ABSTRACT

Title of Dissertation: DEVELOPMENT OF COLLAGEN THIN FILMS FOR INTERVERTEBRAL DISC CELL CULTURE & EXAMINATION OF CELL PHENOTYPES AND INTERACTIONS

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Intervertebral disc degeneration and the associated low back pain lead to a decrease in the quality of life for many individuals, as well as a substantial burden on the health care system. Current therapies are often aimed at resolving the symptoms of degenerative disc disease, rather than addressing the underlying causes of disc degeneration. Researchers have strived to develop cell-based therapies that will not only manage the associated symptoms of disc degeneration but also reverse the degenerative process. To do so, a thorough understanding of the function of each cell type of the disc, both in a healthy and degenerative state, must be reached.

For this project, *in vitro* cell culture substrates for each disc cell phenotype were developed using collagen thin film technology. Hybrid films were created with both type I and type II collagen fibers, the presence of which were confirmed using fluorescently-labeled antibodies. The hybrid films, along with type I and type II collagen thin films, were used to characterize the annulus fibrosus and nucleus pulposus cell phenotypes by

examining morphology and gene expression. Annulus fibrosus cell morphology was observed to be independent of collagen type, but instead on the presence of fibers. Time dependence was also noted. No statistically significant differences were noted for substrate dependent gene expression of either cell type.

Various methods for separating the multiple cell types of the nucleus pulposus were evaluated, and filtration was chosen as the most acceptable. By culturing the two cell types together or individually and examining the gene expression trends, it was observed that chondrocyte-like cells and notochordal cells influence each other *in vitro*. This has significance regarding disc degeneration, as notochordal cells disappear from the disc.

This work was able to develop novel substrates for culture of intervertebral disc cells and utilize these substrates to further characterize the distinct phenotypes of the different cell types found in the disc. It also examined the influence of the disappearance of notochordal cells from the disc has on the remaining chondrocyte-like cells' gene expression. This information could aid in the development of future cell-based therapies for intervertebral disc degeneration.

DEVELOPMENT OF COLLAGEN THIN FILMS FOR INTERVERTEBRAL DISC CELL CULTURE & EXAMINATION OF CELL PHENOTYPES AND INTERACTIONS

by

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ABBREVIATIONS

ADAMTS:	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
AF:	Annulus Fibrosus
AFM:	Atomic Force Microscopy
αSMA:	alpha Smooth Muscle Actin
DPBS:	Dulbecco's Phosphate Buffered Saline
ECM:	Extra-cellular Matrix
EP:	Endplate
FACS:	Fluorescent Activated Cell Sorting
IVD:	Intervertebral Disc
MMP:	Matrix Metalloproteinase
NIST:	National Institute of Standards and Technology
NP:	Nucleus Pulposus
NSAID:	Non-steroidal Anti-inflammatory
RNA:	Ribonucleic acid
RT-PCR:	Reverse Transcription Polymerase Chain Reaction
TCPS:	Tissue Culture Polystyrene
TIMP:	Tissue Inhibitor of Metalloproteinases

CHAPTER 1: INTRODUCTION

Low back pain and the associated lumbar disc disorders represent a huge burden both socioeconomically and on the quality of life of the affected population. It has been estimated that 12-15% of the population in the United States will visit a doctor with complaints of low back pain each year (AAOS 2008). This is accompanied by a decrease in quality of life, as measured by quality-adjusted life years, as well as a substantial cost estimated at over $100 billion annually (Katz 2006). Included in this figure are indirect costs, such as losses of wages, in addition to direct costs including doctor visits and treatments.

Much of this can be attributed to the aging and degeneration of soft tissue between vertebrae in the spinal column: the intervertebral discs. Consisting of an outer annulus fibrosis (AF) surrounding an inner nucleus pulposus (NP), the intervertebral discs act in dissipating loads and in providing range of motion and flexibility for the spine. They are capped top and bottom by cartilaginous end plates, through which necessary molecules – nutrients, oxygen, etc. – diffuse. The AF and the NP are each structurally, chemically, and cellularly distinct. They undergo through different changes during the course of aging and disease. This creates difficulty in understanding the complex interactions between the two, as well as how these interactions alter as both structures undergo degenerative changes. As such, current treatment strategies are aimed at the symptoms rather than at the underlying mechanisms of degeneration, specifically to reduce pain and discomfort. Treatments include minimally or non-invasive options such

as corticosteroids and annuloplasty or surgical intervention, each of which seek to minimize pain without restoring function or reversing degenerative changes (Raj 2008).

Researchers have strived to develop cell-based therapies that will not only manage the associated symptoms of disc degeneration but also reverse the degenerative process and restore the structure, and therefore function, of the intervertebral disc. In order to do this, however, the complex milieu of the AF and NP must be better understood. It is currently established that both a compositional change of the intervertebral disc matrix and a cell population shift occur in the NP through the course of aging and degeneration. These changes, which include variation of collagen and proteoglycan levels throughout the disc, as well as a loss of a major cell type from the nucleus pulposus, present tissue engineers with substantial challenges.

This study seeks to address those challenges by developing an *in vitro* culture substrate that would allow for preservation and observation of the *in vivo* gene expression and protein production of intervertebral disc cells. This substrate would then be used to study the interactions of the intervertebral disc cells and potentially elucidate what a potential cell-based disc degeneration therapy must accomplish to repair the disc.

CHAPTER 2: THE INTERVERTEBRAL DISC

Intervertebral Disc Structure and Function

The vertebral column runs from the skull to the pelvis, consisting of twenty-six vertebrae and the intervertebral discs between them. The vertebral column acts to provide support for the upper body and protection for the spinal cord. The intervertebral disc is the soft tissue structure located between vertebrae in the spine. Its purpose is to absorb the compressive loads placed on the spine due to an upright posture or as a result of muscular forces. It is an integral part of the balance between flexibility and stability of the spine. Together, an intervertebral disc and its adjacent vertebral bodies are called a motion segment. Healthy discs are avascular; in fact, they are the largest avascular structures in the human body. The discs differ in thickness, shape, and cross-section throughout the length of the spine (Pooni, et al. 1986).

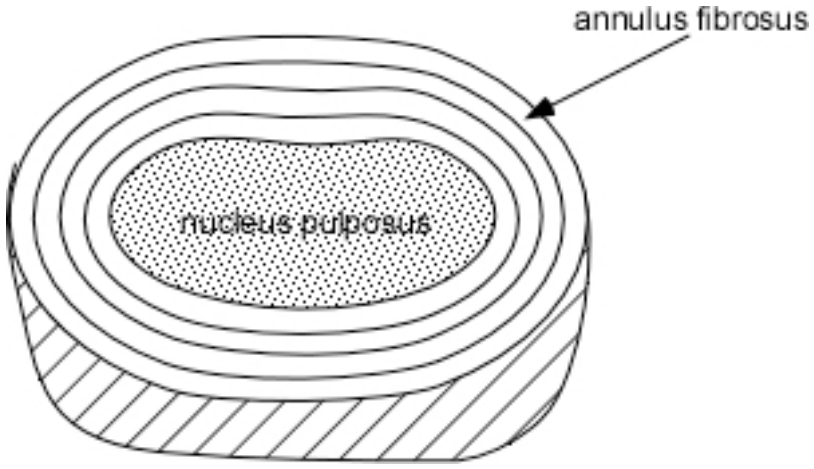


Figure 1: Schematic illustration of an intervertebral disc, showing an inner nucleus pulposus and an outer annulus fibrosus composed of concentric lamellae.

The intervertebral disc (IVD) is comprised of three distinct parts: the cartilaginous endplates (EP), the annulus fibrosus (AF) and the nucleus pulposus (NP). The endplates sit above (cranial to) and below (caudal to) the disc. The NP is the disc's gelatinous core,

and is surrounded by the fibrous AF. When loaded, hydrostatic pressure is generated in the NP, which pushes outward on the AF. This pressure is resisted by the aligned fibers of the AF, thereby dissipating the force. The structures of healthy NPs and AFs are fine-tuned to work together to support the spine, which is reflected in their molecular composition and organization.

The annulus fibrosis is comprised of concentric lamellae made primarily of collagen fibers, aligned at alternating angles. The number of lamellae ranges from fifteen to twenty, and they vary in size (Marchand and Ahmed 1990). It is formed from the embryonic mesenchyme, and is derived from a structure that initially surrounds the nucleus and differentiates into the mature AF and the endplates (Dahia, et al. 2009). The AF is typically thought to have an outer portion with primarily type I collagen, and an inner portion containing a larger amount of type II collagen that transitions into the NP. Proteoglycan content, fiber organization, and mechanical properties of the AF were also found to differ radially. Specifically, the outer annulus showed lower levels of proteoglycans, higher level of fiber organization, and a higher degree of anisotropy with regard to modulus than the inner annulus (Lewis, Hussain and Mao 2008). Work by Yu *et al.* demonstrated that microfibrils also show a higher degree of organization in the outer AF, as well as a higher degree of co-localization with elastin fibers in comparison to the inner AF (Yu, et al. 2007). Differences in proteoglycan contents, particularly for small proteoglycans -such as biglycan, decorin, and fibromodulin- have been found to be dependent not only on annulus location but also on age (K. Singh, K. Masuda and E. J.-M. Thonar, et al. 2008). These various structural and compositional differences manifest in variation of mechanical properties, as demonstrated by Skaggs *et al.*, who found that

tensile properties of the disc were dependent on anatomical location from anterior to posterior in addition to radially (Skaggs, et al. 1994). The cells of the AF are considered to be fibroblast-like. However, variation of AF cells has been observed from inner to outer annulus. Differences in the pericellular matrix (Cao, Guilak and Setton 2007), cell polarity (Gruber, Ingram, et al. 2007), and cell shape and actin cystoskeleton (Bruehlmann, et al. 2002) have all been observed.

The nucleus pulposus is more gelatinous than the annulus fibrosis, with a less organized network of type II collagen. Its high water content and ability to generate hydrostatic pressure are due to the high amounts of proteoglycans, whose charge aide in water retention. Aggrecan, in particular, plays a role in this with its high charge density that helps it retain water. The NP develops from the embryonic notochord, from which the entire cell population present at birth is derived. These notochordal cells are characterized morphologically by their large size and the abundance of vacuoles in the cytosol. In many species, such as rats and pigs, they remain throughout the lifespan of the animal. In other species, including humans, the notochordal cells are thought to disappear by skeletal maturity. They are replaced or supplemented by a second cell phenotype, the chondrocyte-like cell. This cell type is smaller by comparison and lacks the vacuoles of the notochordal cells. Morphologically similar to chondrocytes from articular cartilage, comparisons have shown differences in gene expression but no "on/off" markers to completely distinguish the two cell phentotypes (Lee, et al. 2007). Work by Sakai *et al.* came to similar conclusions using beagles, a species known to lack notochordal cells in adult animals, as well as rats (Sakai, et al. 2009). Interestingly, though there were differences between NP cells and articular cartilage chondrocytes in

each species, three genes that demonstrated significantly different expression levels between cartilage and NP cells in rats failed to do so in dogs that lacked notochordal cells. This suggests that the chondrocyte-like cells are in fact chondrocyte-*like*, rather then chondrocytes, despite several similarities between the structure of the nucleus pulposus and articular cartilage.

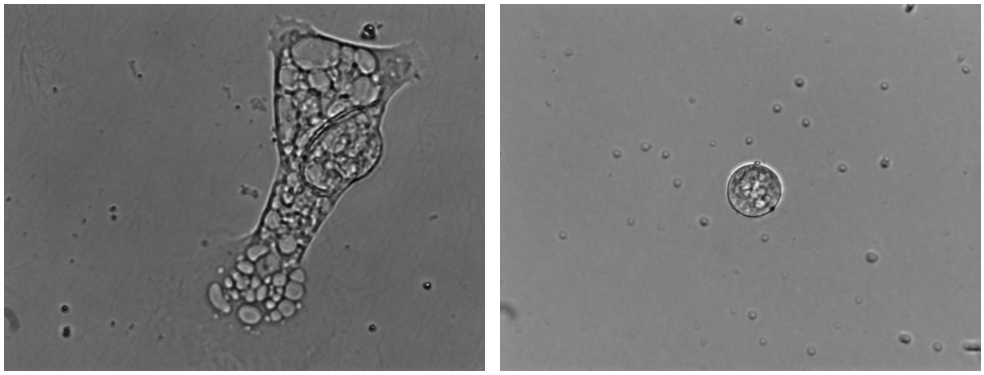


Figure 2: Notochordal cell (left) and chondrocyte-like cell (right) at 400x magnification. Note the large size and abundance of vacuoles in the notochordal cell.

Researchers have been able to separate notochordal cells and chondrocyte-like cells through a variety of methods with varying degrees of success. This has enabled some, though far from complete, examination of the comparative phenotypes of the two cell populations. Notochordal cells, separated by serial filtration, were found to produce more proteoglycan than chondrocyte-like cells. Moreover, more of the proteoglycan produced by the notochordal cells was distributed into the general extracellular matrix rather than retained by the immediate pericellular matrix (Cappello, et al. 2006). This is reinforced by work done by Miyazaki *et al.*, which showed that cells from discs containing notochordal cells produced more glycosaminoglycans than cells from discs containing only chondrocyte-like cells (Miyazaki, et al. 2009). Following separation by fluorescence-activated cell sorting (FACS), analysis has shown differences in gene expression; notochordal cells produced lower amounts of type I collagen and several

proteoglycans, including biglycan, decorin and lumican than smaller cells. Differences in integrin subunits, including α6, α1, and β1 were also observed (Chen, Yan and Setton 2006). CD24, a cell surface marker, was found to be expressed by nucleus pulposus cells identified by FACS as being large with complex cytoplasm (characteristics of notochordal cells) (Fujita, et al. 2005). Notochordal cells and chondrocyte-like cells have been found to respond to environmental factors differently, with notochordal cells showing a higher sensitivity (increased morbidity) to nutrient deprivation and hypoxia while also having higher rates of glucose and oxygen consumption (Guehring, et al. 2009). Likewise, notochordal cells were shown to be more sensitive to loading (Geuhring, et al. 2010).

