

ABSTRACT

Title of Document: THE REGULATION OF INTERVERTEBRAL
DISC CELL INTERACTIONS WITH THEIR
SURROUNDING MICROENVIRONMENT.

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Intervertebral disc degeneration is the major cause of back pain in the US, which can be both physically debilitating and costly to treat. Current treatments include invasive surgeries, which can be effective in ameliorating pain, but also contain the risk of complications. Additionally, these strategies target clinical manifestations of disc degeneration, rather than examine the cause of degenerative changes. Therefore, current research focuses on finding minimally invasive treatments for disc disease such as gene therapy. Regulating intervertebral disc cell interactions with their immediate environment can be a useful tool in the development of therapeutic strategies. This was explored through environmental changes to assess shifts in cell phenotype as well as genetic modulation to elucidate alterations in cell function. Biochemical, nutritional, and physical factors were examined in immature nucleus pulposus cells to assess changes in gene expression, attachment, and proliferation. It was found that nutritional and physical factors can alter gene expression levels of NP

cells, thereby altering cell phenotype. In addition, down-regulation of the proteolytic enzyme MMP-2 was explored through RNAi interference. Five shRNA lentiviral vectors were designed and validated for the sustained gene silencing of MMP-2. Silencing MMP-2 activity resulted in the inability of disc cells to focally degrade gelatin films as well as reduced ability of disc cells to remodel fibers in type I collagen gels, resulting in weakened gel architecture. These functional consequences were further explored in an *in vivo* study utilizing an annular needle-puncture model of disc degeneration. Injection of the shMMP lentiviral construct lead to decreased expression of MMP-2 in the disc, as well as improved disc height and morphology. Thus, the functional consequences of silencing MMP-2 were examined, elucidating its role in the degradative pathway leading to degenerative disc disease. The results of these studies can lay the foundation for developing therapeutic treatments for intervertebral disc degeneration.

THE REGULATION OF INTERVERTEBRAL DISC CELL INTERACTIONS
WITH THEIR SURROUNDING MICROENVIRONMENT

By

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Chapter 1: Background and Significance

The research presented in this dissertation is based on the study of intervertebral disc degeneration. While treatments for this prevalent disorder are well established, current research focuses on the cause of, and preventative strategies for, degenerative changes in the disc. Specifically we are interested in understanding the interplay between intervertebral disc cells and their microenvironment, in terms of changes in gene expression. For this work, we are particularly interested in using RNA interference (RNAi) as an engineering tool to modulate these interactions, and elucidate the consequences of these changes.

Significance

Back pain associated with musculoskeletal disorders of the spine is an extremely common condition that leads to enormous psychosocial and economic ramifications [1]. Current treatments, attempting to reduce pain rather than repair the degenerated disc, include muscle relaxants or injection of corticosteroids, manipulation therapies, discectomy or immobilization of the affected vertebrae [2]. Approaches such as these are not only unpredictable, but also deal almost exclusively with endstage clinical manifestations, and therefore do nothing to alter the disease process itself [1]. The discovery of RNAi presents a promising method to study mechanisms of intervertebral disc degeneration and to engineer cellular function for cell/molecular-based therapies. However, as with any novel technology, the foundation of the research needs to be established in each system. The proposed research is designed to lay out the groundwork for implementing RNAi in the

intervertebral disc, both *in vitro* and *in vivo*. This work will make a significant contribution in characterizing the functional role of MMP-2 in the progression of disc degeneration, and identifying key targets in the development of preventative and therapeutic interventions.

Societal Impact

Intervertebral disc degeneration is one of the conditions that has been linked to back pain. This prevalent medical disorder is the leading source of disability in people under 45 years of age [1] which results in national economic losses exceeding 100 billion dollars annually [3]. Up to 85% of people in the US will experience back pain at some point in their lifetime, and it is the second most common reason for symptomatic physician visits in this country. The vast majority of chronic back pain is associated with degeneration of the intervertebral disc, which can manifest in many different clinical conditions including spinal stenosis and instability, radiculopathy, myelopathy, and disc herniation [1]. Although a direct relationship between disc degeneration and discogenic pain has yet to be established, clinical indications through MRIs and cadaveric studies [4-7] suggest disc degeneration is at least a contributing factor since 90% of surgical procedures performed to alleviate pain involve degenerate discs [8].

Function of the intervertebral disc

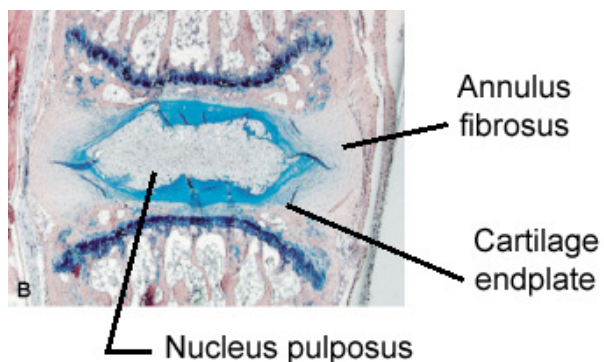
The intervertebral disc plays a mechanical role in the body as it enables the spine to bend and twist as well as support compressive loads. In a normal healthy

disc, the axial loads applied to the spine induce hydrostatic pressure in the nucleus pulposus [9, 10]. To support the load, this pressure distributes the forces evenly on the fibrous collagen network, along the circumference of the inner annulus and keeps the lamellae bulging outward. In the process of disc degeneration, this pressurization is lost, and the inner annulus lamellae collapse inward and become susceptible to degradation [10].

Dynamic loading is important for intervertebral disc physiology and prevention of disc degeneration. Mechanical loads vary across the disc, exposing intervertebral disc cells to complex physical stimuli including compressive, tensile, and shear stresses, fluid flow, hydrostatic pressure, and osmotic pressure. It has been suggested that these different mechanical stimuli influence disc matrix synthesis and turnover. Studies support the hypothesis that loading directly influences disc cell metabolism and that abnormal loading, may accelerate disc degeneration [11].

Structure and composition of the intervertebral disc

Figure 1.1. Intervertebral disc



The disc is composed of the annulus fibrosus, nucleus pulposus and cartilaginous endplates (Fig. 1.1 [12]). The outer annulus fibrosus is firm [13, 14] with high levels of organized collagen

fibers [15], containing fibroblasts towards the outside and fibrochondrocytes towards

the inside [16]. The inner nucleus pulposus is soft and highly hydrated [13, 14], consisting of a viscous proteoglycan gel with small amounts of collagen [15], containing chondrocytes and notochordal cells [16]. The cartilaginous endplates form a transition zone to the adjacent vertebral bodies. Together, these structures form an organic unit [17].

The three major constituents of the disc are water, fibrillar collagens, and aggrecan [9, 18]. The nucleus has a higher concentration of aggrecan and water than other regions of the disc, allowing it to resist compression and distribute forces [13]. In contrast, the annulus has the highest concentration of collagen. Collagen types also vary across the disc [18]. Type I collagen is found mainly in the outer annulus and type II collagen is found primarily in the inner annulus and nucleus [9, 18]. The disc contains many other matrix molecules whose distributions vary across the disc. The concentrations of these components changes with time, as seen in the decrease of water and aggrecan with age [18].

Development of the intervertebral disc

The discs develop from both the embryonic mesenchyme and notochord [15], which guides the embryonic development of the vertebral column [9]. Around day 30, mesenchymal cells begin to condense around the notochord to form the annulus fibrosus [19] and the non-

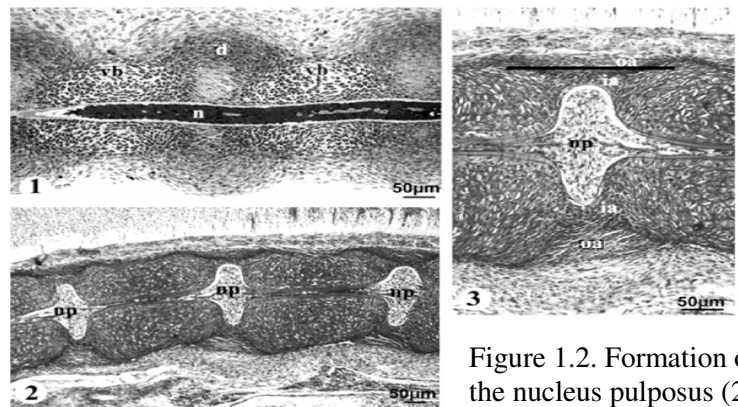


Figure 1.2. Formation of the nucleus pulposus (22)

condensed regions form the osseous vertebral bodies [19-21]. It is believed that the entrapped notochord cells synthesize the nucleus pulposus. (Fig. 1.2 [22]) These cells continue to express genes and proteins characteristic of the embryonic notochord [9, 19-21].

The cell population of the nucleus pulposus varies with age and species. In the early embryo, the cells of the nucleus are notochordal, producing a highly hydrated matrix different from those of mature human discs. Some species retain notochordal cells into adult life, while in others they disappear by birth. In humans, they disappear by 4 to 10 years of age [18]. It is unclear whether the change in cell population is due to differentiation of notochordal cells into chondrocytic cells, or whether it is a result of apoptosis and the migration of cells from the cartilaginous endplates and/or annulus [19-21].

Intervertebral Disc Degeneration

The intervertebral disc is a well encapsulated, essentially avascular organ that depends almost exclusively on diffusion of nutrients from a vascular supply found only at its very peripheral region. Cells of the nucleus are sometimes nearly a full centimeter away from the nearest blood supply [1]. Intervertebral disc degeneration is a multifactorial process involving mechanical, genetic, and biologic factors. Degenerative changes begin relatively early in life and progress through a definable sequence of events. A hallmark of disc degeneration is loss of the normal extracellular matrix in the nucleus pulposus [23], leading to diminished biochemical properties of the disc [17]. In discs containing notochordal cells, collagen content is

low and discs remain hydrated, rarely showing signs of degeneration. The mature human intervertebral disc has low cell density, possibly due to loss of notochordal cells. Intervertebral disc degeneration can first be observed in the nucleus pulposus shortly after the disappearance of notochordal cells although peak incidence in humans occurs much later in life [9].

During the aging process, the low proteoglycan content of the nucleus leads to decreased hydration and the loss of hydrostatic pressure [1]. Therefore, the collagen-rich lamellae of the inner annulus collapse inward and become vulnerable to disease [10]. The collagen content increases and nucleus eventually becomes more fibrous and less hydrated [9]. The annulus fibrosus is then forced to carry larger loads, leading to tears, bulging, rupture, and herniation which can ultimately lead to the failure of the tissue [9]. Other signs of degeneration include the inability to maintain disc height and vertebral body pathology including subchondral sclerosis, end plate ossification and osteophyte formation [1]. If the exact mechanism of the disc pathology was understood, the biologic repair or regeneration of the intervertebral disc could theoretically be achieved by the stimulation of matrix molecules or the prevention of matrix degradation and cell death [24].

Disc repair and remodeling

Damage to the intervertebral disc can result in trauma or arise from degeneration accompanying aging or disease. Healing of such damage often does not occur and degradative processes may lead to herniation of the annulus fibrosus, resulting in pain and predisposing to complete intervertebral disc degeneration [25].

There is currently little information on the healing process of the disc. Most of the research focuses on elucidating the process of disc degeneration, as well as treatments and measures of prevention.

Tissue engineering for the disc may lead to cell-based techniques appropriate for biologic therapies for disc degeneration. The microenvironment surrounding a cell provides not only mechanical support but also a molecular framework, the molecules of which may promote signaling pathways that influence important cell functions, such as proliferation and extracellular matrix production [26]. However, the cell density in normal human discs is low, and many of the cells in degenerate discs are dead, therefore stimulation of the remaining cells using cell based therapies may be insufficient to repair the matrix [2]. Gene therapy is another technique for disc repair that is under investigation. In this strategy, a gene or genes encoding biologically active molecule are introduced into the disc, leading to a stimulation of indigenous disc cells to increase the output of matrix constituents [27]. However, low cell density limits its potential.

Research has also identified many potential molecular targets for biologic intervention in the disc. These molecules can be divided into mitogens, which affect cellular division, and cytokines which can affect a wide variety of intracellular processes by binding to cell surface receptors. At the molecular level, they influence a wide variety of cellular processes including inflammation, matrix stability, vascular ingrowth, and cell viability. Molecular factors may have potential as a means of treatment for symptomatic degenerative disc disease by achieving tissue repair or

symptom reduction [27], however their mode of action is often too diverse to be effective as a therapeutic.

MMP-2

The timely breakdown of extracellular matrix is essential for embryonic development, morphogenesis, reproduction, and tissue resorption and remodeling [28, 29]. The matrix metalloproteinases (MMPs), a family of metallo-dependent proteases capable of degrading all components of the extracellular matrix of connective tissues [30-32], are thought to play a central role in these processes. The expression of most MMPs is transcriptionally regulated by growth factors, hormones, and cytokines [28-30, 32-35]. The proteolytic activities of MMPs are precisely controlled during activation from their precursors, and inhibition by endogenous inhibitors, α -macroglobulins and tissue inhibitors of metalloproteinases (TIMPs) [28]. MMPs have been found to play a role in a number of pathological processes including arthritis, cancer, and cardiovascular disease [28].

All MMPs are synthesized as prepro-enzymes and secreted as inactive zymogens [28-31, 33, 34]. Activation of secreted pro-enzymes requires disruption of the Cys-Zn²⁺ (cysteine switch) interaction and the removal of the propeptide proceeds often in a stepwise manner. The activation of pro-MMP2 is thought to take place primarily on the cell surface [28] through complex formation with TIMP-2 [29, 33-35] and membrane type 1-MMP (MT1-MMP or MMP-14) [29, 30, 35]. In this activation mechanism, TIMP-2 binds to MT1-MMP at the cell surface leaving the TIMP carboxyl terminus available for interaction with the carboxyl terminus of pro-

MMP-2. The resultant TIMP-2:MMP-2 interaction presents the amino terminus of the docked proMMP-2 molecule to an adjacent unoccupied MT1-MMP molecule which converts the MMP-2 to the 62kDa fully active form, from its 72 kDa inactive form [29, 33].

MMP-2 and IVD

High levels of mRNA for a number of MMPs, including MMP-1, MMP-2, MMP-3 and MMP-13, have been found in the degenerating IVD [32] and are thought to be responsible for the remodeling of the disc extracellular matrix components [29, 33]. MMP-2, or gelatinase A, is the major gelatinolytic proteinase constitutively produced by the NP *in vitro* and *in vivo* [30]. It is of particular importance to IVD tissue homeostasis due to its broad substrate specificity [29, 30, 33]. It is therefore an MMP of potential significance in disc degeneration where excessive matrix catabolism is a prominent feature [33]. However, since almost all MMPs are produced as inactive pro-enzymes and may reside in the tissue in their inactive conformation, it is important to measure the actual levels of activity rather than the presence of this enzyme [32].

Intervertebral disc degeneration favors a shift towards primarily catabolic remodeling responses. This shift can arise through changes in enzyme production and activity, matrix production, as well as anti-catabolic agents [36]. Studies have correlated increased MMP-2 production and activation with age and degenerative changes in the NP, as well as alterations in MMP-2 activity in NP cells in response to growth factor stimulation or prolonged loading [30]. In mechanically loaded

specimens, patterns of mRNA levels were consistent with turnover/repair in the nucleus and remodeling and/or injury in the annulus [36]. MMP-2 was shown to be increased in herniated discs [34], and levels of pro- MMP-2 were found to be negatively correlated with collagen type II content in the nucleus [32]. Increases in MMP-2 have also been found in human disc degeneration in autopsy and surgical specimens [32, 36]. In addition, expression of MMPs, specifically the gelatinases, has been shown to be up-regulated after spinal cord injury [37]. Moreover, it has been suggested that MMP-2 contributes to the induction of neovascularization that occurs in the early stages of disc degeneration [30] further implicating MMP-2 in remodeling and degenerative changes in the disc.

Mechanism of RNA interference

The RNA interference pathway was first recognized in *Caenorhabditis elegans* as a response to exogenously introduced long double-stranded RNA (dsRNA), which unexpectedly led to reduced gene expression [38-40]. RNAi is a form of primitive immunity considered to protect the genome against viral infections and genomic instability caused by mobile genetic elements such as transposons [38, 41]. RNAi-mediated gene silencing suppresses gene expression by several mechanisms, including the targeted sequence-specific degradation of mRNA, translational repression, and the maintenance of silenced regions of chromatin [38]. The endogenous RNAi pathway contributes significantly to regulating cellular gene expression [38]. Compared to knockout genetics, RNAi-based silencing is rapid, cost

effective, and can be easily adapted to study homologous gene function in a wide variety of organisms [42].

RNAi involves post-transcriptionally silencing gene expression through double-stranded RNA highly homologous to its own sequence (Fig. 1.3 [43]). Normally, long strands of dsRNA activate the interferon response in cells, leading to apoptosis. However, this response can be bypassed by the introduction of short interfering RNAs in two distinct steps [39, 41, 44]. First, the ribonuclease III enzyme Dicer

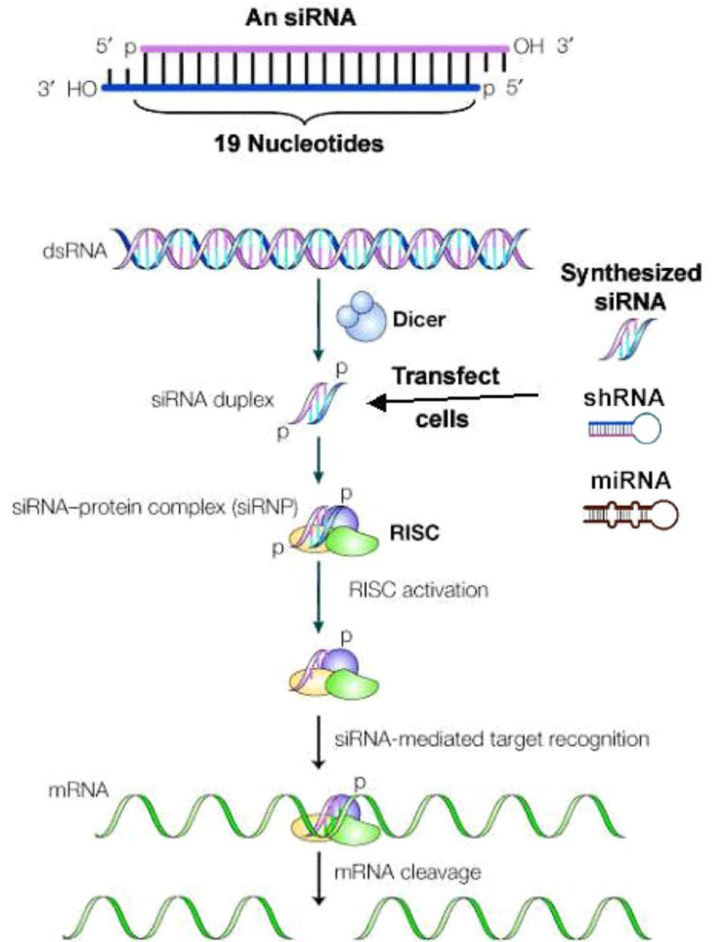


Figure 1.3. RNAi Pathway, adapted from (43)

cleaves long dsRNA into short interfering RNA (siRNA) molecules of 21–23 bp in length [38, 45, 46]. Second, the siRNA duplexes are subsequently unwound [46] and bind to a RNA-induced silencing complex (RISC) which uses the siRNA to guide the sequence-specific cleavage of the RNA transcripts of the target gene [42]. RNAi can also be initiated by introducing chemically synthesized siRNAs in to cells, shRNAs through viral vectors, or miRNAs. Many chemically synthesized siRNAs are commercially

available due to the completion of many animal genome sequencing projects. miRNAs are endogenously produced and involved in development, while shRNAs are usually transfected through a viral vector and offer the advantage of stable silencing in cells[44]. Introducing a single nucleotide change to the siRNA sequence could abrogate siRNA-mediated silencing implying that silencing is highly sequence specific [38].

RNAi in mammalian cells

RNAi is a powerful research tool for reverse genetic studies, which determine the function of a gene by its disruption. Target genes can be silenced by chemically or enzymatically synthesized siRNAs or by DNA-based vector systems that encode short hairpin RNAs (shRNAs) that are processed intracellularly into siRNAs. Typically, RNAi-mediated silencing is said to be incomplete (a “knockdown,” not a “knockout”), however, the targeted mRNA is undetectable even with highly sensitive PCR assays in some cases [38]. In mammalian cells, siRNAs suppress gene expression only for short periods of time, with silencing lasting up to a week depending on cell proliferation, siRNA dilution, and half-life of the target protein [38, 41, 46]. However, siRNA-mediated silencing can persist for several weeks in terminally differentiated, nondividing cells, such as macrophages or neurons. To prolong silencing, plasmid vectors have been developed that effectively express shRNAs, using expression systems based on adenovirus, adeno-associated virus, oncoretroviruses, and lentiviruses [38, 41]. RNAi has now been established as a

standard technique to investigate gene function and can be used routinely in both cell culture models and transgenic animals [41].

shRNA and lentiviruses

Although first discovered with siRNAs, RNAi can also be achieved through the use of short hairpin RNAs (shRNA). The shRNA contains a perfectly double stranded stem of 19–29 bp with one strand identical in sequence to the target mRNA. The two strands of the stems are linked by a loop sequence, and the shRNA is processed by Dicer to generate functional active siRNA. While siRNAs are directly transfected, shRNAs are endogenously transcribed from expression cassettes incorporated into the host cell genome usually from viral gene transfer. Important advantages of shRNAs as compared to siRNAs are (i) lower costs as constructs are continually produced by cells, (ii) the induction of stable gene silencing, and (iii) the ability to track and isolate infected cells based on reporter gene expression [41].

