.63 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.86 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.1 ± 0.5&lt;sup&gt;ab&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td>None</td>
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<td>5.40 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.7 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>FBS</td>
<td>TGF-β1</td>
<td>11.0 ± 0.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.00 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.79 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.3 ± 0.5&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IGF-1</td>
<td>24.6 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.75 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.07 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2 ± 0.8&lt;sup&gt;bcd&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Both</td>
<td>11.3 ± 0.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.98 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.78 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.0 ± 0.4&lt;sup&gt;bcd&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>None</td>
<td>22.6 ± 1.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.09 ± 0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.94 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.8 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 4.2: Phase II construct characteristics after 4 wks in culture
Data are represented as mean ± S.D. Letters refer to statistical analyses performed within each cell ratio. Values not connected by same letter are significantly different.

<table>
<thead>
<tr>
<th>Cell ratio (%MC:AC)</th>
<th>GF treatment</th>
<th>WW (mg)</th>
<th>Diameter (mm)</th>
<th>Thickness (mm)</th>
<th>Cells/construct (10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75:25</td>
<td>Continuous</td>
<td>12.2 ± 0.4c</td>
<td>3.98 ± 0.08b</td>
<td>1.44 ± 0.11b</td>
<td>5.2 ± 0.5a</td>
</tr>
<tr>
<td></td>
<td>Intermittent</td>
<td>14.6 ± 1.0b</td>
<td>4.21 ± 0.05b</td>
<td>1.54 ± 0.08ab</td>
<td>5.0 ± 0.3a</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>18.8 ± 1.3a</td>
<td>4.78 ± 0.23a</td>
<td>1.70 ± 0.15a</td>
<td>4.0 ± 0.4b</td>
</tr>
<tr>
<td>50:50</td>
<td>Continuous</td>
<td>13.1 ± 0.5b</td>
<td>4.28 ± 0.07b</td>
<td>1.46 ± 0.06b</td>
<td>5.8 ± 0.5a</td>
</tr>
<tr>
<td></td>
<td>Intermittent</td>
<td>14.2 ± 0.5b</td>
<td>4.42 ± 0.01b</td>
<td>1.56 ± 0.06b</td>
<td>5.6 ± 0.5ab</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>19.1 ± 1.1a</td>
<td>4.94 ± 0.14a</td>
<td>1.70 ± 0.05a</td>
<td>5.0 ± 0.3b</td>
</tr>
</tbody>
</table>
Figure 4.1: Top and side views of phase I constructs after 4 wks of culture
The 100:0 constructs contracted significantly from the original 5 mm diameter, while serum supplementation led to a spherical morphology. The 75:25 and 50:50 constructs were of similar size. TGF-β1 supplementation led to smaller constructs than IGF-1 supplementation alone or no GF treatment in co-culture constructs. Scale markings are in mm.
Figure 4.2: Top and side views of phase II constructs after 4 wks of culture
Progressively greater contraction was observed with increased TGF-β1 supplementation.
Scale markings are in mm.
Figure 4.3: Collagen staining of constructs from phase I
Picrosirius red staining was more intense in the 100% FC constructs. Among the co-culture groups, TGF-β1 in defined medium led to more intense staining. IGF-1 and GF combination images were not shown due to their similarity to no-GF and TGF-β1 images. Bar represents 250 μm.
Figure 4.4: GAG staining of constructs from phase I
All co-culture groups showed the presence of GAGs, while only the background stained in the 100% FC groups. Among the serum treated groups, TGF-β1 treatment led to decreased GAG staining. IGF-1 and GF combination images were not shown due to their similarity to no-GF and TGF-β1 images. Bar represents 250 μm.
Figure 4.5: Collagen staining of constructs from phase II
Picrosirius red staining was more intense in TGF-β1 treated constructs than in no GF constructs. Bar represents 250 µm.
Figure 4.6: GAG staining of constructs from phase II
Safranin-O staining was more intense in TGF-β1 treated constructs than in no GF constructs. Bar represents 250 μm.
Figure 4.7: Biochemical and biomechanical properties of constructs from phase I
Collagen/WW (a) and GAG/WW (b) were higher for the co-culture groups than for the 100% FC groups. TGF-ß1 treatment significantly increased instantaneous (c), relaxed (d), and Young’s moduli (e) relative to IGF-1 and no GF treatment. Columns and error bars represent means and standard deviations. Capital letters represent statistical significance in a 3-way ANOVA (cell ratio, growth factor, and serum treatment), and lower case letters denote significance within each cell ratio. Groups denoted by different letters are significantly different ($p < 0.05$).
Figure 4.8: Biochemical and biomechanical properties of constructs from phase II
Collagen/WW (a) was greater with increasing TGF-β1 treatment, though GAG/WW (b) was not affected by TGF-β1 supplementation. Instantaneous (c), relaxed (d), and Young’s moduli (e) were higher with continuous and intermittent TGF-β1 supplementation than without GF treatment, and intermittent treatment led to increased biomechanical properties relative to no GF treatment. Capital letters represent statistical significance in a 2-way ANOVA (cell ratio, GF treatment), and lower case letters denote significance within each cell ratio. Groups denoted by different letters are significantly different ($p < 0.05$).
Though skin represents a promising source of autologous cells for cartilage tissue engineering, several obstacles within the current cell culture protocol necessitate further optimization. These include considerable supply costs involved with the current protocol, inhomogeneity of cell differentiation, and low cell yield. This chapter describes a series of refinements that address each of these issues. First, a low-cost surface coating capable of inducing dermis cell chondrogenesis was identified. Second, a procedure was developed and optimized to ensure consistent surface coverage and thus reduce inhomogeneity of cell differentiation. Finally, the effects of multiple passages on dermis cell growth and chondrogenesis were explored. The result is a more effective and cost-efficient process capable of engineering larger tissues.
Introduction

The *in vitro* methodologies for cartilage and fibrocartilage tissue engineering developed in our laboratory have resulted in constructs with clinical dimensions and functional properties approaching native values [154, 155, 160, 191]. The utility of these approaches towards treatments for cartilage regeneration is dependent on the development of clinically relevant cell sources. Extensive research has demonstrated chondrogenesis in non-primary cells, including those derived from bone marrow [211], adipose tissue [212], muscle [213], synovium [214], periosteum [215], and skin [216]. Of these, skin is perhaps the most clinically favorable cell source considering its relative abundance and accessibility. Previous studies have demonstrated the isolation of a dermis cell subpopulation capable of chondrogenesis when plated on surfaces coated with the cartilage-derived proteoglycan, aggrecan [198, 216]. Upon this substrate, the cells, termed dermis-isolated, aggrecan-sensitive (DIAS) cells, aggregate and upregulate expression of collagen type II and aggrecan, while downregulating expression of collagen type I.