The mechanism of notochordal cell loss and the origin of chondrocyte-like cells are subject of debate. One school of thought is that the chondrocyte-like cells originate outside the nucleus pulposus and are of mesenchymal origin, as are articular cartilage chondrocytes. The similarities of the two cells, detailed previously, lend credence to this idea. Work by Kim *et al.* showed that all chondrocyte-like cells in the non-notochordal disc originated in and migrated from the endplate (Kim, et al. 2003). This observation was supported when it was observed *in vitro* that unknown soluble factors secreted by notochordal cells were capable of initiating endplate chondrocyte migration (Kim, et al. 2009). Recently, however, it was demonstrated by Choi *et al.* that all cells in the nucleus pulposus are derived from the notochord (Choi, Cohn and Harfe 2008). It is acknowledged by Choi that the discrepancy could be the result of variation between species. It was also noted that in chondrodystophoid dogs, thought to be similar to humans in that they lack notochordal cell population in mature specimens, that cells

positive for cytokeratin-18 were observed in adult discs despite cytokeratin-18 being typically associated with the notochord (Sakai, et al. 2009). Cells exhibiting notochordal markers, but without the typical notochordal morphology (large with vacuoles), were observed in adult human NPs (Weiler, et al. 2010). Use of live-cell imaging techniques have demonstrated that notochordal cells are capable of producing cells morphologically very similar to chondrocyte-like cells, suggesting that the chondrocyte-like cells may be a subset of notochordal cells (Kim, et al. 2009).

The relevance of the origin of all cell types of the nucleus pulposus is in the approach to treating intervertebral disc degeneration. In humans, the loss of notochordal cells and the transition to a chondrocyte-like cell population can coincide with the onset of disc degenerative disease. Whether this is a case of causation or correlation remains unclear. Understanding the origins of each cell type of the disc and, more importantly, the role each one plays in the maintenance of a healthy nucleus pulposus is critical to the development of cell-based treatments for disc degeneration.

Degeneration

The intervertebral disc undergoes many changes during the natural course of aging, though not all of these changes are related to disc degeneration. It is possible, however, that many of the changes associated with aging may be causes or risk factors for degeneration. In an effort to separate to two processes, Sing *et al.* examined discs of varying age while controlling for level of degeneration through careful selection of disc specimens. It was found that in both the annulus and nucleus the total collagen and proteoglycan content decreased with age. When examining specific small proteoglycans such as biglycan, fibromodulin, or decorin, the trends were found to differ among the

inner annulus, outer annulus, and nucleus (K. Singh, K. Masuda and E. J.-M. Thonar, et al. 2008). Conversely, by controlling for age and examining discs of different degrees degeneration, an initial increase in proteoglycan production was found in the annulus for lower levels of degeneration prior to a decline at higher levels. The nucleus showed a decrease with increasing degeneration (Cs-Szabo, et al. 2002). Nondegenerative discs have been shown to have a decreased amount of tissue inhibitor of metalloproteinases-3 (TIMP-3) with age, indicating a potential increase in catabolic processes over anabolic (Tsuij, et al. 2007). Cell density for both annulus and nucleus was found to decrease with age (Liebscher, et al. 2010). *In vitro*, nucleus cells from older bovine discs produced less aggrecan and type II collagen while producing more MMP-13 matrix metalloproteinase involved in extracellular matrix breakdown (Kandel, et al. 2007). Again, this suggests an imbalance between anabolic and catabolic processes. Together, these changes to the disc may potentially make the disc more susceptible to injury and less able to cope with the loads experienced by the spinal column.

To distinguish the actual degenerative changes and the associated degenerative disc disease from the natural progression of aging, a definition was proposed by Adams and Roughley that narrowed the focus to structural failure. Specifically, degeneration was defined as "an aberrant, cell-mediated response to progressive structural failure" (Adams and Roughley 2006). Thus, mechanical loading can initiate the degenerative state through a structural injury. Factors that weaken the disc, including aging (as discussed above), are elements that may lead to, but not directly cause, disc degeneration. In addition to aging, genetic predisposition plays at least some part in leading to disc degeneration. It has been shown a specific gene polymorphism for aggrecan was

significantly over-represented in persons with multi-level disc degeneration (Kim, et al. 2010). A survey of the literature conducted by Kalichman and Hunter concluded that evidence exists for familial disposition to disc degeneration (Kalichman and Hunter 2008).

The 'structural failure' required by Adams and Roughley can be a variety of injuries to the disc. These can include disc prolapse, disc narrowing, annular tears and clefts, and damage to the endplate. This disruption of the mechanical integrity of either the annulus or the nucleus would, in turn, disrupt the complex manner in which they work together to dissipate loads. The end result is inappropriate mechanical loads in each area of tissue. Trauma has been shown to result in annular tears and spondylolisthesis (a forward displacement of a vertebral body). A survey of 135 discs showed that radiating tears were nearly exclusive to the posterior annulus and closely related to the location of severe degeneration, and that peripheral tears were most likely due to trauma (Osti, et al. 1992). A finite element model revealed that complex loading resulted in high strain in healthy discs, potentially initiating tears (Schmidt, Heuer and Wilke 2009). Similar results were obtained by Veres *et al.* who demonstrated that torsion potentially lowers the pressure required to form annular tears (Veres, Robertson and Broom 2010).

Macrostructural failures of the disc that may initiate degeneration are accompanied by changes in the microstructure and in cellular activity. In general, degeneration features a shift in the balance of catabolic and anabolic process away from remodeling toward matrix degradation. This is exhibited in the relationship between ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin Motifs) and their inhibitors, TIMP-3 (Tissue Inhibitor of Metalloproteinases). TIMP-3 expression was

discovered to remain unchanged from non-degenerate to degenerate discs, while ADAMTS levels increased in degenerative discs (Pockert, et al. 2009). This increase in ADAMTS level was also observed at advanced stages of degeneration in the annulus and the nucleus (Patel, et al. 2007). Reduced proteoglycan synthesis was observed throughout the disc in patients with spondylolisthesis (condition of displaced vertebrae), and changes in gene expression including up-regulation of ADAMTS6 in annulus cells from degenerative discs have been documented (Roberts, Beard and O'Brien 1982), (Gruber, Hoelscher and Hanley, Jr 2010).

Current Treatments

There is an array of treatment options for those suffering from low back pain resulting from degenerative discs. The Spine Patient Outcome Research Trial (SPORT) followed 2505 patients who opted for either surgical or non-surgical treatment (Hanley Jr, et al. 2010). Analysis showed a significant increase in positive outcome at four years for the surgical group compared to the non-operative treatments, such as NSAIDs. However, current surgical approaches such as spinal fusion do not actually heal the disc. For this, tissue engineers are currently looking at cell implantation to stimulate repair and regrowth of damaged and degenerate disc tissue. Several studies have examined the use of mesenchymal stem cells (msc) as a potential cell candidate. Le Maitre *et al.* investigated msc survival and phenotype when injected into nucleus pulposus tissue explants *in vitro*. Their analysis showed msc survival for up to four weeks with increases in expression of aggrecan and type II collagen (Le Maitre, et al. 2009). *In vivo* studies have likewise been conducted, including Sobajima *et al.*, who noted msc survival in rabbit discs up to 24 weeks (Sobajima, et al. 2008). Further investigation of stem cell

response is necessary to fully characterize the potential of this treatment option so that it may be effectively implemented in patients. Moreover, a better understanding must be reached regarding the role that annular, notochordal, and chondrocyte-like cells play in maintaining the health of the intervertebral disc.

CHAPTER 3: THESIS AND OBJECTIVES

The advancement of stem cells as a biological tool presents an opportunity to develop therapies that not only eliminate the symptoms of intervertebral disc degeneration but also potentially restore the disc to its original state. Tissue engineers can use diverse substrates and signals to control stem cell gene expression to direct stem cell differentiation to appropriate cell types. In order for these concepts and techniques to be successfully applied to the intervertebral disc, the end goal for these cells must be clearly defined for both the nucleus pulposus and annulus fibrosus. For the disc, this definition must be in terms of the cells that are present in an adult degenerate disc, the genes that they are (or are not) expressing, and the effects that any cell type that is no longer present may have had when the disc when in a healthy state. However, this is currently unclear due to the complexities of the disc. Why do the notochordal cells disappear, and what role do they play in a healthy disc? How does their disappearance affect the remaining chondrocyte-like cells? The answers to these questions, and many others, must be fully understood for any effective cell-based therapy to be developed.

The goals of this work were to examine the phenotype of each cell type of the intervertebral disc and to attempt to characterize the affects that the disappearance of notochordal cells have on chondrocyte-like cells. This knowledge will be a critical impetus to developing cell-based tissue engineering therapeutic strategies. A series of objectives was established to accomplish this. The first objective was to adapt current collagen thin film technology and techniques to the production of type II collagen films for use with the cells of the nucleus pulposus. The next objectives were to characterize the response of intervertebral disc cells to collagen thin films *in vitro* and to determine

the cell culture substrate that retains *in vivo* gene expression levels for AF and NP cells. Additionally, it was hoped that this would provide insight to the phenotypes of each cell. The last objective was to characterize the effects that the presence of notochordal cells have on chondrocyte-like cells, as well as provide insights to the differences in gene expression between notochordal cells and chondrocyte-like cells.

Aim 1: Development of type II collagen films

Hypothesis: The similar molecular structures of type I and type II collagen will allow for the production of type II collagen thin films.

Aim 2: Evaluation of cell culture substrates and cell phenotypes

Hypothesis A: AF cells will demonstrate differences that are reflective of variances between the inner and outer annulus when cultured on type I and type II collagen films.

Hypothesis B: Type II collagen films will enable NP cells to retain gene expression that is characteristic of a healthy disc.

Aim 3: Establish the effects of notochordal cell loss on chondrocyte-like cells

Hypothesis: Chondrocyte-like cells will exhibit differences in gene expression as compared to notochordal cells, and the gene expression of chondrocyte-like cells will change in the presence/absence of notochordal cells.

CHAPTER 4: DEVELOPMENT OF TYPE II COLLAGEN FILMS

A cell culture option that has been used with success with certain cell types is collagen thin films. By controlling the surface chemistry, researchers at the National Institute of Standards and Technology were able to induce self-assembly of type I collagen into thin (~13-38nm) films to use as a culture substrate for smooth muscle cells (Elliott, Tona, et al. 2003). Fibrils were observed to self-assemble on hydrophobic surfaces, but not on hydrophilic surfaces. Due to the range of hydrophobicities suitable for these films, they were able to form on simple untreated polystyrene surfaces (Elliott, Halter and Plant 2008). The advantage of these culture substrates is that they allow cells to attach to fibrilar collagen as they would *in vivo* while maintaining a two-dimensional surface beneficial for microscopy. As such, collagen thin films have been used in conjunction with quantitative microscopy to evaluate tenascin-C promoter activity (Langenbach, et al. 2006) and focal adhesion kinase regulation (Bhadriraju, et al. 2009).

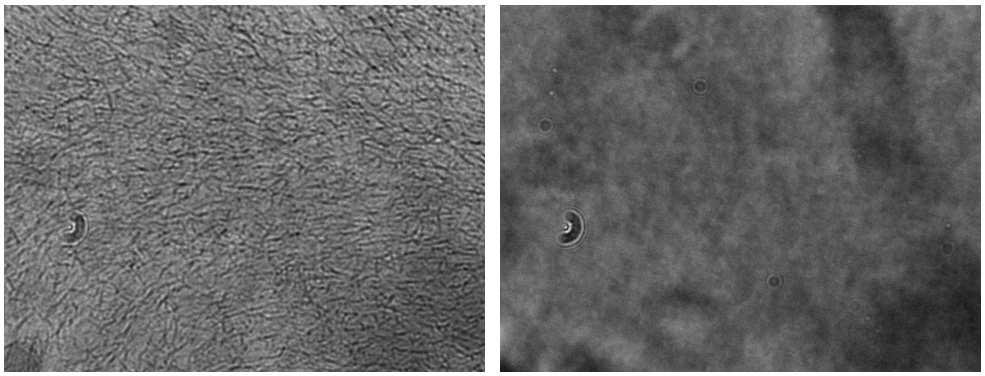


Figure 3: Type I collagen thin film (left) formed on a polystyrene surface showing collagen fibrils indicative of films formation, and an untreated polystyrene well (right) at 100x magnification.

Type I collagen thin films present several advantages as a cell culture surface. Compared to polystyrene, these surfaces present to the cell an accurate replication of the collagen fibrils they would interact with *in vivo*. This is true compared to adsorbed

collagen surfaces as well, which featured a monomeric layer of collagen on a surface, rather than assembled fibrils with supramolecular features similar to native collagen. This includes the 67 nm banding pattern observed on native collagen. The highly reproducible assembly of the collagen thin films can be controlled by the surface chemistry by manipulating the hydrophobicity.

Type I and type II collagen are similar molecules. Both are trimers, though type II collagen is a homotrimer while type I collagen is a heterotrimer. Type II collagen consists of three alpha1 subunits in a triple helix; type I collagen is formed by two alpha1 subunits and one alpha2 subunits for its triple helix. Both molecules are produced inside the cell as a pro-collagen molecule before being secreted and processed outside the cell. Analysis of the molecular structures show that the charge and hydrophobicity of the C- and N- termini of each molecule are similar. Therefore, it was assumed that assembly of type II collagen into thin films would proceed as type I collagen does, and would be governed by the same substrate surface chemistry. As a potential alternative, hybrid films were investigated. Hybrid films were formed by first coating the surface with type I fibrils before type II collagen was be added. The formation of collagen thin films with type II collagen fibrils will allow for investigation of AF and NP cell phenotype and morphology *in vitro* using quantitative microscopy in ways previously impossible with 3D culture methods, or with tissue culture polystyrene surface that do not maintain cell phenotypes.