Efficient delivery and stable transduction of target cells by shRNA-expression cassettes into the host cell genome can be readily achieved by retroviral, lentiviral or adenoviral vectors infecting a broad range of cell types [41, 42]. The lentivirus system can express integrated shRNA efficiently in a wide variety of cell lines and primary cells both *in vitro* and *in vivo* [47]. In addition, lentiviruses can efficiently integrate into the genome of primary dividing and non-dividing cells [41] which allows direct injection into a tissue or organ to knockdown the expression of specific genes [47, 48]. Because lentiviral vectors incorporate into the host chromosome, the succeeding progeny is likely to inherit the provirus and express the appropriate genes

[47]. Moreover, lentiviruses are relatively easy to generate, show minimal immunogenicity, and can be produced with high titers so that it can be applied for large-scale RNAi assays for studying gene functions [48]. Furthermore, the ability to stably express shRNAs in human cells using viral vectors raises the possibility of using RNAi as a form of gene therapy to selectively inhibit the expression of disease specific genes such as oncogenes [45] or MMPs.

RNAi as a therapeutic

RNAi has been said to be the next new class of therapeutics. Since RNAi is a naturally occurring process and all genes are potential targets, the possible applications in medicine are unlimited. The widespread applicability, relative ease of use, and low cost of production makes siRNAs an attractive small-molecule drug, compared to costly antibodies or recombinant growth factors [44, 49]. In addition, siRNAs are chemically stable and can be stored lyophilized without refrigeration. Once in cells, the anticipated duration of silencing is predicted to vary from 5 days to several weeks, which makes dosing as an injectable drug possible. The sequence specificity of RNAi promises potent therapies with little toxicity due to off-target gene silencing [38]. Although this specificity might lead to resistance due to sequence mutations when treating viral infections or cancer, which has been the case in several *in vitro* studies, resistance to RNAi may be overcome by introducing a new siRNA that targets a different site on the same mRNA. Moreover, siRNAs that target conserved sequences or multiple sequences at once may abrogate this problem [38]. The use of shRNAs provides the opportunity for long-term improvement of a

persistent problem by using lentiviral vectors that integrate into the genome and can be used to transduce hematopoietic stem cells and other slowly dividing progenitor cells in a variety of tissues [38, 44]. The therapeutic potential of RNAi achieved through vectored expression of shRNAs has been shown in mouse models using adeno-associated viruses, retroviruses, and lentiviruses [38, 43, 44].

RNAi and IVD

Previous studies in the IVD using RNAi suggest that not only is the disc an immune-privileged site, but disc cells are also capable of transfection. Kakutani et al. [50] demonstrated siRNA mediated gene silencing of reporter plasmids introduced into NP cells from both humans and rats *in vitro*. The inhibitory effects were maintained for 2 weeks, but disappeared completely by 3 weeks. Although the study utilized siRNAs targeting externally produced plasmids, rather than endogenously produced genes, it illustrated the efficacy of RNAi in disc cells. A later study by Suzuki et al. [51] presented long-term gene silencing of both reporter plasmids, as well as an endogenously produced gene *in vivo* in rat coccygeal discs. The inhibitory effects were maintained for up to 24 weeks suggesting that RNAi might be a promising therapy for disc degeneration. Seki et al. [52] explored knockdown of genes associated with disc degeneration with promising results *in vivo* in a rabbit needle-puncture model. Injection of siRNA against ADATS5 induced the suppression of degeneration in NP tissues. These and other studies have reported beneficial properties of gene delivery of therapeutic factors to the IVD as a potential treatment strategy for DDD [53].

Specific Aims and Hypotheses

Disc degeneration is a prevalent medical problem with current research focusing on finding new treatments for back pain. In an effort to further these studies, the global objective of this work is to investigate how cells of the intervertebral disc interact with their microenvironment in order to develop methodologies for novel therapies for disc degeneration. The global hypothesis is that alterations in cellular environment can induce changes in gene expression, and changes in genetic make-up of cells can, in turn, alter cellular response and function. The specific aims of this thesis will test this hypothesis by investigating the role of the cellular microenvironment in the maintenance of disc cell phenotype, and the use of gene silencing as a tool for cellular engineering.

Specific Aim 1 – Determine how biochemical, nutritional and physical factors regulate notochordal gene expression *in vitro*.

Research studies of the intervertebral disc often involve *in vitro* cell culture techniques. It is hypothesized that changes in cell microenvironment can alter cellular phenotype and function, which can confound experimental results. Hence, experiments examining these alterations are needed to lay the foundation for future cell culture work *in vitro*.

Specific Aim 2 – Determine the efficacy of multiple shRNA vectors targeting MMP-2 in disc cells and optimal parameters to implement RNAi *in vitro*.

RNAi is often difficult to achieve in primary cells. The hypothesis is that among multiple shRNA sequences targeting a gene, one will achieve the greatest level of knockdown. Subsequently, *in vitro* experiments will optimize parameters and verify sequences capable of effectively knocking down MMP-2 gene expression in disc cells.

Specific Aim 3 – Determine the functional consequences of silencing MMP-2 in an *in vitro* cell culture model.

It is hypothesized that silencing MMP-2 can initiate a number of molecular changes in disc cells. In order to elucidate the role of MMP-2 in degradation of the disc, cellular alteration of their environment will be examined using gelatin films and three-dimensional collagen gels.

Specific Aim 4 – Determine efficacy of RNAi *in vivo* in degenerate disc models.

MMP-2 has been found in high levels in degenerate discs. In order to examine whether it is the cause of matrix degradation, RNAi studies silencing MMP-2 in a physiologic system are necessary. It is hypothesized that silencing MMP-2 will hinder the degenerative process in IVDs. Furthermore, it is crucial that gene knockdown in a pathological system be accomplished *in vivo* to establish the use of RNAi as a therapeutic tool for disc degeneration.

Chapter 2: Environmental Regulation of Notochordal Gene

Expression in Nucleus Pulposus Cells

(Rastogi A., et al. *Environmental Regulation of Notochordal Gene Expression in Nucleus Pulposus Cells* J Cell Physiol, 2009, **220** p.698–705.)

Abstract

Cells of the nucleus pulposus (NP) in the intervertebral disc are derived directly from the embryonic notochord. In humans, a shift in NP cell population coincides with the beginning of age-related changes in the extracellular matrix that can lead to spinal disorders. To begin identifying the bases of these changes, the manner by which relevant environmental factors impact cell function must be understood. This study investigated the roles of biochemical, nutrient, and physical factors in regulating immature NP cells. Specifically, we examined cell morphology, attachment, proliferation, and expression of genes associated with the notochord and immature NP (Sox9, CD24, and type IIA procollagen). Primary cells isolated from rat caudal discs were exposed to different media formulations and physical culture configurations either in 21% (ambient) or 2% (hypoxic) O₂. As expected, cells in alginate beads retained a vacuolated morphology similar to chordocytes, with little change in gene expression. Interestingly, NP tissues not enzymatically digested were more profoundly influenced by oxygen. In monolayer, α -MEM preserved vacuolated morphology, produced the highest efficiency of attachment, and best maintained gene expression. DMEM and Opti-MEM cultures resulted in high levels of proliferation, but these appeared to involve small non-vacuolated cells. Gene expression patterns for cells in DMEM monolayer cultures were consistent with chondrocyte de-

differentiation, with the response being delayed by hypoxia. Overall, results indicate that certain environmental conditions induce cellular changes that compromise the notochordal phenotype in immature NP. These results form the foundation on which the mechanisms of these changes can be elucidated.

Introduction

The nucleus pulposus (NP) of the intervertebral disc (IVD) represents the fate of the notochord after embryonic morphogenesis. As a developmental organ of chordates, the notochord serves an important function in signaling and in axial support [54] and consists of a laminin- and aggrecan-rich basement membrane sheath [55, 56] that encases a population of highly vacuolated chordocyte cells [57-60]. Small chordoblasts, distinct from chordocytes, line and presumably maintain the sheath. During development of the vertebral bodies, ventrally localized expression of alkaline phosphatase, and chordoblast rearrangement propagates circumferentially [56]. The resulting formation of the vertebral bodies generates islands of notochordal remnants that constitute the immature NP [56, 61].

The fate and regulation of notochordal cells after establishment of the spinal column have not been extensively studied, but an improved understanding may provide clues into aging and disorders of the IVD, such as degenerative disc disease (DDD). It has been well documented that the cellular and extracellular matrix (ECM) makeup in humans is age-dependent. In immature discs, the NP is a highly hydrated, almost fluidic, translucent matrix [18] that confers a unique biological and biomechanical environment for NP cells. Although details are undocumented, the

immature human NP likely contains not only chordocytes but also chordoblasts. The adult NP is populated exclusively by what have been described as “chondrocyte-like cells,” which are smaller and do not possess the vacuolated appearance of chordocytes, within a cartilaginous matrix [18, 62]. There is still no definitive evidence as to whether the change in cell population evolves from the remaining chordoblasts and chordocytes, or results from an influx of chondrocytic cells from the endplates and/or annulus fibrosus [21, 63, 64]. The impetus that brings about these changes in the adult disc and the mechanisms by which they occur are also unknown, but some evidence in the literature point to mechanical and chemical stress as potential mediators of notochordal cell survival and phenotype in the adult organism [12, 65]. In other animals, the retention of the notochordal population depends on species. Some, such as rabbit, rat, mouse, and pig, possess notochordal cells through most of their adult life, as the presence of vacuolated chordocytes indicates [18]. In these discs, cells of the mature NP are described as having a mixed population of notochordal and chondrocyte-like cells, though it is unclear how chordocytes, chordoblasts, and extrinsic cells fit into these classifications [10, 18]. It has been suggested that the co-existence of these cells may pose some biological significance due to cell-cell interactions and paracrine signaling [10].

The relatively few studies on cells isolated from immature NP tissues have yielded large variation in published and anecdotal findings. For instance, some studies have been able to passage NP cells in monolayer [66, 67] while others have observed extremely slow growth kinetics [68]. There have also been conflicting observations regarding the ability of NP cells to maintain viability in hydrogel

cultures after enzymatic isolation from tissues [20, 69]. Finally, because the disc is avascular, nutrient supply – particularly, oxygen – has been thought to be a strong determinant of IVD form and function during growth [70, 71]. Despite findings that hypoxia regulates MAPK signaling and VEGF expression in rat NP cells [66, 72-74], the physiologic environment does not appear to generate oxaemic distress compared with cultures at normal O₂ levels [75-77]. It is not clear how hypoxia might regulate other genes associated with the immature NP such as CD24, type IIA collagen, α_6 integrin subunit, and galectin-3 [9, 15, 78-81]. The use of varied experimental conditions have further made it difficult to reconcile any differences among reported results.

Because of the continually evolving conditions in the aging disc and our currently limited understanding of post-embryonic notochordal cells, this study sought to determine how cells isolated from immature NP might be regulated by environmental variables. Specifically, we utilized NP from rat caudal discs as a model of immature NP cells, and investigated biochemical, nutritional, and biophysical stimuli by varying media formulation, oxygen concentration, and culture configuration. The goal was to determine how NP tissues are affected as a mixed population culture system, and perhaps gain some insight into the relative functions of the two cell types. Based on current views of the NP cell regulation, we hypothesized that monolayer and hypoxia would have detrimental effects, and posed the null hypothesis that cells would be insensitive to media formulation. Surprisingly, we found strong interactions among these three factors, so that outcomes depended on the specific combination of conditions. Maintenance of notochord-associated genes

was supported by alginate bead culture and α -MEM monolayer cultures, while mechanically disrupted tissues in hypoxia and DMEM monolayer cultures promoted a loss of notochordal phenotype.

Materials and Methods

Cell isolation and culture

A total of 56 male Sprague-Dawley rats (6-9 months) were obtained and euthanized using CO₂ asphyxiation, as approved by the Institutional Animal Care and Use Committee at the University of Maryland, College Park. Excised tails were promptly removed of skin and transported into a biosafety cabinet where they were subjected to two washes in sterile PBS. Surrounding soft tissues were completely removed to expose caudal intervertebral discs. For each disc, a cut was made close to one of the vertebral bodies, and the gelatinous NP was scooped out with a microcurette. Care was taken not to disturb the underlying endplate. Six NP tissues from each tail were pooled and prepared for culture using one of three methods (below). Qualitative assessments of cell morphology were made using an Olympus IX81 (Olympus America, Inc., Center Valley, PA) for light microscopy of experimental samples and for fluorescence microscopy using a fluorescein-based cytoplasmic stain (CellTracker; Invitrogen, Carlsbad, CA) in separate identically prepared alginate bead samples.

1. Monolayer culture: Upon removal, NP tissues were digested in 0.4% (w/v) pronase for 1 hour followed by 0.025% (w/v) collagenase overnight. Digests were centrifuged, cells were resuspended in fresh media, and plated.

2. *Disrupted NP in alginate beads*: NP tissues were placed directly into 500 µl of 2% (w/v) alginate in a microcentrifuge tube and then mechanically disrupted using a pestle. The alginate was expelled dropwise from a 22 gauge needle into 102 mM CaCl₂ to form beads. The beads were cured for 10 minutes and then placed in fresh culture media.

3. *Cells dispersed in alginate beads*: Upon removal, NP tissues were digested in 0.4% (w/v) pronase for 1 hour followed by 0.025% (w/v) collagenase overnight. Digests were filtered through a 70 µm cell strainer and centrifuged. Cells were resuspended in 500 µl of 2% (w/v) alginate, expelled dropwise from a 22 gauge needle into 102 mM CaCl₂, and cured for 10 minutes before being placed in fresh culture media.

Hypoxic conditions were maintained by filtering pre-mixed gas consisting of 2% O₂, 5% CO₂, and the balance N₂ and humidifying the gas using a sparger. Continuous inflow of humidified gas terminated in a chamber containing the flasks/dishes of cells, and exhausted.

Cell attachment and proliferation

Twenty Sprague-Dawley rats were used for cell attachment and proliferation measurements, which were based on DNA quantitation by PicoGreen reagent (Invitrogen, Carlsbad, CA). The design involved nine experimental groups: one Day 0 baseline assessment of DNA content, and eight different combinations of FBS (2, 10%) with basal media (DMEM, α-MEM, Opti-MEM, and RPMI) (Invitrogen, Carlsbad, CA). For these experiments, caudal discs from two rats (12 discs total)

were pooled to generate enough material for one replicate containing all nine groups. Cells were isolated according to procedures for monolayer culture described above. Equal volumes of digested tissues were distributed among the nine experimental groups and, with the exception of the Day 0 group, placed in the incubator for 3 days without media exchange. For Day 3 assays (n=5), cells were washed with PBS and trypsinized (0.25% trypsin, 0.1% EDTA; Invitrogen, Carlsbad, CA). For Day 10 assays (n=5), media was exchanged at day 3 and then cultured for 7 additional days with normal media changes before being trypsinized. For all samples, cells were pelleted, resuspended in TE buffer, and subjected to five freeze-thaw cycles for lysis. The PicoGreen reagent was used according to manufacturer's instructions with a calibration curve generated from Lambda DNA. Fluorescence was measured using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). Attachment was defined as DNA content at Day 3 relative to that at Day 0. Proliferation was defined as the ratio of DNA content at Day 10 relative to that at Day 3.

Gene expression

Thirty-six rats were allocated for studying the impact of physical culture configuration, hypoxia, and media conditions. Relative quantitation of gene expression was performed using separate freshly isolated tissues as physiologic reference samples. Effects of physical configuration was examined by comparing (1) cell monolayers, (2) mechanically disrupted NP in alginate beads, and (3) enzymatically digested NP in alginate beads all cultured in DMEM (Gibco/Invitrogen, Carlsbad, CA) containing 2% FBS (Sigma, St. Louis, MO), 100

U/mL penicillin, and 100 μ g/mL streptomycin (Gibco/Invitrogen, Carlsbad, CA). Sensitivity to biochemical environment was studied by including an additional group of cell monolayers cultured in α -MEM also containing 2% FBS (Sigma, St. Louis, MO), 100 U/mL penicillin, and 100 μ g/mL streptomycin (Gibco/Invitrogen, Carlsbad, CA). The role of oxygen concentration was examined in every preparation by culturing in either 2% or 21% O₂, with 5% CO₂ and the balance N₂ at 37°C.

Each tail was assigned to one of the four treatment groups: DMEM monolayer (n=3), DMEM disrupted in alginate (n=3), DMEM digested in alginate (n=3), and α -MEM monolayer (n=3). From each tail, six discs were pooled and divided equally between hypoxic (2% O₂) and normoxic (21% O₂) to form matched pairs cultured in parallel. Cells were maintained with regular media changes for 1, 2, or 4 weeks (n=3 for each time point).

For alginate samples, cells were released from beads by submersion in a sodium citrate solution (55 mM sodium citrate, 0.15 M NaCl, 25 mM HEPES) for 10 minutes, and then collected by centrifugation and lysed. Cell monolayers were lysed directly. RNA purification was performed using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). Samples were reverse transcribed, and real-time PCR using SYBR Green was performed (iCycler, Bio-Rad Laboratories, Hercules, CA) to quantify expression of type IIA procollagen, Sox9, CD24, and GAPDH (Table 2.1).

Table 2.1. Sequences of Primers used for RT-PCR Analysis

Gene	Forward and Reverse Sequence	GenBank accession no.
Type IIA procollagen	5'-AGGAGGCTGGCAGCTG-3' 5'-CACTGGCAGTGGCGAG-3'	NM_012929
Sox9	5'-AATCTCCTGGACCCCTTCAT-3' 5'-TTCCTCGCTCTCCTTCTTCA-3'	XM_343981
CD24	5'-CTTCTGGCACTGCTCCTACC-3' 5'-AGAGACGTTTCTTGGCCTGA-3'	NM_012752
GAPDH	5'-AACCCATCACCATCTTCCAG-3' 5'-GTGGTTCACACCCATCACAA-3'	NM_017008

Data and statistical analysis

Relative quantitation of real-time RT-PCR data was performed using the $\Delta\Delta C_t$ method [82]. Briefly, the C_t values for each triplicate were averaged and used for subsequent calculations. ΔC_t was computed by subtracting the averaged C_t values of the internal control gene GAPDH from those of the gene of interest (i.e. $\Delta C_{t_{col2a}} = C_{t_{col2a}} - C_{t_{GAPDH}}$). $\Delta\Delta C_t$ for each gene of interest was computed by subtracting the ΔC_t of the baseline reference (time zero specimen obtained from tail discs) from the ΔC_t for the preparation and time point of interest (i.e. $\Delta\Delta C_{t_{col2a}; preparation X, time Y} = \Delta C_{t_{col2a}; preparation X, time Y} - \Delta C_{t_{col2a}; baseline}$). These $\Delta\Delta C_t$ values for each gene of interest of each preparation and time combination were then expressed as relative changes in mRNA levels (fold difference) through the exponential relation: $2^{-\Delta\Delta C_t}$. Data are reported as the average value of the range of calculated fold difference, which incorporates the standard deviation of the $\Delta\Delta C_t$ value in the fold difference

calculation as $\Delta\Delta Ct + SD$ and $\Delta\Delta Ct - SD$. Statistical analyses (SPSS 14.0, Chicago, IL) were performed using one-way ANOVA with Tukey's HSD post hoc tests ($\alpha = 0.05$).

Results

Cell morphology

For monolayer cultures, we observed clear distinctions among media conditions. In DMEM, cells were initially small and vacuolated when first plated on polystyrene (Figure 2.1 a, d). Over time, cells formed large complexes as they proliferated and flattened out (Figure 2.1 c). Cell monolayers in α -MEM also started as small cells, although larger in size and containing more pronounced vacuoles than cells in DMEM. There was also noticeably greater cell attachment in α -MEM than DMEM, and cells appeared to form larger vacuole-containing complexes (Figure 2.1 e, h). It is unclear whether the initially higher cell attachment contributed by allowing more cells to be included within each complex. Cells plated in RPMI were very small with few noticeable vacuoles, and attachment characteristics similar to that of DMEM (Figure 2.1 i). No dramatic changes in cell size or features were observed in RPMI over time. Cells plated in Opti-MEM appeared similar to those plated in DMEM, but with what appeared to be less proliferation over time (Figure 2.1 j). In most monolayer cultures, there was clearly the formation of colonies consisting of smaller cells, which expanded faster in 10% FBS media than in 2% (Figure 2.1 b). Fewer of these smaller cells were observed in α -MEM cultures, but this also may have appeared as such due to the relatively greater numbers of vacuolated cells.