While these findings are indeed promising, issues with the DIAS cell culture protocols necessitate further refinement. First, the cost of aggrecan is prohibitive, accounting for over half of the total supply costs for these experiments. The exact mechanism of the aggrecan-mediated chondroinduction process is not well understood, though it was suggested that a crucial step is cell aggregation and formation of nodules, as shown in Fig. 5.1 [198, 216]. In these bodies, the majority of cells have no interaction with the substrate, though they continue to increase upregulation of genes for cartilage-specific matrix components. This suggests that cells continue to undergo phenotypic
changes independent from cell-matrix interactions. Therefore, identification of an alternative substrate that induces nodule formation could lead to similar phenotypic changes. Chondroitin sulfate (CS) is the predominant GAG associated with the aggrecan core protein (Fig. 1.6a). Importantly, it is a relatively inexpensive molecule, and substitution of aggrecan with CS would dramatically reduce the material costs of future studies.

Second, the method for applying aggrecan to surfaces used in the previous studies results in a non-uniform substrate. The aggrecan surfaces were prepared by coating the entire well with a thin layer of aqueous solution. Drying proceeds more quickly at the center of the well leaving that area uncoated while the proteoglycan accumulates around the edges. Unsurprisingly, seeded cells demonstrate location-dependent variation in morphology. Those found on the coated areas assume a rounded morphology and form nodules, while those on the uncoated areas attach and spread. An optimized process would create a consistent surface coating capable of inducing a more homogeneous cell response.

Another obstacle is obtaining a sufficient number of cells for engineering larger tissues. The average dermis cell yield of 5-12 million/cm\(^2\) of skin is not adequate for engineering large tissues such as the TMJ disc. Yield could be increased by greater expansion and passaging, however this can lead to alterations in cell phenotype. For example, prolonged passaging of bone marrow derived mesenchymal stem cells reduces their clonogenicity and chondrogenic potential [217]. Whether and at what passage phenotypic changes occur in DIAS cells is unknown.
To address these issues, efforts to refine the DIAS cell culture protocols were undertaken. DIAS cells were seeded on surfaces coated with aggrecan and CS to compare nodule formation qualitatively and quantitatively. CS solutions were applied drop-wise in the center of the well rather than on the entire well surface to eliminate edge effects. Additionally, the concentration of solution was adjusted to create a consistent and durable substrate. Assessment of the coated surfaces consisted of rinsing with water and staining wells with safranin-O, then examining nodule formation on the optimized coatings. Finally, the optimized surface coating was utilized in a study examining the effects of passaging on DIAS cell expansion and differentiation. At each passage, cell attachment proceeded for only 10 min to continue selecting for the fast-attaching subpopulation. Monolayer cells were assessed for cell morphology and clonogenicity, and 3D cultures were assessed through histological and quantitative biochemical analyses. The specific hypotheses tested in these studies were that nodule formation could be induced by CS coated surfaces, and that passaging would affect DIAS cell morphology, clonogenicity, and chondrogenesis.

Material and Methods

*Dermal Fibroblast Isolation*

Full-thickness abdomen skins from seven adult goats were obtained from a local abattoir. The dermis was isolated from adipose and epidermal tissue, minced, and digested overnight in medium containing 0.2% type II collagenase (Worthington, Lakewood, NJ) at 37°C with gentle agitation. Base medium consisted of DMEM with 4.5 g/L glucose and L-glutamine (Gibco, Grand Island, NY), 1% penicillin/streptomycin/fungizone
(Biowhittaker, Walkersville, MD), and 1% non-essential amino acids (Life Technologies, Gaithersburg, MD). Resultant cell suspensions were diluted with expansion medium (base medium with 10% FBS [Biowhittaker]), filtered through 70 μm cell strainers (BD Falcon, Bedford, MA) and centrifuged at 300g for 5 min. Cell pellets were resuspended in expansion medium, combined, and plated in flasks.

Isolation of the DIAS Subpopulation

Upon reaching 80-85% confluence, cultures were treated with 0.5% Dispase (BD, Franklin Lakes, NJ) for 15 minutes and the non-adherent cells were discarded. After re-expansion to 80-85% confluence, cells were lifted using 0.25% trypsin and 5 mM EDTA (Sigma, St. Louis, MO). To isolate the chondroinducible (DIAS) subpopulation, the cell suspension was allowed to attach to tissue-culture polystyrene (TCP) flasks for 10 min, and floating cells were discarded.[198] The flasks were washed with PBS and cultured in expansion medium until reaching 80-85% confluence, then lifted using trypsin/EDTA.

Preparation and Seeding of Aggrecan and CS Coated Surfaces

Equal concentration solutions of CS and aggrecan were prepared and used to coat the well surfaces of TCP 24-well plates. A control surface of DI H2O was also prepared. After drying overnight, 2 x 10^5 cells were plated in expansion medium. After 24 h, the wells were filled with 1 ml of chondrogenic medium consisting of base medium with 50 μg/ml ascorbic acid-2-phosphate (Acros Organics, Geel, Belgium), 0.4 mM proline (Acros), 50 mg/ml ITS+ Premix (BD Biosciences, Bedford, MA), 10^{-7} M dexamethasone (Sigma), 10 ng/ml transforming growth factor β1 (TGF-β1) (Peprotech, Rocky Hill, NJ), 100 ng/ml recombinant human insulin-like growth factor (Peprotech), and 1% FBS. At 48 h, images
were taken at 5 locations on the surface and nodules were manually counted and normalized to surface area.

*Optimization of Surface Coatings*

CS solutions were prepared in the following concentrations: 0.64%, 0.32%, 0.16%, 0.08%, and 0.01%. A single 20 µl droplet was placed in the middle of each well and allowed to dry overnight. Plates were rinsed with water and stained with safranin-O to visualize coatings.

Only the 0.08% and 0.01% solutions created stable surface coatings, so these groups were selected to examine the temporal progression of nodule formation. As before, surface coatings were prepared drop-wise using 0.08% and 0.01% solutions. A 20 µl suspension of $2 \times 10^5$ DIAS cells in expansion medium was applied to the dried coating, and 500 µl of chondrogenic medium was carefully added at 4 h. Every other day, 250 µl of medium was exchanged for the duration of culture. Images of nodule formation were taken at 2, 12, and 48 h after seeding. By 48 h, all nodules had migrated into a single micromass.

*Passaging Effects*

Dermis cells were obtained from fresh skins and DIAS cells were isolated as described before. During each subculture, DIAS cells were allowed to attach for 10 min before rinsing with PBS. At passages 1, 2, 3, 5, and 8, cells were lifted and divided into groups for differentiation on CS surfaces (as described before) and colony forming units analysis. At passages 4, 6, and 7, cells were carried forward without assessment.

