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Diminished Matrix in Pathologic Intervertebral Discs and Tissue Engineered Solutions

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Diminished Matrix in Pathologic Intervertebral Discs and Tissue Engineered Solutions

by

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DISSERTATION

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ABSTRACT

Diminished Matrix in Pathologic Intervertebral Discs and Tissue Engineered Solutions

by

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Doctor of Philosophy in Bioengineering

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Back pain caused by degenerative disc disease is associated with significant costs and patient morbidity. Although previous studies have thoroughly investigated the matrix of degenerated discs, few studies have investigated the subset of degenerated discs that are specifically painful. Detailed knowledge of how these properties in painful discs compare to those of nonpainful discs will provide guidelines for the development of tissue engineered treatment. The goal of the current dissertation is to characterize the matrix of the painful disc and investigate nucleus pulposus tissue engineering.

We characterized painful and nonpainful discs that were harvested from waste tissue of human surgical patients. The mechanical properties, matrix properties, and matrix synthesis of these tissues were measured using mechanical indentation, hydration, biochemistry, histology, and gene expression. Our data indicated that the painful annulus had altered gene and protein expression of proteoglycan and collagen, and consequent diminished mechanical properties. In contrast, the painful nucleus had elevated gene expression of decorin and higher energy dissipation than the nonpainful nucleus.

Interestingly, gene expression data of several proteoglycans and collagens correlate with indentation and hydration properties.

In addition to characterizing the matrix properties of painful discs, we investigated the differentiation of human mesenchymal stem cells (MSCs) into nucleus pulposus cells for tissue engineering. Specifically, MSCs were seeded into a three-dimensional alginate scaffold, pretreated with growth factor, and stimulated with mechanical compression. The effect of compressive stimulation on cell differentiation was measured by gene expression of several chondrogenic markers, including aggrecan, collagen II, Sox9, collagen I, and collagen X. Our data indicate that growth factor treatment promotes production of chondrogenic matrix proteins, including proteoglycans and collagen II; however, compressive stimulation had no effect on gene markers of chondrogenic differentiation.

The results of this dissertation suggest that painful discs have diminished mechanical and matrix properties. Importantly, these diminished properties have previously been associated with pain mechanisms via disc hypermobility, stress concentrations, and reduced barriers to nerve infiltration. Although our investigation of MSC differentiation with compressive stimulation was inconclusive, our characterization of painful disc matrix may guide future attempts to regenerate painful degenerated discs.

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Chapter 1: Introduction

1.1 Back Pain and the Intervertebral Disc

Back pain is a prevalent problem that causes significant costs and patient morbidity. This symptom is the second most common reason for patient visits to the clinician (1). A previous population based study estimated that 84% of individuals had experienced low back pain during their lifetimes, and 11% of adults had been disabled by low back pain within six months of the survey (2). The costs associated with back pain include direct costs, such as office visits or surgical procedures, and indirect costs, which is typically from lost wages. In the United States, the total costs of back pain exceed $100 billion per year (3). In addition to monetary costs, depression is associated with chronic back pain (4).

Several potential sources of back pain exist. The spine consists of multiple tissues – ligaments, musculature, spinal nerve roots, facet joints, and the intervertebral disc – and several of these tissues can be sources of pain (5). For example, spinal stenosis causes

narrowing of the spinal canal, resulting in nerve irritation and pain. The facet joints or intervertebral disc may degenerate and cause pain. In addition to disc pain from degenerative changes, disc pain can also occur by disc herniation, where the nucleus of the disc bulges through an annular tear. While multiple causes of back pain exist, the following dissertation will focus on pain in the intervertebral disc. More specifically, this dissertation focuses on characterization and treatment of pathologic disc degeneration.

One barrier to disc treatment is a poor understanding of the pathologic disc matrix. Despite decades of research devoted to the intervertebral disc, relatively little information exists regarding the matrix features of painful discs that distinguish them from nonpainful discs. The intervertebral disc is a load bearing joint that provides cushioning to the spine. Given the structural demands of the disc, some important questions are: Do painful discs have compromised mechanical properties? If so, what matrix features specific to painful discs lead to compromised mechanics? The theme of this dissertation is to investigate these questions and set goals for treatments that aim to resolve disc pain.

This chapter introduces the background content required to understand the studies presented in the body of the dissertation. First, the reader is provided with a brief description of the functional anatomy of the disc, followed by the matrix constituents of the disc. Next, the chapter describes disc degeneration and pain. The following section describes current treatment strategies and future treatment with tissue engineering. Finally, a brief outline of the remaining chapters is provided.

1.2 Disc Anatomy

The intervertebral disc consists of three tissues: the nucleus pulposus, annulus fibrosus, and cartilaginous endplates (Figure 1-1). These three tissues work in concert to provide range of motion and withstand compressive loads.

The nucleus pulposus is the gelatinous tissue that resides in the center of the disc. This tissue has a high affinity for water due to its high proteoglycan content (6). The nucleus contains significantly more collagen II than collagen I, which functions to provide the structural framework for containing proteoglycans. The matrix of the nucleus lacks organization and is contained by the surrounding annulus fibrosus. The cells in the nucleus pulposus are chondrocyte-like with a round morphology. They survive in the low nutrition environment of disc while producing collagen II and aggrecan (6,7). As in other cartilaginous tissues, the large water content of the nucleus provides high compressive mechanical properties.

The annulus fibrosus forms the outer ring that surrounds the nucleus pulposus. This highly organized tissue consists of concentric lamellar sheets made up of collagen. The collagen fibers of these lamellar sheets are oriented at 60 degrees to the vertical axis and alternate in direction with each neighboring sheet (8). The primary collagens are collagens I and II, with an increasing ratio of collagen II to collagen I from the outer to inner annulus (9). The ground matrix lies between lamellar sheets, and consists of proteoglycan, elastin, and other collagens (10). Cells in the outer annulus fibrosus are fibroblastic, with an elongated shape and secrete collagen I. The inner annulus fibrosus is a transition zone, containing cells with mixed nucleus and annulus phenotype.

The cartilaginous endplates are located at the superior and inferior edges of the disc, and separate the rest of the disc from the vertebral body. This thin layer of cartilage contains chondrocytes. Because the disc is an avascular tissue, the cartilaginous endplates are the primary medium for nutrient and water transport in the disc (11).

The unique architecture of the intervertebral disc provides mobility and cushioning. The nucleus of the healthy disc retains water, which provides resistance to compressive force (12). Water in the disc pressurizes the nucleus and, as a result, the surrounding annulus experiences circumferential tensile forces. The high collagen content of the annulus provides strong tensile mechanical properties (13). In addition, the range of motion in the disc requires large strains in the annulus that are accommodated by the lamellar organization. In particular, the layered lamellae are able to slide relative to each other and subsequently recoil to due to the elastic fibers between layers (14). The disc is both large and complex, but able to withstand the high mechanical demands of daily living.

Because the disc is both large and avascular, it has poor healing capacity. The blood supply to the disc is confined to the periphery at the outer annulus and endplate. Thus, delivery of nutrients and removal of waste products is mediated by diffusion throughout the disc (11). This mechanism of transport creates a nutrient deprived region in the center of the disc. As a result, disc cells are acclimated to survive in hypoxic and acidic conditions. However, in extreme hypoxia and acid levels, disc cells will produce less matrix or possibly die (15-17).

1.3 Disc Matrix

Collagen

Several collagens contribute to the extracellular matrix of the disc, including collagen types I, II, III, V, VI, IX, X, XI, XII, and XIV (18). All collagens consist of three α chains that bind together forming a triple helix (19). Each α chain is made up of amino acid repeats that always include glycine and often include 4-hydroxyproline. These collagen helices form specific macromolecular structures depending on the collagen type. For example, collagen types I and II form fibrils that provide tensile strength to disc tissues. Collagen types IX, XII, and XIV are fibril-associated collagens, which can bind to the surfaces of collagen I and II fibrils. Collagen X forms a hexagonal network and is expressed by mature chondrocytes during terminal differentiation.

Collagen has a low turnover and is prone to intermolecular crosslinks (20). Collagens in the disc include enzyme-mediated crosslinks (e.g. pyridinoline crosslinks) and nonenzymatic crosslinks (e.g. advanced glycation endproducts (AGEs), such as pentosidine). These crosslinks can affect the mechanical properties of collagenous tissues. For example, prior studies report that AGEs increase the tensile stiffness of the annulus (21).

Proteoglycans

The disc contains large aggregating proteoglycans and small leucine rich proteoglycans (SLRPs). Aggrecan and versican are two of the large aggregating proteoglycans found in the disc. The most abundant proteoglycan in the disc is aggrecan. This proteoglycan contains branches of covalently bound glycosaminoglycans, which

consists of a combination of chondroitin sulfate and keratin sulfate. Hundreds of aggrecan molecules form aggregates by binding to hyaluronan (22). Due to the negative charge of the glycosaminoglycans, the resulting proteoglycan aggregates have a large negative charge that attracts water molecules. This feature of aggrecan is critical to the hydration properties of the nucleus, which enables the disc to bear compressive load. Versican has similar structure to aggrecan; however, it does not contain keratin sulfate and contains less chondroitin sulfate (23). Although it is thought to have functions similar to aggrecan (23), the role of versican in the disc is not well understood (24).

Several SLRPs are found in the disc, including decorin, lumican, fibromodulin, and biglycan. As the name implies, these proteoglycans have approximately 10 repeats of 24 amino acids with leucine residues (25). Several SLRPs are able to bind collagen fibrils and regulate fibril assembly. This has been demonstrated through alterations in collagen in knockout animal models. For example, decorin deficient mice have skin fragility due to irregular collagen fibril size (26). Fibromodulin-null mice have abnormal tendon morphology, with disorganized and abnormal collagen fiber morphology (27). SLRPs not only bind and interact with collagens, they can also interact with transforming growth factor β (TGF-β). In particular, biglycan, decorin, and fibromodulin are able to bind TGF-β, suggesting that SLRPs may be able to regulate TGF-β, a growth factor that regulates matrix synthesis (28).

1.4 Disc Degeneration

Disc degeneration is an age related process characterized by the breakdown of disc matrix and consequent diminished mechanical function. While matrix changes are normal with aging, the rate of degeneration varies between individuals and disc level within the same individual (29). Degenerated discs contain smaller proteoglycan aggregates and an overall decrease in proteoglycan content (30,31). This is accompanied by a loss of distinction between the nucleus and annulus (32). In degenerated discs, the outer annulus contains increased collagen II while the inner annulus and nucleus contain increased collagen I (33). During severe degeneration, the disc has an overall decrease in large aggregating proteoglycans and SLRPs (34). Decreased SLRPs in degenerated discs are associated with enlarged collagen fibrils and annulus disorganization. Other observations in degenerated discs include clefts in the nucleus and radial tears in the annulus (35). In addition to changes in disc matrix, degenerated discs have decreased concentration of viable cells.

The extracellular matrix of the disc is regulated by a balance of matrix synthesis and degradation. During degeneration, annulus and nucleus cells have decreased synthesis of aggrecan (34). Matrix degradation occurs by production and activation of matrix metalloproteinases (MMPs), which are zinc-dependent proteinases capable of degrading collagens and proteoglycans. MMPs are further regulated by tissue inhibitors of metalloproteinases (TIMPs), which function by binding and inhibiting active MMPs. MMPs 1, 2, 3, 7, 9, and 13 are elevated during disc degeneration (31,36,37), indicating that abnormal regulation of matrix is a contributing factor to disc degeneration.

The degenerative changes in disc matrix lead to altered disc mechanics. For example, the degenerated annulus has reduced tensile properties due to changes in matrix structure, organization, and composition (30,38). In addition, the degenerated nucleus has reduced energy dissipation, swelling stress, and aggregate modulus (39,40). The observed changes in mechanical properties with disc degeneration provide some guidance when investigating tissue engineered therapy. In particular, this may suggest a threshold value of proteoglycan content or mechanical properties to restore cushioning properties of the disc.

Further complicating these parameters, however, many asymptomatic individuals have magnetic resonance imaging (MRI) evidence of disc degeneration (41,42). This observation suggests that degenerated discs are not necessarily painful. Consequently, while previous studies have measured diminished mechanical properties that occur during disc degeneration, these studies include degenerated discs from asymptomatic individuals, and the conclusions therefore may not be relevant to patients.

1.5 Pain

If degeneration is not synonymous with pain, then what is the cause of disc pain? Ongoing research suggests that pain is characterized by innervation, inflammation, and hypermobility; however, the exact cause of pain is not well understood (43). Nerves in healthy discs are confined to the periphery of the annulus and have proprioceptive function. In contrast, nerves in painful discs have infiltrated the outer annulus or endplate and can extend into the nucleus. Nerves that penetrate the annulus can be found in

vascular granulation tissue that fills annular fissures. These zones of vascular granulation tissue found in painful discs are believed to be a wound healing response to annular injury (44). Inflammation has been detected in animal injury models, and may modulate innervation and matrix degradation in painful discs. Several animal models indicate that disc injury results in increased expression of several inflammatory cytokines, including interleukin-1 (IL-1), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α) (45,46). Importantly, upregulation of inflammatory cytokines can result in increased nerve growth factor and sensitization of nociceptors (pain sensing nerves) (47,48). Furthermore, inflammation can cause upregulation of MMPs, which degrade matrix and could compromise the mechanical properties of the disc (43). The resulting hypermobility can lead to abnormal tissue stress. Disc cells may respond to these abnormal stresses by secreting inflammatory factors. Future studies investigating the sources of innervation, inflammation, and hypermobility may improve disc pain therapies.

Diagnosis of discogenic back pain is conducted using a combination of clinical assessment, radiographic findings, and provocative discography. MRI can detect degeneration by evaluating hydration in the nucleus. In practice, nucleus hydration is proportional to signal intensity in T2-weighted MRI images (24). Although MRI is sensitive in detection of disc degeneration, it cannot distinguish between symptomatic and asymptomatic degenerated discs. Thus, MRI alone is not a sufficient diagnostic tool (49). Provocative discogram is considered the gold standard for diagnosis of discogenic back pain (50). This invasive procedure involves recreating patient pain by injecting contrast medium and pressurizing the disc of interest. A subsequent computed axial tomography (CT) scan is used to identify annular fissures. If the procedure successfully

provokes pain that is concordant with patient symptoms, then the discogram suggest that the disc of interest is the pain source. Despite the invasive nature of this procedure, it remains the most effective method of localizing the source of pain.

1.6 Treatment

Treatment for discogenic back pain involves conservative or invasive options depending on the case. Conservative treatment options include anti-inflammatory drugs, physical therapy, back school, and massage (51). This initial treatment modality aims to provide relief by addressing inflammation, muscle atrophy, or poor posture. If these conservative therapies fail to provide pain relief, invasive options are considered. Fusion of the adjacent vertebrae is a common surgical procedure used to treat discogenic back pain (52). During this procedure, the relative motion of the vertebrae adjacent to the painful disc is fixed. Generally, this is accomplished using a bone autograft placed between the vertebrae to promote union. Instrumentation (e.g. plates or screws) may be implanted to help stabilize the vertebrae. While fusion may provide pain relief by eliminating pathologic vertebral motion, it may affect the motion of adjacent segments and promote adjacent segment disease (53). Another invasive treatment option is total disc replacement (TDR), which replaces the disc with an implant that preserves some relative motion of adjacent vertebrae. While TDR preserves mobility in the motion segment, the relative improvement in patient outcomes of TDR compared to fusion remains controversial (54). Because treatment options such as spinal fusion or TDR have potentially undesirable outcomes, such as decreased range of motion, altered

biomechanics, and/or posterior muscle atrophy (55,56), alternative treatments that restore disc function are ongoing research topics.

1.7 Tissue Engineering

With continued research, a tissue engineered therapy may pose an alternative to the existing invasive treatments. The goal of a tissue engineered disc is to restore the matrix and mechanical properties of a painful degenerated disc. Such a therapy would restore the normal kinematics of the diseased motion segment. Several questions remain unanswered before these therapies become a reality (57). For example, which tissue(s) within the disc should be targeted for tissue engineered therapy? Potential targets include the annulus, nucleus, endplate, or some combination of the three. Depending on the tissue of interest, such a therapy requires a fibroblastic or chondrogenic cell source. Autologous intervertebral disc cells from adjacent levels would be a poor choice due to potential adjacent level morbidity and low cell density. Due to the lack of autologous disc cells available for transplantation, stem cells are an attractive cell source.

Stem cells are cells with multipotent differentiation capacity that are available for autologous transplantation. These unspecialized cells can self-renew and differentiate into various cell types when exposed to appropriate stimuli. Multiple types of stem cells exist, including embryonic stem cells and adult stem cells. Embryonic stem cells are associated with much controversy because they are harvested from an embryo that will no longer be able to develop into a human life. This technology raises a potential ethical concern that embryos are intentionally created (either through in vitro fertilization or an aborted fetus)

and destroyed for the sole purpose of harvesting embryonic stem cells. Furthermore, therapeutic cloning, a process that generates stem cells by combining patient cells with an enucleated ovum, raises religious and ethical concerns regarding the beginnings of life (58). Adult stem cells are less controversial than embryonic stem cells because their derivation does not involve use of embryos or cloning (59). More specifically, adult stem cells can be isolated from the bone marrow of the patient. This repository of adult stem cells provides the body with a cell source during turnover, and may be a cell source for tissue engineered therapy. In orthopaedics, mesenchymal stem cells (MSCs) are of particular interest because they can differentiate into osteogenic, chondogenic, and fibroblastic cells (60). These MSCs can be autologously harvested from the bone marrow. The subsequent challenge for the tissue engineer is to determine the appropriate cues that cause MSC differentiation into disc cells. Ongoing research indicates that transforming growth factor β (TGF-β) and mechanical stimulation can guide MSC differentiation.

The TGF-β family of cytokines includes TGF-βs, bone morphogenetic proteins (BMPs), activins, and related proteins (61). The TGF-β protein has three isoforms: TGF-β1, TGF-β2, and TGF-β3. TGF-β acts on a variety of cell types regulating cell growth, differentiation, and apoptosis (62). TGF-β can mediate transcription through the Smad signaling pathway. Specifically, TGF-β binds to the TGF-β type II receptor at the cell membrane which phosphorylates and activates TGF-β type I receptor. TGF-β type I receptor phosphorylates receptor-activated Smads, which form complexes with Smad4 that then translocate into the nucleus (62). These complexes can facilitate Sox9 regulation of collagen II (63). Importantly, collagen II is a key marker of chondrogenic

differentiation. Thus, TGF-β regulates MSC chondrogenesis through the Smad signalling pathway.

Studies with TGF-β stimulation of MSCs have demonstrated the chondrogenic potential of MSCs. Chondrogenic cells are characterized by expression of collagen II and aggrecan, and a rounded cell morphology. Treatment of MSCs with TGF-β, while in micromass or three-dimensional culture, induces these key features indicative of chondrogenic differentiation (64-67). Rather than maintaining a functional chondrogenic phenotype, however, they subsequently express markers of hypertrophy and terminal differentiation. In particular, these MSC cultures express collagen X and show histologic signs of calcification (65). This sequence of events is reminiscent of endochondral ossification – the developmental process for the growth of long bones. During endochondral ossification, mesenchymal stem cells differentiate into chondrocytes. These chondrocytes secrete a cartilaginous matrix and subsequently progress to a hypertrophic phenotype, as indicated by expression of specific markers like collagen X. Next, the matrix becomes calcified and the chondrocytes undergo apoptosis, making way for osteoblasts and bone matrix (68). While TGF-β stimulation of MSCs has sparked interest for an autologous chondrogenic cell source, the progression to the hypertrophic phenotype raises questions regarding the stability of MSCs for clinical use (69).