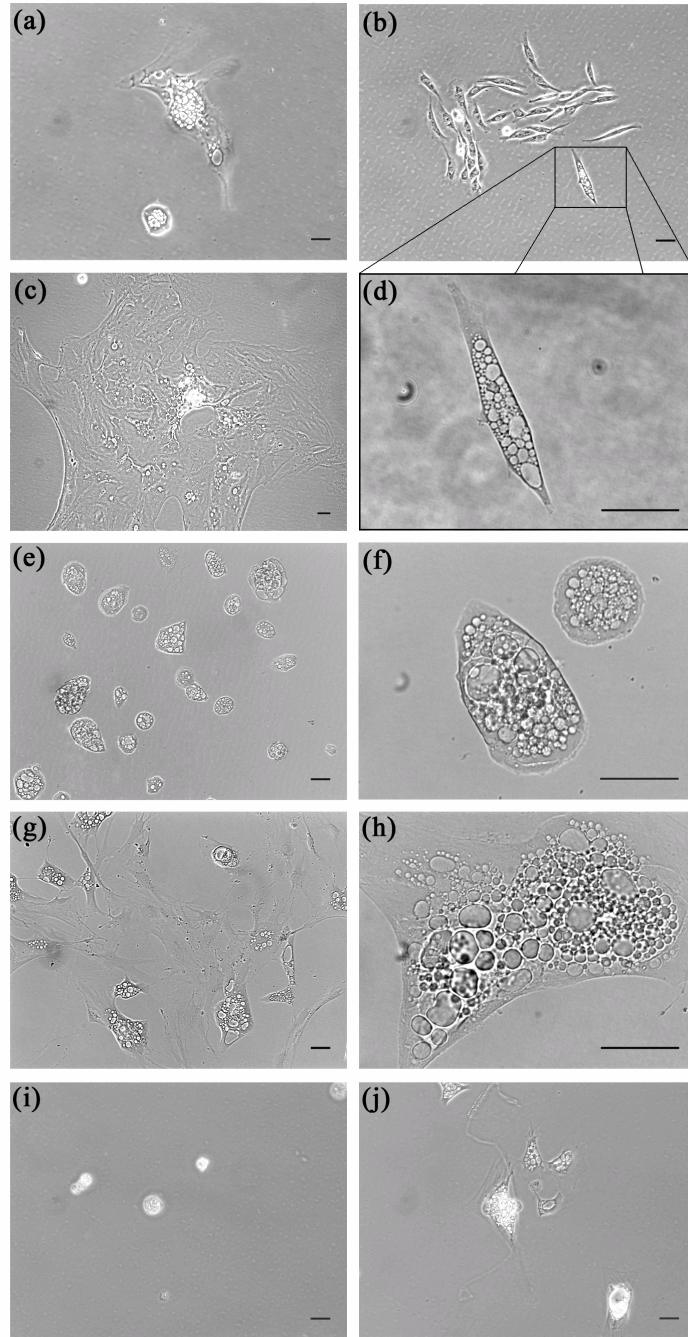


Figure 2.1. Microscopic images of monolayer cell cultures: (a,b) DMEM culture at 1 week in 2%FBS and 10% FBS, respectively; (c) DMEM culture at 2 weeks; (d) magnification of part b; (e,f) α -MEM culture at 1 week; (g,h) α -MEM culture at 2 weeks; (i) RPMI culture at 1 week; (j) Opti-MEM culture at 1 week. Cells maintained a vacuolated morphology in DMEM and α -MEM. Cell attachment was greater in α -MEM culture, while DMEM culture promoted proliferation of small chondrocyte like cells. Cells cultured in RPMI and Opti-MEM had low cell attachment and proliferation, and were unable to maintain their vacuolated morphology. Scale bars represent 50 μ m.

For alginate bead cultures, other than sizes of cell clusters, there were no obvious distinctions between mechanically disrupted and enzymatically digested tissues. In mechanically disrupted NP cultures, large islands of cells were visible through the alginate beads. Despite being in clusters with unique cell-cell and cell-matrix contacts, cells were generally rounded and contained large vacuoles (Figure 2.2 a). Enzymatically digested cells were found mostly as singlets but sometimes in smaller groups than mechanically disrupted samples. Most of the cells were vacuolated, and they were rounded, as one would expect in hydrogel culture (Figure 2.2 b).

There did not appear to be any marked influence of oxygen concentration on cell morphology in any of the preparations and conditions.

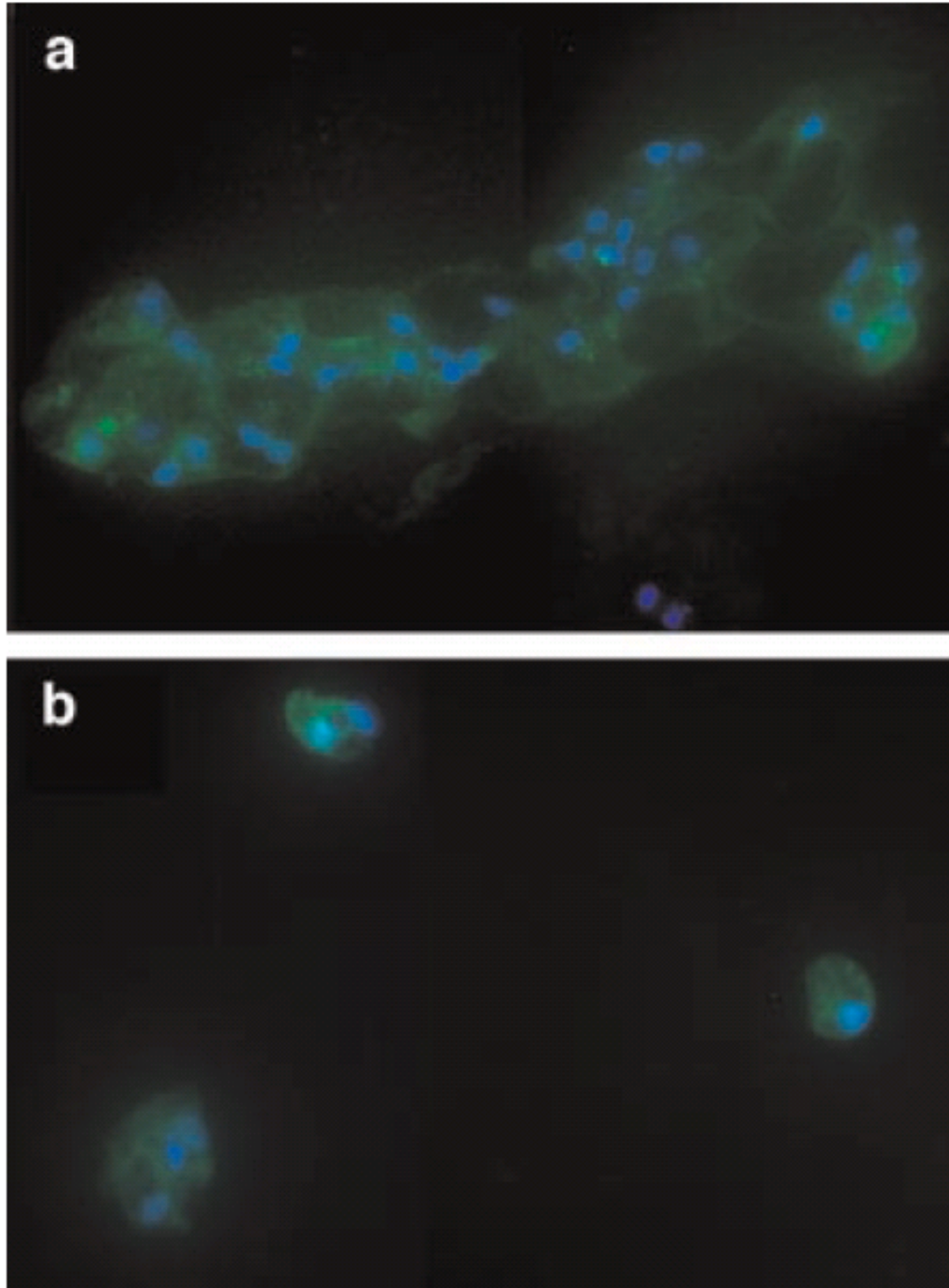


Figure 2.2. Fluorescence micrographs of (a) mechanically disrupted NP tissues and (b) enzymatically digested NP cells, both at 2 weeks in alginate bead culture. Cells are stained with CellTracker Green to indicate viability and DAPI to stain the cell nuclei (400X magnification). Both mechanically disrupted and enzymatically digested NP tissue showed cells which were vacuolated and able to maintain physiologic morphology. Mechanically disrupted tissue in alginate beads contained large clusters of cells, which were able to maintain both cell-cell and cell-matrix interactions, while enzymatically digested cells appeared in small clusters of 1 to 2 cells.

Cell attachment and proliferation

Using DNA quantitation, we observed a strong dependence of cell attachment on biochemical formulation of basal culture media, with a more modest effect of FBS concentration (Figure 2.3 a). In low serum conditions, attachment with α -MEM was 75% compared with approximately 50% for DMEM and RPMI, and 33% for Opti-MEM. Higher levels of serum resulted in mild increases in attachment with α -MEM increasing to 83%. Interestingly, DMEM exhibited lower attachment rates in 10% FBS, and we observed this trend in four of the five trials.

As one would expect, media containing 10% FBS generally resulted in greater cell proliferation (Figure 2.3 2b). Comparable growth characteristics between DMEM and α -MEM were observed for both 2% and 10% FBS conditions. For these media, cell numbers increased approximately 25% from Day 3 to Day 10 in 2% FBS, and 137% in 10% FBS. RPMI yielded much fewer cells at Day 10 compared with Day 3. It is unclear whether the 75% decrease in DNA content is due to cells unable to maintain attachment or to cell death. Culturing in RPMI with 10% FBS resulted in maintenance of cell number. Opti-MEM appeared to exhibit worse proliferative characteristics in 10% FBS; however, this is slightly misleading due to a more pronounced variability in this particular media condition. None of the differences were statistically significant due to the inherent variability of cell isolation procedures in general.

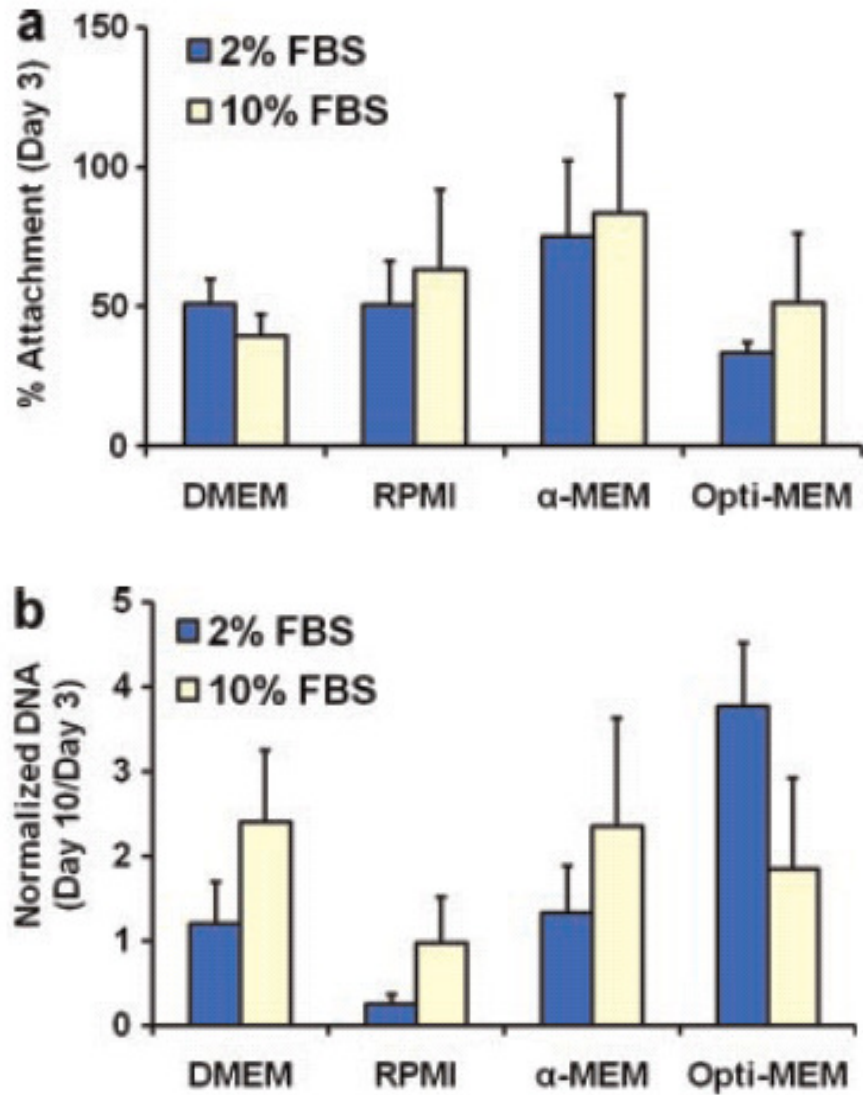


Figure 2.3. Dependence of (a) cell attachment and (b) proliferation on media conditions. Overall, α -MEM provided optimal attachment while Opti-MEM had the lowest cells attachment. Cell culture in DMEM, α -MEM and Opti-MEM provided high growth characteristics, however, DMEM culture allowed proliferation of different cell types, while growth in Opti-MEM was lower in 10% FBS compared to 2% FBS.

Gene expression

The role of culture configuration appeared to serve as an interacting factor with oxygen levels. For monolayers in DMEM (Figure 2.4 a), expression of genes associated with the immature NP began either unchanged or upregulated compared with fresh tissues. However, by four weeks of culture, monolayers exhibited marked decreases in expression of all genes. Hypoxia did not influence trends in gene expression, but did generate more pronounced changes in expression levels. In contrast, cells cultured in alginate beads (Figure 2.4 b) retained modest, but steady, increases in expression for all genes examined, with no apparent effect of hypoxia. Mechanically disrupted NP tissues (Figure 2.4 c) exhibited interesting patterns of gene expression that strongly depended on oxygen levels. In hypoxia, Sox9 and type IIA procollagen were immediately upregulated and showed modest decreases over four weeks. But at normal oxygen levels, these genes were comparable to physiologic, and expression increased over four weeks. CD24 decreased over time in hypoxia, and maintained steady expression levels in normoxia.

The use of α -MEM for monolayer culture induced gene expression patterns drastically different from DMEM monolayers (Figure 2.4 d). As opposed to steady decreases in immature NP cell-associated gene expression over four weeks in DMEM, cells cultured in α -MEM maintained levels of Sox9 and CD24 expression above physiologic throughout 4 weeks. Type IIA procollagen was similar to physiologic over the first two weeks, before an increase observed at 4 weeks. There was a very modest effect of oxygen level on all genes.

Overall, in DMEM culture, the preparation with cells dispersed in alginate exhibited more consistent upregulation of all genes. The two other culture preparations resulted in more complex regulation of the genes of interest that was strongly dependent on time point and/or O₂ level. For monolayer cultures, α -MEM also stimulated a consistent upregulated expression of essentially all genes.

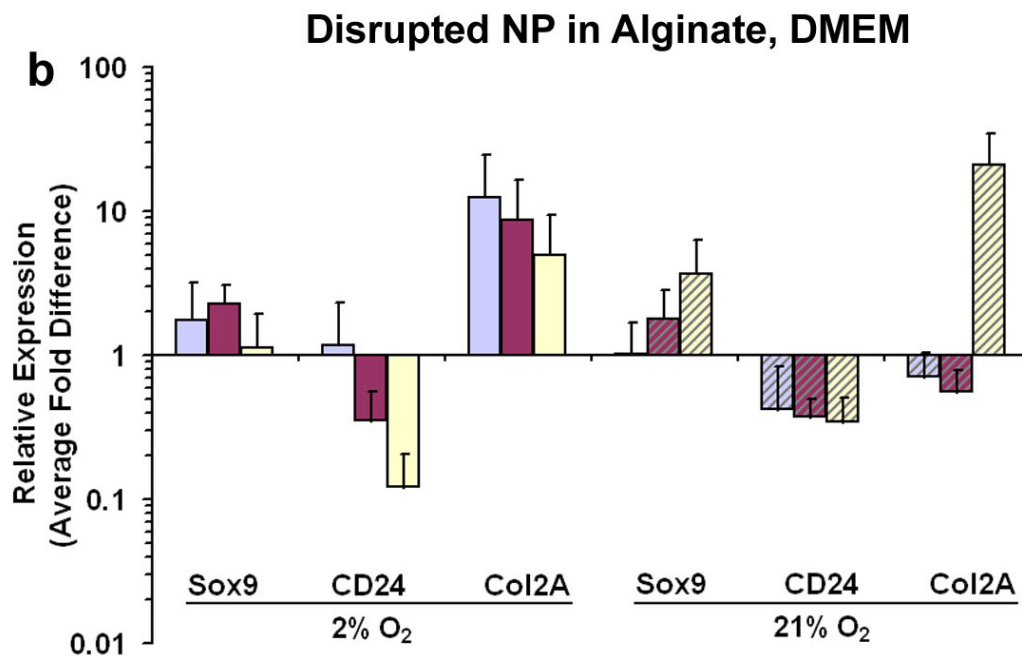
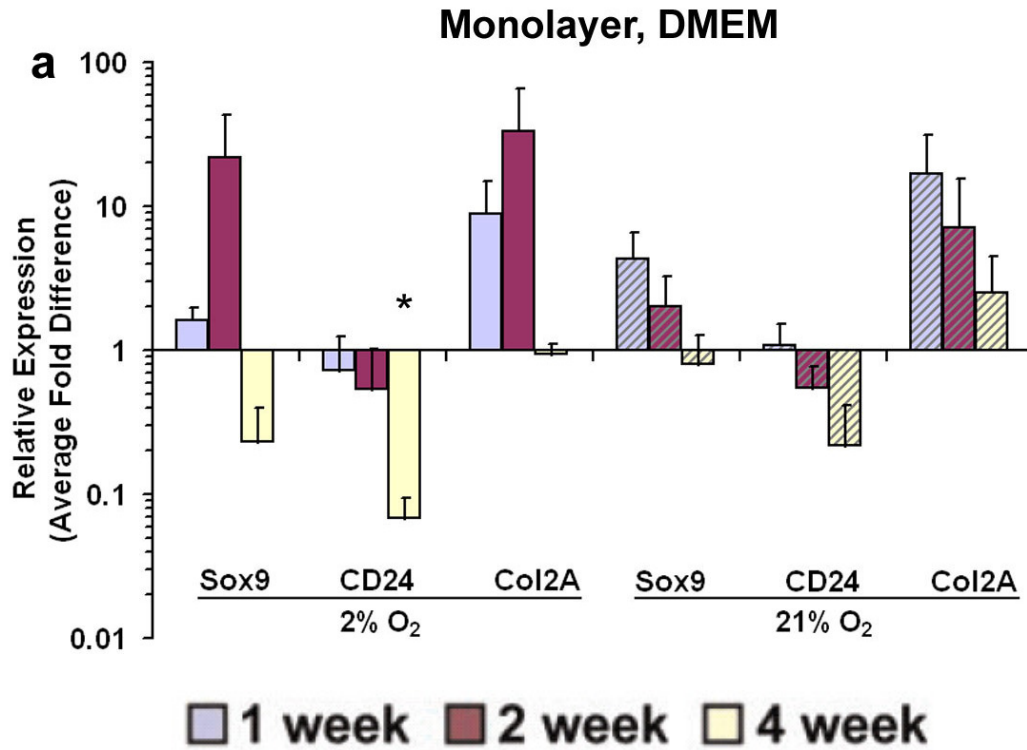


Figure 2.4. PCR data showing relative changes in gene expression at 1, 2, and 4 weeks in 2% and 21% oxygen levels for (a) monolayer culture in DMEM. Changes in gene expression of CD24 at 4 weeks in hypoxia are significantly different from time zero levels ($P = 0.035$); (b) mechanically disrupted NP in alginate beads cultured in DMEM. There are no significant differences in gene expression. Down-regulation of CD24, a marker of notochordal cells, in both configurations demonstrates loss of the notochordal cell phenotype over time in culture.

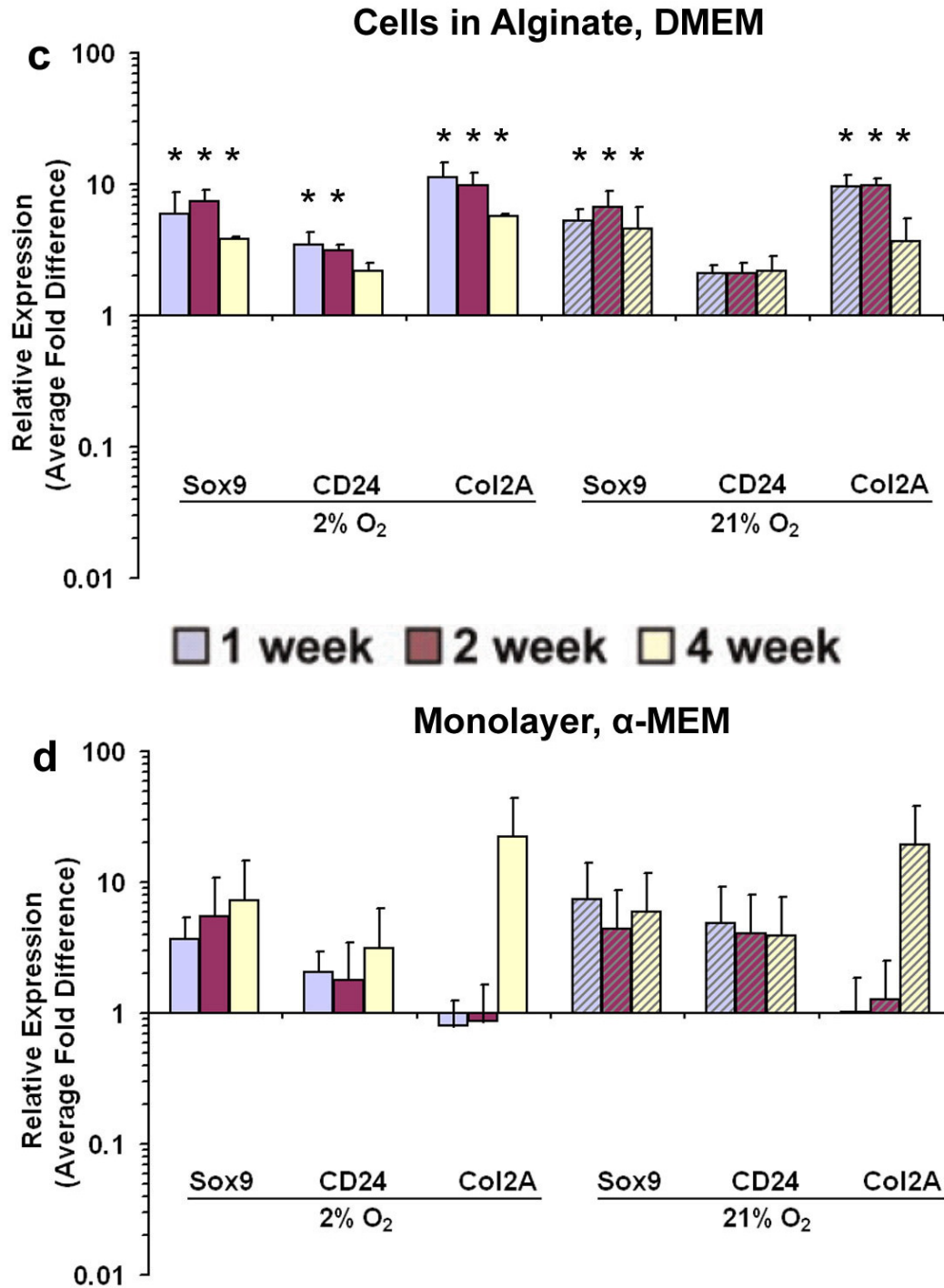


Figure 2.4 cont'd. PCR data showing relative changes in gene expression at 1, 2, and 4 weeks in 2% and 21% oxygen levels for (c) cells embedded in alginate beads cultured in DMEM. Changes in gene expression of Sox9 at all time points ($P < 0.007$), collagen IIA at all time points ($P < 0.011$) except 4 weeks in 21% O₂ ($P = 0.14$), and CD24 at 1 and 2 weeks in 2% O₂ ($P < 0.008$) are significantly different from time zero levels; (d) monolayer culture in α-MEM for 1, 2, and 4 weeks in 2% and 21% oxygen levels. There are no significant differences in gene expression. Both culture configurations resulted in maintenance of notochordal cell gene expression levels, and therefore notochordal cell phenotype.