*Colony Forming Units (CFU) Assay*
To assess for clonogenicity, a CFU assessment was performed at each timepoint. Petri dishes (100 mm diameter) were filled with 10 ml of expansion medium, and $10^2$ cells were added. After 2 wks of undisturbed culture, dishes were fixed with methanol and stained with crystal violet. Colonies greater than 2 mm diameter were counted, and % CFU was determined as the number of colonies normalized to the number of seeded cells.

**Quantitative Biochemistry**

Samples were digested in 125 $\mu$g/mL papain (Sigma) for 18 hours at 60°C. After digestion, the samples were assayed for sulfated GAG content using the Blyscan GAG Assay Kit (Biocolor, Belfast, Ireland), total collagen using a hydroxyproline assay (Sircol™, Newtonabbey, Northern Ireland), and DNA content using the Picogreen® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR).

**Histology and Immunohistochemistry**

After 2 wks of culture on CS, samples were frozen and 14 $\mu$m-thick cryosections were taken. After fixation in 10% phosphate buffered formalin, collagen distribution was examined with picrosirius red, and GAG distribution was examined with safranin-O and fast green. Immunohistochemical (IHC) analyses for collagen types I and II were performed on acetone-fixed sections. Anti-collagen type I (US Biological, Swampsacott, MA) and anti-collagen type II (Cedarlane Labs, Burlington, NC) were applied to slides. Secondary antibodies and avidin-biotinylated enzymes (Vectastain ABC kit, Burlingame, CA) were applied, followed by DAB reagent (Vector labs), and slides were counterstained with hematoxylin.
Statistical Analysis

A total of 6 wells were examined for quantifying nodule formation on aggrecan and CS surfaces, and data were analyzed using a Student’s t-test. For CFU and quantitative biochemistry, n=6 was used. Data were analyzed with a one-way analysis of variance (ANOVA), using Tukey’s HSD post hoc were applicable. Significance was defined as \( p < 0.05 \), and data are reported as mean ± standard deviation.

Results

Nodule Formation on Aggrecan and CS Surfaces

At 48 h, nodules formed on aggrecan and CS, but not on control surfaces (Fig. 5.2). CS nodules where more isolated and showed more cell spreading around their periphery than aggrecan nodules. DIAS cells formed 330 ± 60 nodules/cm\(^2\) on CS, compared with 310 ± 40 nodules/cm\(^2\) on aggrecan, though the values were not significantly different (\( p = 0.7 \)).

Surface Coating Consistency and Stability

Coatings left behind by CS solutions greater than 0.08% released from the surface with washing, while the 0.08% and 0.01% solutions left stable coatings (Fig. 5.3). The 0.01% solution dried in a ring around the periphery of the original droplet area, while the 0.08% coating covered the majority of the original droplet surface area.

The temporal progression of nodule formation was examined on both surfaces (Fig. 5.4). At 2 h, cells on the 0.08% coatings were more aggregated than those on 0.01%. By 12 h, cells on 0.08% had formed distinct nodules, while those on 0.01% were...
attached to the surfaces. At 48 h, both groups had formed a single large cell mass (micromass) in the center of the well.

**Passage Effects – CFU Assessment**

Overall, there was a decrease in % CFU with passage ($r^2 = 0.76$), from a high of 64 ± 13% at P1 to a low of 6 ± 2% CFU at P8 (Fig. 5.5). Colony distribution at P1 and P8 can be seen in Fig. 5.6. Cells at early passages are smaller and less spread than those at later passages (Fig. 5.7).

**Passage Effects – Quantitative Biochemistry**

Cellularity remained constant for the first three passages, but decreased significantly between P3 and P5 (Fig. 5.8). P5 micromasses had 52% fewer cells than P3 ($p < 0.001$), and P8 micromasses had 24% fewer cells than P5 ($p < 0.001$). Total collagen content increased progressively with passage (Fig. 5.9a), from 14.9 ± 3.6 µg at P1 to 32.4 ± 6.3 µg at P8 ($p = 0.008$). Collagen content per cell was constant from P1 to P3, but there was a significant increase between P3 and P5, as it increased from 4.8 ± 2.6 µg per $10^5$ cells to 12.5 ± 4.0 µg per $10^5$ cells (Fig. 5.9b) ($p = 0.008$). Total GAG accumulation increased with passage to a maximum 6.2 ± 0.6 µg at P3, and then declined to 3.4 ± 0.8 µg at P8 (Fig. 5.9c). GAG deposition per cell increased with passage, from 0.6 ± 0.6 µg at P1 to 2.0 ± 0.9 µg at P5 (Fig. 5.9d).

**Passage Effects – Histology and Immunohistochemistry**

Representative images of stained sections from P1, P3, and P8 are shown in Fig. 5.10. Staining intensity for collagen type I increased significantly with passage. Collagen type II stained weakly in P1 groups, while no staining was apparent for P3 and P8. Collagen staining with picrosirius red was more diffuse for P1 and P3 sections than for P8 sections.
Finally, GAG staining was more intense in P3 and P8 sections than in P1 sections, though safranin-O staining was faint in all sections.

Discussion

Issues with the previous methods for chondrogenesis of DIAS cells necessitate refinement of cell culture techniques. The results of these experiments address several aspects of the existing protocol and improve DIAS cell chondrogenesis by reducing cost, improving homogeneity, and increasing potential cell yield. First, CS was identified as a low-cost substrate capable of inducing nodule formation. Second, the CS application process was refined to create a stable and consistent surface. The optimized surface induced temporal changes in cell morphology and aggregation consistent with the previous chondroinduction process on aggrecan, and in addition, showed no evidence of spread cells, which were present using the previous method. Finally, this work identified significant changes in DIAS cell morphology, clonogenicity, proliferation, and matrix synthesis with passage, though the results suggest phenotypic stability up to three passages.

Cell aggregation is a common step in various methodologies for progenitor cell chondrogenesis (e.g., pellet culture, micromass culture, embryoid body formation). In our system, seeded cells aggregate and condense into nodules and proceed to secrete a cartilaginous matrix. These steps mirror the prechondrification stages of cell migration, aggregation, and condensation during in utero cartilage development [218, 219]. The results of this study demonstrate the ability of CS to initiate this sequence. In comparing aggrecan to CS, qualitative differences were observed between nodules on both surfaces;
CS nodules had spread cells around their periphery and were less aggregated than aggrecan nodules (Fig. 5.2). It was hypothesized that this was the result of inferior application of CS in comparison to aggrecan, whose application had been optimized previously. Indeed, nodule formation on the optimized CS coating (Fig. 5.4) more closely resembled that shown on aggrecan.

After identifying CS as an alternative to aggrecan, a method to create a homogeneous and stable coating was sought. Homogeneous coating was achieved by applying the solution drop-wise rather than to the entire well. This eliminated edge effects from the well walls, and as a result, drying proceeded more evenly. The concentration of solution had an effect on both surface coverage and stability. In the lowest concentration (0.01%), the CS was able to accumulate around the edges as the solution dried leaving only a ring. At higher concentrations, the CS adsorbed evenly on the surface, however only the 0.08% solution remained intact after rinsing and staining.