Methods

Type II collagen films

Type I and type II collagen films were prepared on silicone wafers that had been coated first with chromium then with gold. The wafers were then placed in a 0.5mM alkanethiol solution of 1-Hexadecanthiol (C_{16}), 1-Deodecanethiol (C_{12}), 1-Undecanol ($C_{11}OH$), or a 50:50 ratio of C_{12} + $C_{11}OH$ and left overnight to form a monolayer on top of the gold surface. The wafers were then rinsed three times with EtOH. Contact angle measurements of two different drops of water were made to a hydrophobic/hydrophilic surface. By mixing 800 µL of 3 mg/mL type I collagen (Advanced Biomatrix, San Diego, CA) or type II collagen (Sigma-Aldrich, St Louis, MO) with 100 µL 10x DPBS (Sigma-Aldrich, St. Louis, MO) and 100 µL 0.1N NaOH, then adding 7 mL of 1x DPBS (Sigma-Aldrich, St. Louis, MO) a neutralized, buffered 300µg/mL solution was created. The wafers were incubated with 1-2 mL of the resulting solution for at least 16hrs to form the collagen thin films. The wafers were then rinsed with Dulbecco's Phosphate Buffered Saline (DPBS) followed by deionized H_2O (Millipore, Bedford, MA) and dried with inert N_2 gas. The type I and type II films were examined using ellipsometry to determine thickness (J.A. Woollam, model M-44, Lincoln, NE). Two films for each surface were examined, and two gold treated wafers were left for baseline measurements. Three measurements were made from each surface and averaged. Lastly, images were taken with a scanning electron microscope in an attempt to view the fibrils.

Hybrid collagen films

Type I/type II hybrid films were formed on C_{16} treated silicone wafers. Three experimental groups were created: 1) type I collagen solution (300 µg/mL) incubated for

one hour, followed by incubation in DPBS for 16 hrs; 2) type I collagen solution (300 μg/mL) incubated for one hour, followed by incubation in type II collagen solution (300 μg/mL) for 16 hrs, creating hybrid films; or 3) only type II collagen (300 μg/mL) for 16 hrs. All wafers with films were rinsed and dried as previously described. Thicknesses were measured using ellipsometry, and Atomic Force Microscopy (AFM) was used to characterize the surface coverage and the structure of the fibrils (AFM done by Tighe Spurlin at NIST, machine operated in contact mode). Hybrid films were also created in polystyrene wells for use with antibody staining to ensure type II collagen fibril formation. After film formation, 3% BSA (Sigma-Aldrich, St. Louis, MO) was added for 1hr. The primary antibody (mouse type II monoclonal, Fisher Scientific, Pittsburgh, PA) was added in a 1:100 antibody:3% BSA solution for one hour on a rocker. The solution was removed and the films were rinsed three times with 3% BSA. A 1:200 dilution of rabbit anti-mouse with AlexaFluor 488 secondary antibody (Molecular Probes, Eugene, OR) was added, and the films were placed on a rocker for one hour. Films were then imaged using a Coolsnap HQ2 camera attached to an Olympus IX71 microscope. As a control, type I base-layers were formed without type II collagen to test the cross-reactivity of the type II collagen antibody with type I collagen.

Results and statistical analysis

Ellipsometry was used to measure thickness of samples by first modeling the alkanethiol monolayer assembled on the gold-coated prepared wafers as a control. The thickness of the collagen on top of this layer was determined by modeling the layer as a dielectric slab with fixed optical constants (n = 1.46, k = 0) using the manufacturer's software. Three measurements of each surface were made and averaged.

A 2-tailed T-test with a level of significance of 0.05 was used to determine the statistical significance of differences between film thicknesses.

Results

The results show that type II collagen films do not form at 300µg/mL concentrations over the range of hydrophobicities. Table 1 shows the thickness values of the type I and type II collagen films formed on each of the thiol layers on the prepared silicone wafers, along with water contact angles. Water contact angles are a measure of hydrophobicity, with higher values indicating an increased level of hydrophobicity. Lower values are indicative of a hydrophilic surface.

Table 1: Thickness values for type I and type II collagen

	Water Contact Angle	Type I collagen	Type II collagen
C_{16}	105.5°	52.0 nm	6.0 nm
C_{12}	109.5°	55.5 nm	10.0 nm
$C_{11}OH + C_{12}$	69°	53.5 nm	6.0 nm
$C_{11}OH$	21.5°	4.5 nm	7.5 nm

The values for the type I collagen thin films are within the range of expected values for thin films, with the exception of $C_{11}OH$, which is outside the hydrophobic range to form the films and therefore acts as a negative control. The lower values displayed by each of the type II groups demonstrate a lack of substantial fibril formation. Additionally, visual indications typical with thin film formation were absent. Visual indications included hazing of the surface with the drying process and an inability to observe the film under 100x magnification. Scanning electron microscopy also verified the lack of film formation (Figure 4).

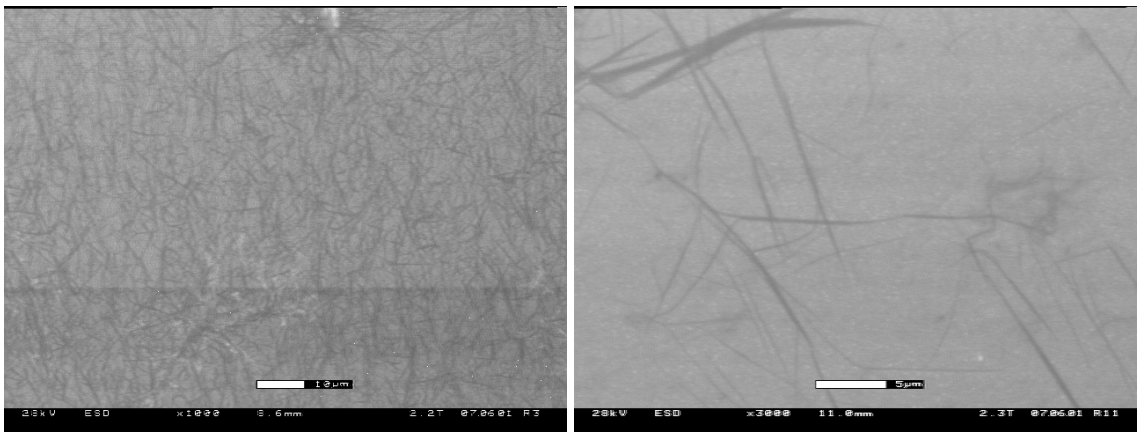


Figure 4: SEM images of collagen thin films: type I collagen (left) and type II collagen (right). The type I film has fibrils present, while no fibril formation is evident for type II collagen (protein present most likely precipitated out of solution).

Hybrid collagen films, with both type I and type II collagen fibrils present, were able to be formed using sequential 300 μg/mL incubations of type I and type II collagen. While not as thick as type I collagen films, the hybrid films were significantly thicker than both the type I collagen base-layer as well as the pure type II collagen films.

Table 2: Film Thickness

Type I baselayer	Hybrid film	Type II collagen
14.5 ± 0.64 nm	18.0 ± 0.44 nm	8.1 ± 0.04 nm

The presence of type II collagen fibrils was confirmed using fluorescent antibodies, as shown in Figure 5.

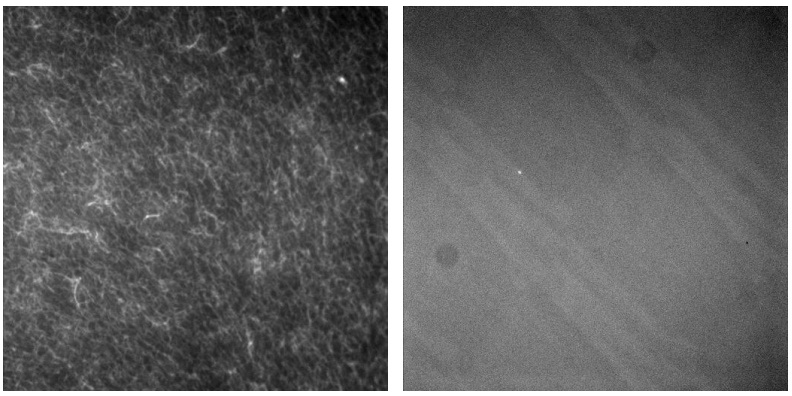


Figure 5: Fluorescent antibody image of type II collagen fibrils from a hybrid film (left) showing the presence of type II collagen, and control image (right) of type I collagen only, showing non-reactivity of type II antibody with type I collagen.

The hybrid film was also analyzed using AFM, which showed an increase in surface coverage from the one hour type I collagen incubation to the final hybrid film (Figure 6) of 42.8% to 63.5%.

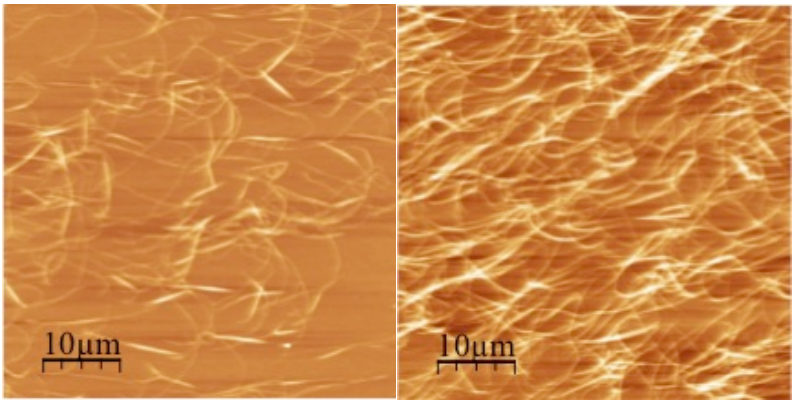


Figure 6: AFM images of type I base-layer (left) and hybrid film (right), showing an higher amount of collagen fibrils following incubation with type II collagen with an increase in percentage of the surface covered (42.8% for type I base-layer, 63.5% for hybrid film).

Discussion

Type I collagen thin films can be formed using a 300μg/mL neutralized, buffered collagen solution. However, this concentration proved unsuitable for type II collagen thin film formation regardless of surface hydrophobicity. Later work by Tighe Spurlin at

NIST demonstrated collagen II film formation by increasing concentration of collagen in the solution, which concurs with results observed by Fertala *et al.*, who saw an increase in lag time and critical concentration necessary for formation of collagen type II fibrils (Fertala, et al. 1994). By increasing the concentration to 600 μg/mL, they observed fibrilogenesis during the twenty-four hour incubation period.

However, type II collagen fibrils were able to assemble by first incubating a type I collagen solution on a surface for one hour, followed by an overnight incubation of a 300μg/mL type II collagen solution. The exact interaction between the initial type I collagen material on the surface and the type II collagen fibers formed during the secondary incubation was uncharacterized at this time. A study of type II collagen fibril assembly noted difficulty inducing fibrilogenesis at 37°C but had some success at lower temperatures. A positive result was also obtained by incubating a 500 μg/mL type II collagen solution at 21°C for 24 hours, followed by an incubation at 37°C for 24hrs. Type II collagen fibrils were able to form at 37°C overnight if there was some material already present on the surface (Dong, et al. 2007). This study offers additional evidence that type II collagen fibrilogenesis can readily occur at lower concentrations at higher temperatures *in vitro* if already initiated. The fact that the charge and hydrophobicity of the end terminals of the two collagen types are similar may provide enough for the type I collagen and type II collagen to interact in forming the hybrid films.

The goal of this aim was to develop type II collagen films for use with intervertebral disc cells. While type II films were unable to be developed specifically through this aim, the knowledge gained allowed for both the future development of the type II films and the development of the hybrid type I/type II collagen films. The

presence of both collagen types in the hybrid films may provide unique insights on the behavior of the inner annulus fibrosus cells, as well as cell response to the transition towards the predominantly type I collagen outer annulus fibrosus. Likewise, nucleus pulposus cells seeded on these films could potentially shed light on the cellular response to the transition of the nucleus from a healthy gelatinous state to a fibrocartilaginous state during degeneration.

While a defining aspect of the collagen thin films is the presence of collagen fibers extending a measurable distance from the surface, the films remain a two-dimensional environment. This presents the possibility of a best-of-both-worlds scenario by giving the cells the matrix interaction typically found in three-dimensional substrates while maintaining superior optical properties for use with quantitative microscopy techniques. Lastly, given the higher cost of type II collagen of appropriate purity to form thin films, hybrid films may provide a low-cost alternative.

CHAPTER 5: EVALUATION OF CELL CULTURE SUBSTRATES AND CELL PHENOTYPES

The chemical and mechanical environment of a cell is critical for it to maintain its phenotype. As the mechanical environment of the disc changes with injury, the cells' gene expression changes. This also occurs when cells are removed from their specific chemical/mechanical environment *in vivo* and cultured *in vitro*. Therefore, finding the cell culture conditions that conserve the distinct phenotypes of the various intervertebral disc cells is critical to enabling the study of the cells in a laboratory environment. A comprehensive study of chemical environment (media, O_2 concentration) and physical environment (polystyrene culture, alginate culture) found differences in morphology and gene expression for certain culture configurations (Rastogi, et al. 2009). Annulus cells were found to have different gene expression profiles when cultured in alginate or on tissue culture polystyrene (Wang, et al. 2001). It has been demonstrated that lovastatin can restore nucleus pulposus cell gene expression to original levels following monolayer culture (Hu, et al. 2010). Explant models have also been used to maintain nucleus pulposus cell phenotype with some success (Feng, Wan, et al., Nucleus Pulposus Explant Culture Model 2009).

The objective of this study was to evaluate the cell culture surfaces developed in the previous objective as culture surfaces for AF and NP cells. Once the collagen films for use with intervertebral disc cells were developed, the cells' response to the presence of the different types of collagen, as well as the presence of fibular collagen (as opposed to adsorbed) was examined. Annulus fibrosus and nucleus pulposus cells were seeded on

prepared substrates and examined for morphological and phenotypic differences. Genes analyzed include:

- GAPDH (AF & NP) – "Housekeeping" gene for AF and NP cells, used as an internal reference for each group.
- Collagen type I (AF & NP) – Structural molecule found predominantly in the outer annulus.
- Collagen type II (AF & NP) – Structural molecule found in the nucleus, as well as the inner annulus.
- Aggrecan (AF & NP) – The predominant proteoglycan in the nucleus, it is also found in the inner annulus.
- CD24 (NP only) – Cell surface marker thought to be indicative of notochordal lineage.
- Keratin 8 (NP only) – An intermediate filament protein found to be expressed by only a fraction of nucleus pulosus cells, presumably by those of notochordal origin (Gilson, Dreger and Urban 2010).
- MMP-2 (AF only) – A matrix metalloproteinase (collagenase) involved in the breakdown of extracellular matrix during remodeling and degradation.
- α-Smooth Muscle Actin (AF only) – Cellular protein found to be upregulated near sites of annular injury (Melrose, et al. 2001).

Quantitative microscopy will be utilized to examine the cell area. Automation of the process enables a large amount of data to be collected in a controlled, uniform way that limits the variability between images.