Discussion

Results of this study demonstrate that physical, nutritional, and biochemical factors interact to generate distinct cellular responses. Although scientific efforts have primarily focused on embryonic development, the significance of notochordal cells likely extends even beyond morphogenesis of the spine. The immature NP derives its mechanical and biological function from these cells [83, 84], and understanding their regulation by environmental variables will provide insight into aging, disease, and potentially repair. Evidence suggests that discogenic spinal disorders may be linked with changes in the NP's abilities to sustain a high internal turgor pressure, maintain flexibility, and provide proper IVD load distribution. For instance, chondrodystrophoid dogs exhibit a loss of notochordal NP cells and are known to develop DDD, while their non-chondrodystrophoid counterparts retain these cells and generally avoid such disorders [21]. Experimental procedures that compromise the notochordal NP likewise have adverse effects on disc health; compressive loads that induce apoptosis and puncture injuries that lead to NP extrusion both result in degenerative changes [12, 65, 85-87]. Importantly, if we consider mechanically disrupted NP tissues as a scaled down version of the immature NP complete with physiologic cellular microenvironment, the results of hypoxic effects are consistent with the enhanced pro-chondrogenic, anti-notochordal phenotype observed in larger animals. Such a postulate would also underscore the potential for some *in vitro* cell culture preparations, like enzymatic cell isolates, to generate results that cannot be translated to the organism.

To our knowledge, there have been no definitive descriptions of cell morphologic changes for notochordal NP cells in culture. Prior reports where notochordal NP have been specifically used either employed different biomaterials [20], examined only short duration cultures [81], or have not provided detailed observations or images [66, 67, 69, 72, 75]. Considering the existence of multiple subpopulations of cells involved, interpreting results from different studies requires adequate standardization of the system.

Using alginate bead culture, we did not observe any detectable loss of cell viability either in mechanically disrupted or enzymatically digested cells, contrary to published data [20]. This may be due to a difference in cell source species or the use of a non-thermal setting hydrogel. In all culture durations and preparations, more heavily vacuolated cells – presumably of chordocyte lineage – was observed as the predominant initial cell type, but their vacuolar appearance varied among different preparations. Thus, there did not appear to be any survival-limiting factor. However, because small chondrocyte-like cells appeared to proliferate faster in monolayer, our observations suggest that vacuolated chordocytes may eventually be overrun over time/passage, making long-term cultures of these cells challenging. Future studies in sorted populations and controlled co-cultures will provide better insight into these effects.

Since the nature of NP cells evolves with age and most likely with time in culture, we examined a subset of genes associated with NP cells: Sox9, CD24, and type IIA procollagen. Type IIA procollagen, a longer alternatively spliced isoform of type II procollagen, is expressed by progenitor cells during chondrogenesis [88], but

not by mature chondrocytes [89, 90]. This isoform has also been identified in the immature NP and the inner annulus of the IVD. The NH₂-propeptide of type IIA collagen has been hypothesized to play a signaling, rather than structural, role, because it does not co-localize with the triple helical region in the NP [15]. Sox9, a transcription factor for type II collagen, is expressed both by chondrocytes [91, 92] and by notochordal cells [93-95], but not hypertrophic chondrocytes [95] or de-differentiating chondrocytes in monolayer [96]. Expression of Sox9 and other chondrogenic processes have been shown to be regulated by hypoxia through the transcription factor HIF-1 α [97, 98]. Finally, CD24 is a cell surface marker predominantly associated with lymphocytes and neuronal development, but recently found to be expressed in the notochordal NP [78]. CD24 appears to be associated with the larger chondrocytes, as identified by FACS analysis, and expression levels are much higher in NP cells than cells from the AF or any other musculoskeletal tissues. Our rationale was to examine relative changes in expression of these three genes as a means to identify key cellular alterations.

Results show that the effects of hypoxia are drastic for mechanically disrupted preparations, but negligible for enzymatic digestions, either in monolayer or in alginate beads. This may be a less acute manifestation of cell-cell or cell-matrix interactions that was deemed necessary for cell survival in other preparations [20], but it is not clear what the nature of these interactions are. Based on our data, normoxia appears to favor notochordal maintenance with steady CD24 levels and upward trends in Sox9 and type IIA procollagen. Conversely, hypoxia may induce chondrogenic differentiation with maintenance of Sox9, reduction of CD24, and high

initial but low long-term expression of type IIA procollagen. Perhaps the organized “micro-mass-like” clusters of cells in mechanically disrupted preparations facilitated hypoxia-induced chondrogenesis.

In DMEM, the NP cell population exhibited changes in gene expression that were consistent with loss of chondrogenic and notochordal phenotypes. For normoxia, all expression levels steadily decreased. For hypoxia, there was a transient increase in Sox9 and type IIA procollagen up to 2 weeks, suggesting an initial hypoxia-induced chondrogenic response, followed by a sharp decline in expression at 4 weeks. Hypoxia appeared to be detrimental for notochordal gene expression, since CD24 continuously decreased. The de-differentiation of primary chondrocytes cultured on tissue culture plastic has been well documented, one of the most notable changes being a shift in the type of collagen produced [99, 100]. Consistent with these previous observations, our present findings also show a decrease in type II collagen and, to our knowledge, is the first reported evidence that this occurs in intervertebral disc NP cells. However, further study is needed to determine whether other collagen species are concurrently upregulated.

In contrast, α -MEM cultures were able to sustain chondrogenic and notochordal phenotypes over time in an oxygen concentration-independent manner. We noted very little difference in the formulations between α -MEM and DMEM, except for one potentially crucial distinction that ascorbic acid (50 mg/L) is present in α -MEM, but not DMEM. It may be that improved procollagen secretion in the presence of ascorbic acid [101, 102] allows cells to recreate a more physiologic substrate to inhibit the de-differentiation response.

In DMEM, enzymatically digested cells embedded in alginate beads exhibited levels of expression that were slightly higher than physiologic, but steady through 4 weeks, just as observed for α -MEM. Similar to what others have observed for chondrocytes, embedding NP cells in hydrogels for cell culture may provide a better microenvironment than tissue culture plastic for maintaining physiologic NP phenotype. Agarose and alginate bead cultures allow cells to maintain a more rounded shape and have been shown to “rescue” de-differentiated chondrocytes, allowing them to re-acquire a chondrocytic phenotype [103-105]. Similarly, mesenchymal stem cells have been found to undergo chondrogenic differentiation by encapsulation in a hydrogel environment alone [106-108]. In terms of oxygen concentration, there was no apparent difference for both Sox9 and type IIA collagen. Insensitivity of enzymatically digested cells to oxygen concentration (both in monolayer and alginate) is consistent with previous findings that HIF-1 α , a regulator of Sox9, remains constant whether monolayer NP cells are in normoxia or hypoxia [66, 72, 75, 76]. Since some signaling pathways in NP cells have been shown influenced by hypoxia [66, 72], genes other than those we examined are likely differentially influenced. It is interesting, however, that two distinct environmental variables (monolayer in α -MEM and alginate bead in DMEM) were able to provide sustained expression of chondrogenic and notochordal genes.

Attachment and proliferation assays were consistent with our morphologic observations and gene expression results in monolayers. Chondrocytes in α -MEM appeared to attach with greater efficiency than in other media formulations, and their prevalence did not change drastically over time. Cells were able to attach in RPMI,

but NP cell growth and/or survival was clearly problematic, consistent with the lack of spreading in vacuolated cells we observed. DMEM and Opti-MEM yielded robust proliferation, but these appeared to favor growth of chondrocyte-like cell colonies. Gene expression results for cells in DMEM are consistent with hypoxia and de-differentiation behaviors in chondrocytes.

Overall, our data demonstrate that hypoxic conditions promoted a chondrogenic response that was detrimental to a notochordal phenotype in immature NP cells, but only in mechanically disrupted cultures. Notochordal gene expression was improved in normoxia. The distinction might be attributed to physiologic cell-cell or cell-ECM interactions, since enzymatically digested cells were not oxygen-sensitive. On the other hand, digested cells maintained notochordal gene expression either in 2% or 21% oxygen if cultured in alginate beads or with α -MEM in monolayer. Low serum levels did not have any striking influence on cell attachment.

One difficulty encountered in this study was identifying and interpreting cell type-specific responses for these cultures that possess at least two known subpopulations. It was not possible to determine whether one subpopulation might be more responsive, and consequently dominate the ensemble response. Pure populations of chondrocytes, and smaller chondroblasts and “chondrocyte-like” cells might provide more precise characterization of their responses. Since reports had indicated the potential significance of cell-cell interactions between these two subpopulations, we chose to sacrifice the interpretive value by examining how immature NP cells, as a whole, would respond to biochemical, nutritional, and physical variables. The current results together with future studies in sorted cell

populations will make it possible to elucidate potential synergistic effects of cell-cell interactions and other microenvironmental variables. These results are important considering how little is understood about the notochord-derived cells of the NP and their age-related changes. Such findings may secondarily be useful for establishing *in vitro* cell culture protocols for NP cells, and potentially their subpopulations.

Acknowledgements

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Chapter 3: Engineering Matrix Metabolism of Intervertebral

Disc Cells using RNA Interference

(Chapters 3 and 4 will be combined for submission to the Journal of Biological Chemistry with revision of technical content)

Abstract

A majority of chronic back pain is associated with degeneration of the intervertebral disc (IVD) and involves a cascade of events influenced by several interacting factors. During degeneration the extracellular matrix is dramatically altered, and this is believed to arise from increased activities of MMPs, ADAM-TSs, and other proteolytic enzymes. Proposed therapeutic and regenerative strategies for the IVD require the ability to modulate cell function, in particular the inhibition of catabolic molecules. This study validates an approach to inhibit matrix degradation by silencing MMP activity using RNA interference (RNAi) in *in vitro* cell culture. Five short hairpin RNAs (shRNAs) were designed to target the cellular mRNA for MMP-2, cloned into a lentiviral vector, and introduced into annulus fibrosus cells. Gene expression was assessed using real-time RT-PCR, and an activity assay was used to measure MMP-2 protein levels in both cell lysates and cell culture media. We found that treated cells had decreased MMP-2 protein levels in both media and cell lysates compared to non-infected control cells. There was also a decrease in the amount of cellular mRNA for MMP-2 in infected cells as compared to non-infected cells. However, the five constructs exhibited differential effectiveness of MMP-2 knockdown. This system suggests a promising method to enable the implantation of

cell-based therapies into a toxic microenvironment. Specifically, silencing MMP-2 activity can suppress matrix breakdown and tissue remodeling, minimizing the degradative events that may be triggered with transplantation of engineered constructs into the disc.

Introduction

There have been numerous studies focusing on the intervertebral disc (IVD) and explicating the factors that may lead to degenerative disc disease (DDD). The etiology of degeneration is not well understood, but is thought to involve biological, chemical, and mechanical changes, and is as well a factor of age and environment [109]. Current treatments can be both debilitating and costly, intensifying the research efforts in developing therapies for treatment of DDD [110]. Intervertebral disc degeneration favors a shift towards primarily catabolic remodeling responses in the extracellular matrix (ECM) [30, 31, 110, 111]. Although the precise mechanisms of disc degeneration are not well understood, it is known that there are several interacting factors involved in the events leading to a diseased state [16, 112].

The use of RNA interference (RNAi) to manipulate genes has become a powerful tool in research over the last few years. This technique can be applied towards discovering or validating gene functions as well as engineering decreases in gene expression [113, 114]. The advantages of RNAi include the ability to induce sequence specific gene silencing, its genome wide capabilities, and the ability to induce both transient and stable effects, all harnessed from a naturally occurring process [38, 113]. Although extensive studies are required to elucidate unanticipated

effects, RNAi is a promising technology that holds tremendous promise for treating human disease. The main goal of this work is to develop the methodology for implementing RNAi in the intervertebral disc *in vitro* in order to elucidate the process of disc degeneration.

The family of matrix metalloproteinases (MMPs) is responsible for the breakdown and synthesis of the extracellular matrix proteins in the body. Thus they are key mediators of tissue remodeling, but also play a role in cell proliferation, migration, differentiation, angiogenesis, apoptosis and host defense [28]. A number of different MMPs have been found in diseased discs, but their functional roles in the degenerative cascade are not clear. MMP-2, a gelatinase thought to participate in the secondary breakdown of collagen, has been found in elevated levels in degenerate discs [30, 32-34]. One of the putative roles of matrix metalloproteinase-2 (MMP-2) is the remodeling of the extracellular matrix. Increased activation of MMP-2 has also been shown to be induced by mechanical stress [36, 115].

As a first step towards improved understanding of MMP function, this current study validates an approach to silence MMP-2 gene expression using RNA interference (RNAi) in an *in vitro* cell culture system. While, many studies have implemented siRNAs to regulate gene expression, to our knowledge this is the first study to utilize small hairpin RNAs (shRNAs) to achieve stable knockdown of MMP-2.

Materials and Methods

Construct preparation

The BLOCK-iT U6 RNAi Entry Vector Kit and the BLOCK-iT Lentiviral RNAi Expression System (Invitrogen, Carlsbad, CA) were used for procedures. Five 21-23 nucleotide sequences (Table 3.1) were identified from the rat MMP-2 gene from which shRNA target sequences were designed using a 4 nucleotide hairpin loop. A nonsense control (shNon) was also constructed which contained a scrambled sequence. Oligonucleotides (oligos) complementary to the shRNA target sequences were designed and designated D-H. The two complementary oligos were annealed together and ligated into the provided pENTR/U6 entry vector, which contained a kanamycin resistance gene. The vector was transformed in OneShot TOP10 *E.coli* and selected on LB agar plates with 50 ug/ml kanamycin. Antibiotic-resistant colonies were cultured overnight in LB broth also containing antibiotic. Plasmid from overnight bacterial cultures was purified and sequenced to ensure no mutations occurred to the target sequence during processing.

Table 3.1. MMP-2 shRNA sequences

Sequence Name	Top and Bottom “stem-loop-stem” sequences, 5’ to 3’
MMP-2 D	CACCGCTGAAGGACACCCTCAAGAACGAATTCTTGAGGGTGTCTTCAGC AAAAGCTGAAGGACACCCTCAAGAATTCGTTCTTGAGGGTGTCTTCAGC
MMP-2 E	CACCGCCGGGATAAGAAGTATGGATTCTCGAAAGAATCCATACTTCTTATCCCGG AAAACCGGGATAAGAAGTATGGATTCTTTCGAGAATCCATACTTCTTATCCCGGC
MMP-2 F	CACCGCTGTGTTCTTCGCAGGGAATCGAAATTCCTGCGAAGAACACAGC AAAAGCTGTGTTCTTCGCAGGGAATTCGATTCCCTGCGAAGAACACAGC
MMP-2 G	CACCGCAATACCTGAACACTTTCTACGAATAGAAAGTGTTCAAGGATTGC AAAAGCAATACCTGAACACTTTCTATTTCGTAGAAAGTGTTCAAGGATTGC
MMP-2 H	CACCGTGGTGGTCACAGCTATTTCTTCCGAAGAAGAAATAGCTGTGACCACCA AAAATGGTGGTCACAGCTATTTCTTCTTCGGAAGAAGAAATAGCTGTGACCACCAC
MMP-2 scrambled	CACCGCCGATTAGCTGATCGTGCTTAGTCGAAACTAAGCACGATCAGCTAATCGG AAAACCGATTAGCTGATCGTGCTTAGTTTCGACTAAGCACGATCAGCTAATCGGC

The purified entry vector was recombined with the provided pLenti6/BLOCK-iT-DEST vector, which contained an ampicillin resistance gene. The resultant expression construct was transformed in OneShot Stbl3 Competent *E.coli* and selected on LB agar plates with 100ug/ml ampicillin. Antibiotic-resistant colonies were cultured overnight in LB broth also containing antibiotic. Purified plasmid was again sequenced to ensure no mutations occurred during processing.

The purified expression construct was then packaged into a replication-deficient lentivirus to obtain a lentiviral stock to be used for subsequent experiments. Briefly, 293FT packaging cells were cotransfected with the purified expression construct and ViraPower Packaging mix using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Virus containing supernatants were harvested 72 hours later, centrifuged to remove cell debris, sterile filtered, and stored at -80°C until use.

Primary cell isolation and culture

Annulus fibrosus (AF) tissue was harvested from Sprague-Dawley rat caudal discs and digested overnight in 3 mg/ml collagenase. The next day, the digests were centrifuged, resuspended in fresh culture media, and plated in tissue culture flasks. The cells were cultured until passage 3 with media changes everyday. The cells were maintained in DMEM containing 10% FBS and 1% Penicillin-Streptomycin at 37°C with 5% CO₂ and balance air.

Cell infection

Passage 3 AF cells were plated in 6-well tissue culture plates in complete media at 50% confluence. Each of the five viral constructs and the nonsense control were added to one well each, at an MOI of 0.1, along with Polybrene (Sigma, St. Louis, MO) to facilitate infection. Virus was removed the next day and replaced with complete culture medium. Blasticidin (Invitrogen, Carlsbad, CA) was added in appropriate samples the day after virus removal, at a concentration of 8 µg/ml. Media was changed every 3 days, each media change contained blasticidin in the appropriate samples. Each time point also included a corresponding non-infected control (plated cells with no virus).

Three separate experiments were conducted to screen the five viral constructs. For experiment 1: Cells were infected and harvested at 1, 4, 7, and 10 days following virus removal. For experiment 2: Cells were infected, then harvested at 1, 4, 7, and 10 days following addition of blasticidin. For experiment 3: Cells were infected and then subjected to a 10 day blasticidin treatment, followed by culture in complete media without blasticidin. Cells were harvested at 1, 4, 7, and 10 days following removal of blasticidin. At each time point, samples were collected by scraping cells using Tris-HCl, pH 7.5-8.0, and a cell scraper. Harvested samples were divided into 3 groups to measure (1) DNA content (2) MMP-2 content, and (3) gene expression. Media was also harvested from samples for measurement of MMP-2 content. The construct with the highest level of knockdown, designated shMMP, was validated for use in future experiments (n=4).

MMP-2 expression

MMP-2 content was quantified using a microplate-based activity assay (R&D Systems Inc., Minneapolis, MN). Cell samples were pulverized with a pestle in Tris-HCl solution then loaded into microplates, along with media samples and standards. Protein levels were obtained according to the manufacturer's protocol. Protein levels were normalized to DNA content, which was determined using PicoGreen assay (Invitrogen, Carlsbad, CA). Cell samples were subjected to 5 freeze-thaw cycles for cell lysis and loaded into a microtiter plate along with standards generated from Lambda DNA. PicoGreen reagent was then added to all wells. All absorbance (MMP-2) and fluorescence (DNA) measurements were made using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA).

For quantitative RT-PCR, RNA was isolated and reverse transcribed for PCR to obtain gene expression levels of GAPDH and MMP-2, using primers designed for rat genes (Table 3.2) from Primer3 software (<http://frodo.wi.mit.edu>). Results were analyzed using the $\Delta\Delta C_t$ method [82]. These $\Delta\Delta C_t$ values were then expressed as relative changes in mRNA levels (fold difference) through the exponential relation: $2^{-\Delta\Delta C_t}$. For validation of shMMP, data are reported as the average value of the range of calculated fold difference, which incorporates the standard deviation of the $\Delta\Delta C_t$ value in the fold difference calculation as $\Delta\Delta C_t + SD$ and $\Delta\Delta C_t - SD$. Statistical analyses (SPSS 14.0, Chicago, IL) were performed using one-way ANOVA ($\alpha = 0.05$).

Table 3.2. Sequences of Primers used for RT-PCR Analysis

Gene Name	Forward and Reverse primers	GenBank accession no.	Length of product (bp)
GAPDH	5'-AACCCATCACCATCTTCCAG-3' 5'-GTGGTTCACACCCATCACAA-3'	NM_017008	197
MMP-2	5'-AGCTCCCGGAAAAGATTGAT-3' 5'-TCCAGTTAAAGGCAGCGTCT-3'	NM_031054	180

Results

Three separate experiments were conducted to screen the five viral constructs, designated D-H, targeting MMP-2. Overall, stable silencing was achieved in each of the 3 experiments. All constructs, except D were able to down-regulate gene expression and protein levels through 10 days, in Experiments 1 and 2 (Figure 3.1 and Figure 3.2). In addition, the constructs maintained stable knockdown of MMP-2 throughout the 3 week culture period in Experiment 3 (Figure 3.3). Similar trends of gene silencing were observed in each of the 3 experiments for each of the 5 constructs. However, the level of knockdown was greater in Experiment 1 as compared to Experiments 2 and 3. Overall, constructs F and G were more successful in knocking down MMP-2 mRNA and protein compared to the other constructs. Up to 80% gene silencing was observed for both constructs. Therefore, construct G, designated shMMP, was validated for use in future experiments.