Passaging DIAS cells led to significant changes in cell characteristics, though cells from P1 through P3 behaved similarly in monolayer and in 3D culture. The CFU assessment indicated that P1, P2, and P3 cells were the most replicative, with a significant decrease observed between P3 and P5, and a further decrease between P5 and P8. This trend was mirrored by the 3D cell proliferation results, which indicated that P1, P2, and P3 cells had the greatest number of cells, and that there was a significant decrease in proliferative capacity between P3 and P5. The opposite trend was apparent with respect to collagen and GAG synthesis, as P1, P2, and P3 cells deposited less collagen and GAG per cell than P5 and P8 cells. Taken together, these results suggest a transition from a proliferative phenotype at early passages to a more synthetic phenotype at later
passages. This change likely reflects a reduction in cell plasticity [217], rendering these cells less useful for the purpose of chondrogenesis. However, given that the primary phenotype persisted through three passages, these results signify that a far greater quantity of chondroinducible cells can be obtained than previously thought.

In conclusion, this work optimized the DIAS cell culture procedure by reducing cost, improving homogeneity, and increasing cell yield. The optimized methods were utilized in an examination of the effects of hypoxia during cell expansion and differentiation, which is described in the next chapter.
Figure 5.1: Nodule formation on aggrecan
(a) DIAS cells form nodules on aggrecan-coated surfaces. (b) DIAS cells attach and spread on uncoated surfaces. From Deng et al.[198]
Figure 5.2: DIAS cell morphology on aggrecan, CS, and control surfaces
Images were taken 48 h after seeding. Cells form nodules on aggrecan and CS substrates, and spread on uncoated substrates.
Figure 5.3: CS coatings stained with safranin-O
Only the 0.08% CS solution left a stable and consistent surface. The 0.01% CS solution and left a ring with an uncoated center, while solutions greater than 0.08% detached with rinsing and staining.
Figure 5.4: Progression of nodule formation on 0.01% and 0.08% CS coatings
Images were taken 2 h, 12 h, and 48 h after seeding. Cells were more aggregated on 0.08% than on 0.01% coatings at 2 h. By 12 h, cells formed nodules on 0.08% substrates but attached to the 0.01% surfaces. At 48 h, both groups contained a single cell mass.
Figure 5.5: Decrease in DIAS cell clonogenicity with passage
Values represent the number of colonies normalized to the number of cells seeded. Data are mean ± S.D. Groups not connected by letters are significantly different.
Figure 5.6: Stained colonies at P1 and P8
Images show single-cell derived colonies at P1 (left) and P8 (right), demonstrating reduction in CFU%.
Figure 5.7: Changes in cell morphology with passage
P1 cells (left) and P8 cells (right). Cells from early passages were smaller, while cells from later passages were larger and more spread. Scale = 100 μm
Figure 5.8: Cell proliferation during 2 wks of 3D culture on CS
Seeding was at $2 \times 10^5$ cells per sample. Cellularity was consistent through three passages, though it dropped significantly between P3 and P5. Data are mean ± S.D. Groups not connected by letters are significantly different.
Figure 5.9: Changes in ECM production with passage during culture on CS
Total collagen production and collagen production per cell increased with greater passage, though P1 through P3 groups were not significantly different. GAG production per cell increased with passage, though total GAG production reached a maximum at P3 and returned to early passage levels at P8. Data are mean ± S.D. Groups not connected by letters are significantly different.
Collagen type I staining became more intense with increasing passage. Collagen type II staining was weak for all groups, though small areas of staining are visible in P1 groups. Picrosirius red stained collagen more diffusely in P1 and P3 groups than in P8 groups. GAG staining (purple) was more intense in P3 and P8 groups than in P1 groups. Scale = 250 μm.
CHAPTER 6: Effects of Hypoxia on Expansion and Chondrogenesis of Dermis Cells *

Abstract

Dermis-isolated, aggrecan sensitive (DIAS) cells, a subpopulation of dermis cells capable of chondrogenesis in the presence of cartilage matrix, are a promising source of autologous cells for tissue engineering. Hypoxia has profound effects on \textit{in vitro} chondrogenesis and could improve the expansion and differentiation processes for DIAS cells. The goal of this study was to enhance chondrogenesis of DIAS cells through exposure to hypoxic conditions (5\% O$_2$) during expansion and/or differentiation. DIAS cells were expanded in hypoxic (5\% O$_2$) or normoxic (20\% O$_2$) conditions, then differentiated for 2 wks on chondroitin sulfate coated surfaces in both environments. Monolayer cells were examined for proliferation rate and clongenicity, and micromasses were assessed for cellular, biochemical, and histological properties. Differentiation in hypoxia following normoxic expansion increased per cell production of collagen type II 2.3 fold and GAGs 1.2 fold relative to continuous normoxic culture ($p < 0.0001$). Groups expanded in hypoxia produced 51\% more collagen and 23\% more GAGs than those expanded in normoxia ($p < 0.0001$). Hypoxia also limited cell proliferation in monolayer and in 3D culture. Hypoxic differentiation following normoxic expansion significantly enhanced chondrogenesis of DIAS cells, improving their utility for cartilage engineering.

Introduction

Hyaline articular cartilage and fibrocartilage of TMJ and knee meniscus lack the intrinsic ability for self-repair. Efforts to engineer tissues in vitro using primary cells have resulted in constructs with clinical dimensions and functional properties approaching those of native cartilage [154, 160] and fibrocartilage [155, 191]. A significant challenge on the path toward clinical translation of these efforts is development of appropriate cell sources. A variety of stem cells, both embryonic [188, 189, 193, 220] and adult [197, 221, 222], have been explored for their chondrogenic potential, and research shows that the dermis contains a population of cells capable of chondrogenesis [147, 216, 223, 224]. Recently, our group found that adult dermal fibroblasts are capable of chondrogenic differentiation when cultured on the aggrecan, the main proteoglycan found in cartilage [216]. We further showed that a subpopulation with high chondrogenic potential, termed dermis-isolated, aggrecan-sensitive (DIAS) cells, could be selected through rapid adherence to tissue culture polystyrene surfaces [198]. Nevertheless, observed differences in ECM content and biomechanical properties between engineered tissue from chondrocytes and DIAS cells motivate further optimization of the expansion and chondroinduction protocols.