Mechanical forces can also stimulate MSC differentiation. In general, musculoskeletal tissues such as cartilage, bone, ligament, and disc experience a combination of tensile and compressive loads depending on tissue location and function. These applied loads promote tissue homeostasis. For example, cartilage experiences compressive loading, which has been shown to regulate chondrocyte matrix synthesis

(70-72). Tissue engineers with interest in finding a chondrogenic cell source have found that MSCs exposed to cyclic compressive loads express chondrogenic markers such as collagen II and aggrecan (73). On the other hand, tensile load, which is the predominant loading direction in the annulus fibrosus and other ligamentous tissues, promotes fibroblast biosynthesis (74,75). Similarly, MSCs exposed to tensile loads express fibroblastic markers such as collagen I, collagen III, and tenascin-C (76,77). With ongoing research, mechanical stimulation of MSCs will be refined to direct cell differentiation and generate functional tissue engineered constructs.

1.8 Dissertation Goals

The overall goal of this dissertation is to investigate the features of painful, degenerated intervertebral discs with the ultimate goal of tissue engineered therapy. It is well established that disc mechanical and matrix properties degrade with degeneration. However, prior studies include cadaveric tissues from donors with undefined back pain history. Since many degenerated discs are nonpainful, the clinical relevance of these prior observations is uncertain. Thus, the following dissertation focuses on painful discs rather than degenerated discs. The first objective of this dissertation is to characterize the matrix of painful discs by measuring mechanical properties, matrix content, and matrix synthesis. Characterization of the painful disc provides a target for the matrix and mechanical properties of tissue engineered treatments. The second objective is to investigate tissue engineered nucleus using MSCs as a cell source.

The remainder of this dissertation contains the following:

a. Chapter 2: In the first study, the mechanical properties and matrix content of painful degenerated discs are characterized and compared to those of nonpainful degenerated discs. These mechanical properties reflect the matrix constituents of the disc. To the author's knowledge, no study to date has measured the mechanical properties of painful degenerated discs.
b. Chapter 3: The second study details the matrix synthesis of painful degenerated discs and elucidates correlations between extracellular matrix synthesis and disc mechanical properties. During this study, gene expression of collagens, large proteoglycans, and small proteoglycans are used to measure synthesis of extracellular matrix constituents. These experiments identify specific matrix constituents that are elevated or suppressed in painful degenerated discs. In addition, the role of each matrix constituent in disc mechanics is clarified using correlation analysis.
c. Chapter 4: The final study of this dissertation investigates tissue engineered treatment of intervertebral discs. Specifically, this study attempts to refine MSC differentiation into nucleus pulposus cells using a combination of TGF-β treatment and mechanical stimulation. Ideally, the resulting treatment would regenerate damaged tissue, such that the matrix has properties resembling those of a nonpainful disc.
d. Chapter 5: Finally, the last chapter discusses concluding remarks and future directions.

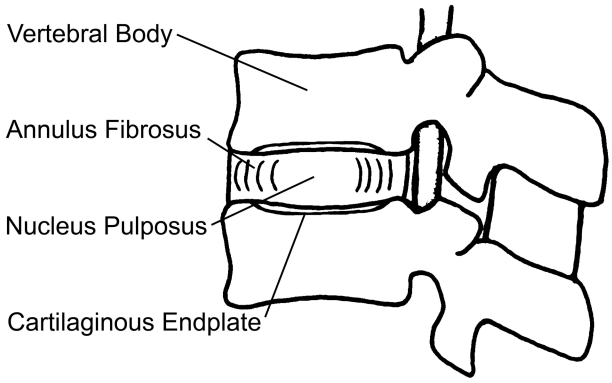

Figure 1-1: Basic anatomy of the intervertebral disc and the surrounding vertebral bodies. Image adapted from Adams et al. 1990 (78).

Chapter 2: Painful Degenerated Intervertebral Discs have Mechanical and Matrix Properties that Differ from Nonpainful Degenerated Discs

2.1 Introduction

Discogenic back pain is a challenging clinical problem to both diagnose and treat. While the etiology is uncertain in most patients, it can often be linked to deficits in tissue structure (79). The healthy intervertebral disc consists of an outer annulus fibrosus that surrounds the inner nucleus pulposus. The annulus consists of concentric lamellar collagenous rings. The nucleus comprises a proteoglycan-rich matrix that osmotically swells to generate hydrostatic pressure that resists spinal compression. Disc degeneration includes changes in matrix composition leading to deterioration of tissue mechanical

properties, such as nuclear depressurization, which can degrade overall spinal biomechanical behavior. Mechanical insufficiency, along with infiltration and sensitization of pain transmitting neurons (nociceptors), may be responsible for discogenic pain (80). Thus, recent biologic therapies aim to stimulate matrix synthesis (59) in attempt to re-establish mechanical properties and eliminate pain.

Differences in mechanical properties of degenerated compared to normal intervertebral discs may therefore provide clues to help direct therapies. We know that degeneration decreases nuclear energy dissipation (39), swelling pressure, and compressive modulus relative to normal nucleus (40). Additionally, the degenerated annulus has a higher compressive stiffness that correlates with tissue dehydration (81). Consequently, degenerated discs have a breakdown in matrix function, resulting in compromised biomechanical behavior.

However, not all degenerated discs are painful as many asymptomatic individuals have MRI evidence of disc degeneration (41,42). This suggests subtle features may be triggering pain that are not reliably quantified with standard diagnostic tests. Histologic data indicate that painful degenerated discs have disordered annulus lamellar structure, innervation, and vascular granulation tissue (44,79,82). However, these qualitative observations have not been supported by a quantitative analyses to assess their biomechancial significance. The goal of this study was to test the hypothesis that painful and nonpainful discs have differing mechanical and biochemical properties. We tested this hypothesis using disc samples collected during surgery for spinal fusion, total disc replacement, or deformity correction.

2.2 Methods

Patient Selection

We analyzed 37 disc samples (18 nucleus pulposus (NP) samples and 19 annulus fibrosus (AF) samples) from 20 patients. Samples were divided into two groups: degenerated/nonpainful (n=8 AF, n=9 NP), and degenerated/painful (n=11 AF, n=9 NP; Table 2-1). The age of the painful group was 49±10 and that of the nonpainful group was 61±14. Males and females were represented in the painful group (4 female/7 male) and nonpainful group (6 female/3 male). Discs were primarily harvested between levels L4-S1, and one disc was harvested from L3-4. We obtained approval by the UCSF Committee for Human Research (H8317-34145-041).

The tissue categorization in the present study indicated if the disc was the source of pain. Specifically, the painful group consisted of discogenic pain patients, whereas the nonpainful group consisted of adult spinal deformity patients. Both of these patient groups experienced symptoms that justified surgical intervention. Importantly, the symptoms of adult spinal deformity patients did not stem from the disc. Instead, these patients experienced other pathology that was associated with deformity, such as spinal stenosis (narrowing of the spinal canal). In contrast, the painful group had discogenic pain, or pathology localized to the disc. Patient diagnosis was provided by the clinician prior to surgery. Diagnosis was based on: a) clinical presentation; b) radiographic findings; and c) discography (Figure 2-1).

a. Clinical Presentation. If the patient's indication for surgery was not dominated by low back pain (Visual Analog Score; VAS <6), radiographic assessment of degeneration (below) determined if the corresponding sample was classified as

degenerated/nonpainful or excluded as normal. If the patient had chronic low back pain (VAS≥6, for six months or longer), then clinical assessment, discography, and/or radiographic evaluation determined whether the pain was discogenic.

b. Radiographic Findings. All patients were evaluated with plain films. MRI images were obtained for all painful patients and six of the nine nonpainful patients, and disc height and Pfirrmann grade (83) were recorded. Criteria defining disc degeneration were one or more of the following: 1) Plain films with >25% loss of height compared to adjacent discs; 2) T2-weighted MRI image demonstrating reduced nucleus signal and degeneration grade >3 using the Pfirrmann scale; and 3) endplate Modic changes.

c. Discography. The final consideration in our classification system was the result of provocative discography, conducted in eight out of eleven painful discs. Provocative discograms are rated by the quality and severity of pain provoked by injection of dye into the intradiscal space. Discography was performed following the guidelines put forth by the International Spine Intervention Society (ISIS; < 50 psi pressure), which has been shown to result in a false-positive rate of only 6% (84). Patients with disc degeneration and concordant pain (>7/10) were classified as painful. Patients with disc degeneration and mild, non-concordant pain (<5/10) were classified as nonpainful. Patients with equivocal results (5-7/10) on discography and/or uncharacteristic clinical presentation were excluded from the study. Patients with pain and without disc degeneration were excluded from the study.

Tissue Preparation

Tissues were frozen on dry ice within ten minutes of removal and subsequently stored at -80°C. Annulus and nucleus tissue were isolated by visual inspection: annulus tissue was apparent given its organized lamellar structure. The connective tissue surrounding the outer annulus was discarded. Nucleus samples were harvested from the central gelatinous region of the disc consisting of unstructured collagen and proteoglycan (Figure 2-2). Tissue regions that were difficult to identify as annulus or nucleus were not analyzed.

Mechanical Indentation

Because the samples were irregularly shaped, mechanical properties were tested using mechanical indentation. Dynamic indentation tests were performed with the samples submerged in 0.15 M PBS at 20°C (BioDent1000, Active Life Technologies, CA (85,86)). Using a reference probe to determine surface contact, a 1.47 mm diameter cylindrical probe continuously indented the tissue in a sinusoidal fashion with an amplitude of 300 μm and frequency of 2 Hz (Figure 2-3A and Figure 2-3B). Each sample was preconditioned for greater than 20 cycles to create a standard reference configuration with consistent local tissue hydration at the measurement site. During continuous indentation, two consecutive indentations were recorded and averaged to create a force-displacement curve. For each sample, five separate force-displacement curves were recorded in one location and output parameters extracted from each force-displacement curve were averaged. Annulus tissue was indented in the anatomic axial direction (i.e. perpendicular to the annular lamella) and nucleus tissue was indented without directional

specification, due to the lack of definitive architectural features. The force-displacement curve exhibited hysteresis (Figure 2-3C). The slope of the loading curve was used to quantify the indentation modulus. The area in the hysteresis loop was used to quantify the energy dissipated. As typical for viscoelastic materials, the displacement curve lagged the force curve (Figure 2-3D). This lag in displacement is called phase shift, δ. The tangent of δ is the ratio of the loss to storage modulus, thus a large tangent of δ indicates a viscous material whereas a small tangent of δ indicates an elastic material.

Equilibrium Dialysis

The disc hydration at physiologic pressure was assessed using equilibrium dialysis – a technique that applies osmotic pressure on samples. Swelling pressure was measured by equilibrium dialysis as described previously (12). Briefly, tissue samples were equilibrated in polyethylene glycol 20000 (PEG) solutions with 0.15 M sodium chloride, which generated an osmotic pressure. A 30-60 mg tissue slice was placed into dialysis tubing of 3500 MW cutoff, and subsequently submerged into the PEG solution. This was repeated such that each specimen was divided into six slices and placed in six different PEG concentrations (5, 10, 15, 20, 25, and 30 g PEG/100 ml sodium chloride). After 48 hours at 4ºC with gentle agitation, the post-dialysis tissue mass was measured. The tissue slices were lyophilized and the resulting dry masses measured. Dry mass was subtracted from the post-dialysis tissue mass to obtain water content. The water content was normalized to dry mass to obtain tissue hydration. After each experiment, a sample of each PEG solution was lyophilized and weighed to obtain the final PEG concentration. The osmotic pressures of the PEG solutions were calculated using the following equation:

Equation 2-1

$$\frac{\pi}{RT} = \frac{c}{19400} + (2.59 \times 10^{-3})c^2 + (13.5 \times 10^{-3})c^3$$

where π is the osmotic pressure, R is 8.31 J/K*mol, T is temperature, c is concentration in g PEG/ml solution (specific volume of PEG is 0.837 ml/g).

Osmotic pressure versus tissue hydration curves were generated and the data were fit to an equation of the form $\pi = A \times h^B$, where h is tissue hydration, and A and B are constants. The hydration at 0.2 MPa (a physiologic disc pressure (87)) was compared between painful and nonpainful samples. In addition, the dependence of hydration on proteoglycan and collagen content in the combined set of tested tissues was evaluated by performing a multiple linear regression with the following equation:

Equation 2-2

$$h = A_1 + A_2 p + A_3 c + A_4 {}^{p}/_{c}$$

where h is tissue hydration, p is proteoglycan normalized to dry mass, c is collagen content normalized to dry mass, and A_1, A_2, A_3, and A_4 are constants. The individual dependence of tissue hydration on proteoglycan, collagen, and the ratio of proteoglycan to collagen was evaluated with three linear regression analyses.

Protein Quantification

Proteoglycan, collagen, and collagen crosslinking were quantified to determine if matrix quantity correlated with mechanical properties.

Proteoglycan: Samples were digested with papain (21 units/ml) at 65°C for 48 hours. Chondroitin sulfate content was assessed by adding 40 ul of the papain digest to 250 ul dimethylmethylene blue dye (DMMB) solution (88). Absorbance at 525 nm was measured and converted using a chondroitin sulfate standard.

Collagen: Samples were digested with 6 N HCl at 110°C for 16 hours. Collagen content was assessed by hydroxyproline, as described by others (89). Absorbance at 570 nm was measured and converted using a hydroxyproline standard. Collagen was assumed to consist of 14% hydroxyproline (90).

Collagen crosslinking was assessed by quantifying fluorescent advanced glycation endproducts (AGEs) (91). Supernatant from the collagen digest was measured by fluorescence at 370 nm excitation and 440 nm emission and converted with a quinine sulfate standard.

Histology

Disc samples were embedded in paraffin, sectioned, and stained with Picrosirius Red. Sections were imaged at 4X under polarized light to visualize collagen birefringence.

Statistical Analysis

All statistical analyses were performed using the JMP statistical software system (JMP V 8.0.1). Student's t-test and analysis of covariance (ANCOVA) procedures were

used to compare specimen group means and to estimate the effect of the specimen variables (tissue type, painful/nonpainful status entered as categorical predictors; and age entered as a continuous predictor) on the measured parameters of interest (energy dissipated; indentation modulus; tan(δ); hydration; proteoglycan, collagen, and collagen crosslinking content). Correlation coefficients (coefficient of determination, R^2), standard deviations, and linear regressions were also determined along with standard p-values for assessing statistical significance. Probabilities between $0.05<p<0.1$ were defined as 'trends' with near statistical significance (92).

2.3 Results

Patient Selection

MRI data was available for 32 out of the 37 tested samples. Disc heights between the painful (11.5±2.5 mm) and nonpainful (12.2±1.2 mm) groups were statistically indistinguishable ($p>0.4$). In addition, Pfirrmann grades between groups were statistically equivalent by Pearson's chi-square test ($p>0.7$).

Mechanical Indentation

The painful and nonpainful groups had different mechanical properties based on indentation testing. For the annulus samples, energy dissipation was significantly lower in the painful group than in the nonpainful group (Figure 2-4A; 1.8±0.9 vs. 3.5±1.7 (SD) μJ, $p<0.05$). This was not statistically significant when accounting for age as a covariate ($R^2=0.47$, $p_{pain}=0.215$, $p_{age}=0.055$). Conversely, in nucleus samples, energy dissipation was significantly higher in the painful group than in the nonpainful group (1.5±0.7 vs.

0.7±0.4 μJ, $p<0.05$). This result remained significant after including age effects ($R^2=0.33$, $p_{pain}<0.05$, $p_{age}=0.985$). Within the painful disc group, energy dissipation between the nucleus and annulus were statistically indistinguishable ($p=0.41$). In contrast, in the nonpainful disc, energy dissipation of the annulus was significantly higher than the nucleus ($p<0.0005$). These trends in energy dissipation were also observed in indentation modulus (Figure 2-4B). Specifically, the indentation modulus of the painful annulus was less than that of the nonpainful annulus ($p=0.066$). While the indentation modulus of the annulus was significantly higher than that of the nucleus in both painful and nonpainful discs ($p<0.05$), the mean difference between nucleus and annulus indentation modulus was larger in the nonpainful disc. According to the phase shift data, the painful annulus was more viscous and less elastic than the nonpainful annulus as indicated by a higher tangent of δ than the nonpainful annulus (Figure 2-4C, $p<0.05$). The tangent of δ was poorly correlated with both pain and age ($R^2=0.25$, $p_{pain}=0.191$, $p_{age}=0.487$). In contrast, the painful and nonpainful nucleus tissues had statistically indistinguishable tangent of δ ($p=0.82$).

Equilibrium Dialysis

The painful and nonpainful annulus samples had different equilibrium water content by equilibrium dialysis. At an osmotic pressure that approximates physiologic pressure in the disc, the hydration of the annulus was significantly lower in the painful group than the nonpainful group (Figure 2-5; 2.6±0.5 vs. 3.1±0.2 MPa, $p<0.05$). This result remained significant after including age effects ($R^2=0.42$, $p_{pain}<0.005$, $p_{age}=0.077$). In contrast, the hydration of the painful and nonpainful nucleus samples were statistically

indistinguishable (p=0.38). In both painful and nonpainful groups, the nucleus had significantly higher hydration than the annulus (p<0.01).

The hydration for the combined set of tested tissues was dependent on proteoglycan and collagen content according to Equation 2-2. Multiple linear regression analysis indicated that constants A_1 through A_4 in Equation 2-2 are 3.78 $\frac{mg\ water}{mg\ dry\ tissue}$ (p<0.0001), 4.86 $\frac{mg\ water}{mg\ proteoglycan}$ (p<0.005), -3.60 $\frac{mg\ water}{mg\ collagen}$ (p<0.0005), and -0.602 $\frac{mg\ water \times mg\ collagen}{mg\ dry\ tissue \times mg\ proteoglycan}$ (p<0.05), respectively. The R^2 value of the curve fit was 0.49. In addition, the linear regression analyses indicated that tissue hydration had individual correlations with proteoglycan (R=0.45, p<0.01), collagen (R=-0.55, p<0.0005), and the ratio of proteoglycan to collagen (R=0.41, p<0.05).

Protein Quantification

The painful and nonpainful groups had similar amounts of proteoglycan and collagen (Table 2-2; annulus: p=0.62; nucleus: p=0.61). Including age as a covariate, however, revealed differences in matrix content between the painful and nonpainful annulus (Figure 2-6). Specifically, the painful annulus had lower proteoglycan and higher collagen than the nonpainful annulus after correcting for age effects (proteoglycan: R^2=0.47, p_{pain}<0.05, p_{age}<0.005; collagen: R^2=0.31, p_{pain}<0.05, p_{age}<0.05). As expected, total collagen was higher in the annulus than the nucleus in both groups (p<0.05). Although not statistically significant, collagen crosslinking was higher in the nonpainful nucleus than in the painful nucleus (p=0.11).