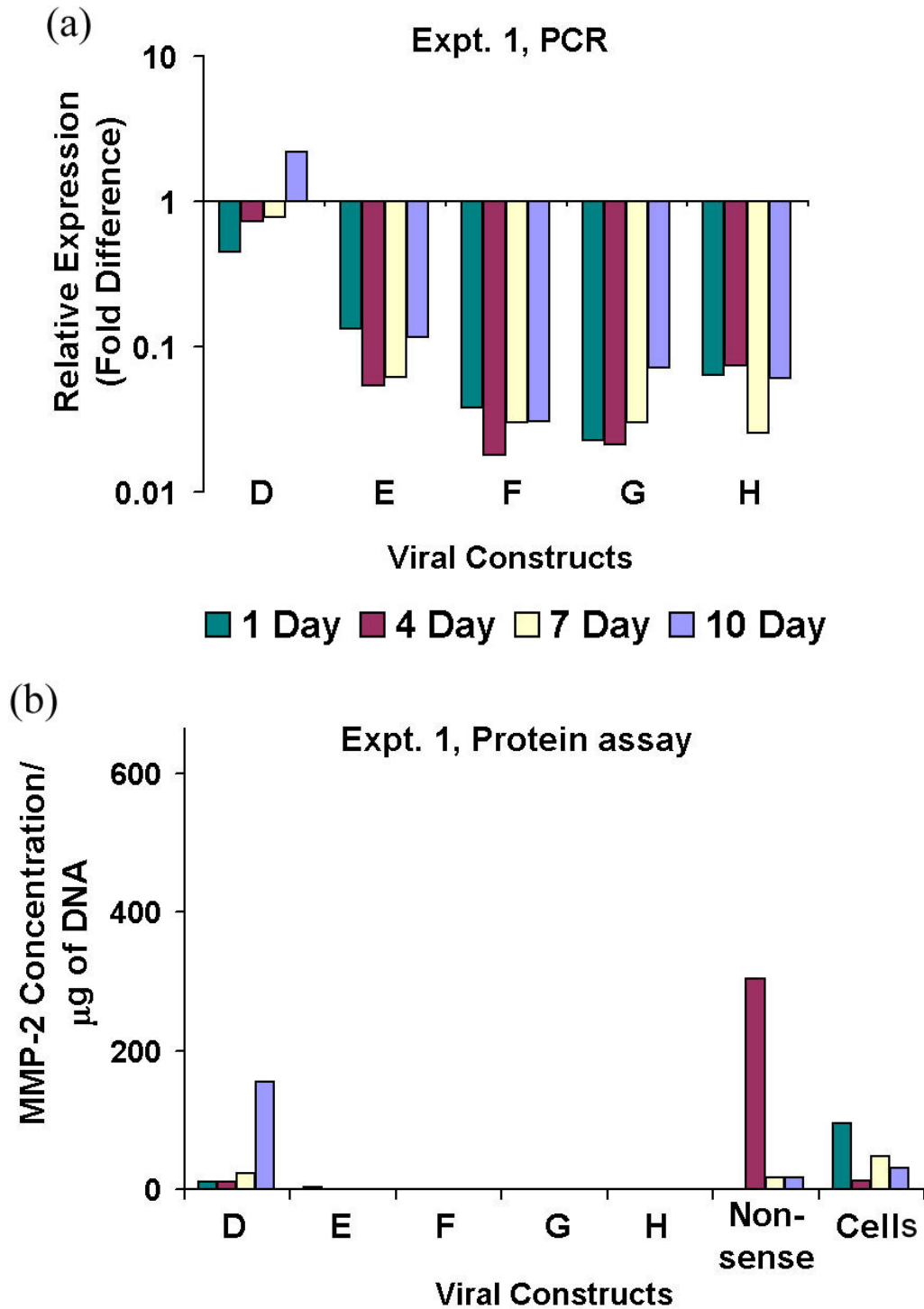


Figure 3.1. Cells were infected with each of the five viral (D-H) constructs and then lysed after 1, 4, 7, and 10 days. (a) PCR results for gene expression of MMP-2 were compared to nonsense controls for each time point. (b) Protein results for cell lysates. The values of nonsense and non-infected controls (Cells) are shown for comparison. Each of the five viral constructs were able to knock down gene expression and protein levels of MMP-2, although to varying degrees through 10 days in culture.

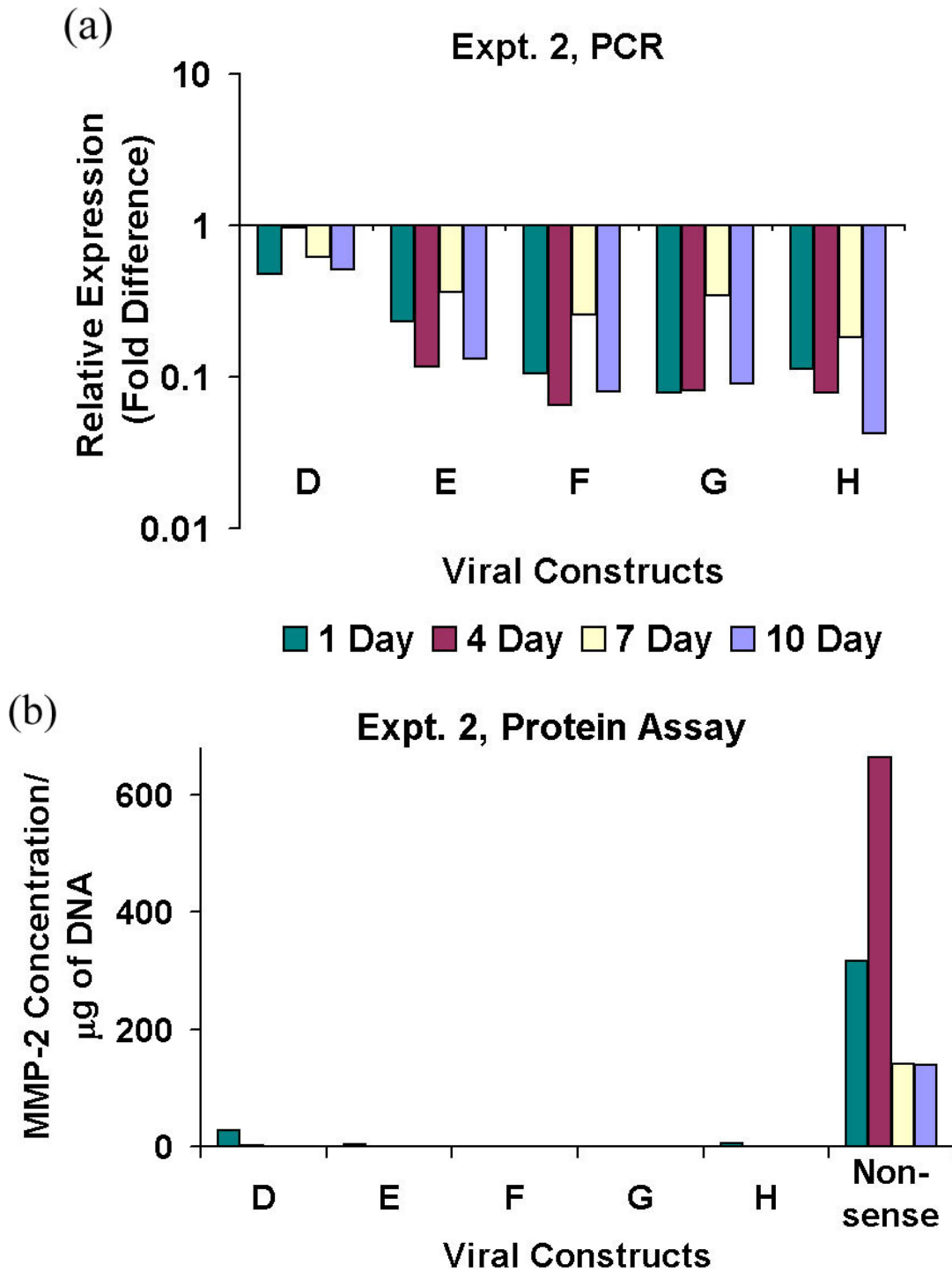


Figure 3.2. Infected cells were treated with blasticidin and then lysed after 1, 4, 7, and 10 days. (a) PCR results were compared to nonsense controls for each time point. (b) Protein results for cell lysates. The values of nonsense controls are shown for comparison. Treatment with blasticidin resulted in a pure population of only infected cells. Gene expression and protein levels of MMP-2 were down-regulated throughout the culture period for each of the five viral constructs.

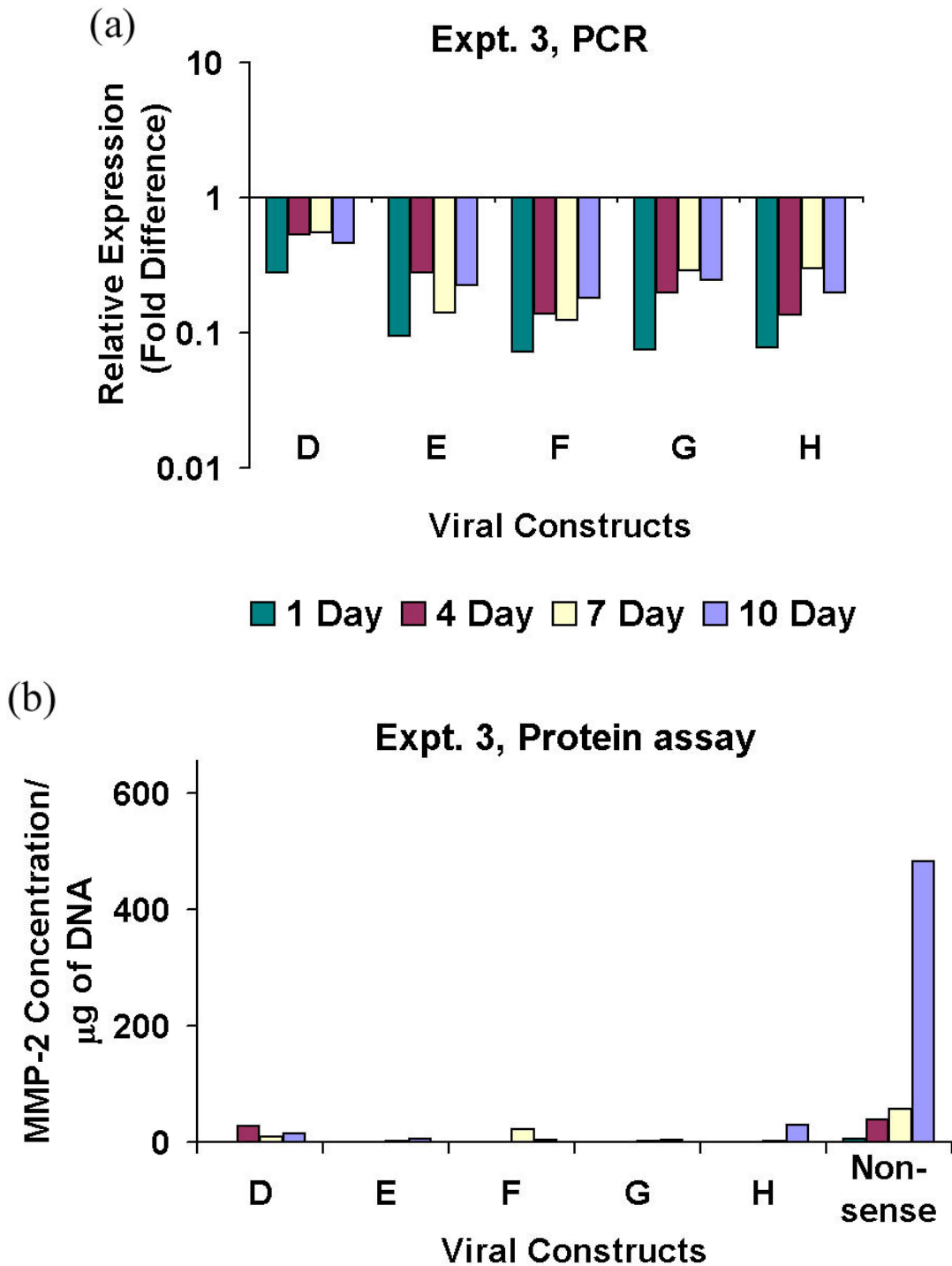


Figure 3.3. Infected cells were treated with blasticidin for 10 days followed by culture in DMEM and then lysed after 1, 4, 7, and 10 days. (a) PCR results for gene expression levels of MMP-2 were compared to nonsense controls for each time point. (b) Protein results for cell lysates. The values of nonsense controls are shown for comparison. Cells were cultured in DMEM following treatment with blasticidin to allow for proliferation of only infected cells. Both MMP-2 gene expression and protein levels were down-regulation by each of the constructs for the culture period.

Experiments 1 and 2 were repeated (n=4) with shMMP and shNon to validate the results from the initial gene knockdown experiments. As before, MMP-2 mRNA levels were down-regulated in shMMP infected cells as compared to shNon infected cells. In addition, MMP-2 protein levels were lower in shMMP infected cells and media as compared to shNon infected cells and media through 10 days in culture (Figure 3.4).

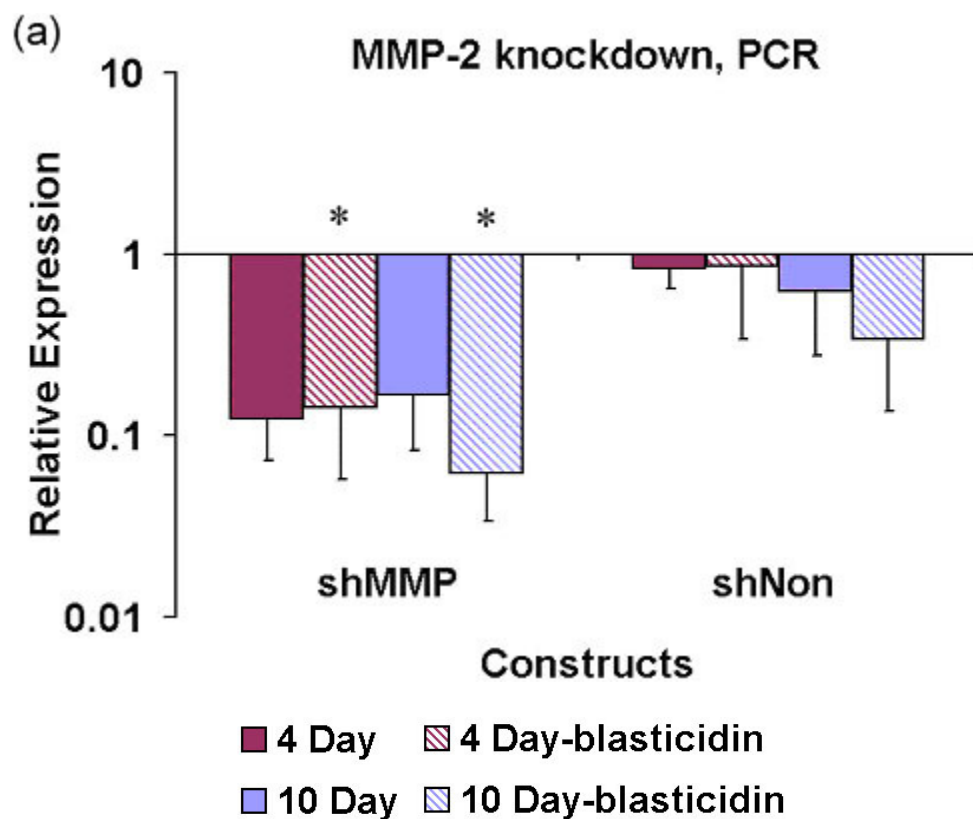


Figure 3.4. Cells infected with shMMP and shNon, with and without treatment with blasticidin. Cells were lysed at 4 and 10 days. (a) PCR results for gene expression levels of MMP-2 were compared to non-infected controls for each time point. Down-regulation MMP-2 with shMMP are significantly different from shNon at 4 ($P = 0.036$) and 10 days ($P = 0.042$) in culture with blasticidin. PCR results from validation studies corroborate results from initial experiments, as there was a greater extent of down-regulation of MMP-2 gene expression levels in shMMP infected cells compared to shNon infected cells.

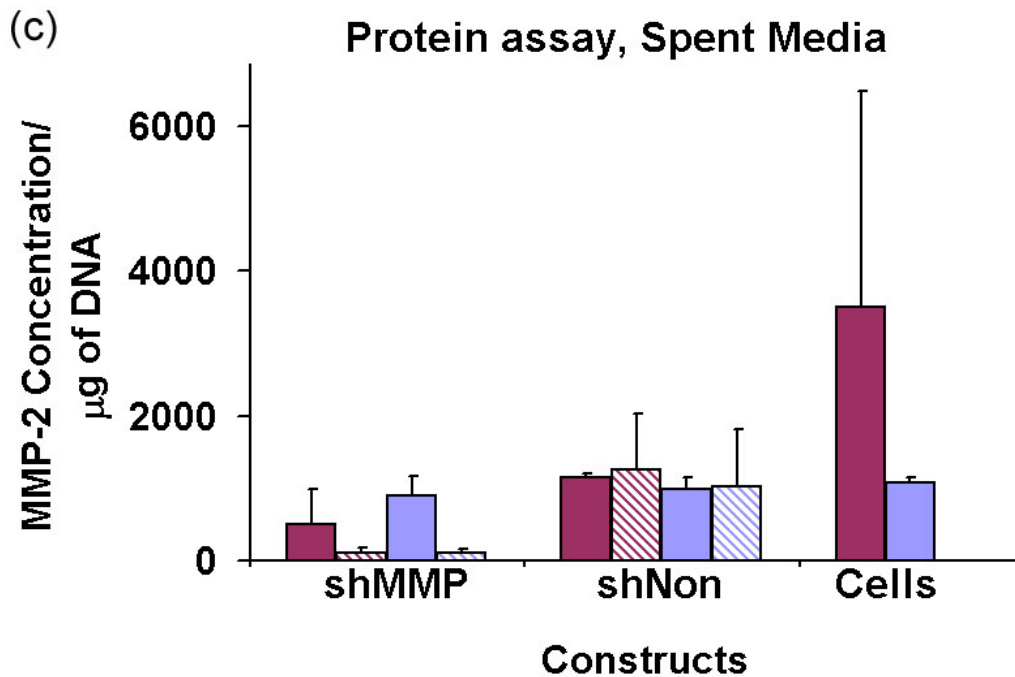
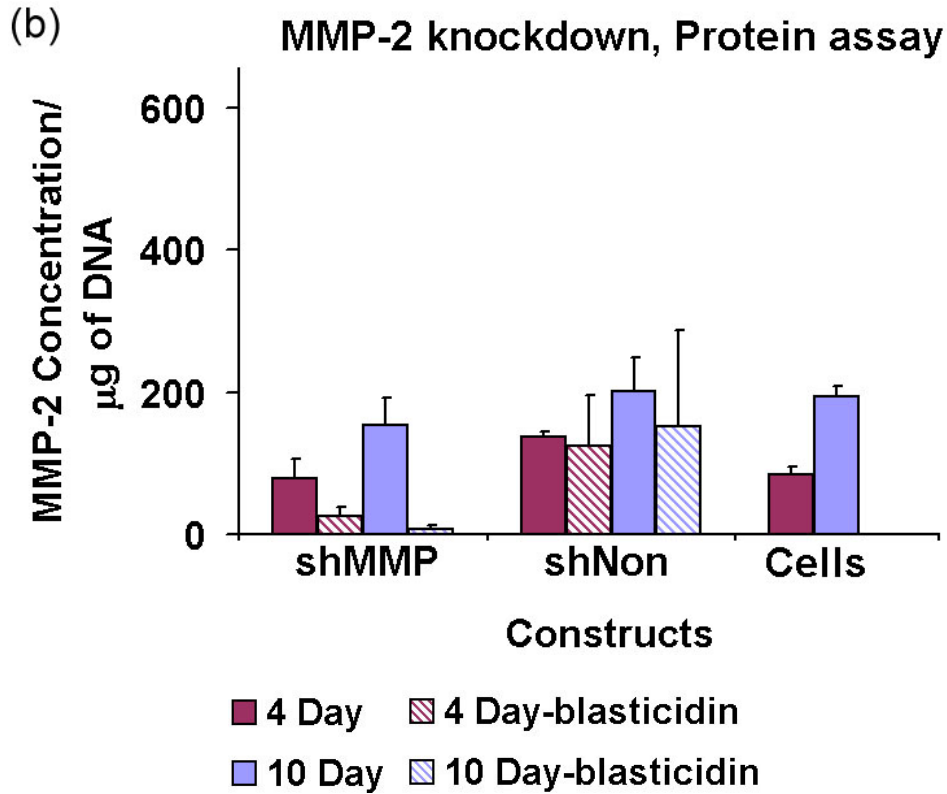


Figure 3.4 cont'd. Cells infected with shMMP and shNon, with and without treatment with blasticidin. Cells were lysed at 4 and 10 days. (b) Protein data for cell lysates and (c) spent media. The values of non-infected controls (Cells) are shown for comparison. Protein results from validation studies corroborate results from initial experiments, as MMP-2 protein levels were down-regulated in shMMP infected cells compared to shNon infected cells.