Mesenchymal stem cells (MSC) are sensitive to ambient oxygen levels during in vitro expansion [225]. Reduced oxygen tension (<5%) has been shown to increase proliferation [226], enhance colony-forming efficiency [227], and prevent phenotypic drift [228] during monolayer culture of bone-marrow derived MSCs (bMSCs). In addition, adipose-derived stem cells (ASCs) expanded in hypoxia (2%) upregulate collagen II gene expression and increase sulfated GAG deposition in high density culture.
relative to normoxic expansion, suggesting that this may select of 'prime' those cells capable of chondrogenesis [229]. In dermal fibroblasts, reduced oxygen tension during monolayer culture modulates expression of a myriad of proteins, including those related to transcriptional control, metabolism, and matrix remodeling [230], though the effect of hypoxia during expansion on subsequent chondrogenic ability of dermis cells is unknown.

Reduced oxygen tension during differentiation of stem cells upregulates expression of cartilage-related genes [220, 231-234] and increases production of cartilage-specific matrix [231, 234-236]. A study by Mizuno et al. [237] showed enhanced chondrogenesis of immature dermal fibroblasts seeded on collagen/demineralized bone powder sponges in low oxygen tension (5%), and increased production of hypoxia inducible factor-1α (HIF-1α), a known regulator of hypoxic chondrogenic differentiation [238]. Based on these results, it was hypothesized that chondrogenesis of the adult dermis cells used in this study would also be enhanced in low ambient oxygen.

In an effort to refine the expansion and differentiation processes for DIAS cells, and to gain an understanding of how hypoxia affects the chondrogenesis of adult dermal fibroblasts, this study examined the effects of hypoxia during expansion and chondroinduction. We hypothesized that exposure to hypoxia during expansion and/or differentiation would enhance chondrogenesis of DIAS cells. The primary criterion for evaluating chondrogenesis was collagen type II production during differentiation, both overall and relative to cell number and total collagen production. Sulfated glycosaminoglycan (GAG) production was also quantified, and deposition of collagen
types I and II, total collagen, and GAGs were examined histologically. In addition, we
examined proliferation and colony forming units (CFU-F) in monolayer to evaluate the
effects of hypoxia on the cell-growth characteristics of DIAS cells.

Materials and Methods

Dermal Fibroblast Isolation

Full-thickness skins from the abdomens of seven adult goats were obtained from a local
abattoir. The dermis was isolated, minced, and digested in medium containing 0.2% type
II collagenase (Worthington, Lakewood, NJ) at 37°C with agitation. Base medium
consisted of DMEM with 4.5 g/L glucose and L-glutamine (Gibco, Grand Island, NY),
1% penicillin/streptomycin/fungizone (Biowhittaker, Walkersville, MD), and 1% non-
essential amino acids (Life Technologies, Gaithersburg, MD). Digests were diluted with
expansion medium (base medium with 10% FBS [Biowhittaker]), filtered, and
centrifuged at 300g. Cells were resuspended in expansion medium, combined, and plated
in flasks.

Expansion in Hypoxia and Normoxia and Isolation of a Chondroinducible Subpopulation

Cells were cultured separately in hypoxic (5% O₂) or normoxic (20% O₂) incubators. All
liquids were preconditioned in 100 mm petri dishes in the respective incubators for >12 h
prior to use on cell cultures to acclimate to environmental O₂ levels [239]. Upon reaching
80-85% confluence, cultures were treated with 0.5% Dispase (BD, Franklin Lakes, NJ)
for 15 minutes and the non-adherent cells were discarded. After expansion to 80-85%
confluence, cells were lifted using trypsin and EDTA (Sigma, St. Louis, MO). To isolate
the chondroinducible subpopulation (DIAS), the cell suspension was exposed to tissue-
culture polystyrene (TCP) flasks for 10 min, and floating cells were discarded [198]. The flasks were washed 3x with PBS and cultured in expansion medium until reaching 80-85% confluence (~5 d), then lifted using trypsin and EDTA for monolayer expansion assays and differentiation culture.

Assessment of Monolayer Proliferation and Clonogenesis

DIAS cells were resuspended and $10^4$ cells were added to 100 mm petri dishes with expansion medium and cultured in hypoxic or normoxic incubators. At each time point (d 0, 1, 3, 5, 7, 9, and 11), cells were collected and frozen at -80°C. After collecting cells from each time point, cell number was determined in using Picogreen® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR).

For fibroblast CFU (CFU-F) assessment, $10^2$ DIAS cells were cultured undisturbed in 100 mm Petri dishes for 2 wks. Dishes were fixed with methanol, stained with 2% crystal violet, and colonies greater than 2mm diameter were counted. The % CFU-F was determined as the number of colonies normalized to seeded cells.

Chondrogenesis on Coated Surfaces

As illustrated in Fig. 6.1, cells from both expansion environments (hypoxia, normoxia) were cultured in both environments during differentiation (Hypoxia -> Hypoxia (HH), Hypoxia -> Normoxia (HN), Normoxia -> Hypoxia (NH), Normoxia -> Normoxia (NN)). Chondrogenesis was initiated on chondroitin-coated substrates. Coated surfaces were prepared in 24-well TCP plates. A sterile 0.08% chondroitin sulfate (Sigma) solution was prepared and 20 μl was dropped into each well and allowed to dry overnight.

DIAS cells were suspended in chondrogenic medium consisting of base medium with 50 μg/ml ascorbic acid-2-phosphate (Acros Organics, Geel, Belgium), 0.4 mM
proline (Acros), 50 mg/ml ITS+ Premix (BD Biosciences, Bedford, MA), $10^{-7}$ M dexamethasone (Sigma), 10 ng/ml transforming growth factor β1 (TGF-β1) (Peprotech, Rocky Hill, NJ), 100 ng/ml recombinant human insulin-like growth factor (Peprotech), and 1% FBS. 2 x $10^5$ cells were seeded in a 20 µl droplet on the dried surface. After 4 hrs, 500 µl of chondrogenic medium was carefully added around the condensed cell mass, and 250 µl of media was exchanged every other day for a total of 2 wks.

Quantitative Biochemistry

After 3 wks, the contents of four wells were combined to make one sample for quantitative biochemical analysis. Samples were digested in pepsin (10 mg/ml) with acetic acid, followed by pancreatic elastase (1 mg/ml) in Tris buffer at 4°C. Cellularity was determined using the Picogreen® kit. Total sulfated GAG content was determined using a dimethylmethylene blue (DMMB) dye-binding assay kit (Biocolor, Newtownabbey, Northern Ireland). Total collagen content was determined with a hydroxyproline assay using Sircol™ standards (Biocolor) [205].