Methods

Cell harvest

Nucleus pulposus and annulus fibrosus cells were harvested from rat caudal discs. Rats (Taconic Farms Inc, Hudson, NY or Harlan Laboratories, Frederick, MD) were euthanized and their tails amputated and skinned. Flexing the tail revealed the disc location, and the surrounding musculature was removed with a scalpel to expose the outer surface of the annulus. A lateral incision was made at what would be the proximal end of the disc at the endplate to expose the disc surface in the transverse plane. The nucleus was then removed with a scooper and placed in a 4 mg/mL pronase (Roche, Basel Switzerland) in α-MEM solution supplemented with 2% FBS and 1% penicillin-streptomycin (Invitrogen, Grand Island, NY). The remaining annulus fibrosus was removed with an incision at the distal end of the disc. It would then be diced into smaller pieces and placed in a 3 mg/mL solution of collagenase in DMEN supplemented with 10% FBS and 1% penicillin-streptomycin (Invitrogen, Grand Island, NY). The AF tissue would be incubated overnight at 37°C (~18hrs), while the NP tissue would be removed from incubation after one hour and centrifuged at 360g for ten minutes. The resulting supernate enzyme solution was removed and the precipitate was resuspended in a 0.25mg/mL collagenase in α-mem supplemented with 10% FBS and 1% penicillin-streptomycin and incubated overnight. Following overnight incubation, both AF and NP tissues were removed from incubation and centrifuged at 360g for ten minutes. The tissues were resuspended in fresh media. The AF cells were plated and expanded before use, while NP cells were plated immediately. By plating and passaging the AF, proper

cell numbers were easily achieved. The NP cells were less responsive to culture on polystyrene surfaces and did not readily increase in numbers.

Cell culture substrate preparation

Type I collagen and hybrid collagen thin films were made in six-well untreated polystyrene plates (VWR, Radnor, PA) as previously described. Type II collagen films were prepared using the same procedure with a high concentration (600 μg/mL) neutralized type II collagen (Elstin Products Company, Owensville, MO). Adsorbed collagen surfaces were prepared by covering a well with ~1mL of a 50 μg/mL collagen solution and incubating at room temperature for one hour.

AF and NP cells on substrates

To examine cell morphology and gene expression on various cell culture substrates, AF and NP cells were seeded on type I, type II, and hybrid collagen films; type I and type II adsorbed collagen surfaces; and tissue culture polystyrene. AF and NP cells were collected from 3 rat tails and pooled for each group. A control disc was taken from each tail, from which AF cell and NP cell RNA were isolated immediately using an RNeasy Micro Kit (Qiagen, Valencia, CA). AF cells were expanded for one passage. All surfaces were calibrated with media for thirty minutes prior to use. AF cells were seeded at 600 cells/cm^2 for 24hrs or 200 cells/cm^2 for 72 hrs. NP cells were seeded at 2000 cells/cm^2 for each time point. For each group of three tails, two wells were plated for each surface. The first well was used for gene expression analysis. RNA was isolated using an RNeasy Micro Kit. Isolated RNA was used in a real-time RT-PCR reaction using a MyIQ Single Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and analyzed using the ΔΔct method to calculate fold difference in expression. The

second well was used for morphological analysis using quantitative microscopy. The cells were fixed in 1% PFA for 45 minutes, then rinsed with DPBS. 1mL of 0.1% Triton-X 100 containing 0.5 µg/mL Texas-Red – Maleimide (Molecular Probes, Eugene, OR) was added to stain the cell cytoskeleton (maleimide is thiol reactive), and the films were left covered on a rocker for one hour. The reaction was quenched by adding 1mL of 3% BSA and the samples were left covered on a rocker for fifteen minutes. For double labeling, 1mL of DPBS containing 2 µg/mL DAPI (Sigma-Aldrich, St Louis, MO) was added, to stain the cell nuclei. The cells were imaged using a Coolsnap HQ2 camera attached to an Olympus IX71 microscope. The microscope was programmed to capture 100 image fields per well, with a Tx-Red, DAPI, and phase contrast image taken and correlated for each image field.

Results and statistical analysis

PCR results were analyzed using the ΔΔct method, which uses the threshold values reported by the PCR software. Each sample was run in triplicate. Comparison between the internal control gene (GAPDH) and each experimental gene are first made (Δct). This value is then compared to the same value for the experimental condition used as a control (ΔΔct). The control for this experiment was the single disc tissue (AF or NP) that was used for immediate RNA isolation, thereby representing the *in vivo* gene expression values. Statistical analysis was performed using ANOVA with Tukey's post-hoc analysis to determine significant differences, specifically by comparing the Δct values for each gene and experimental condition

Images were analyzed for cell area and perimeter, using software written for ImageJ (NIH, Bethesda MD). The software would take user inputs of cell threshold

(taken from the Tx-Red image) and nuclei threshold (taken from the DAPI image) to generate a pixel^2 cell area for each cell with a nucleus. Cell area was recorded for each cell with a single nucleus. Statistical significance between time points was determined using a two-tailed T-test with a level of significance of 0.05, while ANOVA was Tukey's post-hoc analysis was used to among substrates within each time-point.

Results: AF cells

Annulus fibrosus cells showed a morphological dependence on the form of collagen present (either fibrils or adsorbed monolayer). They also displayed a dependence on culture time. However, they did not demonstrate a dependence on the type of collagen fibril present. This is illustrated by Table 3, which displays morphological data for each substrate. No data was able to be collected for polystyrene for Day 3, as the cells had proliferated to the point that morphological measurements were no longer possible.

Table 3: Annulus fibrosus cell area

Substrate	Day 1 Area (μm^2)	Day 3 Area (μm^2)	Day 3:Day 1
Type I (film)	2592 ± 1450	976 ± 1054	0.38
Type II (film)	2804 ± 2250	1038 ± 1198	0.37
Hybrid film	2710 ± 2058	991 ± 804	0.37
Type I (adsorbed)	3175 ± 1793	1558 ± 1109	0.49
Type II (adsorbed)	2318 ± 1666	1319 ± 1133	0.57
Polystyrene	2772 ± 1678	No data	No data

No statistically significant difference was found between AF cell area on any of the types of collagen films for either time point. However, the average area for each film was found to be different than the average area on the adsorbed collagen surfaces. Average area observed on adsorbed type I surfaces was found to be greater than the collagen films,

while adsorbed type II collagen surfaces yielded cells with a lower average area. Cell area on adsorbed type I was different from adsorbed type II collagen for each time point . Moreover, the distribution of cell areas appeared to differ from the adsorbed collagen surfaces to the fibular collagen and polystyrene surfaces.

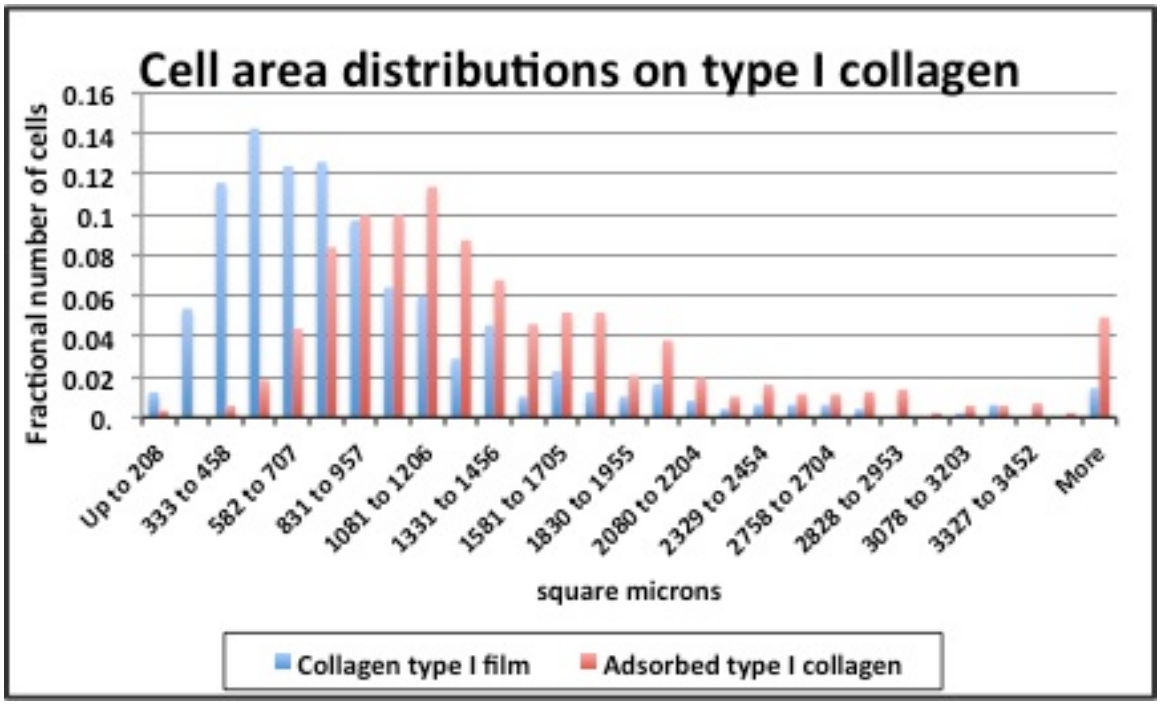


Figure 7: Distribution of AF cell areas on type I collagen thin film and adsorbed surfaces. Note the shift in distribution producing a higher area for cells on adsorbed type I collagen.

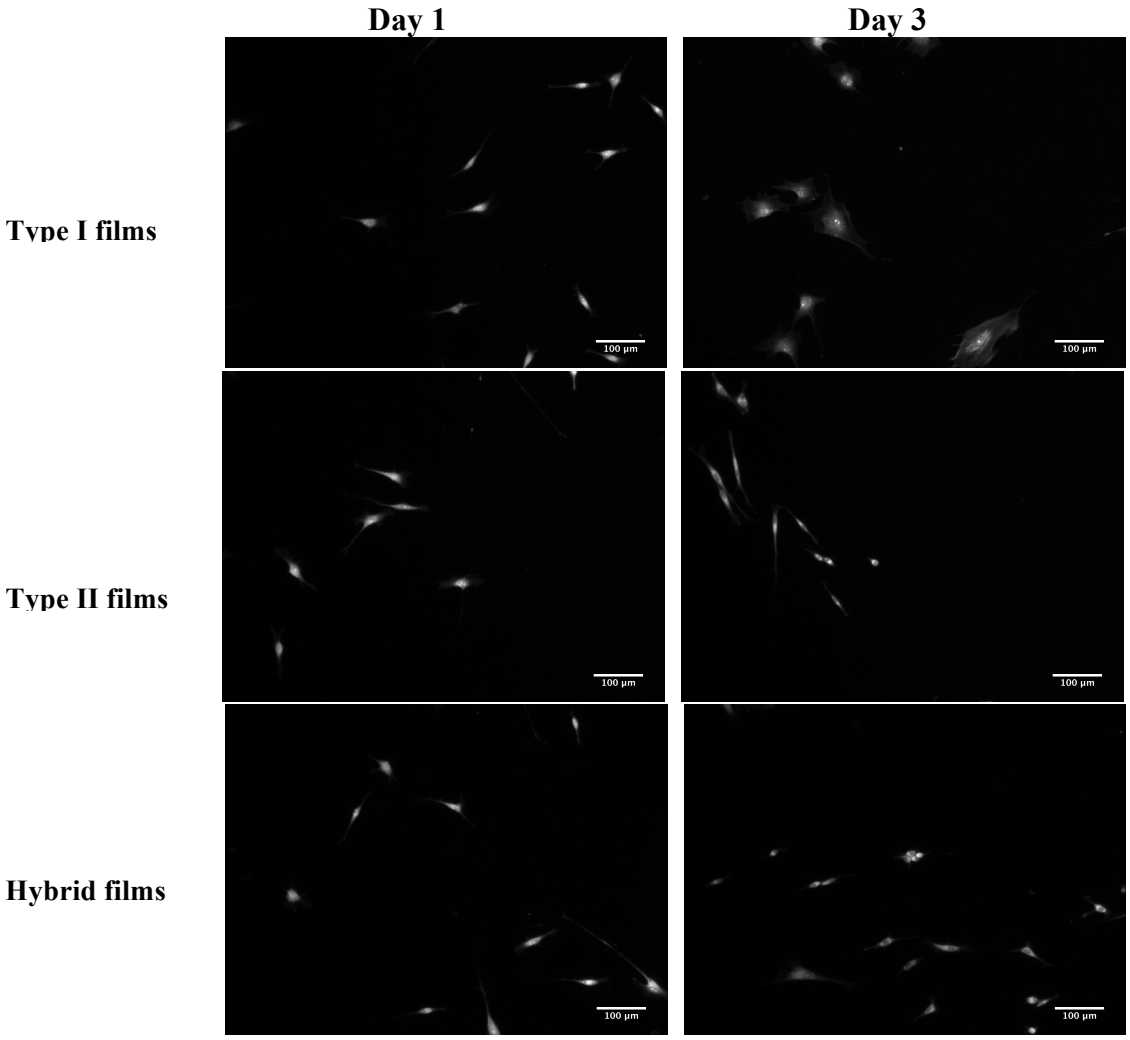
Qualitative observation of the images from which the morphology data was taken showed noticeable differences in cell appearance between substrates. It was common for cells seeded on adsorbed type I collagen to be very large and to have a ruffled-appearing cell edge. This cell type also appeared on adsorbed type II collagen substrates, but was mostly absent from the films. The cells seeded on the films (either type I, type II, or hybrid) tended to have a more compact cell body from which one or more protrusions extended (Figure 8).

The time dependence is evident for each cell culture substrate, in that cells exhibit significantly smaller area on Day 3 than on Day 1 for each. Qualitative observation again confirmed this, as there was a higher amount of smaller cells and an increase in cell density. This was in spite of the fact that the Day 3 substrates were seeded at a lower initial density than the Day 1 substrates. The different seeding densities were chosen in order to account for proliferation and prevent cells from becoming confluent, which would in turn prevent measurements of single cells. This increase in cell population was observed on each of the substrates, particularly for the adsorbed collagen and polystyrene surfaces, suggesting that the cells proliferated (Figure 9). An attempt to quantify cell proliferation was made by calculating the percentage of cells on each surface at 24 hrs out of the total number of cells seeded. This percentage attachment was assumed to be the same for Day 3, and the increase in cells on the surface above the calculated number can be used as an estimate of cell proliferation. Cell proliferation on polystyrene surfaces was to the extant that it prevented morphology measurements, therefore the number cannot be estimated; however, it would therefore be higher than the other surfaces. As Table 4 shows, the proliferation rates for the film surfaces were similar to each other, but different from the adsorbed and polystyrene surfaces.