Histology

The matrix organization of the painful annulus was compromised compared to that of the nonpainful annulus. This has been reported in the literature, and confirmed with histology in the current study (79). In particular, three painful and two nonpainful annulus specimens were randomly selected for histological processing and analysis. Representative sections indicate that the painful annulus had disorganized collagen lamellae (Figure 2-7). In contrast, the organization of the nonpainful annulus was evident from the clear structure of the lamellae.

2.4 Discussion

Our data demonstrate that painful discs have different mechanical properties and annular matrix quantity, while maintaining similar levels of nuclear matrix quantity, as nonpainful discs. Surprisingly, this is despite having similar levels of degeneration—assessed by MRI Pfirrmann grade, disc height, nucleus viscoelasticity, nucleus hydration, and nucleus proteoglycan quantity. We observed that the painful annulus had decreased energy absorption, decreased stiffness, and higher tangent of δ when compared to nonpainful annulus. In addition to possessing inferior mechanical properties, the painful annulus had a diminished ability to imbibe water. The painful nucleus demonstrated the opposite trend in mechanical properties with higher energy dissipation than the nonpainful nucleus. Taken together, these results indicate that subtle mechanical and biochemical changes are coincident with discogenic pain.

Data from current study do not indicate whether altered matrix properties are the cause or effect of pain. Instead, our data suggests that the painful disc has micro-instability, with significantly altered annular matrix after correcting for age differences. According to previous studies, micro-instability can either be a cause or an effect of pain (43). In one scenario, micro-instability can cause pain by creating abnormal tissue stress, which can lead to secretion of proinflammatory factors and pain causing sensitization of nociceptors. Another scenario, however, is that inflammation is the underlying mechanism of both pain and matrix degradation. Specifically, disc injury can result in elevated levels of inflammatory cytokines, such as TNF-α and IL-1β (45). These cytokines can cause pain by upregulating nerve growth factor, resulting in sensitization of nociceptors (48). Importantly, another consequence of inflammation is the upregulation of matrix metalloproteinases (93), which degrade matrix and compromise the mechanical properties of the disc. While the results from the current study provide insight in the characteristics of painful discs, the mechanisms of pain provocation remain indeterminate.

While compressive indentation is not a widely used testing technique to measure disc mechanical properties, this method produced comparisons of painful and nonpainful tissue based on energy dissipation, indentation modulus, and phase shift. One advantage to indentation testing is that it has fewer requirements for specimen geometry. In particular, indentation requires a flat indentation surface and that the specimen is large and thick relative to the indentation probe and amplitude. In contrast, alternate methods, such as confined compression and tensile testing, require precise specimen geometry. Cadaveric disc tissues, for example, are initially intact and can be trimmed to a precise

geometry. Therefore, confined compression and tensile testing with cadaveric tissues can provide insight into nucleus compressive properties or annulus tensile properties, respectively. Given the limitations in size associated with the surgical waste tissues used in the current study, indentation was an appropriate option. We observed differences in energy dissipation, indentation modulus, and tangent of the phase shift between painful and nonpainful samples. During testing, the indentation probe generates a loading curve as the probe indents the tissue and an unloading curve as the probe retracts (Figure 2-3C**)**. Energy dissipation is a measure of the difference in energy between the loading and unloading curves. Because nucleus and annulus tissue are viscoelastic, the loading and unloading curves do not coincide and the tissue dissipates energy during cyclic indentation. The dissipated energy is primarily energy required to exude water out of the proteoglycan and collagen matrix during loading that is not available during unloading. Energy dissipation is affected by tissue stiffness and hydration. Indentation modulus is the slope of the loading curve. This represents the stiffness of the tissue as the tissue is indented. Phase shift is the lag in force response to the indentation probe displacement. The tangent of the phase shift is the ratio of loss to storage modulus. Thus, a large tangent of the phase shift suggests that the specimen is viscous, whereas a small tangent of the phase shift suggests that the specimen is elastic. These output parameters provide insight into the mechanical properties of painful and nonpainful discs. However, this indentation data is difficult to compare with previous studies that have investigated healthy or degenerated discs using alternative methods like confined compression or tensile tests.

Consistent with prior observations, our results indicate unique mechanical and matrix properties in the painful annulus. Previous reports demonstrate that the painful

annulus has distinct histological features, including disordered annulus lamellar structure and infiltration of vascular granulation tissue (79). These findings were accompanied by observations of increased connective tissue growth factor in the painful disc, which suggests fibrosis in the disc matrix. Our histologic data verify the previously observed disorganized annulus lamellar structure. Furthermore, our results indicate that these morphologic changes in the painful annulus are accompanied by quantifiable changes including decreased proteoglycan and increased collagen. By measuring the structural properties of proteoglycan and collagen, our indentation tests detected altered matrix in the painful annulus. Mechanical indentation, like other compressive testing protocols, measures compressive properties of the annulus which are dominated by proteoglycans. However, mechanical indentation also measures surface tensile properties of collagen fibrils located at the superficial tissue region (94). In fact, others have demonstrated that the distribution of indentation modulus correlated with prior measures of tensile strength distribution in the annulus (95). Because the painful annulus samples of the current study have increased collagen content, we may expect that they also have superior indentation properties; however, these tissues also have disordered annular structure and decreased proteoglycan content, which diminish the indentation properties. The combined effects of these tissue parameters results in decreased energy dissipation and indentation modulus of the painful annulus. In addition, vascular granulation tissue could result in a less elastic, more viscous annular tissue, which would explain the increased tangent δ.

In the nucleus of the painful disc, we noted increased energy dissipation without associated changes in proteoglycan or collagen quantity. Proteoglycan and collagen quantification by DMMB and hydroxyproline do not fully define the matrix properties of

the nucleus. In painful discs, the presence of fibrosis and infiltration of vascular granulation tissue extends into the nucleus (79), which could cause elevated energy dissipation without associated changes in proteoglycan or collagen quantity. In addition, several less abundant extracellular matrix molecules in the nucleus affect matrix integrity and mechanical properties. For example, collagen IX covalently binds collagen II (96) and its role in matrix function has been demonstrated in studies of genetic polymorphisms. These studies have identified links between collagen IX polymorphisms and disc degeneration or compromised mechanical properties in the nucleus (97,98).

Altered matrix in the painful disc leads to diminished hydration characteristics. When exposed to an osmotic pressure of 0.2 MPa during equilibrium dialysis tests, the painful annulus was less hydrated than the nonpainful annulus. These results and our indentation test data suggest that the painful annulus had inferior mechanical properties. As expected, tissue hydration correlated with proteoglycan and collagen quantity (Equation 2-2), confirming that matrix quantity approximately predicts matrix function. In addition, altered collagen structure, which is found in the painful disc, can affect collagen intrafibrillar water content and disc hydration (99).

Mechanical indentation data indicate that the annulus and nucleus have similar mechanical properties in the painful disc, but different properties in the nonpainful disc. Specifically, in the nonpainful disc, the nucleus had lower energy absorption and indentation modulus than the annulus. These properties are consistent with the existing knowledge of disc biomechanics, where the hydrated nucleus is surrounded by the organized annulus, evenly distributing load along the neighboring vertebral bodies. As the disc degenerates, the nucleus dehydrates and the annular collagen infiltrates the

nucleus, resulting in loss of distinction between the annulus and nucleus (83,100). These degenerative changes were evident in the painful disc group from our mechanical indentation results. The similar mechanical properties of the nucleus and annulus in painful discs are a departure from typical nucleus and annulus function, where the structured annulus contains the gelatinous nucleus. While the painful and nonpainful discs had similar degeneration grades (indicated by Pfirrmann grade, disc height, nucleus viscoelasticity, nucleus hydration, and nucleus proteoglycan quantity), these mechanical properties indicating a change in nucleus and annulus function were specific to painful discs. Thus, our data suggest subtle degenerative changes in painful discs that are independent of the standard indicators of disc degeneration.

This study included two limitations that arose from the challenges associated with surgical patient selection and group designation. The first was that the nonpainful group consisted primarily of adult scoliotic discs, which may have had tissue asymmetries or altered matrix. The use of scoliotic discs is a practical limitation since degenerated nonpainful discs are infrequently removed from patients other than those with deformity. These scoliotic discs have a history of asymmetric loading and the potential for regional variation in tissue properties. However, prior studies that compare matrix from the convex and concave sides of the scoliotic curve have poor agreement. Some studies have indicated heterogeneity in collagen content and crosslinking between the convex and concave scoliotic disc (101,102). Other studies, however, have indicated no differences between the convex and concave matrix by histology, collagen content, or proteoglycan content (103-105). We did not control for tissue asymmetry, which possibly contributed to variability in our mechanical measures. Nonetheless our results were statistically

significant when compared to the painful disc group. Aside from tissue asymmetry, scoliotic discs may also have altered matrix. Others have shown that scoliotic discs have reduced annular elastin and collagen organization (103); however, this would suggest mechanical trends opposite to those reported here. Consequently, we do not believe the presence of scoliosis biases our mechanical data. The second limitation was the age difference between groups. Previous studies have shown that older age results in increased AGEs and degeneration (102,106). Despite the age differences between groups, AGEs were statistically indistinguishable between painful and nonpainful groups for both nucleus and annulus tissue. The degeneration level between groups was similar, as demonstrated by Pfirrmann grade and disc height measures. Furthermore, the nonpainful annulus group (higher mean age) had higher tissue hydration and energy dissipation than the painful annulus group. This is contrary to an expected degenerative-age effect in the annulus, which has been shown by others to result in increased compressive modulus from tissue dehydration (81).

While the source of disc pain remains under investigation, we have identified compromised mechanical and matrix properties in the painful disc, which has been associated with biomechanical hypermobility, nerve activation, and pain. Discs with moderate degeneration have abnormal motion segment kinematics indicative of mechanical instability or hypermobility (107,108). Results from an animal study, where hypermobility was induced by incision of musculature and removal of spinous processes, suggested that hypermobility causes endplate degeneration and cell apoptosis (109). Our indentation test data indicates that painful discs have loss of distinction between nucleus and annulus, and our histological data is consistent with prior findings indicating loss of

annular organization (79). These features in the painful disc compromise load bearing and induce stress concentrations in the disc (110). Importantly, stress concentrations in the disc at sites of innervation can cause pain (110,111). These degenerative matrix changes can be initiated by injury resulting from mechanical overloading (112). To the authors' knowledge, this is the first study comparing the mechanical properties of painful and nonpainful discs.

Table 2-1: Specimen summary

Case #	Group	Gender	Age	Level	Tissue Tested
1	Nonpainful	Female	39	L4-L5	NP
2	Nonpainful	Female	42	L4-L5	AF and NP
3	Nonpainful	Male	56	L5-S1	AF and NP
4	Nonpainful	Female	58	L5-S1	AF and NP
5	Nonpainful	Female	61	L5-S1	AF and NP
6	Nonpainful	Female	69	L5-S1	AF and NP
7	Nonpainful	Female	71	L3-4	AF and NP
8	Nonpainful	Male	74	L5-S1	AF and NP
9	Nonpainful	Male	78	L5-S1	AF and NP
10	Painful	Male	37	L5-S1	AF and NP
11	Painful	Female	39	L4-5	AF and NP
12	Painful	Male	42	L4-5	AF and NP
13	Painful	Male	43	L4-5	AF and NP
14	Painful	Male	44	L5-S1	AF and NP
15	Painful	Male	44	L4-5	AF and NP
16	Painful	Male	50	L5-S1	AF and NP
17	Painful	Female	54	L5-S1	AF and NP
18	Painful	Female	57	L5/S1	AF and NP
19	Painful	Female	61	L5-S1	AF
20	Painful	Male	67	L5-S1	AF

Table 2-2: Matrix quantification of painful and nonpainful discs for nucleus and annulus. Values are means ± SD.

	Nucleus Pulposus		Annulus Fibrosus	
	Painful	*Nonpainful*	*Painful*	*Nonpainful*
Proteoglycan/Dry Mass (μg/mg)	160±77	138±102	134±66	150±70
Collagen/Dry Mass (μg/mg)	228±45	187±70	344±154*	285±46*
Collagen Crosslinking (ng Quinine/μg Collagen)	5.21±1.52	9.15±6.39	3.67±2.85	4.16±0.43

* Indicates significant difference between annulus and nucleus. $p<0.05$.

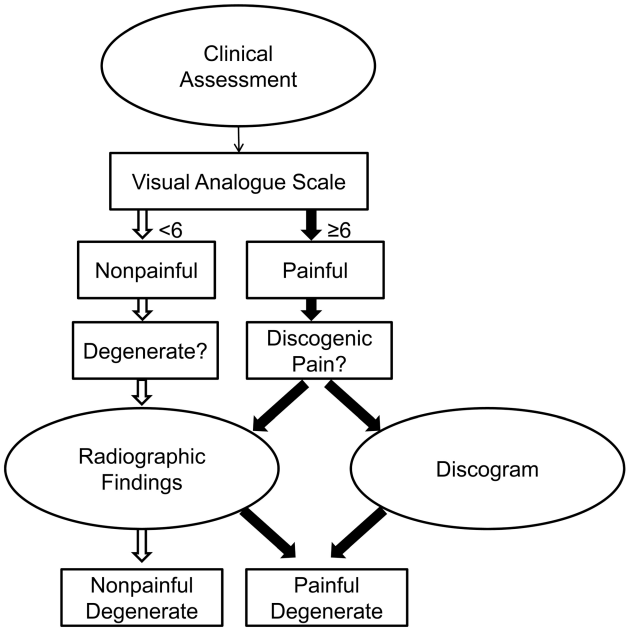

Figure 2-1: Tissues were categorized as nonpainful degenerated or painful degenerated based on clinical assessment, radiographic findings, and discogram.

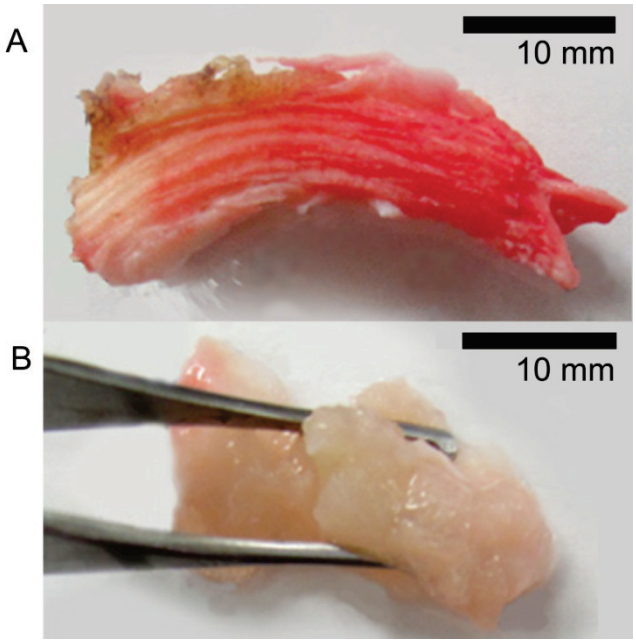

Figure 2-2: Representative annulus (A) and nucleus (B) samples.

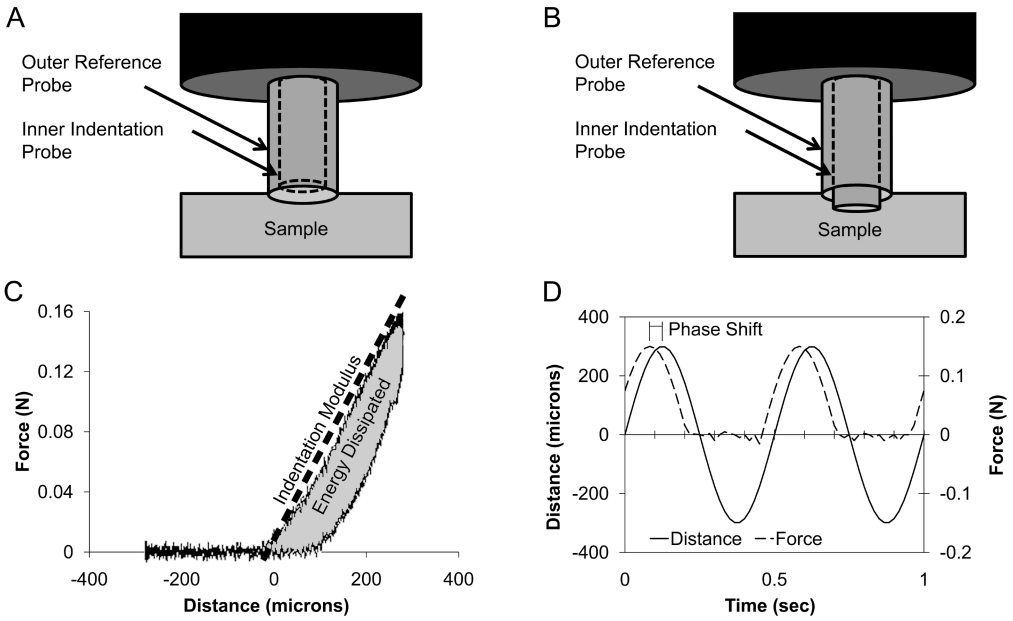

Figure 2-3: Mechanical indentation test. The outer reference probe contacted the test tissue (A) and the inner indentation probe indented the tissue (B) while recording force-displacement data. Energy dissipation and indentation modulus were extracted from the resulting force-displacement curve (C). The phase shift, δ, was recorded from the lag between force and distance (D).

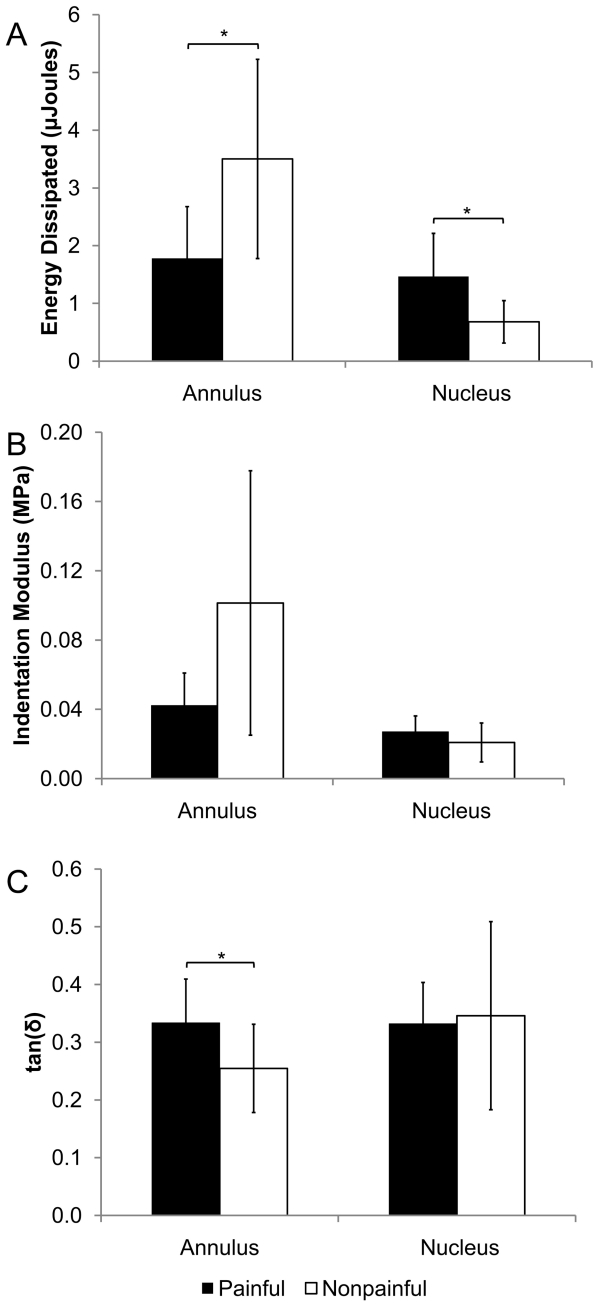

Figure 2-4: Mechanical indentation test output parameters of painful and nonpainful discs for nucleus and annulus. (A) Energy dissipation. (B) Indentation modulus. (C) tan(δ). Values are means ± SD. *$p<0.05$ by t-test.