Collagen type II was quantified using an indirect enzyme-linked immunosorbent assay (ELISA). Samples and standards were incubated in a 96-well plate overnight at 4°C. Wells were blocked with BSA overnight at 4°C, then exposed to a primary antibody, anti-collagen type II IgG (Cedarlane Labs, Burlington, NC) for 1 h at 20°C. The secondary antibody, anti-IgG horseradish peroxidase (Millipore, Temecula, CA) was then applied for 1 h at 20°C. Between each incubation step, wells were washed 3x with 0.05% Tween-20. Results were visualized at 450 nm using a TMB substrate.
Histology and Immunohistochemistry

Micromasses were cryo-sectioned at 14 µm. Histology sections were fixed in 10% phosphate buffered formalin and stained with safranin-O/fast green to examine GAG distribution, and picrosirius red to examine collagen. Immunohistochemical (IHC) analyses for collagen types I and II were performed on acetone-fixed sections using primary antibodies from US Biological (anti-collagen type I, Swampsacott, MA) and Cedarlane Labs (anti-collagen type II). Secondary antibodies and avidin-biotinylated enzymes (Vectastain ABC kit, Burlingame, CA) were applied, followed by DAB reagent (Vector labs), and slides were counterstained with hematoxylin.

Statistical Analyses

For proliferation assays, n=3 was used at each time point, while n=5 was used for CFU-F and quantitative biochemistry. Data were analyzed with a two-factor analysis of variance (ANOVA), using Tukey’s HSD post hoc where applicable. Significance was defined as $p < 0.05$, and data are reported as mean ± standard deviation.

Results

Cell Growth and CFU-F

The examination of cell proliferation revealed differences in cell growth kinetics in hypoxic and normoxic culture (Fig. 6.2a). There was no statistical difference between the groups over the first 5 d of growth, though normoxic culture resulted in a statistically significant increase in cell number at days 7, 9, and 11, compared with hypoxic culture.
Cells cultured in normoxia also had a higher CFU-F (63 ± 8%) than hypoxic cultured cells (35 ± 4%) (Fig. 6.2b).

**Cellularity of Micromass Cultures**

Cellularity increased during the 14 d of 3D culture for all treatments (Fig. 6.3). Hypoxic differentiation decreased cell growth 26% ($p < 0.0001$) relative to normoxic differentiation, while hypoxic expansion led to a 9% decrease ($p = 0.04$) in cellularity of micromasses relative to normoxic expansion. There were no statistical differences between the NN and NH groups ($p = 0.8$), or NH and HH groups ($p = 0.2$).

**Total Collagen Production**

Data for total collagen accumulation during differentiation are shown in Figs. 6.4a and 6.4c. Cells expanded in hypoxia produced 51% more total collagen than normoxia-expanded cells ($p < 0.0001$), while hypoxic differentiation increased collagen production 17% ($p = 0.004$). Hypoxic differentiation also led to a per cell increase in collagen production of 59% ($p < 0.0001$) relative to normoxic differentiation.

**Collagen Type II Production**

Data for collagen type II accumulation during differentiation are shown in Figs. 6.4b and 6.4d. Hypoxic expansion decreased total accumulation of collagen type II 39% and per cell accumulation 38%, while hypoxic differentiation increased total accumulation of collagen type II 62% and per cell accumulation 85% ($p < 0.0001$ for all comparisons). The NH group produced 1.6 fold more collagen type II ($p < 0.0001$) and 2.3 fold more collagen type II per cell ($p < 0.0001$) than the NN group. HN micromasses contained 95% more collagen type II ($p < 0.0001$) and 37% more collagen type II per cell ($p = 0.02$) than NN micromasses. Collagen type II/total collagen (Fig. 6.4e) was 1.3 fold greater for
groups expanded in normoxia \((p < 0.0001)\) relative to hypoxic expansion and the ratio was increased 23\% \((p = 0.003)\) with subsequent hypoxic differentiation. The NH group had a collagen type II/total collagen ratio of 0.15, statistically greater than all other groups.

**GAG Production**

Data for sulfated GAG content are shown in Fig. 6.5. Hypoxic expansion increased total sulfated GAG production by 23\% \((p < 0.0001)\), while hypoxic differentiation increased GAGs by 15\% \((p < 0.0001)\). Similar trends were apparent when normalized to cell number. For normoxia-expanded groups, hypoxic differentiation increased GAG by 73\% and increased GAG per cell by 124\% over normoxic differentiation \((p < 0.0001)\). For the hypoxia-expanded groups, hypoxic differentiation decreased total GAG by 16\% \((p < 0.0001)\) but increased per cell GAG production by 19\% \((p = 0.01)\).

**Histological Evaluation**

Representative pictures from the histological examination of micromass ECM from all groups are presented in Fig. 6.6. Immunostaining revealed the presence of collagen types I and II in all constructs. Collagen type I staining was strongest in the HH group, and weakest in the NN group. Collagen type II staining was more intense in the NH group, while staining in the NN and HH groups was the least intense. All groups stained brightly for collagen with picrosirius red and there were no apparent differences among groups. The HN and NH groups appeared purple with safranin-O/fast green, indicating greater GAG staining relative to the HH and NN groups which stained blue.
Discussion

The overall aim of this study was to enhance in vitro chondrogenesis of DIAS cells by examining the effects of reduced oxygen tension. Cells were cultured in hypoxic (5% O₂) and normoxic (20% O₂) environments during expansion and differentiation, and chondrogenesis was assessed through analysis of ECM production and cell-growth characteristics. Hypoxic differentiation (NH) dramatically increased production of collagen type II and increased the ratio of collagen type II to total collagen production compared to continuous normoxic culture (NN), and was therefore identified as the best treatment to enhance chondrogenesis of DIAS cells. In addition, hypoxic expansion (HN and HH) significantly increased total matrix production relative to continuous normoxic culture (NN). These results demonstrate the potent effects of oxygen tension on the chondrogenic ability of DIAS cells, and improve the utility of these cells for cartilage and fibrocartilage tissue engineering.

This study is the first to quantify collagen type II production by dermis cells. The group that produced the greatest amount (NH) synthesized 1.3 μg of collagen type II per $10^5$ cells, on par with reported values for ASC chondrogenesis at 2 wks [240]. The quantity and composition of matrix produced by stem cells is among the most important factors for evaluating their suitability for tissue engineering applications. Collagen type II is produced in significant quantities almost exclusively in cartilaginous tissues. It is the most abundant protein in hyaline articular cartilage, while fibrocartilages from the TMJ and knee meniscus contain varying ratios of collagen type II to other collagens [31]. The production of this protein in comparison with other matrix constituents can provide a quantitative measure of the chondrocytic character of stem cells following chondrogenic
treatments. In this study, 15% of the total collagen produced by the NH group was type II, compared to 8% for the NN and HN groups and 3% for the HH group. These values are all indicative of a fibrochondrocytic phenotype, though hypoxic differentiation advanced the cells further towards a chondrocytic phenotype.