Table 4: Cell attachment and proliferation

Substrate	Cell Attachment %	Cell Proliferation %
Type I (film)	11.6	247
Type II (film)	9.7	348
Hybrid film	9.3	316
Type I (adsorbed)	11.5	532
Type II (adsorbed)	16.3	718
Polystyrene	12.1	High

The morphological distinctions between surfaces present at Day 1 remained at Day 3.



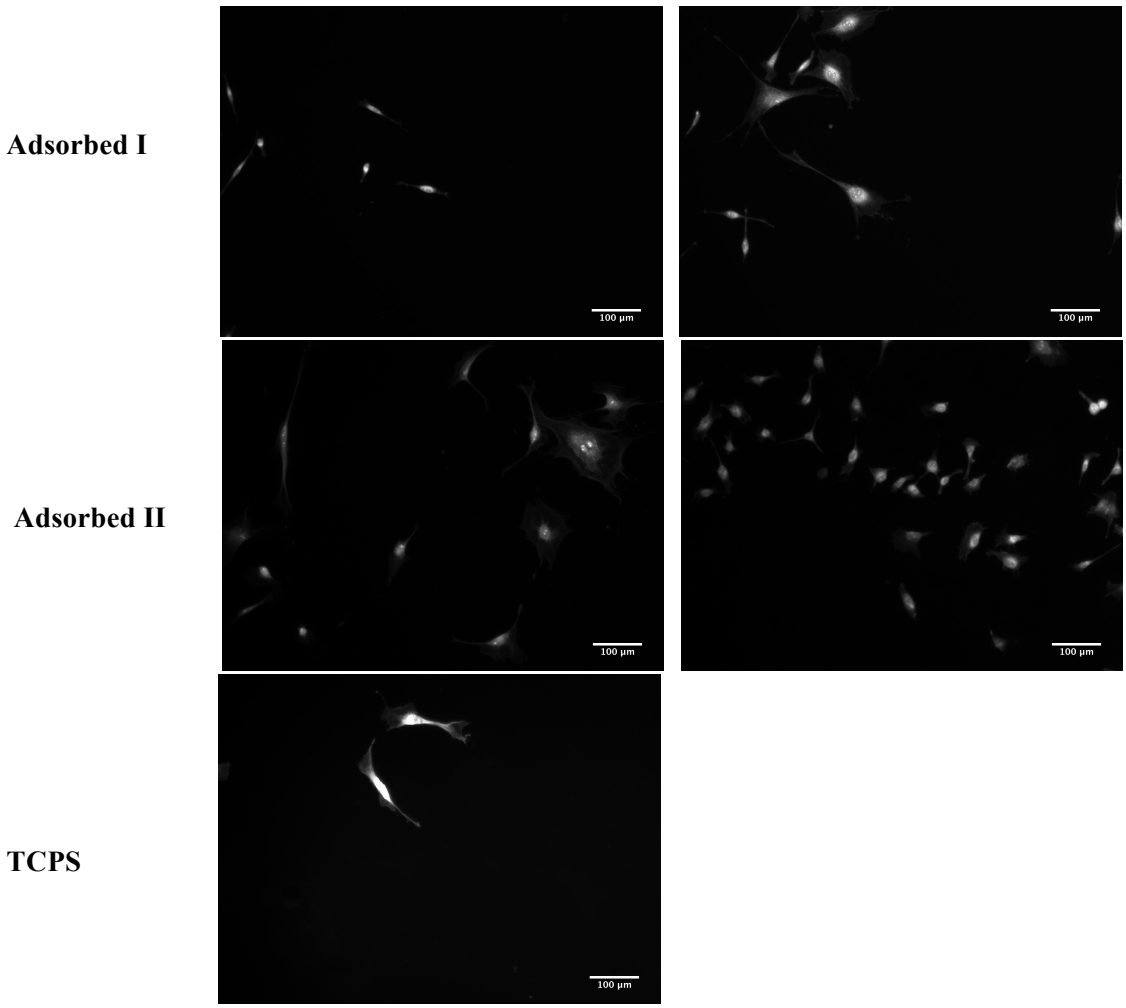


Figure 8: Images of annulus fibrosus cells on each of the surfaces for each time point (Day three on polystyrene not available). The cells seeded on polystyrene or adsorbed collagen surfaces appeard to be more spread than those on fibrillar collagen surfaces.

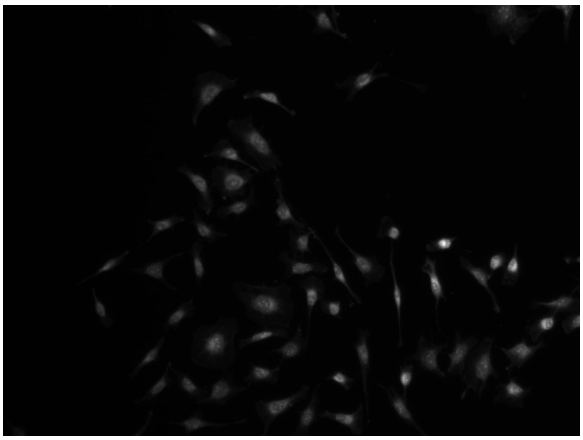


Figure 9: Annulus fibrosus cells on type I adsorbed collagen surface at Day 3 showing a higher density of cells than was observed at Day 1.

This time dependence was not reflected on gene expression, however, as no statistical significance was found for any of the genes, substrates, or time points examined. For the RT-PCR data, significant differences were calculated by comparing Δct values among experimental groups. The graphs, however, show the fold difference in expression as compared to the *in vivo* control discs, calculated using the ΔΔct method. The *in vivo* control levels are therefore valued as 1 in the following plots. Error bars are standard deviations based on multiple samples that are each analyzed in triplicate.

Collagen 1

Collagen 1 was observed to be significantly up-regulated compared to *in vivo* control levels for all culture conditions and for each time-point. It was also found to be insensitive to culture condition type, as no significant differences were found among the different surfaces.

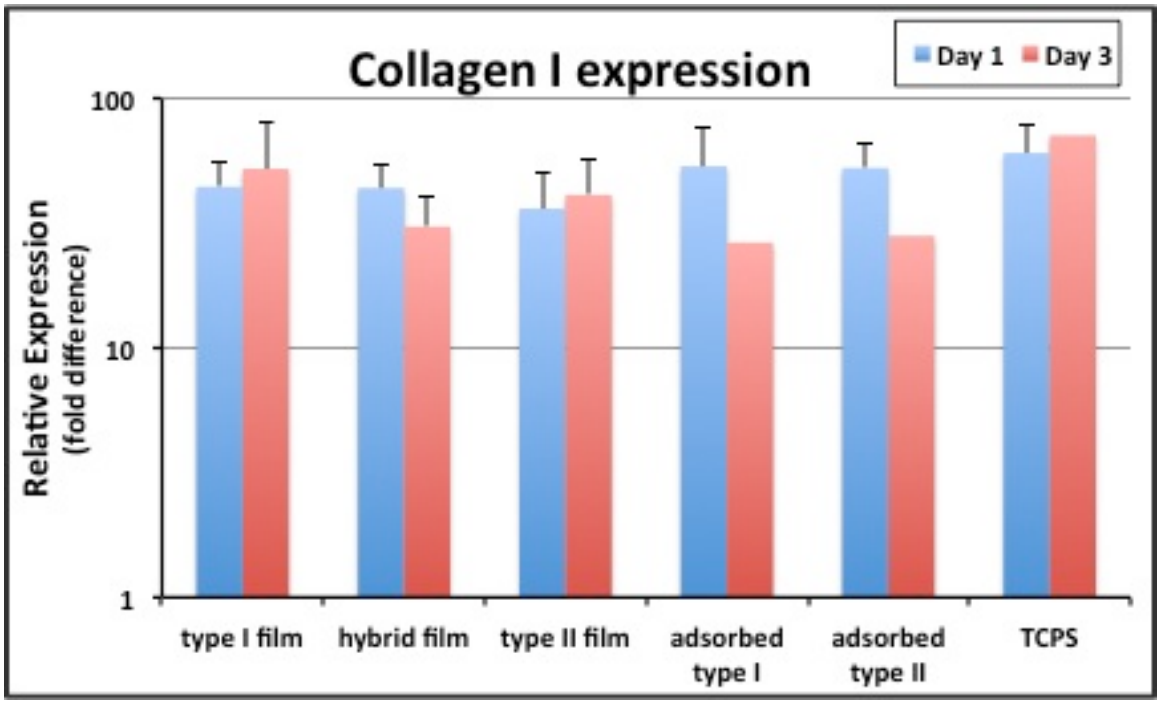


Figure 10: AF cell type I collagen expression, showing an up-regulation compared to *in vivo* control levels. Collagen 1 was found to not be dependent on culture surface or time in culture.

Collagen 2

Collagen 2 was observed to significantly down-regulated compared to *in vivo* control levels for all culture conditions except for type I collagen films at Day 3 and for type II collagen films at Day 3. No significant differences between groups were observed; however, lack of data from some samples (lack of error bars on graph) mean that differences might be observed with more trials.

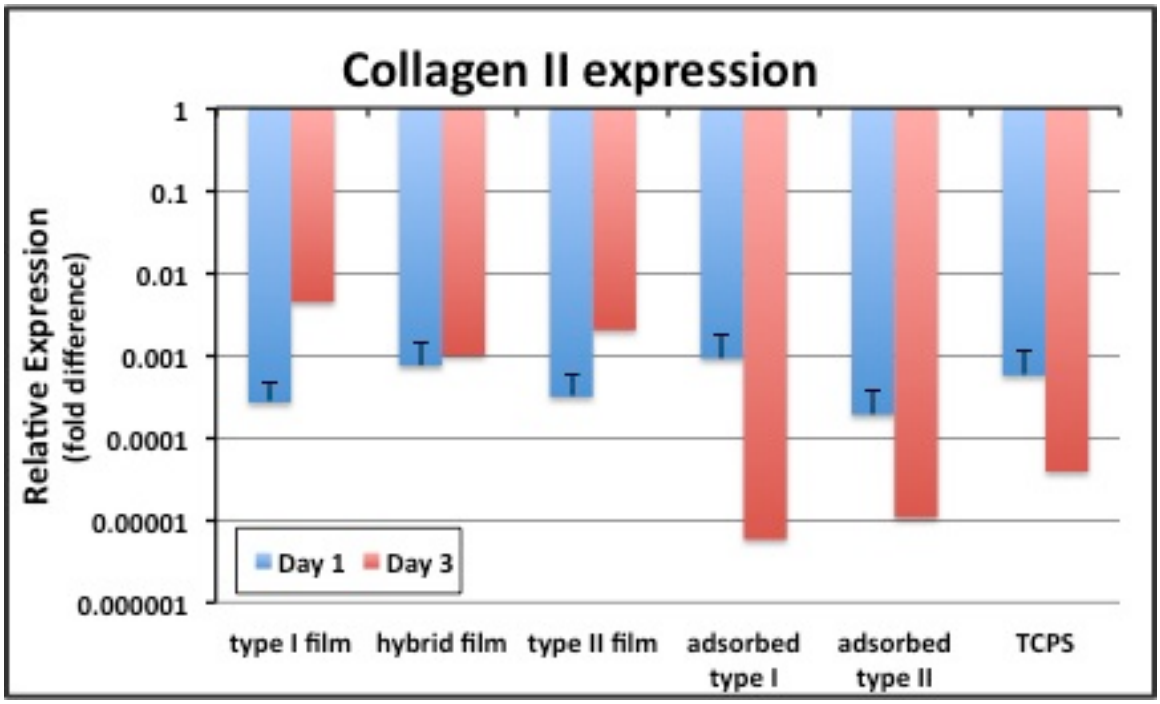


Figure 11: AF cell type II collagen expression, showing significant down-regulation compared to *in vivo* controls for all culture conditions except Day 3 values for type I collagen films and type II collagen films.

Aggrecan

Aggrecan expression was found to be significantly down-regulated compared to *in vivo* control levels for all culture conditions except for each collagen film at Day 3. This is similar to the results observed for Collagen 2; likewise, lack of data points to generate standard deviation prevents statistical significance for trends between groups.

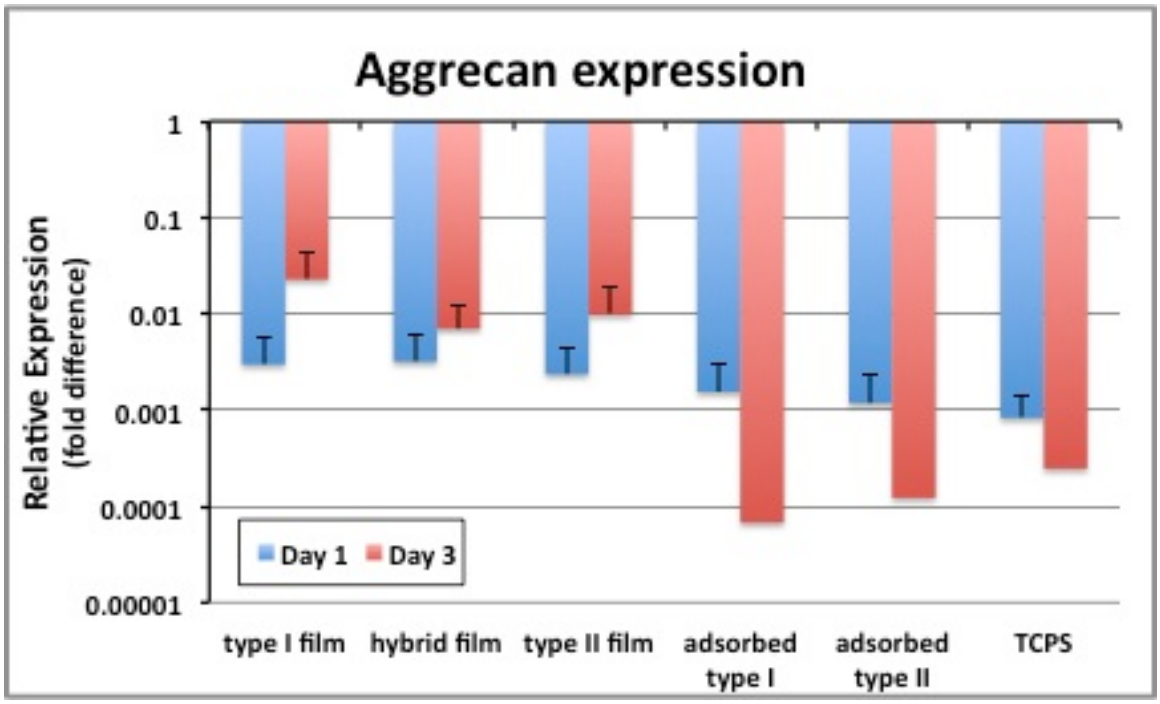


Figure 12: AF cell aggrecan expression, demonstrating significant down-regulation compared to *in vivo* control values for all culture conditions except Day 3 values for type I, type II and hybrid films.