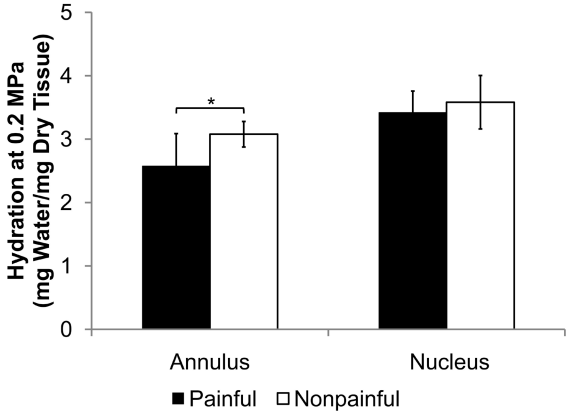

Figure 2-5: Hydration at 0.2 MPa of painful and nonpainful discs for nucleus and annulus. Values are means ± SD. *$p<0.05$ by t-test.

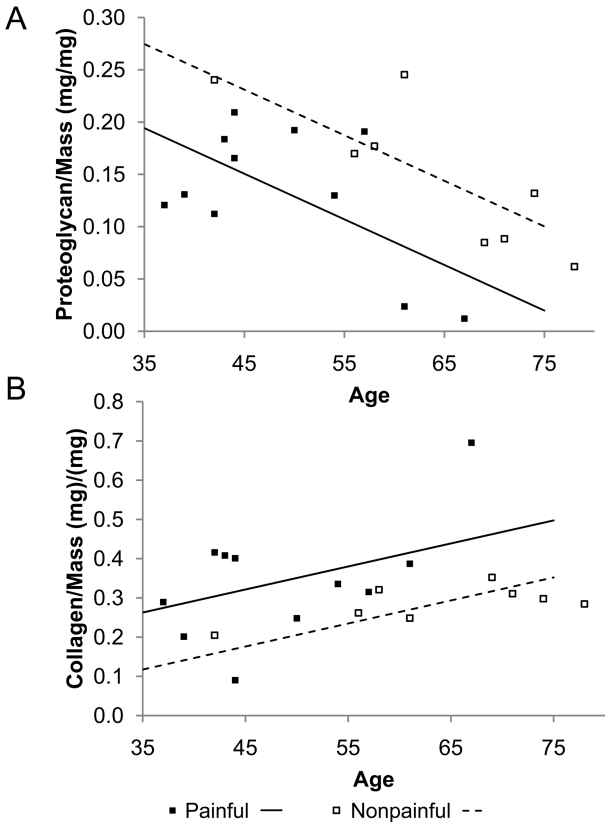

Figure 2-6: The effect of age and pain on matrix quantity in the annulus. The painful annulus had less proteoglycan (A) and more collagen (B) than the nonpainful annulus.

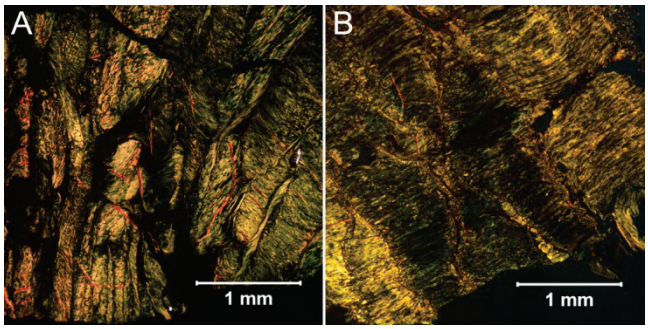

Figure 2-7: Annulus samples stained with Picrosirius Red under polarized light. The painful annulus (A) had disorganized lamellar structure compared to the nonpainful annulus (B).

Chapter 3: Matrix Gene Expression is Altered in Painful Intervertebral Discs and Correlates with Mechanical Properties

3.1 Introduction

Symptomatic intervertebral disc degeneration is a source of back pain with unknown cause. Previous efforts have investigated the characteristics of disc degeneration; however, recent reports indicate that disc degeneration is not always accompanied with pain (41). While the features that distinguish symptomatic from asymptomatic degenerated discs are unclear, hypermobility is a possible contributing factor to disc pain. Hypermobility may generate abnormal tissue stress, which leads to inflammation, nerve sensitization, and ultimately pain. The breakdown of extracellular matrix is one cause of hypermobility (43,113).

The extracellular matrix, consisting primarily of proteoglycans and collagens, undergoes biochemical changes during degeneration. Disc proteoglycans include large aggregating proteoglycans (aggrecan and versican) and small leucine rich proteoglycans (SLRPs; decorin, biglycan, and fibromodulin). Each of these matrix molecules serves specific functions. For example, aggrecan attracts water, and provides the necessary mechanical properties to withstand compressive force (23). SLRPs contribute to matrix organization by facilitating collagen fibril assembly and matrix remodeling (25). These large and small proteoglycans work in concert with collagens to define the mechanical properties of the disc. During degeneration, however, these properties are diminished as the disc exhibits reduced proteoglycan and collagen II, and increased collagen I (34,106,114,115). The extracellular matrix of healthy and degenerated discs have been extensively studied.

In contrast, the extracellular matrix of painful degenerated discs are not well understood. In Chapter 2, we found that painful degenerated discs have diminished mechanical properties compared to nonpainful degenerated discs. This suggests that specific matrix features may accompany disc pain. In a recent study, histological data showed that painful discs have disorder annular lamellae (79). Diminished mechanical properties and matrix organization may result from altered matrix synthesis. To investigate this possibility, we tested the hypothesis that painful discs have unique expression profile of extracellular matrix genes as compared to nonpainful discs. In addition, we hypothesized that matrix gene expression is correlated with disc mechanical properties.

3.2 Methods

Patient Selection

We analyzed 35 samples (approximately one nucleus pulposus (NP) and one annulus fibrosus (AF) sample per disc) from two patient groups: degenerated/nonpainful (n=8AF, n=8NP), and degenerated/painful (n=11AF, n=8NP; Table 3-1). We obtained approval by the UCSF Committee for Human Research (H8317-34145-041). The age of the nonpainful group was 61±14 and that of the painful group was 49±10. Diagnosis was based on clinical presentation, radiographic findings, and discography as described in Section 2.2.

Tissue Preparation

Tissues were frozen on dry ice within ten minutes of removal and subsequently stored at -80°C. Annulus and nucleus tissue were isolated by visual inspection: annulus tissue was apparent given its organized lamellar structure. The connective tissue surrounding the outer annulus was discarded. Nucleus samples were harvested from the central gelatinous region of the disc consisting of unstructured collagen and proteoglycan. Tissue regions that were difficult to identify as annulus or nucleus were not analyzed.

Gene Expression

Tissue was pulverized in liquid nitrogen using a mixer mill [MM301, Retsch] and suspended in Trizol reagent. RNA was extracted using the PureLink RNA mini kit [Invitrogen] and reverse transcribed using iScript cDNA synthesis kit [BioRad]. Gene expression was measured by quantitative real-time PCR [CFX96, BioRad] using primers

designed with NCBI Primer-BLAST. Gene expression (aggrecan, versican, fibromodulin, decorin, biglycan, collagen I, and collagen II) was normalized to GAPDH and converted to fold change by calculating $2^{-\Delta Ct}$ (116).

Mechanical Properties

Mechanical indentation and equilibrium dialysis were performed as described in Section 2.2. Using these testing protocols, we measured indentation modulus, energy dissipation, viscoelasticity, and hydration at 0.2 MPa.

Statistical Analysis

All statistical analyses were performed using the JMP statistical software system (JMP V 8.0.1). A logarithmic transformation was used on gene expression fold change to create a Gaussian distribution. Analysis of covariance (ANCOVA) was used to compare specimen group means and to estimate the effect of the specimen variables (painful/nonpainful status entered as categorical predictors; and age entered as a continuous predictor) on gene expression data. Coefficient of determination (R^2), standard deviations, and linear regressions were also determined along with standard p-values for assessing statistical significance. Pearson's product-moment correlations (R) were individually determined for gene expression and mechanical properties. The significance of each R-value was determined using a t-test with the null hypothesis that R equals zero. Multiple R-values were not compared and, therefore, no adjustment was made for multiple comparisons. Probabilities between $0.05<p<0.1$ were defined as 'trends' with near statistical significance (92).

3.3 Results

Painful and nonpainful discs have distinct proteoglycan and collagen gene expression profiles. When compared to the nonpainful annulus, the painful annulus had lower expression of proteoglycans (Figure 3-1; aggrecan (R^2=0.48, p_{pain}<0.01, p_{age}<0.005), versican (R^2=0.20, p_{pain}<0. 1, p_{age}<0.2), decorin (R^2=0.52, p_{pain}<0.005, p_{age}<0.005), and fibromodulin (R^2=0.41, p_{pain}<0.05, p_{age}<0.01)). In addition, the painful annulus had higher expression of collagen I than the nonpainful annulus (R^2=0.28, p_{pain} <0.05, p_{age}<0.2). The painful nucleus had a trend with higher expression of decorin than the nonpainful nucleus (Figure 3-2; R^2=0.21, p_{pain} <0.1, p_{age}<0.2).

Annulus and nucleus cells have differences in matrix gene expression. In nonpainful discs, the annulus had higher expression of decorin than the nucleus (Figure 3-3; p<0.05). In contrast, the nucleus and annulus of painful discs had statistically indistinguishable levels of decorin expression (p>0.5). In painful discs, the nucleus has higher gene expression of aggrecan (p<0.05), versican (p<0.05), and collagen II (p<0.1) than the annulus.

When the annulus and nucleus tissues were analyzed separately, individual gene expression of extracellular matrix molecules correlated with mechanical properties. In the annulus, aggrecan, fibromodulin, and decorin correlated positively with tissue hydration (Table 3-2). Biglycan correlated negatively with energy dissipation and elastic modulus, but positively with tangent of δ. Collagen I correlated negatively with elastic modulus. In the nucleus, aggrecan correlated positively with elastic modulus (Table 3-3). Versican

correlated positively with energy dissipation, tangent of δ, and hydration. Decorin correlated positively with energy dissipation and tangent of δ. Collagen II correlated positively with energy dissipation.

The nucleus tissues of painful and nonpainful discs had statistically indistinguishable hydration properties ($p>0.2$) and aggrecan gene expression levels ($p>0.3$).

3.4 Discussion

Our data suggest that cells in painful discs have altered expression of extracellular matrix genes compared to nonpainful discs. In addition, our data indicates a correlation between matrix gene expression and disc mechanical properties. We previously found that painful discs have diminished mechanical properties compared to nonpainful discs (Chapter 2). Interestingly, elevated or suppressed expression of several genes may explain the compromised mechanical properties of painful discs. Specifically, aggrecan, versican, fibromodulin, and decorin expression correlated with annulus hydration, and were lower in the painful than nonpainful annulus. In addition, collagen I expression correlated negatively with annulus indentation modulus, and was higher in the painful than nonpainful annulus. These trends suggest that aggrecan, versican, fibromodulin, decorin, and collagen I expression were contributing factors to the diminished hydration and indentation modulus in the painful annulus that were observed in Chapter 2. In nucleus samples, decorin expression correlated with energy dissipation and tangent of δ, and was higher in the painful samples than nonpainful samples. Elevated decorin

expression in the painful nucleus could contribute to the elevated energy dissipation in the painful nucleus that was observed in Chapter 2.

Altered expression of SLRPs in painful discs may lead to disorganized extracellular matrix with inferior mechanical properties. The role of SLRPs in matrix organization and tissue mechanics has been observed in other tissues. In particular, decorin, biglycan, and fibromodulin knockout animal models exhibit dysregulation of collagen fibril size and assembly in skin, bone, and tendon, respectively. These matrix changes lead to diminished mechanical properties (25,117). Results from these knockout animal models shed light on data from the current study. We observed that the painful annulus had decreased expression of SLRPs compared to the nonpainful annulus. The decreased expression of SLRPs is a potential culprit for the disorganized annular lamellar architecture (79) and consequent diminished mechanical properties in painful discs (Chapter 2). Our results indicating positive correlations between SLRP expression and mechanical properties fits with this model of matrix organization and tissue mechanics.

We observed an abnormal distribution of decorin between the annulus and nucleus in painful discs. Typically, decorin is more abundant in the annulus than the nucleus (24,118). Our observations of decorin expression in nonpainful discs are consistent with this expected distribution. In painful discs, however, the decorin expression in the annulus and nucleus are statistically indistinguishable. Because of decorin's role in collagen fibril formation, this abnormal distribution of decorin suggests that the annulus and nucleus of painful discs have matrix that are less organizationally distinct than the nonpainful disc. Interestingly, this deviation of matrix distribution is specific to decorin in painful discs. Other matrix molecules that are known to be

differentially expressed in the annulus and nucleus include aggrecan and collagen II. These matrix constituents are responsible for tissue hydration. Our data indicate that the distribution of these matrix molecules in painful discs follows the expected pattern of higher abundance in the nucleus than annulus.

Our correlation data also suggests roles of specific large proteoglycans and collagens in disc mechanics. For example, versican is a large proteoglycan with potential roles in disc mechanics that are not fully defined. Recent studies suggested that versican may have organizational roles contributing to the viscoelastic properties. In particular, versican may interact with elastin between adjacent annular lamellae to facilitate the viscoelastic properties of the annulus (24). The association of versican with elastin is not unique to the annulus (119,120) and may also contribute to viscoelastic properties of the nucleus. Interestingly, our data indicates that within the nucleus, versican is significantly correlated with energy dissipation, tangent of δ, and hydration. Because the tangent of δ is the ratio of loss to storage modulus, it is a measure of tissue viscoelasticity. Together, these data suggest that versican plays a role in the viscoelastic properties of nucleus tissue. In addition, our data of several matrix molecules are consistent with their previously understood roles in disc mechanics. Aggrecan is well known as a large proteoglycan that attracts water, which is critical for the compressive mechanical properties of the disc. As expected, aggrecan correlated with hydration and indentation modulus. Compressive mechanical properties in the nucleus are largely determined by aggrecan and collagen II. Collagen II forms the structural framework for proteoglycans (31). Thus, our data indicating a correlation between collagen II expression and energy dissipation supports the known function of collagen II in the nucleus. Surprisingly,

collagen I is negatively correlated with annular indentation modulus. This finding is counterintuitive when considering that collagen I is an important structural matrix protein of the annulus. Further examination of our data, however, indicates that collagen I is also negatively correlated with aggrecan, versican, fibromodulin, and decorin (data not shown). These proteoglycans act synergistically with collagen I to resist mechanical loads. Thus, increased expression of collagen I is accompanied by decreased expression of proteoglycans and reduced mechanical properties.

The current study focuses on how pain, rather than age or degeneration, affects matrix gene expression. Due to the subjective nature of pain, categorization of samples as painful or nonpainful is challenging. We therefore confirmed the presence of discogenic pain using discography with ISIS standards, which has a low false-positive rate of 6% (84). Although we categorized patients by pain, the resulting experimental groups had age differences; nonpainful patients were older than painful patients. Our analyses account for this potentially confounding variable using ANCOVA with age as the covariate. Furthermore, to address the potentially confounding effect of degeneration in this study, we used gene expression of aggrecan and hydration as measures of degeneration. The decline of these parameters in the nucleus is a key feature of degeneration (7,121). Gene expression of aggrecan and hydration were statistically indistinguishable between painful and nonpainful nucleus samples, indicating similar levels of degeneration.

This study is not without limitations. In particular, we have focused our analysis on gene expression data; however, gene expression is not necessarily representative of protein quantity or disc morphology. Extracellular matrix is regulated by matrix synthesis

and degradation. Our analysis of mRNA quantity of matrix genes is an indicator of matrix synthesis. The resulting matrix can be degraded by matrix metalloproteinases, which are regulated by tissue inhibitors of metalloproteinases (31). Although gene expression data do not provide a definitive picture of matrix constituents in painful discs, they indicate features of the painful disc phenotype that contribute to disc matrix. Furthermore, the matrix genes of the current study correlate with measured mechanical properties, indicating that matrix gene expression translates to matrix protein and affects disc mechanics. Thus, gene expression data supported by mechanical measurements provides insight into the unique features of painful discs.

Our characterization of the painful disc matrix may provide insight to previously described mechanisms of pain. In particular, changes in disc matrix may facilitate nerve infiltration—a requirement for pain—and disc hypermobility. In healthy discs, nerves are typically confined to the outer annulus with axons that align with concentric annular lamellae (122). However, in degenerated discs, nerves can enter the inner annulus at sites of annular disruption (123). The reduced SLRP expression that we observed in painful discs may cause disorganization of the annular matrix and contribute to nerve infiltration. In addition, aggrecan within the annulus or nucleus inhibits nerve and vascular cell growth (124,125). Nerves often follow vasculature into the disc, and aggrecan may act as a barrier to innervation. Our data demonstrating reduced aggrecan expression in the painful annulus suggest that painful discs have diminished barriers for nerve entry. Matrix changes in painful discs may also cause hypermobility. Our data demonstrates that altered expression of matrix in painful discs correlates with changes in mechanical properties. Furthermore, prior studies indicate that diminished matrix leads to abnormal

disc kinematics (113) and stress concentrations (111). These factors contribute to hypermobility, which can cause pain by sensitizing and stimulating nerves that have entered the disc (43). The altered expression of extracellular matrix molecules in painful discs found in the current study fits with the existing model of disc pain.

Table 3-1: Specimen summary

Case #	Group	Gender	Age	Level	Tissue Tested
1	Nonpainful	Female	39	L4-L5	NP
2	Nonpainful	Female	42	L4-L5	AF and NP
3	Nonpainful	Male	56	L5-S1	AF and NP
4	Nonpainful	Female	58	L5-S1	AF
5	Nonpainful	Female	61	L5-S1	AF and NP
6	Nonpainful	Female	69	L5-S1	AF and NP
7	Nonpainful	Female	71	L3-4	AF and NP
8	Nonpainful	Male	74	L5-S1	AF and NP
9	Nonpainful	Male	78	L5-S1	AF and NP
10	Painful	Male	37	L5-S1	AF and NP
11	Painful	Female	39	L4-5	AF and NP
12	Painful	Male	42	L4-5	AF and NP
13	Painful	Male	43	L4-5	AF and NP
14	Painful	Male	44	L5-S1	AF and NP
15	Painful	Male	44	L4-5	AF
16	Painful	Male	50	L5-S1	AF and NP
17	Painful	Female	54	L5-S1	AF and NP
18	Painful	Female	57	L5/S1	AF and NP
19	Painful	Female	61	L5-S1	AF
20	Painful	Male	67	L5-S1	AF

Table 3-2: Correlation of gene expression with mechanical properties in the annulus.