Discussion

Intervertebral disc degeneration is the leading source of morbidity and a major cause of work disability [116]. Treatment strategies for disc degeneration include increasing levels of anabolic growth factors, or blocking the activity of catabolic factors, or both [52]. RNAi technology offers the advantage of stable silencing of catabolic factors which can be of therapeutic value in treating disc degeneration. To this extent, lentiviral vectors have provided an advancement in RNAi and offer the means to achieve significant levels of gene transfer both *in vitro* and *in vivo* [117]. Although various studies have used siRNAs for gene therapy in IVD cells, to our knowledge this is the first study to utilize shRNAs to achieve stable knockdown of an endogenously produced gene in the intervertebral disc.

Many proteinases are capable of degrading ECM components, but MMPs appear to be particularly important for matrix degradation [117]. MMP-2 has been widely studied in the treatment of cancer. siRNAs against MMP-2 resulted in decreased tumor invasion, migration and angiogenesis, with a 60% reduction in tumor size [118]. Since MMP-2 has also been implicated in matrix degeneration and tissue remodeling in the intervertebral disc [28, 36, 115], evaluating its role in the degenerative cascade that leads to DDD can serve as an important step in the development of therapeutic treatments. Five viral vectors were made targeting MMP-2 in rat AF cells. Silencing of MMP-2 gene expression was achieved with each of the constructs, in each of the three screening experiments. In addition, down-regulated levels of MMP-2 were maintained up to 3 weeks in culture.

Some constructs were more successful in silencing MMP-2 than others, however. While constructs F and G achieved up to 80% knockdown, suppression of gene expression was diminished by 10 days in culture for construct D. During the transfection protocol, not all cells are transduced with the shRNA. It is possible that cell metabolism slows for transduced cells, therefore proliferation rates for untransduced cells may be greater than transduced cells. In addition, it is known that siRNAs that target different regions of the same gene vary markedly in their effectiveness [38]. siRNA efficacy depends largely on multiple factors, including the secondary structure of the mRNA target, the binding of the RNA-binding proteins, and thermodynamic stability of the duplex [38, 44]. Together, the lesser extent of gene silencing and greater proliferation of untransduced cells and a may have masked the effects of MMP-2 knockdown in construct D.

Treatment of cells with blasticidin yielded a pure population of only infected cells, eliminating masking effects by untransduced cells. It was expected that the pure population would exhibit higher levels on knockdown compared to a mixed population. However, both Experiments 2 and 3 resulted in a lower extent of gene silencing. It is possible that the 10 day blasticidin treatment used to kill untransduced cells in these experiments may trigger an increase in MMP-2 gene expression leading to a lower degree of knockdown. During antibiotic treatment, multiple metabolic processes are interfered with or changed [119], which may trigger an up-regulation of genes. Since overall gene expression levels may be higher in these cells, the level of MMP-2 knockdown is effectively reduced.

Although the shNon contained a scrambled sequence, some level of knockdown was seen in these cells as well. It is known that the interferon response may be triggered in the process of cell transfection with a viral vector [38, 44]. Thus, there are changes occurring in cells at the molecular level. The infection may cause overall changes in the gene expression profile in cells as well [120]. These factors together could lead to some level of gene knockdown observed in cells infected with the shNon vector. In addition, there is some debate as to the specificity of sequences required for effective RNAi [121]. It is possible that various bases in the scrambled nonsense sequence may be able to match the MMP-2 mRNA found in cells causing some knockdown effect. Compared to the nonsense construct however, shMMP was successful in knocking down MMP-2 mRNA and protein levels to a higher degree. Therefore the chosen sequence for shMMP was considered effective for use in future RNAi experiments.

The present study validates lentiviral shRNA delivery and the targeting of MMP-2 in intervertebral disc cells. We have demonstrated significantly reduced mRNA and protein levels in both cells and media samples *in vitro*. These results help establish RNAi protocols using lentiviral delivery of shRNA to study the function of MMP-2 in disc degeneration. Studies using transfection of siRNA allow for only the transient down-regulation of gene expression, making it difficult to adapt for therapeutic purposes. However, shRNA delivery can allow for long-term studies to determine the functional role of genes relevant to intervertebral disc degeneration, such as MMP-2. These techniques can be applied to future *in vitro* studies elucidating the effects of silencing MMP-2 on cellular interaction with their

surrounding microenvironment, as it is held responsible for remodeling and degradation of extracellular matrix components. Improvements in shRNA delivery and reduction of off-target effects can make RNAi an attractive method for gene silencing to treat intervertebral disc degeneration.

Acknowledgements

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Chapter 4: Functional Consequences of Silencing MMP-2 in Intervertebral Disc Cells using RNA Interference

Abstract

One of the putative roles of matrix metalloproteinase-2 (MMP-2), a gelatinase, is the remodeling of the extracellular matrix. We have previously designed and validated an shRNA sequence cloned into a lentiviral vector to knockdown MMP-2 expression in cells from the annulus fibrosus (AF) of the intervertebral disc. To further the investigation of the functional role of MMP-2 in AF cells, this study utilized RNA interference to examine the effects of MMP-2 silencing on gelatin degradation and type I collagen gel remodeling. Infected and non-infected AF cells were seeded on gelatin films and analyzed for degradation. Additionally, type I collagen gels were embedded with infected and non-infected cells and mechanically tested to examine differences in material properties. The ability of cells to remodel collagen fibers was also examined through histological staining. Silencing MMP-2 resulted in the inability of cells to degrade gelatin and remodel collagen gels. Gels were also weaker, possibly due to a lesser degree of remodeling. The findings of this study will help to elucidate the interactions between MMP-2, collagen and gelatin. The breakdown of the collagen found in the AF is thought to play a major role in intervertebral disc degeneration. Knowledge of the functional significance of MMP-2 is essential to understanding and treating this prevalent disorder.

Introduction

Various physiologic and pathophysiologic processes, including tissue growth, repair, and degeneration, involve cell-mediated tissue remodeling events. During remodeling, extracellular matrix (ECM) turnover rates are increased [122], causing shifts in tissue architecture and composition. While a number of molecules are thought to regulate this process, the family of matrix metalloproteinases (MMPs) is believed to be particularly important in processing ECM protein degradation [30-32]. In addition to being key mediators of tissue remodeling, however, MMPs are also known to be involved in cell proliferation, migration, differentiation, angiogenesis, apoptosis and host defense [28].

One example of chronic pathophysiologic remodeling occurs in the intervertebral disc (IVD) of the spine with degenerative disc disease (DDD). In the annulus fibrosus (AF) subregion of the disc, there is an acceleration of age-related degradative changes, which are thought to be caused by enhancement of catabolic processing of the ECM [30, 31, 110, 111]. A number of different MMPs have been shown to be expressed at elevated levels in diseased discs [32]. One of these is MMP-2, a gelatinase thought to participate in the secondary breakdown of collagen critical for remodeling [30, 32-34]. Activation of MMP-2 above endogenous baseline levels has been shown to be induced by mechanical stress [36, 115] and by inflammation [27]. However, the functional role of MMP-2 in disc health and degeneration remain unclear. Current treatments can be both debilitating and costly, intensifying the research efforts in developing therapies for treatment of DDD [110]. An improved understanding of the molecular mechanisms involved in DDD would

contribute to the development of interventional strategies to decelerate the degenerative processes.

In this study we examine the effects of silencing MMP-2 on collagen remodeling and gelatin degradation in an *in vitro* cell culture system. With the use of RNAi technology, we can investigate the functional role of MMP-2 in annulus fibrosus cells. Many studies have effectively silenced various genes using RNAi in the disc; however few have examined the functional consequences. Elucidating the action of MMP-2 can aid in designing more specific therapies for DDD.

Materials and Methods

Primary cell isolation and infection

Annulus fibrosus tissue was harvested from Sprague-Dawley rat caudal discs and digested overnight in 3 mg/ml collagenase. The next day, the digests were centrifuged, resuspended in fresh culture media, and plated in tissue culture flasks. The cells were cultured until passage 3 with media changes everyday. AF cells were maintained in DMEM containing 10% FBS and 1% Penicillin-Streptomycin at 37°C with 5% CO₂ and balance air.

Passage 3 AF cells were plated in tissue culture flasks in complete media at 50% confluence. A validated lentiviral vector containing an shRNA sequence targeting MMP-2, designated shMMP, and a vector containing a nonspecific scrambled sequence, designated shNon, were added to one flask each, at an MOI of 0.1, along with Polybrene (Sigma, St. Louis, MO) to facilitate infection. Virus was removed the next day and replaced with complete culture medium. To obtain a pure

population of infected cells, blasticidin (Invitrogen, Carlsbad, CA) was added the day after virus removal, at a concentration of 8 $\mu\text{g/ml}$. Media was changed every 3 days, each media change contained blasticidin when necessary.

Gelatin Films

A 5% gelatin solution was pipetted onto glass slides and allowed to air dry. Once cooled, the slides were chemically cross-linked in 4% formalin for 1 hour at room temperature. The slides were then washed extensively with sterile PBS, and stored overnight in PBS at 4°C. The next day, the PBS was removed and slides were equilibrated in complete cell culture media for 30 minutes before cell seeding.

Separate populations of AF cells infected with the shMMP construct and the shNon construct were treated with blasticidin to obtain a pure population of infected cells. Infected cells (n=4 for shMMP and shNon each) and non-infected control cells (n=4) were seeded onto the films at a density of 200,000 cells/film. The cell suspension was allowed to settle for 30 minutes before cell culture media was added. Gelatin films without cells served as negative controls (n=4). Films were cultured for 4 days at which time media was removed from the dish and films were washed once with PBS. Films were stained with 1% Ponceau S for 1 minute and washed with DIH_2O for 5 minutes on a shaker. Films were visualized immediately after staining. Percent degradation of gelatin was quantified using Image J (U.S. National Institutes of Health, Bethesda, MD) analysis. Statistical analysis (SPSS 14.0, Chicago, IL) was performed using Kruskal-Wallis test with Games-Howell post hoc analysis.

Collagen Gels

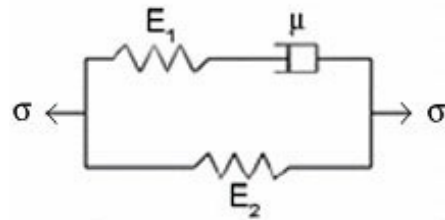
Three-dimensional type I collagen gels were made from rat tail collagen (BD Biosciences, San Jose CA) at a concentration of 3 mg/ml following manufacturer's protocols. All the components were pre-chilled and sterile. Briefly, the type I collagen stock solution was diluted in 10X PBS (Gibco/Invitrogen, Carlsbad, CA), sterile water, and 1N NaOH to achieve a final concentration of 3 mg/ml collagen in 1X PBS at pH 7.4. The collagen solution was kept on ice until use.

Passage 3 AF cells were infected with the shMMP and shNon viruses, as described previously. Two separate populations of cells were used for each of the 2 viruses, one of which was treated with blasticidin to obtain a pure population of only infected cells, while the other was used as a mixed population of infected and non-infected cells (n=3 for each). Non-infected AF cells were used as positive controls (n=3) while gels without cells were used as negative controls (n=3). Cells were trypsinized, pelleted and resuspended in collagen solution at 500,000 cells/ml. Cells were pipetted into custom-made dog-bone shaped molds (3mm thick) fabricated to be 15mm in width at the grips with a 5mm wide x 10mm long gauge region, placed in petri dishes. Polypropylene mesh (Small Parts, Inc., MA) with approximately 300 μ m pore size was embedded in the collagen at each end of the mold for gripping. The gels were incubated for 90 minutes at 37°C incubator to allow polymerization, then placed in 6-well plates in complete culture media. Gels were cultured for 7 days. At the time point, gels were used either for mechanical testing to ascertain changes in material properties, or for histology to evaluate structural changes.

Mechanical Testing and Histology

For mechanical testing, gels were removed from culture and their cross-sectional area was measured using calipers since they exhibited varying degrees of contraction during culture. Gels were clamped on each end at the mesh, and creep testing was performed in tension using hanging weights. An initial weight of 1.5g was added, followed by increments of 0.5g, each at 2 minute intervals. The gels were loaded until failure. Deformations were captured using a video system, and strain computed by image analysis of individual frames. Ten frames per second were

captured for the first 10 seconds, and two frames were second were captured for the remainder of the first increment. Changes in gel length in each frame were evaluated using ImageJ. The material properties of each gel were evaluated by fitting data to the standard



$$\epsilon(t) = \sigma \left[\frac{1}{E_2} + \left(\frac{1}{E_1 + E_2} - \frac{1}{E_2} \right) e^{-\left(\frac{E_1 E_2}{\mu E_1 + \mu E_2} \right) t} \right]$$

Figure 4.1. Standard linear viscoelastic solid model.

linear viscoelastic solid model (Figure 4.1) using Matlab. Values were obtained for E1 and E2 (measures of strength), and μ (a measure of viscosity). Thus changes in the viscoelastic properties of the gels were evaluated.

For histology, gels were fixed overnight in 10% buffered formalin. The collagen gels were then infiltrated with paraffin before being embedded in paraffin blocks. 7 μ m sections were cut and stained with Hematoxylin, Fast Green and Safranin-O to evaluate changes in collagen structure.

Results

Gelatin films

After culture for 4 days, blank films which served as negative controls stained homogeneously red with the Ponceau S (Figure 4.2 a). In contrast, gelatin films seeded with non-infected control AF cells showed focal degradation of the gelatin immediately surrounding the cells. This area appeared lighter pink to white, compared to the red-stained gelatin in areas without cells (Figure 4.2 b). Films seeded with cells infected with shNon also appeared to have focal degradation surrounding the infected cells, similar to what was seen in the films with non-infected AF cells (Figure 4.2 d). However, in films seeded with cells infected with shMMP, little to no focal degradation was observed around the cells. The gelatin was stained more homogeneously red, similar to the blank films (Figure 4.2 c). Quantification of degradation (Figure 4.3) verified visual results, with non-infected cells and shNon infected cells having similar percent degradation, while shMMP infected cells showed minimal percent degradation.

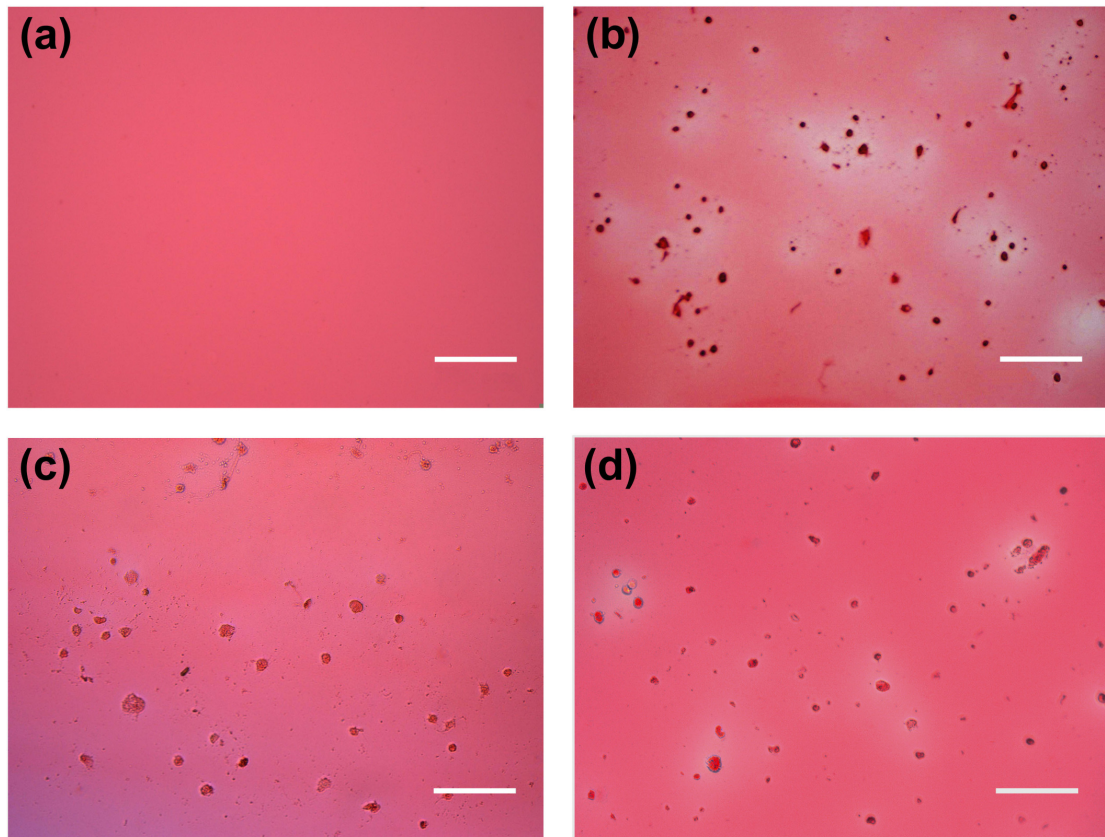


Figure 4.2. Microscopy images of gelatin films seeded with: (a) no cells, (b) non-infected AF cells, (c) cells infected with shMMP, (d) cells infected with shNon. Films were stained at 4 days in culture with Ponceau S, and visualized immediately. Scale bars represent 200 μm . All images were taken at 100X. Films with no cells (a) stained evenly red, while films seeded with non-infected AF cells (b) showed focal degradation of gelatin in the areas immediately surrounding cells, which is shown by the lighter stained regions. Similar results are seen with cells infected with shNon (d), while cells infected with shMMP (c) show little to no focal degradation of gelatin, implicating that MMP-2 is directly involved in gelatin breakdown.

Gelatin Film Degradation

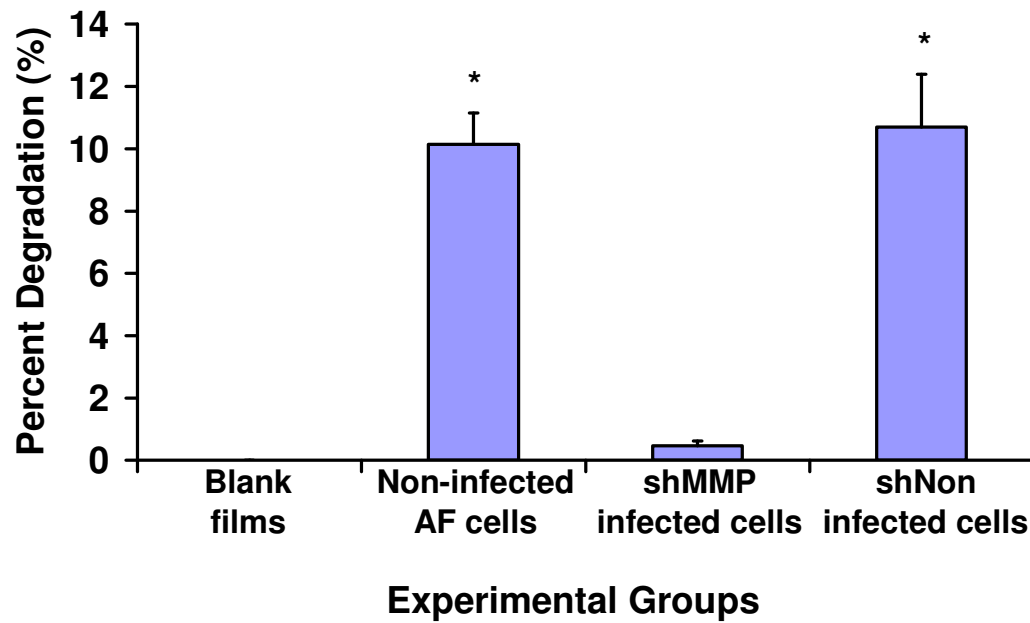


Figure 4.3. Quantification of gelatin degradation. Gelatin films seeded with non-infected AF cells and shNon infected cells were similar in percent degradation of gelatin, which corroborated visual results. Non-infected AF cells were significantly different than shMMP infected cells ($P = 0.001$), with non-infected cells having a higher percentage of gelatin degradation, as was seen in microscopic images. In addition, shNon infected cells were significantly different than shMMP infected cells ($P = 0.002$), with a higher percentage of degradation in films seeded with shNon infected cells, as seen visually.

Collagen Gels

After the 7 day culture period, gels were mechanically tested or fixed. Visually, blank gels remained the same size through culture while gels embedded with non-infected AF cells contracted to approximately 17% of their original size. Gels embedded with cells infected with shMMP contracted to a lesser degree than gels with cells infected with shNon.

Collagen gels without cells exhibited the highest strength and viscosity, but low rupture stress. In contrast, collagen gels with non-infected AF cells had the highest rupture stress, but lowest viscosity, while gels with cells infected with shMMP had among the lower values for strength, but higher values for viscosity (Figure 4.4).