In this study, we also examined the effect of hypoxic expansion on subsequent chondrogenesis and explored whether pre-differentiation exposure to hypoxia would select for or 'prime' the chondrogenic subpopulation as has been demonstrated for ASCs [229]. A similar result was not evident from these data. Continuous hypoxic exposure (HH) increased overall matrix production but reduced the collagen type II to total collagen ratio. Coupled with the monolayer expansion assessments that showed decreased proliferation and clonogenesis, the results indicate a phenotypic switch from mitotic to biosynthetic activity. Hypoxic monolayer culture of fibroblasts is known to upregulate expression of TGF-β1[241], and α1(I) procollagen [242, 243], and increase synthesis of collagen type I[244], while limiting cell proliferation [245, 246]. In this study, we can speculate that exposure of cells to hypoxia during monolayer expansion limited the chondrogenic ability of DIAS cells prior to differentiation in 3D by reducing cell plasticity and committing cells to a synthetic phenotype. Interestingly, the collagen type II per total collagen ratio of the HN was unchanged relative to the NN group, indicating (at least partial) reversal of this response upon reoxygenation, a phenomenon supported by the literature [246].

Methodologies that facilitate in vitro stem cell chondrogenesis typically have a basis in physiological processes or characteristics of native cartilage. In this and our previous study [198], DIAS cells were grown on physiological surfaces; the cartilage-
derived matrix molecule aggrecan, and its predominant GAG, chondroitin sulfate. These surfaces induce a physiological response in which cells assemble into high-density aggregates, reminiscent of mesodermal cell condensation which precedes nascent cartilage development in utero [247]. The reduced oxygen level utilized in this study is similar to that experienced by native chondrocytes, estimated to be 1-8% depending on tissue location [248, 249]. In this study, concurrent application of signals derived from native cartilage physiology, comprising high-density cell culture, cell-matrix interactions, soluble chondrogenic agents, and reduced oxygen tension, enhanced chondrogenesis. Exploration into temporal application of these and additional stimuli, motivated by native cartilage physiology, will likely enhance this process further.

In conclusion, this study demonstrates enhancement of DIAS cell chondrogenesis by applying hypoxic culture conditions. Translation of in vitro cartilage regeneration models will require identification of clinically useful cells, and the use of skin as a donor tissue is especially promising due to the ease of procurement and negligible damage to the donor site. The final goal of this work is to use patients' own skin cells to create functional, autologous tissues for cartilage repair and replacement. Building on our previous study, which demonstrated the isolation and differentiation of a chondroinducible subpopulation from the dermis, these findings provide an important advancement towards that goal.
Figure 6.1: Schematic diagram of the hypoxia study
All steps are carried out in both oxygen environments. 1) Expansion of dermis cells in monolayer. 2) Isolation of a chondroinducible subpopulation. 3a) Differentiation of DIAS cells in micromass culture on CS surfaces. 3b) Concurrent culture of DIAS cells in monolayer to examine proliferation and clonogenesis.
Figure 6.2: Proliferation and clonogenesis of cells in monolayer
Cells proliferated faster in normoxia than hypoxia (a). Cell number was greater in normoxia at days 7 ($p = 0.04$), 9 ($p = 0.04$), and 11 ($p = 0.002$). CFU-F was greater in normoxia than hypoxia ($p < 0.0001$) (b).
Figure 6.3: Cell proliferation during 3D culture
Hypoxia limits proliferation during 3D culture. Cells were seeded at $2 \times 10^5$ per micromass. Data are mean ± S.D. Groups not connected by letters are significantly different.
Figure 6.4: Collagen accumulation during micromass culture
Hypoxic expansion led to increased total collagen accumulation (a) and collagen accumulation per cell (c) during differentiation. Overall collagen type II production was greatest in the NH group (b) as was collagen type II production per cell (d) and relative to total collagen production (e). Data are mean ± S.D. Groups not connected by letters are significantly different.
Figure 6.5: GAG accumulation during micromass culture
Cells exposed to hypoxia (HH, HN, NH) produced more GAG overall (a) and per cell (b) relative to continuous normoxic culture (NN). Data are mean ± S.D. Groups not connected by letters are significantly different.
Figure 6.6: Histological and IHC of the micromasses after 2 wks
Collagen type I staining was strongest for HH (first row), while collagen type II staining was strongest for NH (second row). All groups stained for collagen (third row), while greater GAG staining was apparent for HN and NH (fourth row). Scale = 250 μm.
CONCLUSIONS

The cumulative results of this work address many aspects of TMJ tissue engineering research. Characterization studies are the roadmap by which tissue regeneration efforts are guided, and the interspecies characterization study represents the most comprehensive effort to date. By applying consistent testing methodologies to multiple animal models, direct quantitative comparisons can be made with human tissue for the first time. The second aspect addressed by this thesis is the development of a methodology for engineering fibrocartilage. Application of an optimized growth factor regimen to primary cell co-cultures was shown to recapitulate several characteristics of the native tissue. Finally, this thesis addressed a need to develop clinically relevant cell sources by enhancing the potential utility of dermis-derived cells toward fibrocartilage regeneration. Taken together, these findings represent a significant step in the development of technologies for TMJ fibrocartilage regeneration.

The interspecies characterization study (Chapter 2) highlighted differences among human and animal tissues. Human discs were significantly stiffer than animal models under tension, though under compression, they were softer. The human discs also contained fewer cells overall, and did not display topographical variation in cellularity as the others did. In contrast, notable similarities between the species were also apparent, especially in the topographical variation of mechanical properties. All species displayed anisotropic tensile properties; the intermediate zone was nearly 2.5 times weaker and 5 times softer under mediolateral strain than under anteroposterior strain overall. Also, the instantaneous and relaxed compressive moduli were generally higher in the bands than in the intermediate zone. A primary objective of this study was to identify the animal model
most similar to the human based on quantitative comparisons of functional properties. While no model was found to be equivalent to the human, the pig had similar compressive properties, collagen and GAG content, and gross dimensions. Therefore, these results support the prevailing assumption that the pig is the most appropriate animal model.

Though the pig was identified as the ‘gold standard’ animal model in this study, these results do not preclude the use of other animal models for TMJ research. Performing \textit{in vivo} cartilage regeneration studies in pigs may not be the best choice for early-stage investigations due to the substantial practical limitations of this species related to its size and cost. In such cases, the use of small animal models, such as the rabbit, may be warranted. Therefore, an equally important outcome of this study is the identification of design criteria for engineering animal-specific tissues.

In as much as TMJ disc cells have shown inherent limitations, specifically their relative paucity in the tissue and inability to produce sufficient matrix \textit{in vitro} [129], this study for the first time identifies cells from the knee as useful for TMJ cartilage regeneration. Primary TMJ disc cells are unlikely to fulfill the cell needs for autologous tissue regeneration, as those individuals in need of treatment will likely have diseased tissue. Therefore, the focus of this study was to establish a methodology for fibrocartilage regeneration using a readily available and functionally appropriate cell source (immature bovine chondrocytes and fibrochondrocytes) to serve as a model for future exploration with autologous cells. After establishing an appropriate animal model, a methodology for engineering biomimetic tissue was developed (Chapter 4) using these cells. This work utilized a scaffoldless approach, well established for engineering articular cartilage [154,
160, 204, 250-252], and applied it to the TMJ disc by examining co-cultures of fibrochondrocytes and chondrocytes.