R Values: Annulus

Gene log(fold change)	Energy Dissipated	Elastic Modulus	tan(δ)	Hydration
Aggrecan	0.016	-0.025	-0.040	0.42*
Versican	0.19	0.16	-0.12	0.31
Biglycan	-0.42*	-0.44*	0.53**	0.012
Fibromodulin	0.15	0.12	-0.080	0.49**
Decorin	0.17	0.098	-0.036	0.52**
Collagen I	-0.21	-0.42*	0.32	-0.38
Collagen II	0.20	0.13	-0.16	0.31

*p<0.1; **p<0.05

Table 3-3: Correlation of gene expression with mechanical properties in the nucleus.

R Values: Nucleus

Gene log(fold change)	Energy Dissipated	Elastic Modulus	tan(δ)	Hydration
Aggrecan	0.38	0.43*	0.30	0.36
Versican	0.45*	0.29	0.66***	0.51*
Biglycan	0.37	0.31	0.022	0.27
Fibromodulin	0.37	0.40	0.32	0.40
Decorin	0.57**	0.30	0.44*	0.18
Collagen I	-0.070	-0.38	-0.18	-0.23
Collagen II	0.54**	0.38	0.26	0.40

*p<0.1; **p<0.05; ***p<0.01

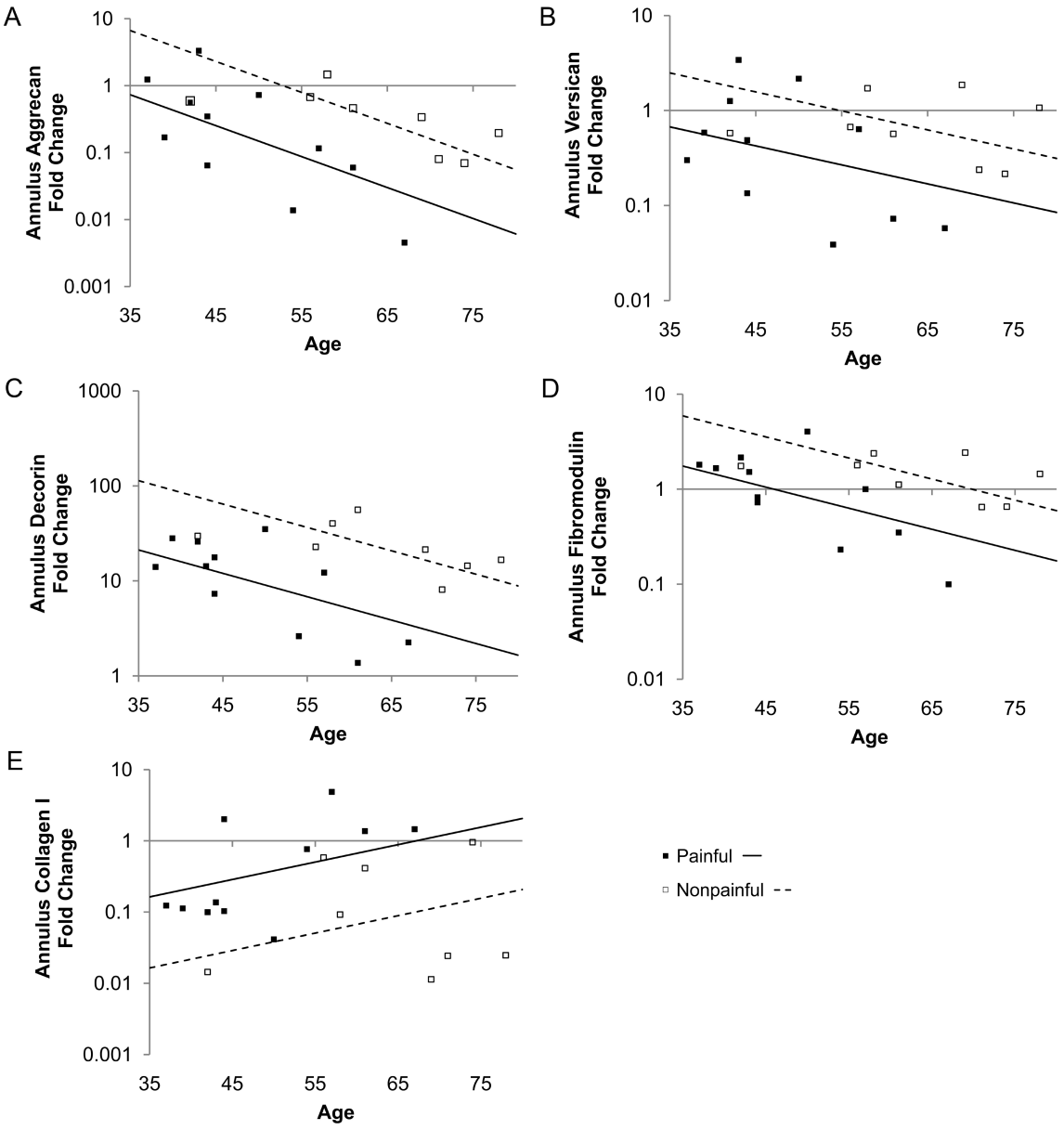

Figure 3-1: The effect of age and pain on expression of extracellular matrix genes in the annulus. The painful annulus had lower gene expression of aggrecan (A), versican (B), decorin (C), and fibromodulin (D), but higher gene expression of collagen I (E) than the nonpainful annulus.

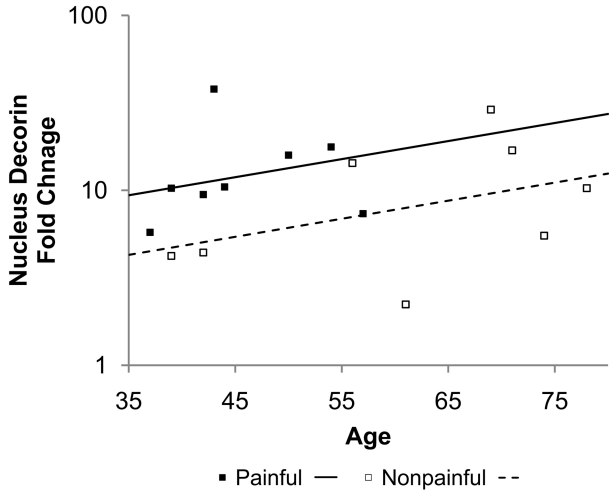

Figure 3-2: The effect of age and pain on expression of decorin in the nucleus. The painful nucleus had higher gene expression of decorin than the nonpainful annulus.

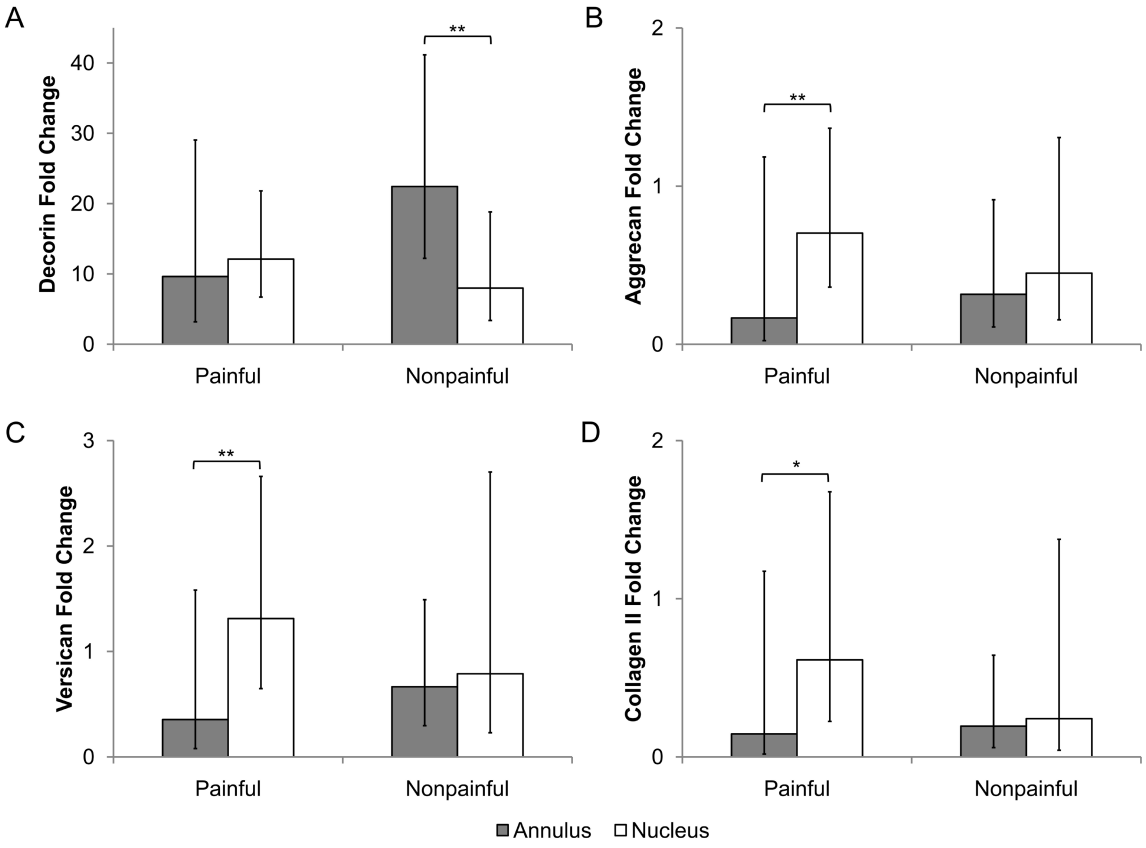

Figure 3-3: Gene expression of decorin (A), aggrecan (B), versican (C), and collagen II (D) with annulus compared to nucleus in painful and nonpainful discs. Values are means ± SD. *p<0.1; **p<0.05 by t-test.

Chapter 4: Mechanical Stimulation of Mesenchymal Stem Cells

4.1 Introduction

Tissue engineering presents promising treatment options for intervertebral disc degeneration. During disc degeneration, changes in matrix composition and deterioration of mechanical properties results in depressurization of the nucleus pulposus and instability. A tissue-engineered nucleus pulposus requires a source of cells that can generate extracellular matrix and restore the mechanical properties of the disc. Human mesenchymal stem cells (MSCs) are a potential cell source capable of differentiating into multiple cell types, including chondrocytes (60,126).

Previous investigators have induced chondrogenic differentiation of MSCs *in vitro* using three-dimensional aggregate cultures treated with TGF-β1, -β2, or TGF-β3. While all three isoforms of TGF-β induce chondrogenic differentiation, TGF-β2 and TGF-β3 stimulation results in increased proteoglycan and collagen II synthesis compared

to TGF-β1 (64). Chondrogenic differentiation may be categorized into multiple stages that mimic endochondral ossification (64,66,127). Such studies successfully induce MSC differentiation as evidenced by gene expression and protein synthesis of chondrogenic markers, such as aggrecan, collagen II, and Sox9. Importantly, the differentiating MSCs eventually express MMP-13, collagen X, and alkaline phosphatase, which are indicative of cell hypertrophy and terminal differentiation during endochondral ossification (127). *In vitro*, MSC cultures gradually generate proteoglycan-rich matrix, which improve mechanical stiffness. However, proteoglycan content and mechanical stiffness deteriorate during long-term cultures (128).

Compressive mechanical stimulation alters cell metabolism as well as gene expression, and may guide chondrogenic differentiation. Several studies involving cartilage explants and three-dimensional chondrocyte/gel constructs report that dynamic loading increases proteoglycan synthesis (129-131). In addition, dynamic compression of chondrocytes may slow terminal differentiation as evidenced by downregulation of MMP-13 (132), a protease found in hypertrophic chondrocytes that degrades collagen II (133). Such research raises the question whether mechanical stimulation suppresses hypertrophy in differentiating MSCs. Both mechanical and biologic stimuli may be used together when optimizing MSC differentiation (134).

Chondrocyte response to mechanical stimulation varies with tissue maturation. For example, mechanically stimulated cartilage explants from calf bovine increases proteoglycan synthesis, whereas fetal and adult bovine explants show no response (135). In addition, dynamic compressive stimulation of chondrocytes *in vitro* preferentially stimulates proteoglycan production with relatively longer culture duration prior to load

(129). Similarly, MSCs cultured for longer duration have increased proteoglycan synthesis in response to mechanical stimulation (136). The goal of this study is to optimize tissue engineered constructs by combining mechanical loading with TGF-β3 stimulus. We believe that MSCs optimally respond to mechanical stimulation at a specific stage of TGF-β3 induced chondrogenesis.

4.2 Methods

Cell culture

Human MSCs and cell culture medium were purchased from Lonza Walkersville, Inc (Walkersville, MD). MSCs were received at passage 2 and expanded in monolayer using mesenchymal stem cell growth medium. Cells were expanded to passage 8 or under, detached using trypsin/EDTA, and resuspended in alginate (Protanol LF200S alginate, FMC Biopolymer, Drammen, Norway, reconstituted in Ca^{+2} and Mg^{+2} free PBS) at a cell density of 4 million cells per mL. A slow gelling $CaCO_3$-GDL (D-glucono-δ-lactone) alginate system (137,138) was used. An aqueous $CaCO_3$ slurry is an insoluble calcium source that can be distributed throughout the alginate, and fresh aqueous GDL releases the calcium ions to induce controlled alginate crosslinking. The $CaCO_3$ slurry and fresh aqueous GDL (Sigma, St. Louis, MO) were added to a final concentration of 30 mM $CaCO_3$, 60 mM GDL, and 2% alginate. The alginate was cast into a 2 mm thick sheet and punched into cylinders (6mm diameter, 2mm thickness) for unconfined compression experiments. The alginate polymerized 15 minutes after adding GDL, and was washed with PBS before culture in serum free medium. After 72 hour recovery in

serum free basal medium, alginate gels were considered day 0 and cultured in chondrogenic media (10ng/mL TGF-β3). Medium was changed every 2-3 days with 3 mL medium for every million cells. At days 0, 6, 18, and 24, chondrogenic cultures were treated with mechanical stimulation.

Mechanical Stimulation

A custom bioreactor was constructed inside a 37° C, 5% CO_2-95% air incubator using a high precision actuator (0.1 μm resolution) and motion controller assembly (PI Polytech, Auburn, MA) with a 1 kg load cell (Sensotec, Columbus, OH). Labview software (National Instruments, Austin, TX) was used to control the actuator, and custom polysulfone fixtures were machined to hold samples submerged in basal medium and generate compressive loads (Figure 4-1). The actuator was driven in displacement control with sinusoidal dynamic loading with 1 Hz frequency for a duration of 4 hours. Compressive strain amplitude was 10% peak-to-peak, consistent with other studies observing biosynthetic response from MSCs (73). Contact control specimen were placed in identical loading chambers with a 2 mm spacer to generate an unloaded condition with the same diffusion constraints as the loaded samples. After mechanical stimulation, gel constructs were cultured for 24 hours or 3 hours in basal medium before harvest.

Viability/Histology

Viability was assessed using a fluorescent live/dead assay with calcein AM and ethidium homodimer-1 (Invitrogen). Histology was used to assess proteoglycan production and cell morphology after stimulation with chondrogenic media for 24 days.

A modified alcian blue protocol was used to preferentially stain proteoglycans in alginate matrix (139). Hematoxylin was used as a nuclear counterstain.

Dot Blot

Dot blot was performed to assess collagen II production after stimulation with chondrogenic media for 24 days. Cells were released from alginate by incubating for 20 minutes at 4°C in 5 volumes in dissolving buffer (55 mM sodium citrate, 0.15 M sodium chloride, pH 6.8). Cells and alginate supernatant were separated and protein was extracted using 9 volumes of lysis buffer (30mM Tris, pH 7.5; 30mM NaCl; 1% Triton X-100). Solutions were incubated for 15 minutes and centrifuged at maximum speed for 5 minutes at 4°C. The supernatant was extracted and blotted onto a nitrocellulose membrane (0.2 um, Bio-Rad). The membrane was blocked using TBS-T in milk. The primary antibody (University of Iowa Developmental Studies Hybridoma Bank, CIIC1) for collagen II was diluted 1:10000 in milk/TBS-T and applied to the membrane for 30 minutes at 4°C. The membrane was washed with TBS-T (3 x 5 minutes) and incubated with the secondary antibody. The membrane was scanned using a Li-Cor Odyssey and background was subtracted using ImageJ software.

Gene expression analysis

Gene expression analysis was used to assess MSC response to mechanical stimulation. Cells were released from alginate by incubating for 20 minutes at 4°C in 5 volumes in dissolving buffer (55 mM sodium citrate, 0.15 M sodium chloride, pH 6.8) (140). RNA isolation and DNase treatment was performed using RNeasy Mini Kit

(Qiagen, Valencia, CA). The extracted RNA was reverse transcribed (Bio-Rad iScript, USA) and resulting cDNA was used for real time quantitative RT-PCR, performed by the Genome Analysis Core Facility at UCSF. Sybr primers for aggrecan, collagen Iα2, collagen IIα1, collagen Xα1, Sox9, and GUS were purchased from Integrated DNA Technologies, Inc (San Diego, CA). An ABI7900 was used to perform real-time quantitative RT-PCR in triplicate. All genes of interest were normalized to GUS levels and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. The number of replicates varied between 0 and 4 depending on the duration of post-load incubation and the gene of interest (Table 4-1).

Statistics

Statistical analysis of gene expression analysis was tested using one-sample Wilcoxon Signed Rank Test. Specifically, ΔΔCt was calculated by normalizing the load group to control group. The ΔΔCt value was compared to a mean value of zero using the one-sample Wilcoxon Signed Rank Test. Significance was defined as $p<0.05$.

4.3 Results

Viability/Histology

Cell viability at day 0 was 60-80% where green fluorescence indicates live and red fluorescence indicates dead cells (Figure 4-2). Similar viability was observed at all other time points tested. Histology with alcian blue and hematoxylin counterstaining indicated that 24 days with TGF-β3 treatment resulted in synthesis of proteoglycan

(Figure 4-3). Specifically, the cells synthesized proteoglycans that were localized to the pericellular matrix. Little or no proteoglycans were found in the alginate scaffold further from the cells.

Dot Blot

Collagen II protein was secreted by the MSCs after 24 days in chondrogenic media (Figure 4-4). This collagen II was localized to the cell pellet and may have been present as pericellular matrix. The alginate supernatant did not stain for collagen II, suggesting that collagen II was not secreted into the alginate scaffold.

Gene Expression

Treatment duration with chondrogenic media (+TGF-β3) had minimal effect on MSC response to mechanical stimulation. After either 24 or 3 hours after the mechanical stimulation, all genes tested had fold changes that were statistically indistinguishable from a mean value of 1 (Figure 4-5 and Figure 4-6; $p>0.25$). The 24 hour post-load experiment demonstrates that the day 0 group had fold changes that slightly deviated from 1; however, none of these values were significant due to the large variability in the data. Because of the undetectable cell response with mechanical stimulation, we discontinued experiments early. Thus, our dataset lacks data at the day 18 group.