MMP-2

MPP-2 was up-regulated for each culture condition in comparison to *in vivo* controls, and was observed to be insensitive to the surface the cells were cultured on.

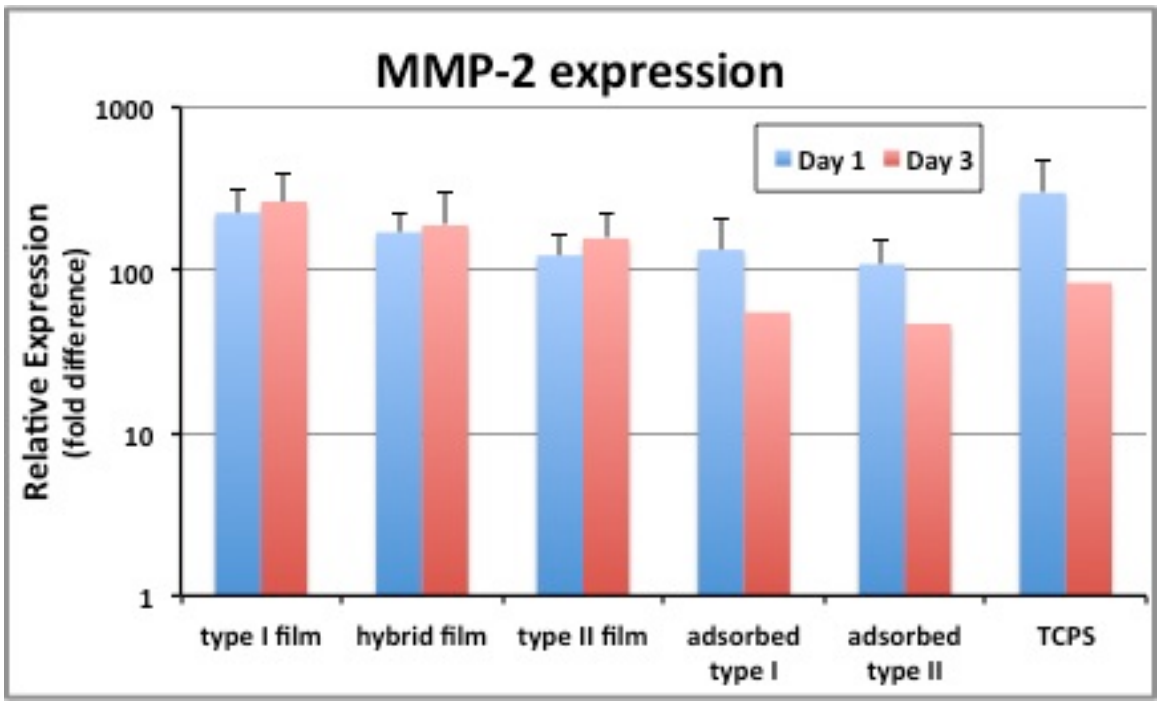


Figure 13: AF MMP-2 expression was significantly up-regulated for each culture condition compared to the *in vivo* controls. MMP-2 was also observed to be independent of cell culture substrate.

αSMA

αSMA was found to be unresponsive to cell culture condition, as no significant differences were found among groups or between any group and the *in vivo* control.

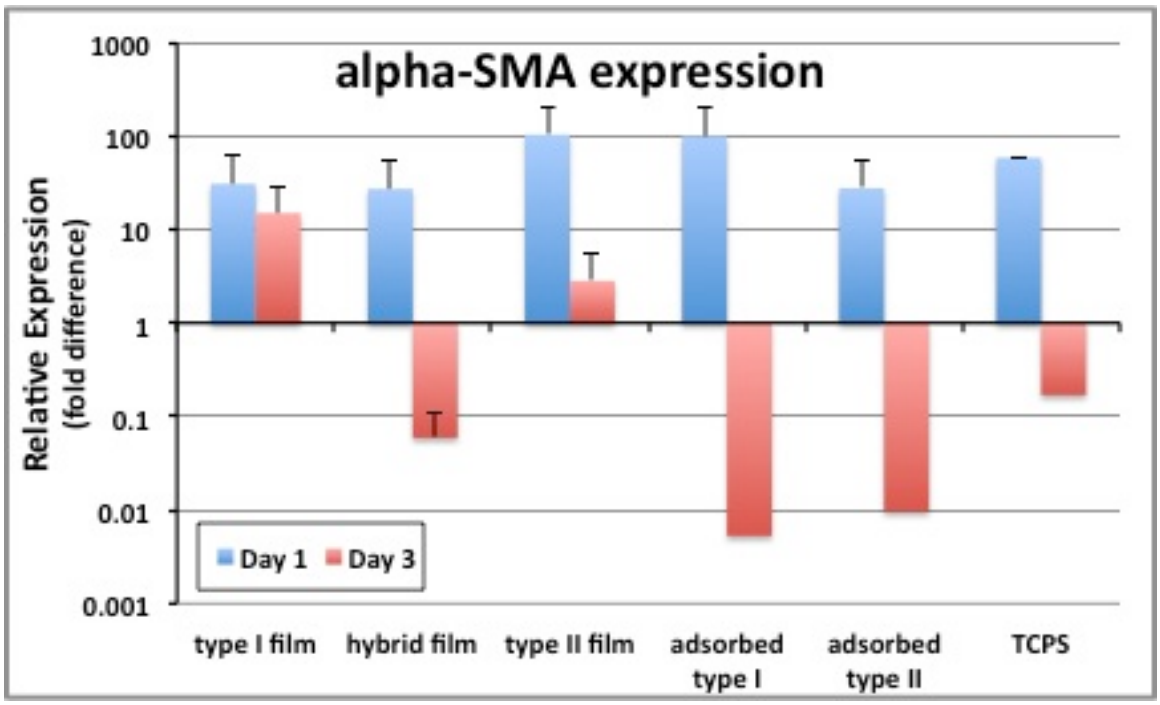


Figure 14: AF αSMA expression was found to be independent of cell culture conditions or time in culture.

Trends

While no significant differences were observed among groups, trends show similarities to the morphology data in the differences between fibrillar collagen and adsorbed collagen surfaces. Trends for aggrecan, type II collagen, and αSMA suggest that the differences between the collagen films and adsorbed collagen might persist. Specifically, there was a decreasing trend for expression levels of each of those genes for cells seeded on adsorbed collagen surfaces in comparison to cell seeded on thin film surfaces.

Discussion: AF cells

The results show that annulus fibrosus cells were not influenced by changes in collagen fibril type. Similarities among type I, type II, and hybrid collagen films show that collagen type does not drive either morphology or gene expression in annulus fibrosus cells. This has implications regarding the structural differences of the inner and outer annulus and the phenotypic differences observed *in vivo*. As previously described, annulus fibrosus cells exhibit differences in cell shape and actin cytoskeleton *in vivo* from inner to outer annulus. The results presented here suggest that these differences are due to factors other than extracellular matrix collagen. Possibilities include non-collagenous extracellular matrix components, matrix organization, and mechanical loading environment. Interestingly, the Day3:Day1 cell area for the type I, hybrid, and type II collagen films were approximately the same (0.38, 0.37, and 0.37, respectively). This strengthens the idea that the same mechanism of cell attachment governs AF cell adhesion to each the three different films. Overall, the cells on all films show large increases in type I collagen and MMP2, suggesting that the cells are building and remodeling matrix.

While there was no observed difference in AF cell response between collagen films, there was a difference between collagen films and collagen coated surfaces, as well as between different types of collagen coating. This suggests the cells attach to fibular collagen using a potentially different mechanism than when attaching to a collagen-coated surface. Moreover, either different mechanisms are used to attach to different collagen coatings, or the single mechanism triggers different downstream events when attached to type II or type I adsorbed collagen that resulting in morphological differences.

Integrins are an important class of molecule that cells use to attach to their surrounding. They consist of an α subunit and β subunit. *In vitro*, it has been discovered that AF cells use similar integrin subunits to attach to type I and type II collagen coatings (Gilchrist, et al. 2007). The β1 subunit was determined to be almost entirely responsible for AF adhesion to both type I and type II collagen. For the α integrin subunit, blocking the α1 subunit was found to inhibit AF attachment to type II collagen by 83%, but only by 49% on type I collagen, for adsorbed collagen. This indicates that additional α subunits may be involved in AF cell attachment to adsorbed type I collagen. The data presented here indicate that it may be engagement of these other α subunits that lead to the observed morphological differences and gene expression differences trends, Future research should explore this concept, particularly for fibular collagen.

Results: NP cells

Nucleus pulposus cell area was independent of culture substrate. The cell area observed on the collagen films were similar to each other and were each lower than the area observed for cells seeded on adsorbed collagen surfaces, as illustrated in Table 5. This is similar to results obtained with annulus fibrosus cells.

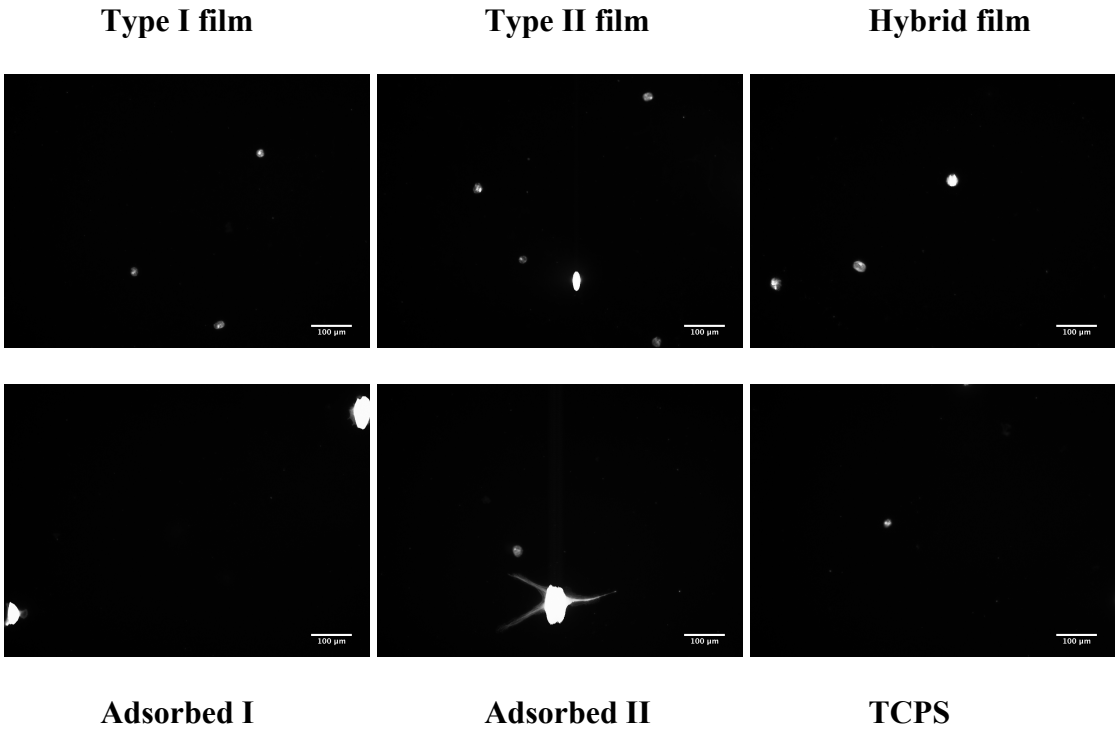


Figure 15: Nucleus pulposus cells at Day 3 on each of the cell culture substrates.

However, the differences were not statistically significant due to the large standard deviations associated with a mixed population of cells. The limited amount of cell attachment at Day 1 meant that morphology data was unable to be obtained. Visual inspection showed a low number of cells attached, each spherical in shape and similar in size.

Table 5: Nucleus pulposus cell area

Substrate	Day 3 Area (μm^2)
Type I (film)	1030 ± 933
Type II (film)	866 ± 1026
Hybrid film	946 ± 851
Type I (adsorbed)	2514 ± 2185
Type II (adsorbed)	2008 ± 1610
Polystyrene	1344 ± 1293

Examination of the area distributions shows that there may be differences between the cell substrates. The distributions are similar for type of substrate, regardless of collagen type. However, differences are notable for cells seeded on a film, adsorbed collagen surfaces, or on tissue culture polystyrene (Figure 16). Cells seeded on films tend to have a single peak, while cells seeded on adsorbed collagen tend to have a more flat distribution.