Important conclusions can be deduced from the results in Chapter 4. First, fibrochondrocyte constructs are not able to maintain their shape in this culture system. A minimum percentage of articular chondrocytes (< 25%) must be included in the cultures, or a feature that accounts for and opposes this contraction, like a center post [155], must be incorporated into the mold design. Second, application of growth factors and serum are powerful mediators of construct characteristics and properties. In serum-free medium, TGFβ treatment increased collagen production 25%, tensile stiffness 130%, and instantaneous compressive modulus 170% over controls. Values for compressive modulus reached 790 kPa (instantaneous) and 120 kPa (relaxed), both overlapping native values (Chapter 2). The greatest tensile modulus obtained was 1.9 MPa, approaching the low end of native values. Furthermore, serum treatment had profound effects of construct morphology, especially in 100% fibrochondrocyte constructs.

The foreseeable applications of the results from Chapter 4 are two-fold. The first is towards an autologous approach using differentiated adult stem cells. The studies described in Chapters 5 and 6 are designed to recapitulate, with dermis cells, the behavior of primary fibrochondrocytes and chondrocytes. In addition, tremendous research is underway examining the chondrogenic potential of adult stem cells from bone marrow [194, 195], adipose tissue [196, 197], and other sources [198, 199]. Considering the rapid advancement of these technologies, this application is indeed promising. The second potential application is in a xenogenic approach, where bovine-cell derived constructs would be used directly for in vivo regeneration of TMJ fibrocartilage. Research supports
the hypothesis that articular cartilage is immuno-privileged as a result of its avascularity [253, 254], though similar studies have not been performed on TMJ fibrocartilage. This application is inherently more risky, though decellularization followed by repopulation with autologous cells could mitigate an immune response.

The final facet of TMJ tissue engineering addressed by this thesis was the development of a viable cell source (Chapters 5 and 6). There are not enough primary cells in the body to support full-scale tissue regeneration, with the added complication that patients seeking treatment are unlikely to have viable donor tissue. Of the adult cell sources under investigation (bone marrow, adipose tissue, synovium, etc.), skin is the most easily accessible and abundant source. Specific aim 3 refined dermis cell chondrogenesis procedures by reducing cost, improving homogeneity, increasing potential cell yield, and enhancing differentiation.

The results from specific aim 3 (Chapters 5-6), which describe the development of an improved methodology for expansion and differentiation of dermis cells, highlight several topics for discussion and future exploration. The first is the effect of the substrate on dermis cell chondrogenesis. Previously, our group demonstrated nodule formation and chondroinduction on aggrecan substrates, thus the name dermis-isolated, aggrecan-sensitive (DIAS) cells [198]. The first experiment in Chapter 5 demonstrated that chondroitin sulfate (CS) is also capable of inducing nodule formation. CS was chosen not only for its low cost, but also for its prominence on the aggrecan molecule, and therefore, the plausibility that aggrecan-induced nodule formation is a result of cellular interaction with CS. Supporting this hypothesis is recent work by Varghese et al. [255] demonstrating condensation and differentiation of mesenchymal stem cells (MSCs) on
CS-modified PEG scaffolds. If future studies can elucidate the mechanism of this cellular response, CS substrates could be fabricated to promote favorable cell-matrix interactions.

Another path for future exploration would be further examination of the phenotypic changes during monolayer culture of DIAS cells as a result of multiple passages (Chapter 5) and reduced oxygen tension (Chapter 6). Both manipulations reduced proliferation and clonogenicity significantly, and increased fibroblast-like matrix production, signaling a loss of plasticity and stem-like characteristics. Indeed, reduction of proliferation and clonogenesis, accompanied by a loss of plasticity, was observed in MSCs with multiple passaging steps [217]. With bone marrow-derived MSCs, similar phenotypic changes can be attenuated if monolayer expansion is performed at reduced oxygen levels [226-228]. Since the in vivo microenvironment of those cells is hypoxic [225], it was hypothesized that high-oxygen tensions were physiologically unfavorable. The oxygen levels experienced by DIAS cells in their native environment are unknown, but the results of Chapter 6, which suggest a loss of stem-like phenotype with hypoxic expansion, put forth the hypothesis that in vivo levels are closer to 20% O₂ than 5% O₂. Better knowledge of the native physiology of DIAS cells would guide further refinement of expansion conditions.

The chondrocyte microenvironment is better understood, and knowledge of its low oxygen levels guided the refinements described in Chapter 6. In that study, exposure of DIAS cells to hypoxia during differentiation significantly enhanced chondrogenesis, as demonstrated by a doubling of collagen type II production relative to total collagen synthesis. This builds upon a strategy designed to recapitulate aspects of native cartilage physiology during differentiation, including cartilaginous matrix (aggrecan, CS), high-
density culture, and chemical signals. A continuation of this approach would incorporate mechanical loading from hydrostatic pressure or direct compression.

While the refinements described in Chapters 5 and 6 represent important advancements in the dermis cell chondrogenesis procedures, significant challenges remain. One is to obtain a fully chondroinduced cell population. Further refinement of differentiation conditions will likely yield additional gains; however, any contamination of the chondrogenic subpopulation with terminal cells would limit the effectiveness of these efforts. Therefore, future studies should characterize the DIAS subpopulation toward developing more efficient isolation methods. A recent study by Bilgen et al. [199] demonstrated the isolation of a chondrogenic subpopulation of synovial fibroblasts by removing cells expressing CD14 (a marker for type-A macrophage-derived synoviocytes not present on MSC-like type-B synovial fibroblasts). Identification of markers that identify DIAS cells could lead to isolation methods that exploit these characteristics. Alternatively, purification could be accomplished at the back end of chondrogenesis by exploiting differences between differentiated and undifferentiated cells. Concurrent studies from our group achieved separation of differentiated embryonic stem cells using a density gradient [256], and highlighted differences in cellular mechanics between differentiated and undifferentiated cells [257]. Utilization of novel isolation methods at either end would further enhance the applicability of the methodologies developed in specific aim 3.

In summary, this thesis addressed many aspects of TMJ cartilage regeneration by enhancing our knowledge of native tissue structure and function, and by providing a framework for development of technologies for fibrocartilage regeneration using a
clinically favorable cell source. Though the primary focus of this research was the TMJ, the results of aims 2 and 3 are applicable to fibrocartilage engineering in general. The conceivable culmination of this and future work is to engineer animal-specific fibrocartilage for *in vivo* studies, and finally, for treatment of temporomandibular disorders.
REFERENCES