4.4 Discussion

Our data indicate that mechanical stimulation did not significantly affect the chondrogenic differentiation of mesenchymal stem cells at any stage of TGF-β induced differentiation. Consistent with prior studies, TGF-β in three-dimensional alginate culture induced chondrogenic differentiation as evidenced by secretion of proteoglycan and collagen matrix (65). However, compressive stimulation did not induce chondrogenic differentiation; chondrogenic genes— aggrecan, Sox9, and collagen I, II, and X—were unaffected by compressive stimulation. Several of our gene expression measurements have large standard deviations, suggesting large variability between replicates. This variability may stem from a variety of sources, including inconsistent or inadequate delivery of load.

Although mechanical stimulation did not induce cell differentiation, the specific protocol used in this study may have been the cause. Previous research indicates that MSCs differentiate in response to mechanical loading (136). Our data, however, indicates little change in gene expression with load. The contrasting results observed in this study and previous reports suggest that specific load doses are required to yield a response from MSCs. One possibility is that mechanical stimulation via contact with the hydrogels in displacement control has inherent variability in load delivery. Such variability could prevent consistent stimulation of cells and, thus, is a culprit in the lack of gene expression changes. Another possibility is that the potent induction of chondrogenic differentiation by TGF-β may have dwarfed the effects of mechanical stimulation. Previous reports suggest that mechanical stimulation may modulate MSC

differentiation through the TGF-β pathway (141). Although mechanical stimulation could promote chondrogenesis, exogenous TGF-β used in our experiment could mask these effects. When recalling that exogenous TGF-β is used in the culture medium for up to 24 days and the duration of mechanical stimulation is four hours, this is a plausible scenario. In addition, one result of TGF-β induced chonrogenic differentiation is the development of a pericellular matrix. The observed pericellular matrix could potentially facilitate mechanosensitivity by providing integrin attachment points. However, the pericellular matrix can also modulate or limit cell deformation (142,143). Because the amount of pericellular matrix increased with duration of TGF-β culture, cell deformation would be reduced at later time points of loading. Thus, these later time points of loading may not have had sufficient load transmission to stimulate the MSCs.

Although previous studies have reported changes in gene expression with mechanical doses similar to those used here, nuances in study design may be responsible for the differences in cell response to mechanical stimulation. For example, Mouw and coworkers reported changes in gene expression with three hours of mechanical stimulation (136). Although this dose of mechanical stimulation was comparable to that of the current study, Mouw and coworkers used MSCs harvested from immature bovine rather than from adult humans as in the current study. The cells of the prior study may have been more plastic than the adult human cells used in the current study and, therefore, more responsive to mechanical stimulation. While we were unable to induce chondrogenic differentiation using the stimulation protocol in the current study, human MSCs may require more potent mechanical stimuli to differentiate.

Future studies that investigate mechanotransduction could include several modifications. In particular, the delivery of mechanical stimulus could be more consistent using hydrostatic pressure rather than contact loading. Compressive loading via hydrostatic pressure allows a higher degree of control where the operator can control the force delivered to the cells and leaves no doubt that every cell in the experiment receives the same mechanical stimulation. Previous studies have used hydrostatic compressive load to induce differentiation of human MSCs (134,144,145). In contrast, contact loading used in the current study leaves the possibility that asymmetries in the loading jigs create uneven loading between cell-gel constructs. Furthermore, the contact load protocol required uniform hydrogel geometry, which we were able to achieve at the cost of suboptimal cell viability. We used a hydrogel polymerization protocol developed by others (138), which used GDL to release Ca^{+2} ions from $CaCO_3$ and polymerize alginate. While this process involved curing the alginate in a mold that controlled the construct geometry, it resulted in ~60-80% viability with some associated cell death. Other three-dimensional culture conditions, such as alginate beads (146) or cell pellets (134), may have assembly methods that are gentle in comparison to that of the current study and have higher cell viability. These alternative methods are compatible with mechanical stimulation by hydrostatic loading. The consistency of force magnitude and delivery to cell samples with compressive hydrostatic load may yield a more potent cell response than that of the current study.

The histology and dot blot data suggest that the proteoglycan and collagen II matrix are localized to cell pericellular matrix. After 24 days of TGF-β3 treatment, alcian blue staining for proteoglycans was localized to the cell and indicated little to no staining

in the alginate matrix distant from the cell. In addition, the dot blot suggests that collagen II was specific to the pericellular matrix. However, our use of Triton X-100 to solublize the collagen matrix was potentially inadequate. Exposure of the collagen II epitope may require heat treatment with more aggressive reagents. Thus, collagen II may have been present in the alginate supernatant, but undetectable. Nonetheless, the positive collagen II signal in the dot blot of the cell lysates suggests that collagen II was present in the pericellular matrix.

In summary, we have investigated compressive stimulation as a tool to induce chondrogenic differentiation of human MSCs. Our data indicated little to no gene response to compressive stimulation at any stage of TGF-β3 treatment. While we were unsuccessful in stimulating cell differentiation with mechanical compression, other studies using different stimulation protocols or different cell types suggest that compressive stimulation can induce cell differentiation. With these previous studies as a guide, we have highlighted future directions that may result in a potent cell response to mechanical stimulation.

Table 4-1: Number of replicates for gene expression analysis. Other genes includes aggrecan, collagen I, collagen II, and collagen X.

	24 Hours Post-load		**3 Hours Post-load**	
	Sox9	*Other Genes*	*Sox9*	*Other Genes*
Day 0	n=1	n=3	n=0	n=4
Day 6	n=2	n=3	n=0	n=3
Day 18	n=1	n=1	n=0	n=0
Day 24	n=2	n=4	n=0	n=2

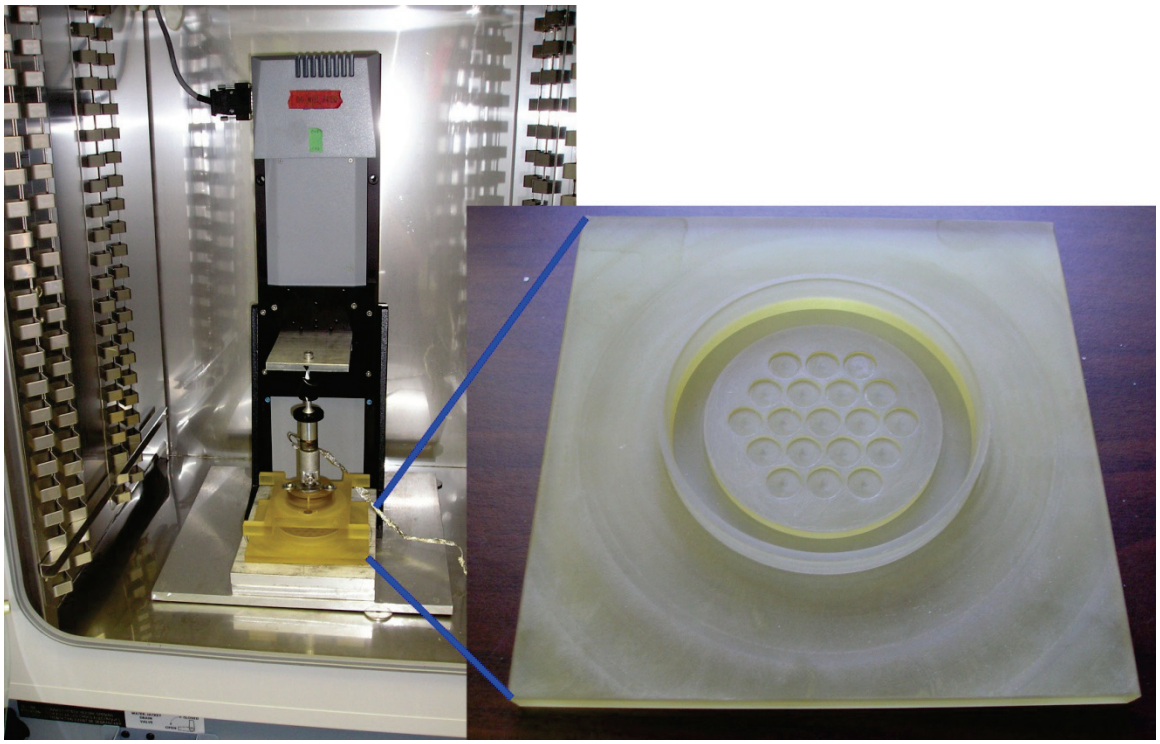

Figure 4-1: Custom bioreactor is housed in an incubator (left). Bottom loading platform with cylindrical depressions that hold the gel constructs is shown with a zoom image (right).

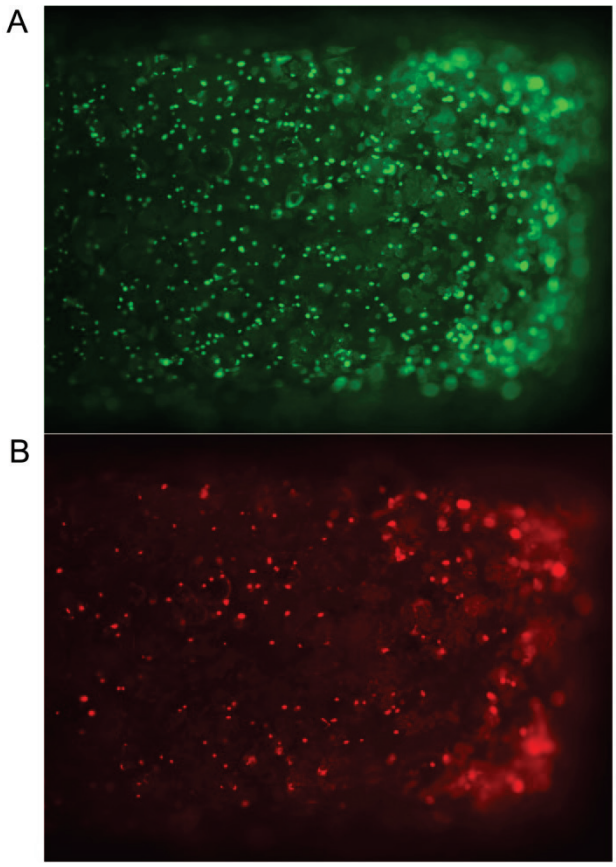

Figure 4-2: Viability at day 0. Green fluorescence indicates live (A) and red fluorescence indicates dead (B).

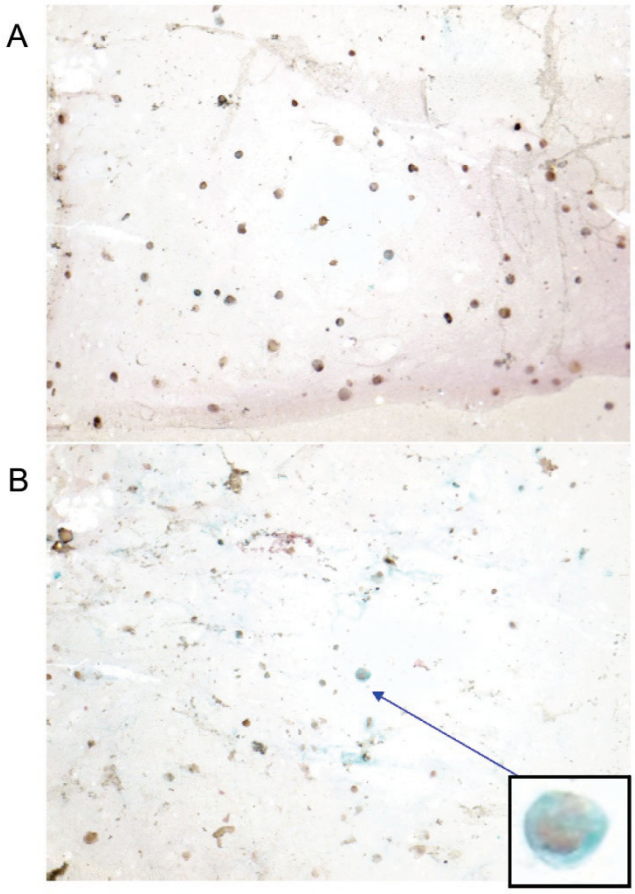

Figure 4-3: Alcian blue with hematoxylin counterstain. Cells at day 0 of alginate culture have no visible matrix (A). Cells cultured for 24 days in chondrogenic media supplemented with TGF-β3 have visible pericellular matrix (B).

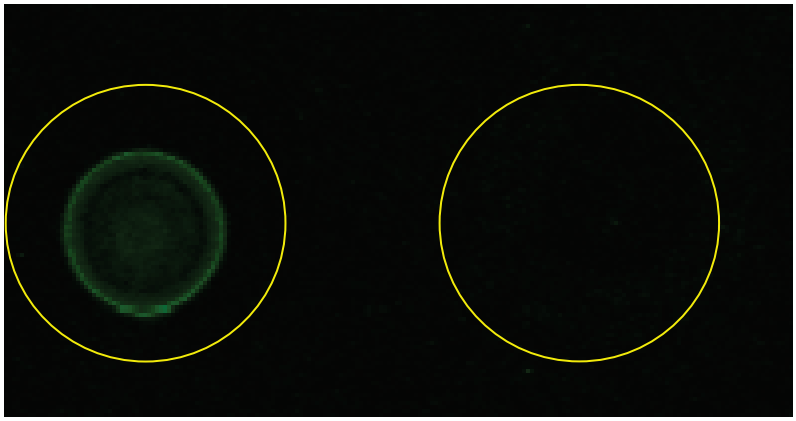

Figure 4-4: Dot blot for cells and alginate supernatant for collagen II antibody. Constructs were cultured for 24 days in chondrogenic media.

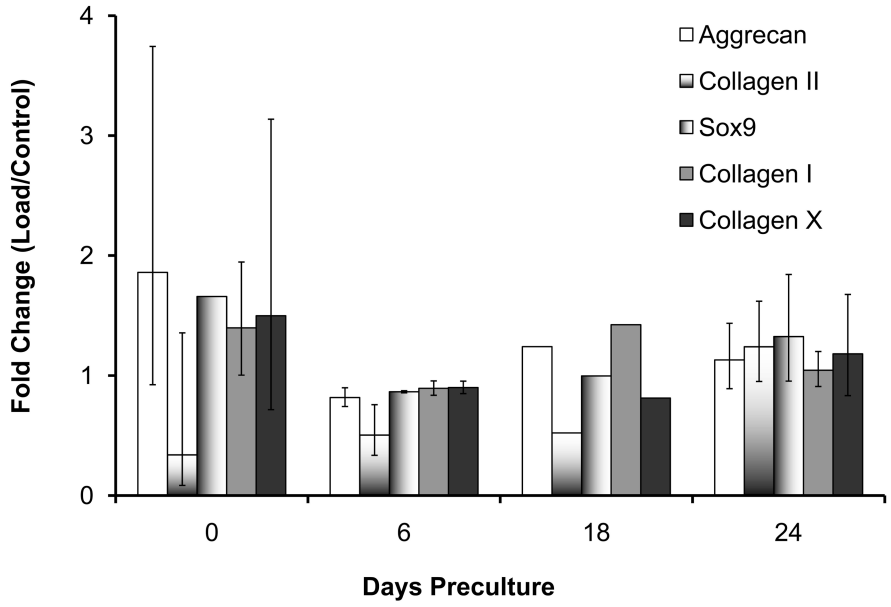

Figure 4-5: Gene expression for cells harvested 24 hours after load. Each gene normalized to GUS housekeeping gene. Each load sample normalized to contact control sample. Values are means ± SD.

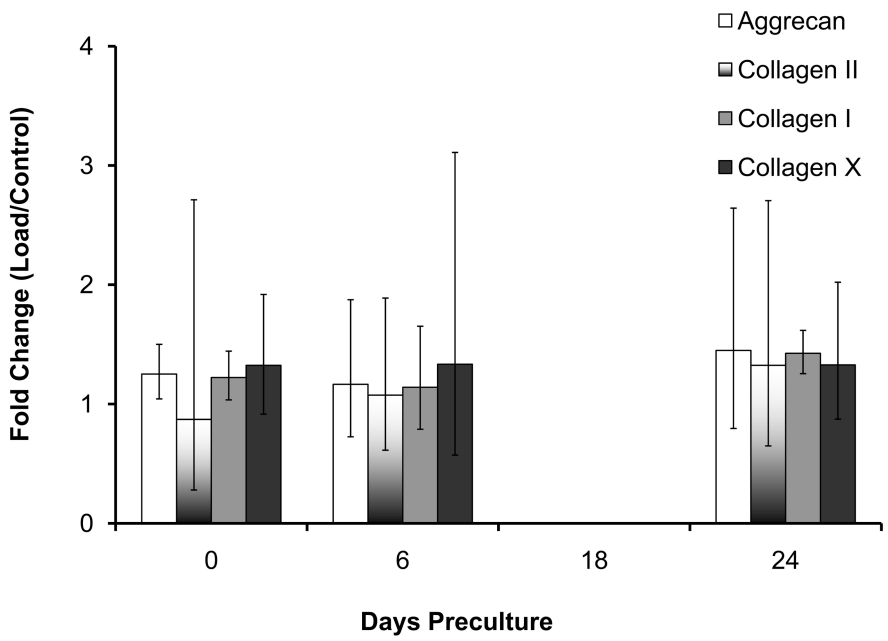

Figure 4-6: Gene expression for cells harvested 3 hours after load. Each gene normalized to GUS housekeeping gene. Each load sample normalized to contact control sample. Values are means ± SD.

Chapter 5: Conclusion

5.1 Research Summary

The overall goal of this dissertation is to characterize the mechanical and matrix properties of painful discs to guide tissue engineered therapy. This body of work progresses the existing knowledge of disc pain. From a research perspective, we have brought attention to the concept that the matrix of painful discs has unique features that cause diminished mechanical properties. In addition, we have investigated techniques to promote stem cell differentiation into chondrogenic cells. From a clinical perspective, these concepts lay the groundwork for future treatment, such as tissue engineering. For a tissue engineered therapy to succeed, parameters that define efficacy must be clarified. From a patient perspective, efficacy is synonymous with disc range of motion and load bearing without pain. The clinical design parameters required to achieve efficacy are less obvious. This dissertation helps in establishing threshold design parameters for tissue engineered constructs by comparing the matrix properties of painful and nonpainful discs.

While several prior studies have investigated the properties of healthy or degenerated discs, the current dissertation fills a gap in knowledge of painful disc properties.

In Chapter 2, we analyzed the mechanical properties and matrix content of painful and nonpainful degenerated discs. Mechanical properties were measured using compressive indentation and equilibrium dialysis. The extracellular matrix structure was assessed by histology and quantified by for proteoglycan, collagen, and collagen crosslinking. We observed that energy dissipation was significantly lower in the painful annulus than in the nonpainful annulus. In contrast, energy dissipation was significantly higher in the painful nucleus than in the nonpainful nucleus. Although not statistically significant, similar trends were observed for indentation modulus. The painful annulus was more viscous and less elastic than the nonpainful annulus. Equilibrium dialysis experiments showed that annulus swelling was significantly lower in the painful group. Consistent with this, we observed that the painful annulus had lower proteoglycan and higher collagen contents after adjusting for donor age. Our data indicate that painful discs have diminished mechanical properties that are partially explained by matrix biochemical composition for the annulus, but not for the nucleus. The results suggest that compromised matrix quality is a potential contributing factor to low back pain, possibly via inadequate material properties that lead to hypermobility or tissue stress concentrations. To the author's knowledge, this is the first study to characterize the mechanical properties of painful discs.