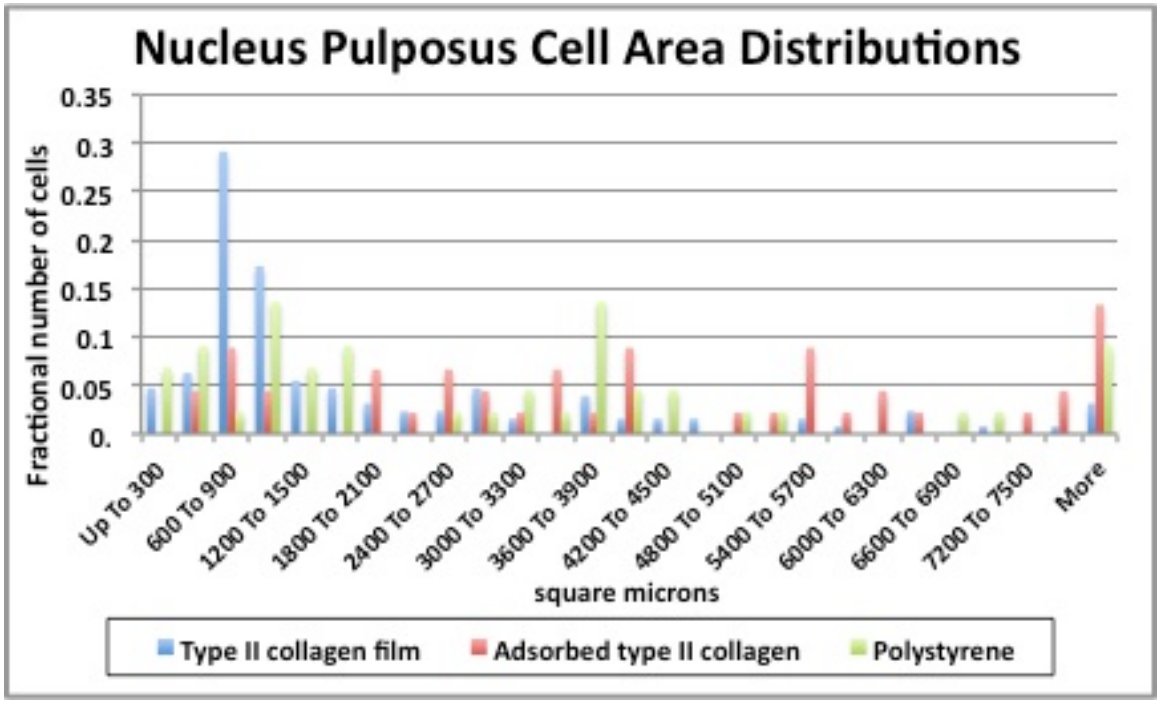


Figure 16: Distribution of Nucleus pulposus cell areas on type II collagen films, adsorbed type II collagn, and polystyrene. Note the large distributions generated by a mixed population of cells, indicating that cells may respond differently to fibrillar or adsorbed collagen surfaces.

The cumulative cell area distributions were examined in an effort to further examine the cell area distributions and separate the cell response to each culture surface as detailed by Elliott *et al* (Elliott, Halter and Plant 2008). However, the experimental setup used did not allow a full comparison of *D*-statistic values. Nonetheless, differences

in the cumulative distributions suggest that may a differential morphological response of the nucleus pulposus cells to the cell culture surfaces.

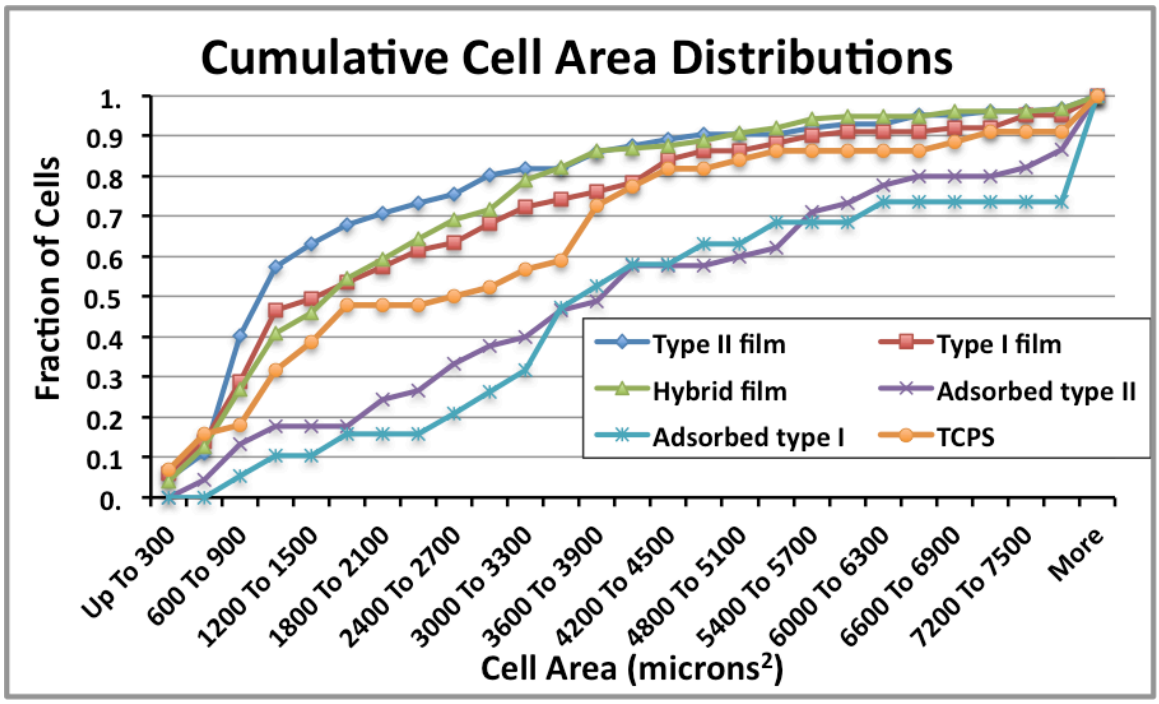


Figure 17: Cululative cell area distibutions of nucleus pulposus cells on each cell culture surface. The cells on adsorbed collagen surfaces demonstrate a higher fraction of cells of a larger area than the cells on fibrillar collagen films.

Gene expression for NP cells was found to be dependent on culture surface some some genes and surfaces, but independent for others. For the RT-PCR data, significant differences were calculated by comparing Δct values among experimental groups. The graphs, however, show the fold difference in expression as compared to the *in vivo* control discs, calculated using the ΔΔct method. The *in vivo* control levels are therefore valued as 1 in the following plots. Error bars are standard deviations based on multiple samples that are each analyzed in triplicate.

Collagen 1

Collagen 1 expression was significantly up-regulated on all surfaces except polystyrene (Day 1) when compared to *in vivo* controls. Likewise, expression was significantly up-

regulated at Day 3 for each collagen film and for type II adsorbed collagen surfaces when compared to Day 1 expression. Non-significant increases were observed among surfaces for Day 1 or Day 3.

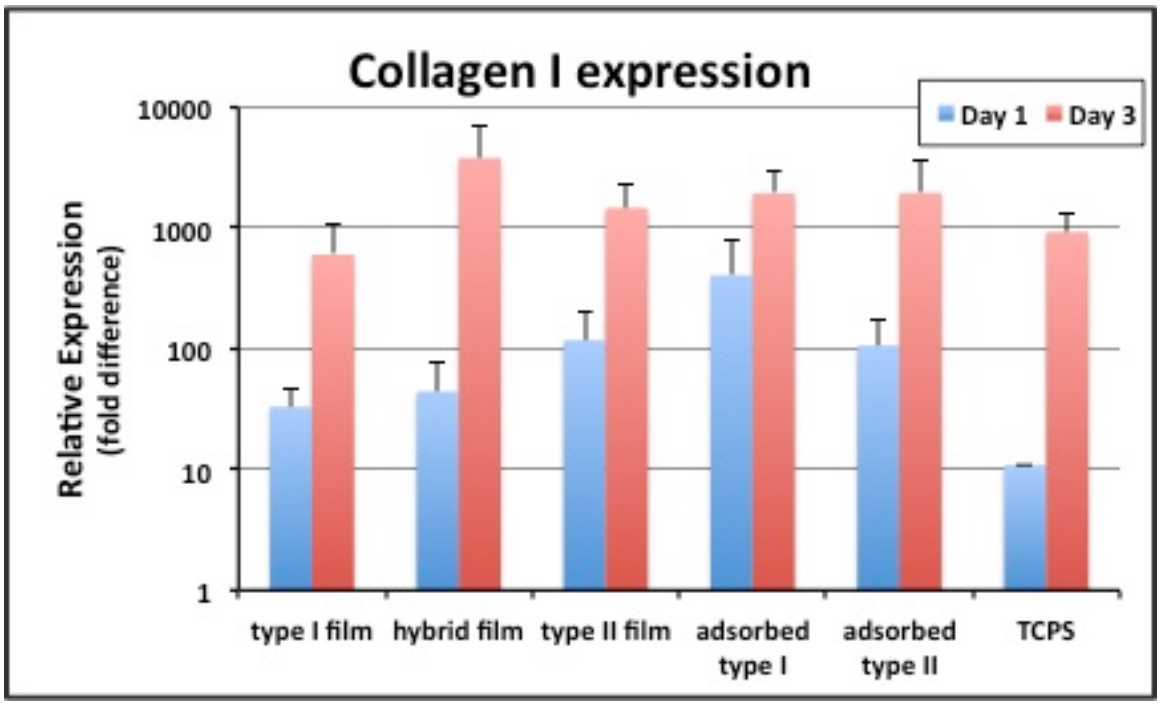


Figure 18: NP cell collagen I expression was found to up-regulated for all surfaces except TCPS at Day 1 compared to *in vivo* controls. Day 3 expression was also found to be significantly higher than Day 1 for type I, type II, and hybrid films as well as for adsorbed type II collagen.

Collagen 2

No significant differences were found for Collagen 2 expression among groups or between any group and the *in vivo* control. This may be due to a low number of data points for that gene.

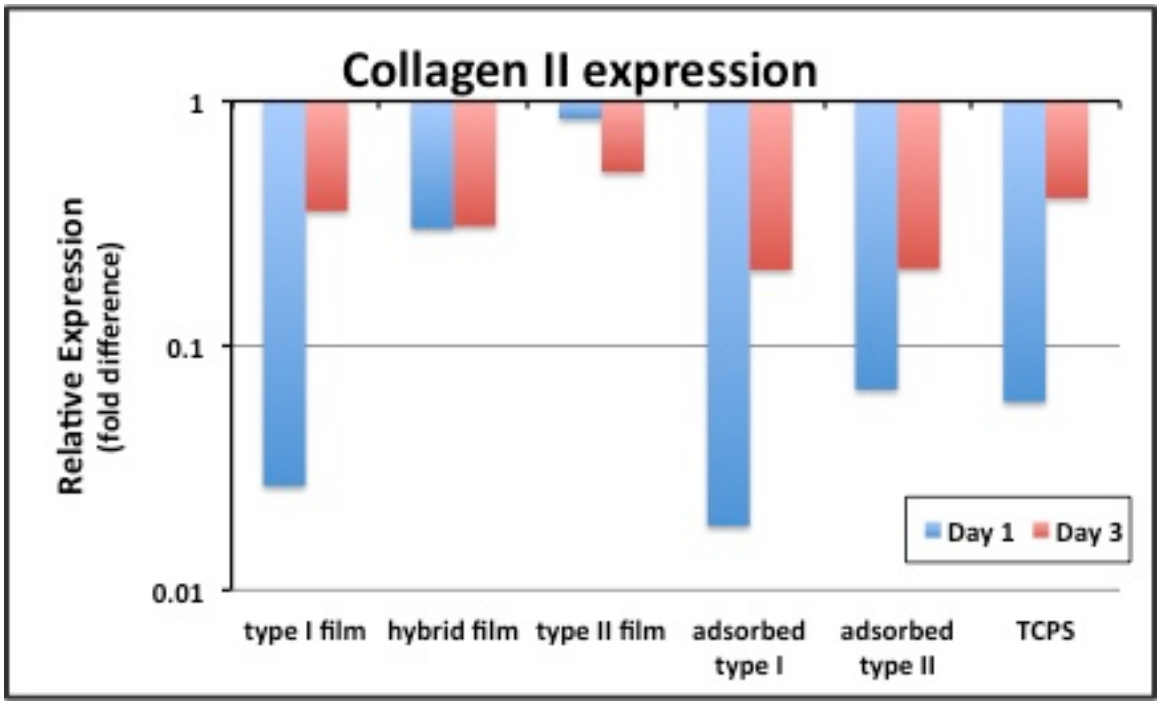


Figure 19: NP Collagen 2 expression was observed to be independent of cell culture conditions or time in culture.

Aggrecan

Aggrecan was found to be independent of culture substrate with the exception of type I adsorbed collagen at Day 3, which was significantly lower than *in vivo* control levels. Aggrecan was found to significantly decrease from Day 1 to Day 3 on adsorbed type II collagen surfaces, and increase from Day 1 to Day 3 on polystyrene.

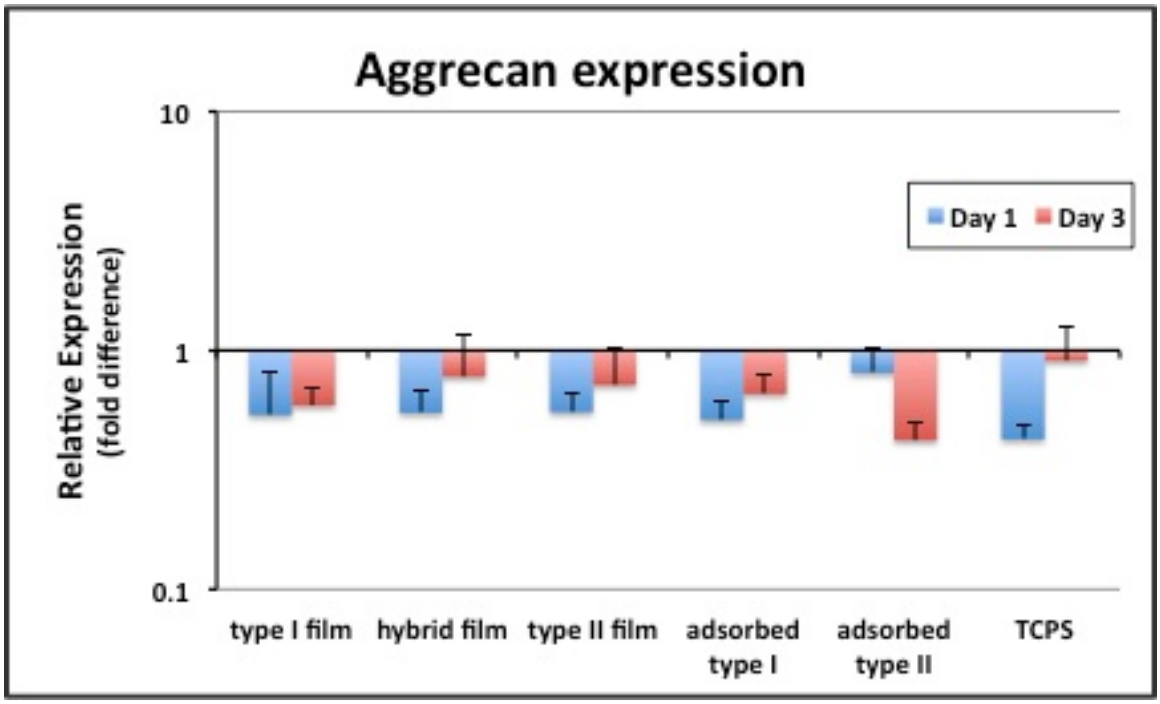


Figure 20: NP cell Aggrecan expression on adsorbed type II collagen was significantly lower than *in vivo* control levels. Significant differences were also observed between Day 1 and Day 3 for adsorbed type II collagen and TCPS surfaces.