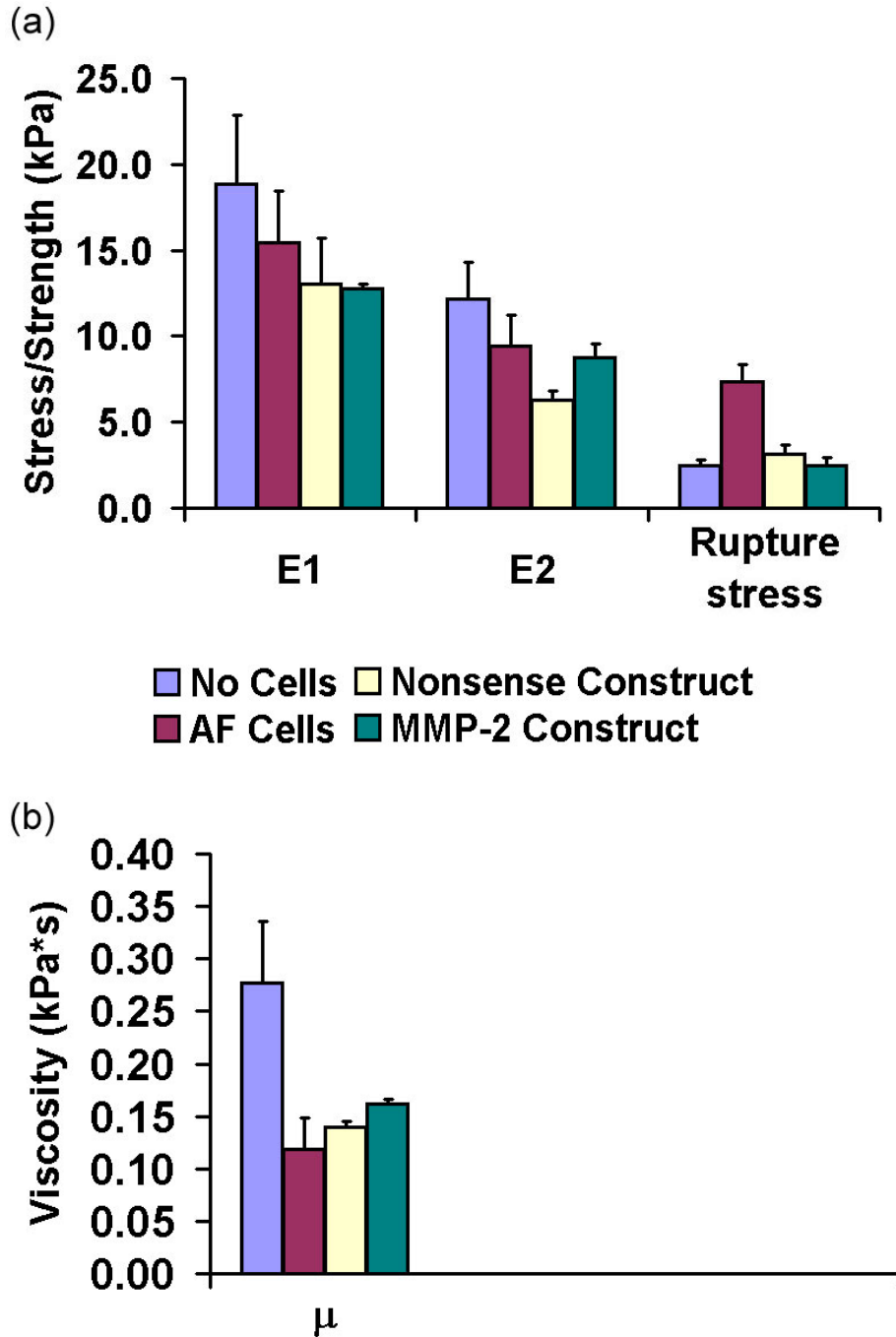


Figure 4.4. Material properties of collagen gels containing no cells, non-infected AF cells, and AF cells infected with shMMP construct or shNon construct. (a) E1 and E2 represent gel strength and (b) μ represents viscosity. Gels containing non-infected AF cells had among the higher values of strength and lower values for viscosity, possibly due to the ability of AF cells to restructure collagen fibers within gels, giving them higher strength, and carry out both catabolic and anabolic processes, giving them lower viscosity values. In contrast, gels containing cells infected with shMMP had lower values for strength, and higher values for viscosity. These cells were unable to restructure their surrounding collagen network, making gels weaker, and were also unable to breakdown gelatin, making them more viscous.

Histological staining of blank gels revealed unstructured collagen fibers with heterogeneous porous structure throughout (Figure 4.5 a). Gels with non-infected control cells contained much denser regions of collagen surrounding the cells, on which they seemed to be anchored. The collagen fibers appeared to be restructured in the entire gel (Figure 4.5 b). However, in gels embedded with shMMP infected cells, minor changes in dimensions were seen over the culture period. The collagen fibers appeared much less structured, similar to the blank gels. The cells did not appear to be anchored on the fibers, and instead were rounded in appearance (Figure 4.5 c). Gels containing cells infected with shNon were also contracted, containing restructured collagen fibers and denser regions surrounding the cells (Figure 4.5 d). Collagen gels imbedded with mixed populations of cells exhibited qualities similar to the non-infected controls, as they were contracted with dense areas of fibers (Figure 4.5 e, f). However, shMMP infected cells were visible in the gels as rounded and unattached to their surrounding matrix (Figure 4.5 e, circled).

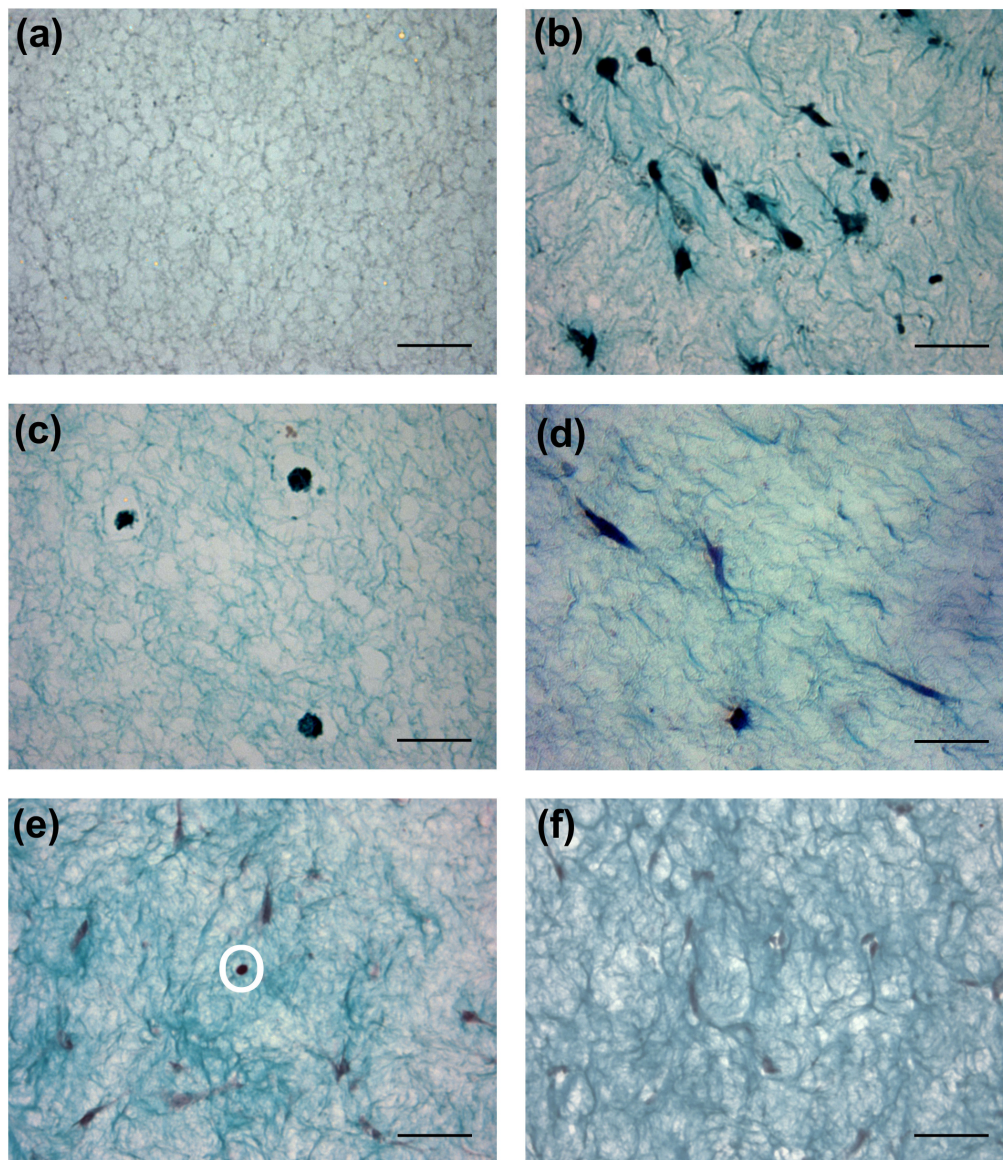


Figure 4.5. Histology images of collagen gels containing: (a) no cells, (b) non-infected AF cells, (c) a pure population of cells infected with shMMP, (d) a pure population of cells infected with shNon, (e) a mixed population of cells infected with shMMP, and (f) a mixed population of cells infected with shNon. Each gel was fixed at 7 days. Images for (a)-(d) were taken at 400X, scale bars represent 50 μm . Images for (e)-(f) were taken at 100X, scale bars represent 200 μm . Non-infected AF cells (b) and shNon infected cells (d) were shown to restructure collagen fibers within gels. These cells appeared elongated and anchored to the fibers. Cells infected with shMMP (c) appeared similar to gels without cells (a) with little to no restructuring of the collagen network and rounded cells. In gels containing mixed populations of cells, collagen fibers were reorganized, however in the shMMP mixed population gels (e), rounded cells were clearly visible without reorganization of fibers in their immediate surroundings. These were not found in shNon mixed population gels (f).

Discussion

MMP-2 is one of the gelatinases in the family of MMPs, responsible for the secondary breakdown of collagen. It is thought to be involved in general ECM breakdown in the process of disc degeneration [30-32]. The functional role of MMP-2 in this process can be evaluated through RNAi technology. Lentiviral delivery of shRNA allows stable silencing of gene expression [47], which can be beneficial in the development of therapies. By silencing MMP-2, we can evaluate changes in the interaction of AF cells with their surrounding matrix, and therefore elucidate their role in the degenerative cascade. Seeding infected and non-infected cells on gelatin films allows us to evaluate the effectiveness of the gelatinase activity of MMP-2. In addition, embedding these cells in type-I collagen gels can help explicate the role of MMP-2 in tissue remodeling. To our knowledge, this study is the first to utilize shRNA technology to ascertain the functional role of a catabolic molecule in the process of disc degeneration. Together these results can be applied to designing interventional strategies to decelerate IVD degeneration.

Gelatin is a substrate widely used for cell adhesion and growth [123, 124]. It can also be used to measure gelatinase activity of enzymes such as MMP-2. A gelatinase in the family of MMPs, it is directly responsible for the secondary breakdown of collagen, along with MMP-9 [30, 33]. AF cells seeded on gelatin films were capable of degrading the area immediately surrounding them. Films were stained evenly red with areas of white immediately surrounding the cells. This focal degradation suggests that MMP-2 has a greater role in degradation of immediate cellular surroundings, rather than general degradation capabilities. These results are

similar to other studies performed with cancer tissues. Fujiwara et al. [125] found a significant correlation between gelatinolytic activity and the detection of MMP-2 by immunocytochemistry in breast tumor samples. Alternatively, silencing MMP-2 in AF cells through RNAi resulted in diminished gelatinolytic activity, implicating MMP-2 as the major enzyme responsible for breakdown of gelatin. These cells exhibited little to no degradation of the gelatin films, compared to non-infected cells. However, cells infected with shNon resembled non-infected cells in their ability to focally degrade gelatin. It was interesting to note that while MMP-2 may be secreted by cells into the surrounding media, there was little to no general degradation of the films seen. However, it is possible that with a longer culture time, enough MMP-2 may be secreted to cause complete breakdown of the films, rather than the focal degradation that was seen.

In *in vitro* cell culture, collagen gels are widely used as a three-dimensional cell scaffold [126]. In this study, we evaluated structural changes in collagen gels, which would lead to a shift in material properties of the gels, in order to assess the functional significance of silencing MMP-2. Infected and non-infected cells were embedded in collagen gels which were polymerized in dog-bone shaped molds to facilitate testing. In many cases, the weakened architecture of the gels caused them to break immediately, making it difficult to obtain reproducible results. A shorter incubation time or higher concentration of collagen may be able to abate these problems. However, creating too stiff of a scaffold may deter changes in structure as well. Although there was no significant difference between experimental groups, some variation in material properties between groups was visible.

Collagen gels without cells had the highest strength and viscosity but lowest rupture stress. In contrast, gels with non-infected AF cells had among the highest values for rupture stress and lowest values of viscosity. These cells were able to reorganize collagen fibers (Figure 4.5 b), giving gels higher strength, and therefore the highest rupture stress. These cells are capable of implementing both catabolic and anabolic events as well. Since MMP-2 is thought to play a role in the remodeling and degradation of ECM components [29, 33], it is likely to be involved in this process. These cells can also actively remove broken down collagen with active MMP-2, resulting in lower viscosity. Collagen gels containing cells infected with shMMP had among the lowest values for strength and rupture stress and higher values for viscosity. By silencing MMP-2, it is possible these cells have the inability to restructure collagen fibers; however the cells may still be degrading their surrounding scaffold through other proteinases. These cells are also potentially producing ECM, but have compromised collagen turnover, leaving them not only weaker, but also more viscous. Cells infected with shNon were able to reorganize collagen fibers, but to a lesser extent compared to non-infected cells; therefore, gels were weaker and viscosity higher, comparatively. This data, combined with histological analysis, may be able to give us some insight into the consequences of silencing MMP-2.

Through histology, we can evaluate the structural changes in the gels over time in culture. These physical changes can help corroborate the differences seen in material properties between the experimental groups. As expected, non-infected AF cells embedded in collagen gels contracted the gels to a great extent. The cells were found anchored on the collagen fibers present in the gel, with denser areas of collagen

surrounding them. Reorganization of the collagen fibers was clearly visible throughout the gels. Since MMP-2 is implicated in reorganization and remodeling of matrix components, it was likely a key mediator of this process. In contrast, gels containing cells infected with shMMP did not contract as much and were similar in appearance to gels without cells. Silencing MMP-2 resulted in the inability of cells to restructure their surrounding collagen scaffold. This further identifies the role of MMP-2 in ECM remodeling.

Through these experiments, we can elucidate the functional significance of MMP-2 in intervertebral discs. Our results have shown that AF cells have the capability to focally degrade gelatin films restructure collagen fibers in type I collagen gels. Upon silencing MMP-2 through RNAi interference technology, these cells lose their ability to do either. Therefore, these results help substantiate the theory that MMP-2 is the key enzyme responsible for gelatin degradation in the ECM as well as remodeling of tissue architecture. Since higher levels of MMP-2 have been associated with diseased discs, it is likely playing an important role in the process of intervertebral disc degeneration. These results suggest that MMP-2 may be a qualified candidate for gene therapy techniques to treat DDD using RNA interference.

Acknowledgements

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Chapter 5: RNA Interference Mediated Gene Silencing of MMP-2 *In Vivo* in an Annular Needle-Puncture Model of Disc Degeneration

Abstract

Degenerative disc disease is a leading source of morbidity and disability, the etiology of which is unknown. Several studies have presented the beneficial effects of gene delivery in the intervertebral disc as a potential treatment strategy. While these studies have mainly focused on the use of siRNA technology, there has been little research in sustained down-regulation of endogenously produced genes through shRNAs in the intervertebral disc. This study utilizes a validated shRNA sequence against MMP-2 to down-regulate gene expression in order to decelerate the process of disc degeneration in an annular needle-puncture model in rats. Lentiviral vectors for MMP-2 and a nonsense sequence were injected into rat caudal discs, punctured with an 18G needle to induce disc degeneration. Discs were compared to non-punctured, and puncture only discs for changes in disc height and morphology, as well as immunopositivity of MMP-2. Injection with shMMP resulted in decreased MMP-2 presence in the disc, and disc height and overall disc morphology was similar to non-punctured control discs. While results were inconsistent, they are promising in terms of successful down-regulation MMP-2 expression in degenerating discs. These results can be applied towards the development of strategies to decelerate the degenerative process in intervertebral discs.

Introduction

Intradiscal gene therapy is one approach that could potentially serve as a minimally invasive treatment of intervertebral disc (IVD) degeneration. Manipulating cell behavior to slow or reverse degeneration has been the focus of much of the current research [53, 127-129]. The physiology of the disc, with its avascularity and lack of direct nutrient supply [50, 51], make the disc a good target for lentiviral-based RNAi as there is low probability of exposure to adjacent tissues [53]. Regeneration of the disc through extracellular matrix (ECM) stimulating factors may prove difficult as the processes are energy consuming [51] in an already depleted environment. Therefore, decreasing catabolic factors in the ECM is a favorable alternative for slowing the degenerative process.

The ECM of the disc is comprised of mainly type I collagen in the annulus fibrosus (AF) and type II collagen in the nucleus pulposus (NP). An array of proteoglycans exists in the NP of the disc, with a main component being aggrecan [9, 18]. Matrix-degrading enzymes such as aggrecanases, collagenases and gelatinases contribute to disc homeostasis through timely breakdown of the ECM, essential for matrix turnover [36, 122]. However, excessive activity of catabolic processes has been implicated in degenerative conditions, in which ECM composition is inappropriately altered. Data have specifically implicated MMP-2, a gelatinase, to be involved in the process of disc degeneration. Studies have correlated increased MMP-2 production and activation with age and degenerative changes in the NP, as well as alterations in MMP-2 activity in NP cells in response to growth factor stimulation or prolonged loading [30]. In addition, MMP-2 was found to be

negatively correlated with collagen type II content in the NP, making it a potentially useful candidate for gene therapy [32].

Previous studies have proven successful in terms of delivering vector-based therapies to disc cells both *in vitro* and *in vivo* [50-52, 116]. Seki et al. [52] recently suppressed IVD degradation through injection of ADAMTS siRNA. However, inhibition of a catabolic gene through shRNA, which can offer the advantage of sustained silencing, has not yet been studied. The purpose of this study was to evaluate the effectiveness of injections of MMP-2 shRNA *in vivo* in a rat caudal disc needle-puncture model. Modulating the catabolic activity of MMP-2 might be a valid approach for gene therapy of intervertebral disc degeneration.

Materials and Methods

Virus preparation

All virus preparation procedures were carried out in a biological safety cabinet. A validated lentiviral vector containing an shRNA sequence targeting MMP-2, designated shMMP, and a vector containing a nonspecific scrambled sequence, designated shNon were sterile filtered, and concentrated 10X. A sterile 1½ inch 27G hypodermic needle was fitted to each of two sterile 20 µl Hamilton glass syringes. 6 µl of each virus was drawn up in one syringe each. The needles were capped, removed from the biological safety cabinet, and placed in the surgical area.

Surgical Procedures

Surgical procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland, College Park. Four discs per rat were used for surgical procedures corresponding to:

- (1) 18G needle puncture, disc c4-c5
- (2) 18G needle puncture with shMMP injection, disc c5-c6
- (3) Control disc, disc c6-c7
- (4) 18G needle puncture with shNon injection, disc c7-c8

Five Sprague-Dawley retired breeder male rats (Harlan Laboratories, Frederick, MD) were placed in an induction chamber.

Rats were anesthetized using 5% isoflurane vaporization in a 2 L/min flow of oxygen. A subcutaneous injection of analgesic (Buprenorphine 0.03mg/0.1ml) and an intramuscular injection of antibiotic (Baytril 2.5mg/0.1ml) were given to the rat. The rat was then placed on an isothermic heating pad in the surgical area containing a miniature C-arm fluoroscopic imager (Fluoroscanner Imaging System Inc., Northbrook, IL). Ocular lubricant (Artificial Tears, Webster, Sterling, MA) was applied to both eyes to prevent desiccation of corneas. The rat tail was swabbed with 10% Betadine, followed by rinsing with 70% isopropyl alcohol pads. The surgical site was draped with sterile cloth drape with an opening, exposing the tail. The individual vertebrae of the rat tail were palpated and marked, to identify the intervertebral disc space from c4-c5 to c7-c8. Positioning was

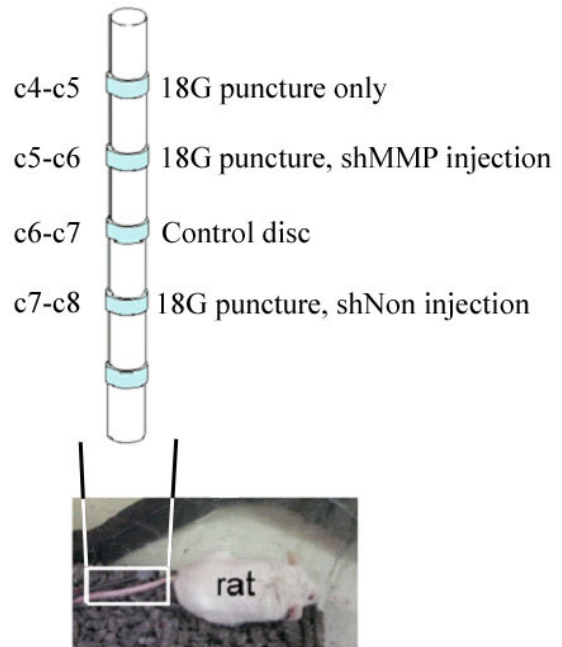


Figure 5.1 Schematic representation of rat tail for surgical procedures.

confirmed using fluoroscopic imaging. A 4 cm cut was made exposing all 4 discs and the surrounding fascia was removed from the skin. A sterile 18G hypodermic needle was marked to the depth corresponding to the radius of the tissue, obtained from radiographic images, and inserted into the c4-c5 disc. Needle location and depth of puncture was confirmed using radiography, and the needle was removed. A new sterile 18G hypodermic needle was marked, inserted into the c5-c6 disc, and again placement was confirmed via x-ray. The 27G needle loaded with shMMP was inserted through the 18G needle and the 18G needle was withdrawn. After radiographic confirmation of the position of the 27G needle, virus was injected into the disc and the needle removed after 2 minutes. The c6-c7 disc served as non-punctured control. The procedures were repeated for the c7-c8 disc, injecting the disc with shNon. The surgical area was rinsed with 50ml of sterile saline (DPBS, Invitrogen, Carlsbad, CA) and the skin closed with tissue glue (VetBond Tissue Adhesive, 3M, St. Paul, MN).