In Chapter 3, we characterized the synthesis of specific extracellular matrix molecules in painful discs. More specifically, we measured the gene expression of aggrecan, versican, fibromodulin, decorin, biglycan, collagen I, and collagen II using

quantitative real-time PCR. We found that the painful annulus had lower expression of proteoglycans (aggrecan, versican, fibromodulin, and decorin) and higher expression of collagen I than the nonpainful annulus. The painful nucleus had higher expression of decorin than the nonpainful nucleus. Interestingly, expression of these proteoglycans and collagens correlated with the mechanical properties measured in Chapter 2. We therefore concluded that painful discs have altered expression of matrix genes that may be the cause of their diminished mechanical properties. Importantly, the resulting matrix may be permissive to nerve infiltration and joint hypermobility.

In Chapter 4, we adjusted our focus to tissue engineering with mesenchymal stem cells (MSCs) as a cell source for nucleus pulposus cells. Specifically, we investigated the chondrogenic differentiation of MSCs using compressive stimulation at different stages of TGF-β3 mediated chondrogenesis. We assessed gene markers of chondrogenic differentiation, including aggrecan, collagen II, and Sox9. In addition, we measured the fibroblast marker collagen I and the hypertrophic marker collagen X. Contrary to prior studies demonstrating chondrogenic effects of compressive stimulation, we found that compressive stimulation did not significantly affect cell differentiation. Our surprising results do not diminish the conclusions of prior work. Instead, our results suggest that the details of a compressive stimulation protocol may have dramatic effects on cell response. Thus, future studies are needed to fine tune parameters that optimize chondrogenic differentiation of MSCs using compressive stimulation.

The approaches taken in these studies feature strengths that distinguish this dissertation from prior work. Because of our unique collaboration with UCSF clinicians, we had access to surgical waste tissue with specimen data that would be unavailable from

cadaveric specimen. In particular, data regarding the presence or lack of discogenic pain are provided by clinicians along with the surgical samples. Many of these valuable samples have size limitations that preclude other mechanical test protocols like tensile or confined compression testing. Given these constraints, we performed compressive indentation and equilibrium dialysis tests. Our data indicate that these tests possessed the sensitivity to detect altered mechanical properties in painful discs. In addition to mechanical characterization, we have quantified matrix content and analyzed matrix synthesis in painful discs. Human samples, such as those analyzed in the current dissertation, are the best option to study disc pain because no alternative model of disc pain exists.

5.2 Future Directions

The current dissertation did not analyze inflammation and innervation, which are important factors that contribute to pain. A future study that measures inflammatory factors and assesses innervation in painful discs could shed light on the findings of this dissertation. In particular, clarification of the inflammatory factors and consequent MMP levels in painful discs would explain the role of matrix degradation in the mechanical properties that we measured. Such analyses would provide a more complete perspective of both matrix synthesis and degradation in painful discs. Importantly, inflammation and hypermobility may cause pain by stimulating or sensitizing nerves in the disc (43). Increased depth of nerve infiltration and the presence of the neurotrophic factor, nerve growth factor, with receptor trk-A have been previously observed in painful discs

(147,148). A future study that combines current measures of disc matrix and mechanics with measures of inflammation and innervation would elucidate correlations between these parameters. Such a correlation analysis would clarify the relative roles of mechanical properties and inflammation in nerve infiltration and sensitization.

The effect of altered matrix in painful discs on nerve infiltration can be assessed in an *in vitro* study. In Chapters 2 and 3, we concluded that the painful annulus has decreased proteoglycan quantity and aggrecan synthesis. Because aggrecan inhibits nerve ingrowth (124), our data suggest that painful discs have decreased barriers to nerve entry. However, features of aggrecan besides quantity may affect nerve inhibition. Instead, subtle features of the aggrecan molecule – e.g. the degree of chondroitin sulfate or keratin sulfate glycosylation, or interactions with hyaluronan-link proteins to form aggregates – may affect its ability to inhibit nerve and blood vessel ingrowth. A future study investigating the ability of aggrecan from painful discs to inhibit nerve growth would provide insight on the cause of increased innervation in painful discs. These experiments would apply similar methods as those used previously to detect inhibition of nerve growth by aggrecan (124). Briefly, aggrecan from annulus samples is extracted and coated on a culture dish. A human neuronal cell line is seeded onto the dish and growth is stimulated using growth factor. Inhibition of nerve growth is assessed by measuring the reduction in neurite outgrowth compared to control groups without aggrecan. Such an experiment could shed light on how the altered proteoglycan matrix in painful discs affects nerve ingrowth.

The future directions discussed above could help direct treatment strategies. In particular, an improved understanding of how extracellular matrix influences

inflammation and innervation would help to focus existing design criteria for tissue engineered treatments. The specific correlation of innervation with inflammation and mechanical properties would determine the relative importance of each factor in a tissue engineered construct. In addition, if future studies indicate that aggrecan from painful discs have reduced inhibition of nerve growth, we would include an analysis of aggrecan on tissue engineered constructs as an indicator of success.

Tissue engineering of disc requires further study before it becomes clinically available. In Chapter 4, we investigated MSCs as a source for nucleus pulposus cells. Although our efforts were inconclusive, future work may clarify the stimuli that encourage nucleus pulposus differentiation. The goal of these efforts would be to create a replacement tissue that can be implanted into a patient and restore a proteoglycan rich nucleus. While the nucleus is a clinically relevant area of study, the annulus may also require treatment. Our data suggest that the annulus has compromised properties in painful discs and may exacerbate pain by causing hypermobility. Therefore, the annulus is also a potential therapeutic target. Moreover, if a therapy could successfully restore pressurization in the nucleus, then an annulus therapy may also be necessary to bolster the existing matrix, enabling it to support the newly pressurized nucleus (149).

Currently, tissue engineering of the annulus is in its infancy; few studies have addressed cell source, scaffold, or implantation. Ideally, cells that secrete annulus matrix, such as collagen I, would be seeded into a scaffold and incorporated into an annular defect. Tissue engineering efforts of the annulus lack a suitable cell source. MSCs are a promising candidate; however, further investigation is necessary to determine the stimuli necessary to promote differentiation into annulus cells (150). Another necessary item is a

cell scaffold that supports the mechanical environment of the annulus. Scaffolds composed of various polymers or gels have proved to be hospitable to cell attachment and matrix synthesis (149). Although these results are promising, an annulus scaffold must also be implanted into the patient. Implantation of the tissue engineered construct is a major obstacle that has received little attention (149). Future efforts to fix scaffolds to the existing annulus or endplate will help bring tissue engineered annulus into the clinic.

5.3 Closing Remarks

In conclusion, this dissertation characterizes the matrix of painful intervertebral disc and investigates MSCs as a cell source for tissue engineered nucleus. These experiments provide insight into the clinical requirements of tissue engineered treatment.

References

1. Cypress BK. Characteristics of physician visits for back symptoms: a national perspective. Am J Public Health. 1983 Apr;73(4):389-395.

2. Cassidy JD, Carroll LJ, Côté P. The Saskatchewan health and back pain survey. The prevalence of low back pain and related disability in Saskatchewan adults. Spine. 1998 Sep 1;23(17):1860-1866; discussion 1867.

3. Katz JN. Lumbar disc disorders and low-back pain: socioeconomic factors and consequences. J Bone Joint Surg Am. 2006 Apr;88 Suppl 2:21-24.

4. Rush AJ, Polatin P, Gatchel RJ. Depression and chronic low back pain: establishing priorities in treatment. Spine. 2000 Oct 15;25(20):2566-2571.

5. Deyo RA, Weinstein JN. Low back pain. N. Engl. J. Med. 2001 Feb 1;344(5):363-370.

6. Roughley PJ, Alini M, Antoniou J. The role of proteoglycans in aging, degeneration and repair of the intervertebral disc. Biochem. Soc. Trans. 2002 Nov;30(Pt 6):869-874.

7. Sive JI, Baird P, Jeziorsk M, Watkins A, Hoyland JA, Freemont AJ. Expression of chondrocyte markers by cells of normal and degenerate intervertebral discs. MP, Mol. Pathol. 2002 Apr;55(2):91-97.

8. Cassidy JJ, Hiltner A, Baer E. Hierarchical structure of the intervertebral disc. Connect. Tissue Res. 1989;23(1):75-88.

9. Eyre DR, Muir H. Types I and II collagens in intervertebral disc. Interchanging radial distributions in annulus fibrosus. Biochem. J. 1976 Jul 1;157(1):267-270.

10. Smith LJ, Fazzalari NL. The elastic fibre network of the human lumbar anulus fibrosus: architecture, mechanical function and potential role in the progression of intervertebral disc degeneration. Eur Spine J. 2009 Apr;18(4):439-448.

11. Urban JPG, Smith S, Fairbank JCT. Nutrition of the intervertebral disc. Spine. 2004 Dec 1;29(23):2700-2709.

12. Urban JP, McMullin JF. Swelling pressure of the lumbar intervertebral discs: influence of age, spinal level, composition, and degeneration. Spine. 1988 Feb;13(2):179-187.

13. Adams MA, Green TP. Tensile properties of the annulus fibrosus. I. The contribution of fibre-matrix interactions to tensile stiffness and strength. Eur Spine J. 1993 Dec;2(4):203-208.

14. Yu J. Elastic tissues of the intervertebral disc. Biochem. Soc. Trans. 2002 Nov;30(Pt 6):848-852.

15. Ishihara H, Urban JP. Effects of low oxygen concentrations and metabolic inhibitors on proteoglycan and protein synthesis rates in the intervertebral disc. J. Orthop. Res. 1999 Nov;17(6):829-835.

16. Razaq S, Wilkins RJ, Urban JPG. The effect of extracellular pH on matrix turnover by cells of the bovine nucleus pulposus. Eur Spine J. 2003 Aug;12(4):341-349.

17. Urban JPG. The role of the physicochemical environment in determining disc cell behaviour. Biochem. Soc. Trans. 2002 Nov;30(Pt 6):858-864.

18. Roughley PJ. Biology of intervertebral disc aging and degeneration: involvement of the extracellular matrix. Spine. 2004 Dec 1;29(23):2691-2699.

19. Myllyharju J, Kivirikko KI. Collagens and collagen-related diseases. Ann. Med. 2001 Feb;33(1):7-21.

20. Avery NC, Bailey AJ. The effects of the Maillard reaction on the physical properties and cell interactions of collagen. Pathol. Biol. 2006 Sep;54(7):387-395.

21. Wagner DR, Reiser KM, Lotz JC. Glycation increases human annulus fibrosus stiffness in both experimental measurements and theoretical predictions. J Biomech. 2006;39(6):1021-1029.

22. Feng H, Danfelter M, Strömqvist B, Heinegård D. Extracellular matrix in disc degeneration. J Bone Joint Surg Am. 2006 Apr;88 Suppl 2:25-29.

23. Handley CJ, Samiric T, Ilic MZ. Structure, Metabolism, and Tissue Roles of Chondroitin Sulfate Proteoglycans. In: Chondroitin Sulfate: Structure, Role and Pharmacological Activity. Academic Press; 2006. p. 219-232.

24. Melrose J, Ghosh P, Taylor TK. A comparative analysis of the differential spatial and temporal distributions of the large (aggrecan, versican) and small (decorin, biglycan, fibromodulin) proteoglycans of the intervertebral disc. J. Anat. 2001 Jan;198(Pt 1):3-15.

25. Iozzo RV. The biology of the small leucine-rich proteoglycans. Functional network of interactive proteins. J. Biol. Chem. 1999 Jul 2;274(27):18843-18846.

26. Danielson KG, Baribault H, Holmes DF, Graham H, Kadler KE, Iozzo RV. Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility. J. Cell Biol. 1997 Feb 10;136(3):729-743.

27. Svensson L, Aszódi A, Reinholt FP, Fässler R, Heinegård D, Oldberg A. Fibromodulin-null mice have abnormal collagen fibrils, tissue organization, and altered lumican deposition in tendon. J. Biol. Chem. 1999 Apr 2;274(14):9636-9647.

28. Hildebrand A, Romarís M, Rasmussen LM, Heinegård D, Twardzik DR, Border WA, et al. Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. Biochem. J. 1994 Sep 1;302 (Pt 2):527-534.

29. Buckwalter JA. Aging and degeneration of the human intervertebral disc. Spine. 1995 Jun 1;20(11):1307-1314.

30. Acaroglu ER, Iatridis JC, Setton LA, Foster RJ, Mow VC, Weidenbaum M. Degeneration and aging affect the tensile behavior of human lumbar anulus fibrosus. Spine. 1995 Dec 15;20(24):2690-2701.

31. Le Maitre CL, Pockert A, Buttle DJ, Freemont AJ, Hoyland JA. Matrix synthesis and degradation in human intervertebral disc degeneration. Biochem. Soc. Trans. 2007 Aug;35(Pt 4):652-655.

32. Lotz JC, Hsieh AH, Walsh AL, Palmer EI, Chin JR. Mechanobiology of the intervertebral disc. Biochem. Soc. Trans. 2002 Nov;30(Pt 6):853-858.

33. Nerlich AG, Schleicher ED, Boos N. 1997 Volvo Award winner in basic science studies. Immunohistologic markers for age-related changes of human lumbar intervertebral discs. Spine. 1997 Dec 15;22(24):2781-2795.

34. Cs-Szabo G, Ragasa-San Juan D, Turumella V, Masuda K, Thonar EJ-MA, An HS. Changes in mRNA and protein levels of proteoglycans of the anulus fibrosus and nucleus pulposus during intervertebral disc degeneration. Spine. 2002 Oct 15;27(20):2212-2219.

35. Boos N, Weissbach S, Rohrbach H, Weiler C, Spratt KF, Nerlich AG. Classification of age-related changes in lumbar intervertebral discs: 2002 Volvo Award in basic science. Spine. 2002 Dec 1;27(23):2631-2644.

36. Le Maitre CL, Freemont AJ, Hoyland JA. Localization of degradative enzymes and their inhibitors in the degenerate human intervertebral disc. J. Pathol. 2004 Sep;204(1):47-54.

37. Crean JK, Roberts S, Jaffray DC, Eisenstein SM, Duance VC. Matrix metalloproteinases in the human intervertebral disc: role in disc degeneration and scoliosis. Spine. 1997 Dec 15;22(24):2877-2884.

38. Fujita Y, Duncan NA, Lotz JC. Radial tensile properties of the lumbar annulus fibrosus are site and degeneration dependent. J. Orthop. Res. 1997 Nov;15(6):814-819.

39. Iatridis JC, Setton LA, Weidenbaum M, Mow VC. Alterations in the mechanical behavior of the human lumbar nucleus pulposus with degeneration and aging. J. Orthop. Res. 1997 Mar;15(2):318-322.

40. Johannessen W, Elliott DM. Effects of degeneration on the biphasic material properties of human nucleus pulposus in confined compression. Spine. 2005 Dec 15;30(24):E724-729.

41. Boden SD, Davis DO, Dina TS, Patronas NJ, Wiesel SW. Abnormal magnetic-resonance scans of the lumbar spine in asymptomatic subjects. A prospective investigation. J Bone Joint Surg Am. 1990 Mar;72(3):403-408.

42. Borenstein DG, O'Mara JW, Boden SD, Lauerman WC, Jacobson A, Platenberg C, et al. The value of magnetic resonance imaging of the lumbar spine to predict low-back pain in asymptomatic subjects: a seven-year follow-up study. J Bone Joint Surg Am. 2001 Sep;83-A(9):1306-1311.

43. Lotz JC, Ulrich JA. Innervation, inflammation, and hypermobility may characterize pathologic disc degeneration: review of animal model data. J Bone Joint Surg Am. 2006 Apr;88 Suppl 2:76-82.

44. Peng B, Wu W, Hou S, Li P, Zhang C, Yang Y. The pathogenesis of discogenic low back pain. J Bone Joint Surg Br. 2005 Jan;87(1):62-67.

45. Ulrich JA, Liebenberg EC, Thuillier DU, Lotz JC. ISSLS prize winner: repeated disc injury causes persistent inflammation. Spine. 2007 Dec 1;32(25):2812-2819.

46. O'Neill CW, Liu JJ, Leibenberg E, Hu SS, Deviren V, Tay BK-B, et al. Percutaneous plasma decompression alters cytokine expression in injured porcine intervertebral discs. Spine J. 2004 Feb;4(1):88-98.

47. Kawakami M, Hashizume H, Nishi H, Matsumoto T, Tamaki T, Kuribayashi K. Comparison of neuropathic pain induced by the application of normal and mechanically compressed nucleus pulposus to lumbar nerve roots in the rat. J. Orthop. Res. 2003 May;21(3):535-539.

48. Woolf CJ, Allchorne A, Safieh-Garabedian B, Poole S. Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. Br. J. Pharmacol. 1997 Jun;121(3):417-424.

49. Zhang Y-gang, Guo T-mao, Guo X, Wu S-xun. Clinical diagnosis for discogenic low back pain. Int. J. Biol. Sci. 2009;5(7):647-658.

50. Raj PP. Intervertebral disc: anatomy-physiology-pathophysiology-treatment. Pain Pract. 2008 Feb;8(1):18-44.

51. Quittan M. Management of back pain. Disabil Rehabil. 2002 May 20;24(8):423-434.

52. Schizas C, Kulik G, Kosmopoulos V. Disc degeneration: current surgical options. Eur Cell Mater. 2010;20:306-315.

53. Harrop JS, Youssef JA, Maltenfort M, Vorwald P, Jabbour P, Bono CM, et al. Lumbar adjacent segment degeneration and disease after arthrodesis and total disc arthroplasty. Spine. 2008 Jul 1;33(15):1701-1707.

54. Freeman BJC, Davenport J. Total disc replacement in the lumbar spine: a systematic review of the literature. Eur Spine J. 2006 Aug;15 Suppl 3:S439-447.

55. Goins ML, Wimberley DW, Yuan PS, Fitzhenry LN, Vaccaro AR. Nucleus pulposus replacement: an emerging technology. Spine J. 2005 Dec;5(6 Suppl):317S-324S.

56. O'Halloran DM, Pandit AS. Tissue-engineering approach to regenerating the intervertebral disc. Tissue Eng. 2007 Aug;13(8):1927-1954.

57. Kandel R, Roberts S, Urban JPG. Tissue engineering and the intervertebral disc: the challenges. Eur Spine J. 2008 Dec;17 Suppl 4:480-491.

58. Daar AS, Bhatt A, Court E, Singer PA. Stem cell research and transplantation: science leading ethics. Transplant. Proc. 2004 Oct;36(8):2504-2506.

59. Leung VYL, Chan D, Cheung KMC. Regeneration of intervertebral disc by mesenchymal stem cells: potentials, limitations, and future direction. Eur Spine J. 2006 Aug;15 Suppl 3:S406-413.

60. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999 Apr 2;284(5411):143-147.

61. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature. 2003 Oct 9;425(6958):577-584.

62. Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature. 1997 Dec 4;390(6659):465-471.

63. Furumatsu T, Tsuda M, Taniguchi N, Tajima Y, Asahara H. Smad3 induces chondrogenesis through the activation of SOX9 via CREB-binding protein/p300 recruitment. J. Biol. Chem. 2005 Mar 4;280(9):8343-8350.

64. Barry F, Boynton RE, Liu B, Murphy JM. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components. Exp. Cell Res. 2001 Aug 15;268(2):189-200.

65. Ichinose S, Yamagata K, Sekiya I, Muneta T, Tagami M. Detailed examination of cartilage formation and endochondral ossification using human mesenchymal stem cells. Clin. Exp. Pharmacol. Physiol. 2005 Jul;32(7):561-570.

66. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp. Cell Res. 1998 Jan 10;238(1):265-272.

67. Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester CO, Pittenger MF. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. Tissue Eng. 1998;4(4):415-428.

68. Hojo H, Ohba S, Yano F, Chung U-il. Coordination of chondrogenesis and osteogenesis by hypertrophic chondrocytes in endochondral bone development. J. Bone Miner. Metab. 2010 Sep;28(5):489-502.

69. Koga H, Engebretsen L, Brinchmann JE, Muneta T, Sekiya I. Mesenchymal stem cell-based therapy for cartilage repair: a review. Knee Surg Sports Traumatol Arthrosc. 2009 Nov;17(11):1289-1297.

70. Guilak F, Meyer BC, Ratcliffe A, Mow VC. The effects of matrix compression on proteoglycan metabolism in articular cartilage explants. Osteoarthr. Cartil. 1994 Jun;2(2):91-101.

71. Wong M, Siegrist M, Cao X. Cyclic compression of articular cartilage explants is associated with progressive consolidation and altered expression pattern of extracellular matrix proteins. Matrix Biol. 1999 Aug;18(4):391-399.

72. Palmoski MJ, Brandt KD. Effects of static and cyclic compressive loading on articular cartilage plugs in vitro. Arthritis Rheum. 1984 Jun;27(6):675-681.

73. Huang C-YC, Hagar KL, Frost LE, Sun Y, Cheung HS. Effects of cyclic compressive loading on chondrogenesis of rabbit bone-marrow derived mesenchymal stem cells. Stem Cells. 2004;22(3):313-323.

74. Vunjak-Novakovic G, Altman G, Horan R, Kaplan DL. Tissue engineering of ligaments. Annu Rev Biomed Eng. 2004;6:131-156.

75. Hsieh AH, Tsai CM, Ma QJ, Lin T, Banes AJ, Villarreal FJ, et al. Time-dependent increases in type-III collagen gene expression in medical collateral ligament fibroblasts under cyclic strains. J. Orthop. Res. 2000 Mar;18(2):220-227.

76. Altman GH, Horan RL, Martin I, Farhadi J, Stark PRH, Volloch V, et al. Cell differentiation by mechanical stress. FASEB J. 2002 Feb;16(2):270-272.

77. Juncosa-Melvin N, Matlin KS, Holdcraft RW, Nirmalanandhan VS, Butler DL. Mechanical stimulation increases collagen type I and collagen type III gene expression of stem cell-collagen sponge constructs for patellar tendon repair. Tissue Eng. 2007 Jun;13(6):1219-1226.

78. Adams MA, Dolan P, Hutton WC, Porter RW. Diurnal changes in spinal mechanics and their clinical significance. J Bone Joint Surg Br. 1990 Mar;72(2):266-270.

79. Peng B, Chen J, Kuang Z, Li D, Pang X, Zhang X. Expression and role of connective tissue growth factor in painful disc fibrosis and degeneration. Spine. 2009 Mar 1;34(5):E178-182.

80. Lotz JC, Kim AJ. Disc regeneration: why, when, and how. Neurosurg. Clin. N. Am. 2005 Oct;16(4):657-663, vii.

81. Iatridis JC, Setton LA, Foster RJ, Rawlins BA, Weidenbaum M, Mow VC. Degeneration affects the anisotropic and nonlinear behaviors of human anulus fibrosus in compression. J Biomech. 1998 Jun;31(6):535-544.

82. Peng B, Hao J, Hou S, Wu W, Jiang D, Fu X, et al. Possible pathogenesis of painful intervertebral disc degeneration. Spine. 2006 Mar 1;31(5):560-566.

83. Pfirrmann CW, Metzdorf A, Zanetti M, Hodler J, Boos N. Magnetic resonance classification of lumbar intervertebral disc degeneration. Spine. 2001 Sep 1;26(17):1873-1878.

84. Wolfer LR, Derby R, Lee J-E, Lee S-H. Systematic review of lumbar provocation discography in asymptomatic subjects with a meta-analysis of false-positive rates. Pain Physician. 2008 Aug;11(4):513-538.

85. Hansma P, Yu H, Schultz D, Rodriguez A, Yurtsev EA, Orr J, et al. The tissue diagnostic instrument. Rev Sci Instrum. 2009 May;80(5):054303.

86. Schultz DS, Rodriguez AG, Hansma PK, Lotz JC. Mechanical profiling of intervertebral discs. J Biomech. 2009 Apr 2

87. Wilke HJ, Neef P, Caimi M, Hoogland T, Claes LE. New in vivo measurements of pressures in the intervertebral disc in daily life. Spine. 1999 Apr 15;24(8):755-762.

88. Farndale RW, Sayers CA, Barrett AJ. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. Connect. Tissue Res. 1982;9(4):247-248.

89. Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch. Biochem. Biophys. 1961 May;93:440-447.

90. Colgrave ML, Allingham PG, Jones A. Hydroxyproline quantification for the estimation of collagen in tissue using multiple reaction monitoring mass spectrometry. Journal of Chromatography A. 2008 Nov 28;1212(1-2):150-153.

91. Monnier VM, Kohn RR, Cerami A. Accelerated age-related browning of human collagen in diabetes mellitus. Proc. Natl. Acad. Sci. U.S.A. 1984 Jan;81(2):583-587.

92. Desbiens NA. A novel use for the word “trend” in the clinical trial literature. Am. J. Med. Sci. 2003 Aug;326(2):61-65.

93. Studer RK, Aboka AM, Gilbertson LG, Georgescu H, Sowa G, Vo N, et al. p38 MAPK inhibition in nucleus pulposus cells: a potential target for treating intervertebral disc degeneration. Spine. 2007 Dec 1;32(25):2827-2833.

94. Korhonen RK, Laasanen MS, Töyräs J, Rieppo J, Hirvonen J, Helminen HJ, et al. Comparison of the equilibrium response of articular cartilage in unconfined compression, confined compression and indentation. J Biomech. 2002 Jul;35(7):903-909.

95. Umehara S, Tadano S, Abumi K, Katagiri K, Kaneda K, Ukai T. Effects of degeneration on the elastic modulus distribution in the lumbar intervertebral disc. Spine. 1996 Apr 1;21(7):811-819; discussion 820.

96. Diab M, Wu JJ, Eyre DR. Collagen type IX from human cartilage: a structural profile of intermolecular cross-linking sites. Biochem. J. 1996 Feb 15;314 (Pt 1):327-332.

97. Annunen S, Paassilta P, Lohiniva J, Perälä M, Pihlajamaa T, Karppinen J, et al. An allele of COL9A2 associated with intervertebral disc disease. Science. 1999 Jul 16;285(5426):409-412.

98. Aladin DMK, Cheung KMC, Chan D, Yee AFY, Jim JJT, Luk KDK, et al. Expression of the Trp2 allele of COL9A2 is associated with alterations in the mechanical properties of human intervertebral discs. Spine. 2007 Dec 1;32(25):2820-2826.

99. Sivan S, Merkher Y, Wachtel E, Ehrlich S, Maroudas A. Correlation of swelling pressure and intrafibrillar water in young and aged human intervertebral discs. J. Orthop. Res. 2006 Jun;24(6):1292-1298.

100. Thompson JP, Pearce RH, Schechter MT, Adams ME, Tsang IK, Bishop PB. Preliminary evaluation of a scheme for grading the gross morphology of the human intervertebral disc. Spine. 1990 May;15(5):411-415.

101. Bushell GR, Ghosh P, Taylor TK, Sutherland JM. The collagen of the intervertebral disc in adolescent idiopathic scoliosis. J Bone Joint Surg Br. 1979 Nov;61-B(4):501-508.

102. Duance VC, Crean JK, Sims TJ, Avery N, Smith S, Menage J, et al. Changes in collagen cross-linking in degenerative disc disease and scoliosis. Spine. 1998 Dec 1;23(23):2545-2551.

103. Yu J, Fairbank JCT, Roberts S, Urban JPG. The elastic fiber network of the anulus fibrosus of the normal and scoliotic human intervertebral disc. Spine. 2005 Aug 15;30(16):1815-1820.

104. Pedrini-Mille A, Pedrini VA, Tudisco C, Ponseti IV, Weinstein SL, Maynard JA. Proteoglycans of human scoliotic intervertebral disc. J Bone Joint Surg Am. 1983 Jul;65(6):815-823.

105. Bibby SRS, Fairbank JCT, Urban MR, Urban JPG. Cell viability in scoliotic discs in relation to disc deformity and nutrient levels. Spine. 2002 Oct 15;27(20):2220-2228; discussion 2227-2228.

106. Adams MA, Roughley PJ. What is intervertebral disc degeneration, and what causes it? Spine. 2006;31(18):2151.

107. Miyazaki M, Hong SW, Yoon SH, Zou J, Tow B, Alanay A, et al. Kinematic analysis of the relationship between the grade of disc degeneration and motion unit of the cervical spine. Spine. 2008 Jan 15;33(2):187-193.

108. Kong MH, Hymanson HJ, Song KY, Chin DK, Cho YE, Yoon DH, et al. Kinetic magnetic resonance imaging analysis of abnormal segmental motion of the functional spine unit. J Neurosurg Spine. 2009 Apr;10(4):357-365.

109. Ariga K, Miyamoto S, Nakase T, Okuda S, Meng W, Yonenobu K, et al. The relationship between apoptosis of endplate chondrocytes and aging and degeneration of the intervertebral disc. Spine. 2001 Nov 15;26(22):2414-2420.

110. Adams MA. Biomechanics of back pain. Acupunct Med. 2004 Dec;22(4):178-188.

111. McNally DS, Shackleford IM, Goodship AE, Mulholland RC. In vivo stress measurement can predict pain on discography. Spine. 1996 Nov 15;21(22):2580-2587.

112. Adams MA, Freeman BJ, Morrison HP, Nelson IW, Dolan P. Mechanical initiation of intervertebral disc degeneration. Spine. 2000 Jul 1;25(13):1625-1636.

113. Gertzbein SD, Seligman J, Holtby R, Chan KH, Kapasouri A, Tile M, et al. Centrode patterns and segmental instability in degenerative disc disease. Spine. 1985 Apr;10(3):257-261.

114. Sztrolovics R, Alini M, Mort JS, Roughley PJ. Age-related changes in fibromodulin and lumican in human intervertebral discs. Spine. 1999 Sep 1;24(17):1765-1771.

115. Antoniou J, Steffen T, Nelson F, Winterbottom N, Hollander AP, Poole RA, et al. The human lumbar intervertebral disc: evidence for changes in the biosynthesis and denaturation of the extracellular matrix with growth, maturation, ageing, and degeneration. J. Clin. Invest. 1996 Aug 15;98(4):996-1003.

116. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc. 2008;3(6):1101-1108.

117. Reed CC, Iozzo RV. The role of decorin in collagen fibrillogenesis and skin homeostasis. Glycoconj. J. 2002 Jun;19(4-5):249-255.

118. Götz W, Barnert S, Bertagnoli R, Miosge N, Kresse H, Herken R. Immunohistochemical localization of the small proteoglycans decorin and biglycan in human intervertebral discs. Cell Tissue Res. 1997 Jul;289(1):185-190.

119. Zimmermann DR, Dours-Zimmermann MT, Schubert M, Bruckner-Tuderman L. Versican is expressed in the proliferating zone in the epidermis and in association with the elastic network of the dermis. J. Cell Biol. 1994 Mar;124(5):817-825.

120. Bode-Lesniewska B, Dours-Zimmermann MT, Odermatt BF, Briner J, Heitz PU, Zimmermann DR. Distribution of the large aggregating proteoglycan versican in adult human tissues. J. Histochem. Cytochem. 1996 Apr;44(4):303-312.

121. Lyons G, Eisenstein SM, Sweet MB. Biochemical changes in intervertebral disc degeneration. Biochim. Biophys. Acta. 1981 Apr 3;673(4):443-453.

122. Palmgren T, Grönblad M, Virri J, Kääpä E, Karaharju E. An immunohistochemical study of nerve structures in the anulus fibrosus of human normal lumbar intervertebral discs. Spine. 1999 Oct 15;24(20):2075-2079.

123. Johnson WE, Evans H, Menage J, Eisenstein SM, El Haj A, Roberts S. Immunohistochemical detection of Schwann cells in innervated and vascularized human intervertebral discs. Spine. 2001 Dec 1;26(23):2550-2557.

124. Johnson WEB, Caterson B, Eisenstein SM, Hynds DL, Snow DM, Roberts S. Human intervertebral disc aggrecan inhibits nerve growth in vitro. Arthritis & Rheumatism. 2002 Oct;46(10):2658-2664.

125. Johnson WEB, Caterson B, Eisenstein SM, Roberts S. Human intervertebral disc aggrecan inhibits endothelial cell adhesion and cell migration in vitro. Spine. 2005 May 15;30(10):1139-1147.

126. Young RG, Butler DL, Weber W, Caplan AI, Gordon SL, Fink DJ. Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J Orthop Res. 1998 Jul;16(4):406-13.

127. Sekiya I, Vuoristo JT, Larson BL, Prockop DJ. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. Proc Natl Acad Sci U S A. 2002 Apr;99(7):4397-402.

128. Mauck RL, Yuan X, Tuan RS. Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. Osteoarthritis Cartilage. 2006 Feb;14(2):179-89.

129. Buschmann MD, Gluzband YA, Grodzinsky AJ, Hunziker EB. Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. J Cell Sci. 1995 Apr;108 (Pt 4):1497-508.

130. Grodzinsky AJ, Levenston ME, Jin M, Frank EH. Cartilage tissue remodeling in response to mechanical forces. Annu Rev Biomed Eng. 2000;2:691-713.

131. Mauck RL, Soltz MA, Wang CC, Wong DD, Chao PH, Valhmu WB, et al. Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. J Biomech Eng. 2000 Jun;122(3):252-60.

132. Wong M, Siegrist M, Goodwin K. Cyclic tensile strain and cyclic hydrostatic pressure differentially regulate expression of hypertrophic markers in primary chondrocytes. Bone. 2003 Oct;33(4):685-93.

133. Wu CW, Tchetina EV, Mwale F, Hasty K, Pidoux I, Reiner A, et al. Proteolysis involving matrix metalloproteinase 13 (collagenase-3) is required for chondrocyte differentiation that is associated with matrix mineralization. J. Bone Miner. Res. 2002 Apr;17(4):639-651.

134. Miyanishi K, Trindade MCD, Lindsey DP, Beaupré GS, Carter DR, Goodman SB, et al. Effects of hydrostatic pressure and transforming growth factor-beta 3 on adult human mesenchymal stem cell chondrogenesis in vitro. Tissue Eng. 2006 Jun;12(6):1419-28.

135. Li KW, Williamson AK, Wang AS, Sah RL. Growth responses of cartilage to static and dynamic compression. Clin Orthop Relat Res. 2001 Oct;(391 Suppl):S34-48.

136. Mouw JK, Connelly JT, Wilson CG, Michael KE, Levenston ME. Dynamic compression regulates the expression and synthesis of chondrocyte-specific matrix molecules in bone marrow stromal cells. Stem Cells. 2007 Mar;25(3):655-63.

137. Draget KI, Østgaard K, Smidsrød O. Alginate-based solid media for plant tissue culture. Applied Microbiology and Biotechnology. 1989;31(1):79-83.

138. Kuo CK, Ma PX. Ionically crosslinked alginate hydrogels as scaffolds for tissue engineering: part 1. Structure, gelation rate and mechanical properties. Biomaterials. 2001 Mar;22(6):511-21.

139. Scott JE, Dorling J. Differential staining of acid glycosaminoglycans (mucopolysaccharides) by alcian blue in salt solutions. Histochemie. 1965 Oct 1;5(3):221-233.

140. Masuda K, Sah RL, Hejna MJ, Thonar EJ-MA. A novel two-step method for the formation of tissue-engineered cartilage by mature bovine chondrocytes: the alginate-recovered-chondrocyte (ARC) method. J Orthop Res. 2003 Jan;21(1):139-48.

141. Li Z, Kupcsik L, Yao S-J, Alini M, Stoddart MJ. Mechanical load modulates chondrogenesis of human mesenchymal stem cells through the TGF-beta pathway. J. Cell. Mol. Med. 2010 Jun;14(6A):1338-1346.

142. Han S-K, Federico S, Herzog W. A depth-dependent model of the pericellular microenvironment of chondrocytes in articular cartilage. Comput Methods Biomech Biomed Engin. 2010 Jul 27;:1.

143. Julkunen P, Wilson W, Jurvelin JS, Korhonen RK. Composition of the pericellular matrix modulates the deformation behaviour of chondrocytes in articular cartilage under static loading. Med Biol Eng Comput. 2009 Dec;47(12):1281-1290.

144. Wagner DR, Lindsey DP, Li KW, Tummala P, Chandran SE, Smith RL, et al. Hydrostatic pressure enhances chondrogenic differentiation of human bone marrow stromal cells in osteochondrogenic medium. Ann Biomed Eng. 2008 May;36(5):813-820.

145. Angele P, Yoo JU, Smith C, Mansour J, Jepsen KJ, Nerlich M, et al. Cyclic hydrostatic pressure enhances the chondrogenic phenotype of human mesenchymal progenitor cells differentiated in vitro. J Orthop Res. 2003 May;21(3):451-7.

146. Ma H-L, Hung S-C, Lin S-Y, Chen Y-L, Lo W-H. Chondrogenesis of human mesenchymal stem cells encapsulated in alginate beads. J Biomed Mater Res A. 2003 Feb;64(2):273-81.

147. Freemont AJ, Peacock TE, Goupille P, Hoyland JA, O'Brien J, Jayson MI. Nerve ingrowth into diseased intervertebral disc in chronic back pain. Lancet. 1997 Jul 19;350(9072):178-181.

148. Freemont AJ, Watkins A, Le Maitre C, Baird P, Jeziorska M, Knight MTN, et al. Nerve growth factor expression and innervation of the painful intervertebral disc. J. Pathol. 2002 Jul;197(3):286-292.

149. Bron JL, Helder MN, Meisel H-J, Van Royen BJ, Smit TH. Repair, regenerative and supportive therapies of the annulus fibrosus: achievements and challenges. Eur Spine J. 2009 Mar;18(3):301-313.

150. Evans C. Potential biologic therapies for the intervertebral disc. J Bone Joint Surg Am. 2006 Apr;88 Suppl 2:95-98.

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