Post-Surgical Procedures and Tissue Harvest

After full recovery, the rats were returned to their cages with unrestricted activity, food, and water. The rats were monitored for 2 weeks daily to ensure complete closure of the surgery site, as well as pain or infection. Analgesia (Buprenorphine at 0.05 mg/kg rat), anti-inflammatory (Carprofen at 5 mg/kg rat), and antibiotic (Cephalexin at 60 mg/kg rat) was administered if needed in accordance with our pain/distress assessments and consultation with the veterinarian.

After 2 weeks, rats were euthanized by carbon dioxide asphyxiation (6-8 L/min until loss of consciousness, followed by 12 L/min for 10 min) followed by bilateral thoracotomy. The tail was cut at c3-c4 and c8-c9 and the skin and surrounding muscle was removed. The tissues were placed in 10% buffered formalin overnight to fix the tissue. The next day, the tissues were placed in 10% formic acid buffered with 10% sodium citrate for decalcification. The solution was changed daily for 1 week. Motion segments were then separated, rinsed in water overnight before paraffin infiltration, then embedded in paraffin blocks. 7 μ m sections were cut and dried overnight on a slide warmer. Sections were used for both immunohistochemical and histological analysis.

Immunohistochemistry and Histology

A commercially available kit, Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and monoclonal antibody to MMP-2 (Lab Vision, Fremont, CA) were used. Paraffin sections were rehydrated through xylene and a series of graded ethanol incubations. Endogenous peroxidase was inactivated by incubation in 3% H₂O₂ for 15 min. After washing with PBS, antigen retrieval with 0.05% trypsin was conducted at 37°C for 15 minutes. This was followed by a 40 minute block step with normal horse serum. Sections were incubated with anti-MMP-2 primary antibody for one hour. After washing, a biotinylated secondary antibody was applied for 30 minutes. The reaction was visualized using DAB (3,3-diaminobenzidine) and the sections were counterstained with Gill's hematoxylin. Procedures for negative

controls remained the same except tissue sections were not incubated with primary antibody.

For histology, paraffin sections were rehydrated through xylene and a series of graded ethanol incubations. Sections were stained with Weigert's hematoxylin, Fast Green, and Safranin-O to visualize cells, collagen and proteoglycan content in the disc as well as changes in the overall structure of the IVD. The slides were mounted with Permount before visualization.

Results

Immunohistochemical staining of tissue sections revealed apparent immunopositive staining (seen as brown stain in sections) for both control discs without needle puncture, as well as discs punctured with an 18G needle (Figure 5.2 b, d). Negative controls for each section were not reactive, demonstrating that positive results were associated only with presence of MMP-2 (Figure 5.2 a, c, e, g). The reactivity was found mainly in the NP of the disc, with little to no staining in the AF. Within the NP, MMP-2 was associated mainly with the ECM surrounding the cells. Discs punctured with the 18G needle, followed by injection of shNon, also showed immunopositive staining in the NP (Figure 5.2 h). In contrast, discs punctured with the 18G needle then injected with shMMP exhibited little to no immunoreactivity, comparatively (Figure 5.2 f).

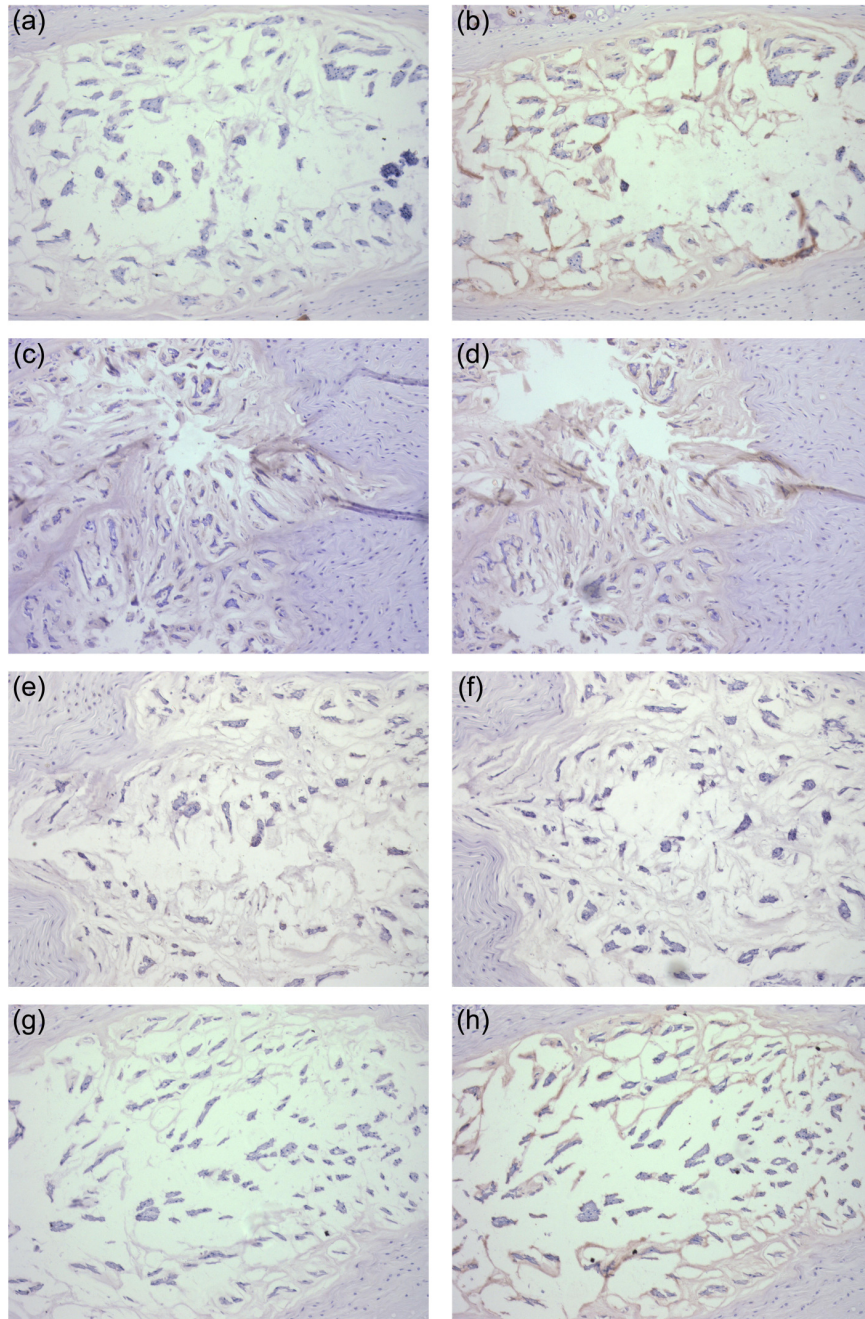


Figure 5.2. Immunohistochemical staining with anti-MMP-2 antibody of: (a,b) Non-punctured control disc, (c,d) 18G punctured disc, (e,f) 18G punctured disc injected with shMMP, and (g,h) 18G punctured disc injected with shNon. (a), (c), (e), (g) are negative control sections without MMP-2 antibody, while (b), (d), (f), (h) were incubated with MMP-2 primary antibody. Negative control sections were all negative for MMP-2, as they did not stain brown. Non-punctured control disc (b), 18G punctured disc (d), and 18G punctured disc injected with shNon (h), were all immuno-positive for MMP-2 as brown staining was clearly visible in these sections. The 18G punctured disc injected with shMMP (d) showed little to no brown staining, demonstrating down-regulation of MMP-2 in this disc.

Histological examination of sections stained with Fast Green and Safranin-O revealed compromised tissue architecture in discs punctured with the 18G needle. Tears and disorganization in the AF, decrease in proteoglycan content in the NP, as well as decreases in overall NP area were visible (Figure 5.3 b). In contrast, the control discs displayed well organized AF lamellae, a rounded NP, well defined boundaries of the disc (Figure 5.3 a). Discs injected with shMMP also exhibited tears in the AF, however, NP area and proteoglycan content and was similar to control discs (Figure 5.3 c). Discs injected with shNon appeared similar to control discs, with little damage visible in the AF (Figure 5.3 d)

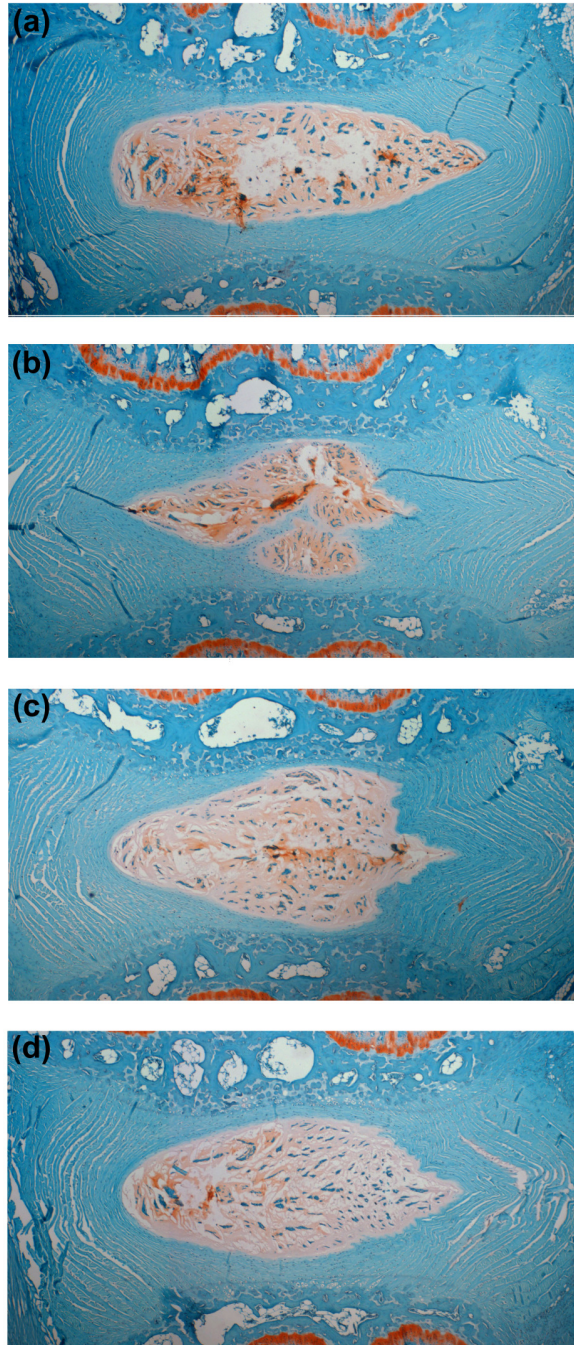


Figure 5.3. Histological images of (a) Non-punctured control discs, (b) 18G punctured disc, (c) 18G punctured disc injected with shMMP, and (d) 18G punctured disc injected with shNon. 7 μ m sections were stained with Fast Green and Safranin-O. The non-punctured control disc (a) had an intact NP and structured AF lamellae, while the 18G punctured disc (b) showed a disrupted NP and AF, with tears clearly visible in the lamellae of the AF. The 18G punctured disc injected with shMMP (c) seemed to be able to retain disc morphology, and appeared similar to the control disc. The shNon injected disc (d) also appeared similar to the non-punctured control disc, although disorganized outer AF lamellae were clearly visible.

Discussion

In this study we explored the efficacy of MMP-2 shRNA in a needle-puncture model of rat caudal intervertebral disc degeneration. We anticipated that silencing the catabolic activity of MMP-2 would attenuate the progression of the degenerative process. Thus it was expected that disc height, NP content, as well as overall disc structure would be similar to that of non-punctured control discs. In addition, we hypothesized that MMP-2 immunoreactivity would be negative in discs injected with shMMP, as compared to punctured discs with and without shNon.

RNAi studies have shown that efficacy of gene silencing depends largely on transduction efficiency and the stability of the transduced gene [49]. Many different methods of transduction have been explored including systemic transduction [130], local gene delivery [131], electroporation [132, 133], intradermal administration [134], and ultrasound [51, 116]. In this study, we injected the lentiviral vector containing shMMP directly into the intervertebral disc since lentiviruses are able to infect both dividing and non-dividing cells. While some samples displayed little to no reactivity with MMP-2 antibody, some samples displayed positive, although reduced, immunoreactivity. This inconsistency was cause for some concern, as our previous studies were successful in silencing MMP-2. However, our previous work with the shMMP construct was performed *in vitro*, where matrix components are digested away before cells are plated in monolayer. It is possible that viral transduction is greater in this case, as there is direct access to cells. In contrast, transduction efficiency may be reduced *in vivo* as cells are surrounded by the ECM in the disc. In addition, since the disc is pressurized and NP content was not removed in

order to maintain disc integrity, it is possible that some of the injected virus was expelled from the disc upon needle withdrawal. This could reduce the amount of virus introduced into the disc space, reducing the overall effect of the injected shMMP. This could be a cause for the reduction of MMP-2 expression in some of the animals, as opposed to silencing, as was expected.

Previous *in vitro* studies with the shMMP vector used a specific MOI for each experiment, with controlled methods for counting and plating cells. *In vivo* studies differ in that there are inherent differences between discs within a tail, as well as differences between animals within a study. Indeed, the size of the disc decreases down the tail of the rat. Although the virus was concentrated, it is possible that there were not sufficient virus particles to transduce the majority of the cells within the disc. As such, further studies in dosing and concentration of injections are required in order to obtain definitive results.

Histological staining revealed that the structures of the punctured discs were indeed compromised 2 weeks after needle puncture. The lamellae of the AF appeared disorganized, the disc height was reduced, and the overall structure of the disc was altered. These results are consistent with other needle-puncture models to induce degeneration of the disc [128, 135-138]. However, in some animals, discs appeared healthy, with little degradative changes after needle puncture. It is possible that, although needle placement was measured via x-ray, needles were not inserted to the correct depth to fully puncture the AF, and reached only the outer boundaries. In most animals, discs injected with shNon resembled the puncture only discs. In contrast, discs injected with shMMP were similar in appearance to the non-punctured

control disc. As these discs also exhibited reduced immunopositivity for MMP-2, it is possible that progression of degeneration was decelerated, making them appear healthier.

Although these results are promising, of the animals tested, the outcomes were not consistent. While surgical procedures remained the same between animals, and depth and positioning of inserted needles was confirmed via radiograph, some discs exhibited complete loss of NP, while others revealed non-degenerated structures. In addition, immunoreactivity of MMP-2 in shMMP injected discs was variable. Further studies, modifying surgical procedures, and taking dosing and virus concentration into consideration, are required to ascertain the effects of MMP-2 shRNA injection into needle-puncture models of degeneration in rat tail discs.

Nevertheless, this study is the first to utilize shRNAs to down-regulate an endogenously produced catabolic gene associated with intervertebral disc degeneration. In addition, this study demonstrates that suppression of MMP-2 was associated with a healthier disc structure, suggesting that MMP-2 contributes to the degeneration of IVD tissues. Although further studies are required, this is a first step towards development of lentiviral based gene therapies for treatment of disc disease. The use of shRNA allows for sustained suppression of genes, which can be essential for designing therapies for clinical use.

Acknowledgements

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Chapter 6: Conclusions and Future Work

The work presented in this dissertation aims to investigate the interaction of intervertebral disc cells with their surrounding microenvironment. The objective of this work is to explore the factors involved in degeneration of the intervertebral disc. In order to do so, this research examined how environmental changes can cause a shift in the genetic profile of cells as well as how modulating endogenous gene expression can alter cell function.

The first study examined the effects of biochemical, nutrient, and physical factors on immature NP cells. Specifically, cell morphology, attachment, proliferation, and gene expression of these cells was investigated. NP cells were cultured in different media formulations, with and without a three-dimensional scaffold, in both hypoxic and ambient O₂ conditions. Cells in alginate beads retained the vacuolated morphology associated with notochordal cells, with little change in gene expression. In monolayer, NP cells cultured in α -MEM resulted in the highest attachment efficiency, maintained gene expression levels, as well as preserved vacuolated morphology. While the highest proliferation levels were observed with DMEM and Opti-MEM cultures, these appeared to involve small non-vacuolated cells. Through these results, we can ascertain that certain environmental conditions induce cellular changes that can alter the notochordal phenotype in immature NP. These results can be used to establish a framework that can be followed for tissue engineering techniques in *in vitro* cell culture.

In another aspect of observing cellular interplay with their surrounding environment, RNA interference technology was explored in silencing matrix-

degrading enzymes in intervertebral disc cells. While RNAi is now a widely used research tool, few studies have utilized shRNAs for sustained down-regulation of genes. In this study, five shRNA sequences were designed against MMP-2, an ECM degrading enzyme associated with intervertebral disc degeneration. These were cloned into lentiviral vectors to facilitate transfection of annulus fibrosus cells *in vitro*. Gene expression levels using real-time RT-PCR were analyzed, as well as MMP-2 protein levels in both cell lysates and cell culture media. It was found that cells infected with virus had decreased MMP-2 protein levels in both media and cell lysates compared to non-infected control cells. Additionally, cellular mRNA for MMP-2 was down-regulated in infected cells compared to non-infected cells. Although the five constructs exhibited differential effectiveness of MMP-2 knockdown, some were able to silence MMP-2 levels up to 80%. One construct, designated shMMP, was chosen and validated for future RNAi work. These results present a promising method to enable the implantation of cell-based therapies for disc degeneration. In addition, this study is the first to utilize shRNA-mediated gene silencing in IVD cells.

MMP-2 function and its potential role in intervertebral disc degeneration were then further investigated in the following study. MMP-2 is a gelatinase, capable of degrading most components of the extracellular matrix, and also responsible for tissue remodeling. Reverse gene studies using RNAi can help elucidate the role of MMP-2 in these processes. With the previously designed and validated shMMP lentiviral vector, this study examined the effects of MMP-2 silencing on gelatin degradation and type I collagen gel remodeling. Annulus fibrosus cells from the disc were

infected with the shMMP vector and a nonsense vector, seeded on gelatin films, and analyzed for degradation. Additionally, infected and non-infected cells were embedded in type I collagen gels and mechanically tested to examine differences in material properties. Changes in gel structure and remodeling of collagen fibers were also examined through histological staining. It was found that silencing MMP-2 resulted in the inability of cells to degrade gelatin, compared to non-infected cells which exhibited a focal degradation in their immediate surroundings. Collagen gels with shMMP infected cells were found to be marginally weaker, possibly due to the lesser degree of remodeling observed through histology. The findings of this study will help to elucidate the interactions between MMP-2, collagen and gelatin, and the functional role of MMP-2 in the degradative pathway leading to disc degeneration. Knowledge of the functional significance of MMP-2 is essential to understanding this disorder and designing preventative therapies to treat it.

Finally, the results and techniques from these studies were applied to an *in vivo* model of disc degeneration. Since MMP-2 has been found associated with degenerated discs, it can be a promising candidate for gene silencing to slow the degenerative cascade. Previous studies have validated needle-puncture of the annulus fibrosus as a model for intervertebral disc degeneration. In this study, the shMMP vector and a nonsense vector were injected into rat caudal discs, punctured first with an 18G hypodermic needle to induce degenerative changes in the disc. These discs were compared to non-punctured control discs as well as puncture only discs. Immunohistochemical analysis revealed that discs injected with shMMP showed little to no staining of MMP-2 compared to the other discs. Control, punctured, and

nonsense vector injected discs were clearly immunopositive comparatively. shMMP injected discs also exhibited a similar disc morphology to control discs compared to puncture only discs. However, these results were not observed among all of the animals tested. Nonetheless, they indicate that silencing MMP-2 can be of potential therapeutic value in designing treatment strategies for intervertebral disc degeneration.

Together these studies further our understanding of the interplay of intervertebral disc cells with their surrounding environment and can lay the foundation for future research in this field. Reverse gene studies using RNAi can be a useful tool in understanding gene function and how it relates to degenerative changes in the disc. While this work focused specifically on elucidating the functional role of MMP-2 in intervertebral disc cells, genes associated with MMP-2 can also be explored. MMP-9, a gelatinase homologous to MMP-2, has been found associated with MMP-2 in degenerated discs. In addition, MMP-14, and TIMP-2 are both genes responsible for the activation of MMP-2, while TIMP-2 is involved in its inhibition as well. RNAi-mediated silencing of these genes may lead to an understanding of the upstream events involved in the process of disc degeneration.

Furthermore, MMP-2 stimulation via cytokine exposure, in conjunction with gene silencing studies can further our understanding of MMP-2 function within the disc environment. While the *in vitro* studies presented here focused mainly on AF cells of intervertebral disc, the research can be carried out using cells from the NP as well. In addition, the effect of silencing MMP-2 on the genetic profile of disc cells is another area of exploration. There are numerous potential applications for RNAi in

the advancement of knowledge of intervertebral disc degeneration. These studies would not only be of scientific value, but can aide in the development of preventative therapies for the treatment of disc disease.

Glossary

α -MEM – Minimum Essential Medium alpha

ADAMTS - A Disintegrin And Metalloproteinase with Thrombospondin Motifs

AF – annulus fibrosus

Ct – cycle threshold

DDD – degenerative disc disease

DMEM – Dulbecco's Modified Eagle Medium

dsRNA – double stranded RNA

ECM – extracellular matrix

G – gauge

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

IVD – intervertebral disc

miRNA – microRNA

MMP – matrix metalloproteinase

MOI – multiplicity of infection

MT-MMP – membrane-type matrix metalloproteinase

NaOH – sodium hydroxide

NP – nucleus pulposus

Opti-MEM – reduced serum medium

PBS – phosphate buffered saline

RNAi – RNA interference

RPMI – cell culture medium

RT-PCR – reverse transcriptase polymerase chain reaction

SD – standard deviation

shMMP – lentiviral vector containing an shRNA sequence targeting MMP-2

shNon – lentiviral vector containing a scrambled sequence

shRNA – small hairpin RNA

siRNA – small interfering RNA

TIMP – tissue inhibitor of metalloproteinase

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