CD24

CD24 was found to be independent of culture surface examined, as no significant differences were found among groups or between any group and the *in vivo* controls.

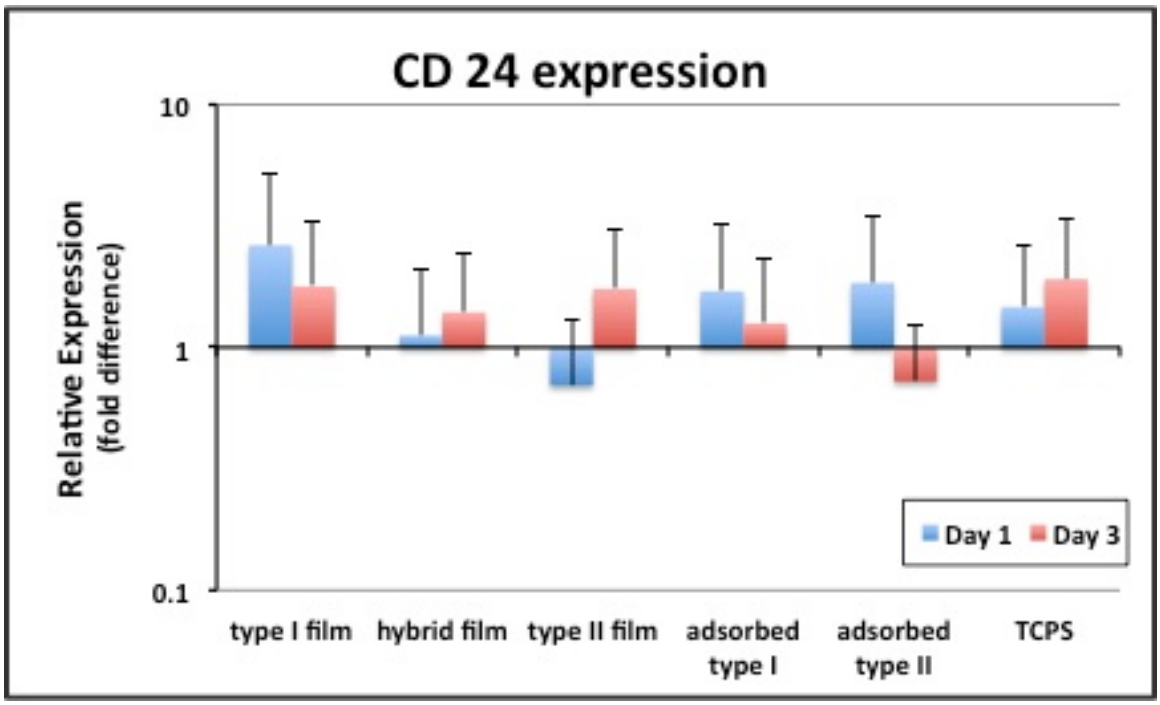


Figure 21: NP cell CD24 expression was observed to be independent of culture surface or time in culture.

Keratin 8

Only Day 1 values for type I collagen films and hybrid films were observed to be significantly different from *in vivo* controls, as both were found to be down-regulated. No differences were observed among surfaces, however, or between time-points.

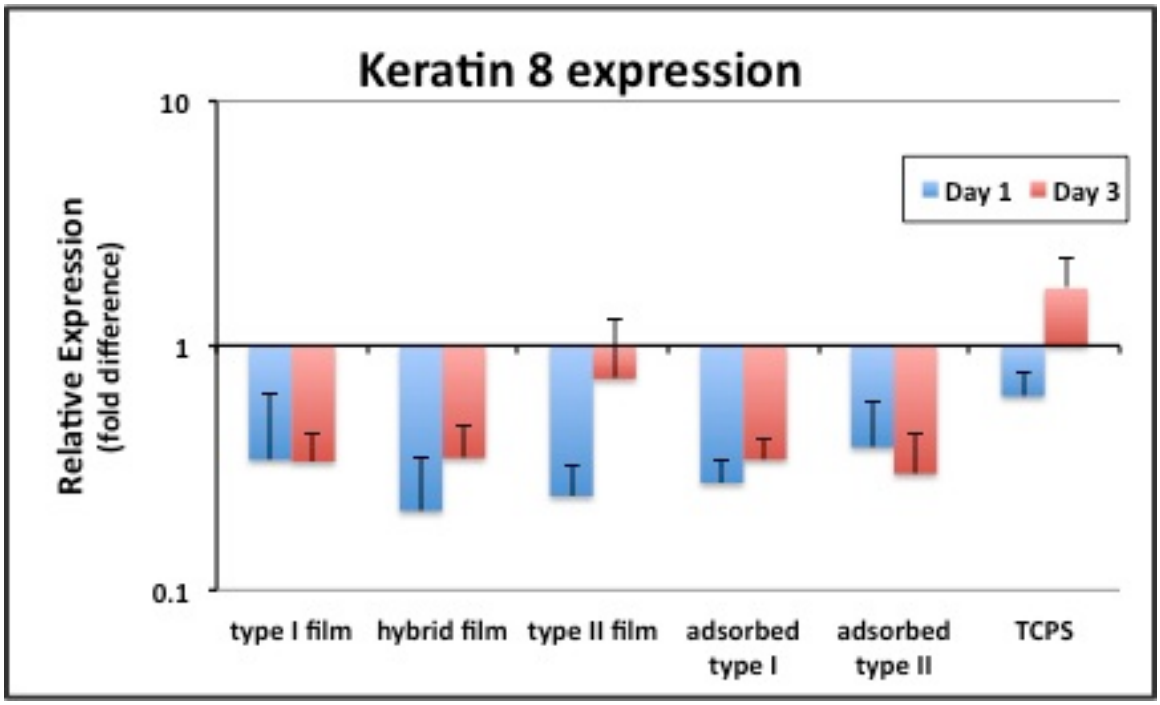


Figure 22: NP cell Keratin 8 expression was down-regulated compared to *in vivo* controls for cells on type I and hybrid films at Day 1.

Trends

Type II collagen expression tended to be lowest on type I collagen surfaces (either film or adsorbed) and highest on type II collagen films, with hybrid film expression in between. Though significance was not found for that gene on account of the number of samples that produced sufficient RNA for PCR analysis, the trend suggest a more substantial down-regulation of collagen type II expression with a the decreasing presence collagen type II fibrils present.

Overall, type II collagen films had expression levels of type II collagen and keratin 8 that were closest to the *in vivo* control.

Discussion: NP cells

Morphological observations of nucleus pulposus cells show behavior similar to annulus fibrosus cells, in that the cells are more responsive to collagen form (adsorbed or thin film) rather than collagen type. While no statistical significance was observed, this may be due to the mixed population used in this part of the study. Chondrocyte-like cells are smaller than notochordal cells; therefore, a large deviation from the population average is expected. Examination of the area distributions also indicates that the cells behave differently on the various substrates. However, distinct peaks in the distribution corresponding to each cell type were not observed on any of the surfaces. Cell area would therefore not work as a discriminator between notochordal and chondrocyte-like cells. Low cell attachment was observed; in fact, not enough cell attached for morphological analysis at Day 1. This is typical for these cells in an *in vitro* environment for culture in monolayer. Other ECM components, such as or laminin or aggrecan, may aide in cell attachment.

Gene expression levels overall show an increase in type I collagen with a decrease in type II collagen, though aggrecan expression was relatively close to *in vivo* control levels. This indicates that nucleus pulposus cells require additional cues or signals to maintain their *in vivo* phenotype in an *in vitro* environment. However, the trends for type II collagen expression show that with an increasing amount of type II fibers present, as in the pure type II collagen films, there may be a corresponding increase in type II collagen expression. Likewise, trends suggest keratin 8 values closest to control on type II films,

as well as expression levels of aggrecan and CD24 that are close to *in vivo*. Therefore, type II collagen films were chosen as the substrate to be used in the subsequent studies.

The results shown here are similar to those obtained by Feng *et al*. using an explant culture model with chondrogenic media (Feng, Wan, et al. 2009). Specifically, type II collagen and aggrecan levels were able to be maintained for up to seven days. While the results presented here do not cover the same time period, the trends observed on the hybrid and type II films indicate continued correlation. Examination of collagen 1, collagen 2, and aggrecan in monolayer culture has shown evidence of initial reduced expression for each (Preradovic A 2005). The collagen 1 results from this study are in contrast to this, as the gene was up-regulated for each culture condition, though use of different culture media may cause this discrepancy. Likewise, decreased expression of collagen 2 and aggrecan during monolayer culture was observed by Kluba *et al.* (Kluba T 2005). That the fibrillar collagen surfaces used in this study were able to maintain collagen 2 and aggrecan levels, despite being in monolayer culture, is significant.

Future work for this aim should investigate the trend of increasing collagen type II expression with increasing type II collagen fiber presence if it could be found to be significant. Similar to the results obtained for annulus fibrosus cells, Gilchrist *et al.* observed that NP cell attachment to type II collagen was entirely governed by the β1 and α1 subunits, while α1 only partially inhibited NP cell attachment to type I collagen (Gilchrist, et al. 2007). The additional α subunits that govern attachment may influence gene expression while not influencing cell morphology. For porcine NP cells, moderate to high levels of α5 and α6 were observed by Nettels *et al.* (Nettles, Richardson and Setton 2004). Investigation of these subunits and their corresponding effects on gene

expression and cell attachment could shed light on how nucleus pulposus cells respond to the environmental changes during degeneration.

CHAPTER 6: ESTABLISH THE EFFECTS OF NOTOCHORDAL CELL LOSS ON CHONDROCYTE-LIKE CELLS

As previously stated, the nucleus pulposus of the intervertebral disc has two different cell types: notochordal cells and chondrocyte-like cells. However, while recent strides have been made in elucidating the differences between them, the exact function of each cell type has not been explicitly defined. This is particularly true of the notochordal cells, which disappear in the human adult intervertebral disc while remaining in the discs of other species. Different approaches have been used in an attempt to quantify differences between chondrocyte-like cells and notochordal cells. One approach involves comparing the cells from an animal with a mixed population of notochordal and chondrocyte-like cells to an animal without notochordal cells. This method is flawed, however, in that it is unable to account for inter-species variation. Nonetheless, observations have been made. This technique was utilized by Miyazaki *et al.* in comparing rat, rabbit in bovine tails, inferring differences in energy metabolism and proteoglycan production by comparing cells from discs with notochordals cells to cells from discs without notochordal cells (Miyazaki, et al. 2009). Inter-species comparison was also used to garner data on the higher sensitivity of notochordal cells to nutrient deprivation by comparing bovine cells (lacking notochordal cells) to procine cells (both notochordal and chondrocyte-like cells) (Guehring, et al. 2009).

A second approach to examine differences between notochordal and chondrocyte-like cells involves sorting the mixed population of cells from a single species into two distinct and separate cell populations. Two methods have been used with some success in this regard: fluorescent activated cell sorting (FACS) and filtering. FACS utilizes several features of a cell, such as its size and complexity, its autofluorescence, or cell-

specific markers that have been tagged with fluorescently-labeled antibodies. As notochordal cells are both larger and possess more complex cytosol, FACS is a potential candidate for sorting mixed populations of nucleus pulposus cells. Indeed, Chen *et al.* used FACS to separate notochordal cells and chondrocyte-like cells to some success (Chen, Yan and Setton 2006). More accurate results could be obtained with a cell-specific marker, but none have yet been found that reliably distinguish between notohcordal and chondrocyte-like cell populations. This may be due to the previously discussed issue that chondrocyte-like be of the same origin as notochordal cells, rather than of mesenchymal origin.

A second approach to sorting the two cell populations of the nucleus pulposus is filtering. This methods uses one or more filters, typically around 10μm in size, to discriminate between the larger notochordal cells and the small chondrocyte-like cells. Kim *et al.* used serial filtration with 40μm, 25μm, and 15μm to separate the two cell populations and observed comparable expression of type II collagen and aggrecan (Kim, et al. 2009). Likewise, Capello *et al.* used sequential 15μm, 10μm, and 8μm filters to separate the cells and demonstrate that notochordal cells produced more proteoglycan than chondrocyte-like cells (Cappello, et al. 2006).

The third hypothesis for this study was that chondrocyte-like cells will exhibit differences in gene expression as compared to notochordal cells, and their gene expression will be changed in the presence/absence of notochordal cells. To investigate this hypothesis, a method of sorting needed to be established in order to examine the individual phenotypes of notochordal and chondrocyte-like cells, and to attempt to observe and quantify the influence of notochordal cells on chondrocyte-like cells.

Therefore, several methods established in the literature were evaluated based on yield and purity of results. Once an established method was chosen, notochordal and chondrocyte-like cells were cultured both together and separately. Cells cultured together as a mixed population were then sorted, yielding four groups for analysis: 1) notochordal cells cultured alone; 2) notochordal cells cultured with chondrocyte-like cells; 3) chondrocyte-like cells cultured alone; and 4) chondrocyte-like cells cultured with notochordal cells.

Methods

Cell Sorting

Cell sorting using FACS was done according to procedures adapted from Chen *et al.* (Chen, Yan and Setton 2006). NP cells were harvested as previously described from the tails of at least three rats or from one macaque tail (University of Maryland School of Medicine, Baltimore, MD), and were suspended in DPBS. AF cells were used to establish a maximum size for chondrocyte-like cells based on forward- and side-scatter measurements (indicative of size and complexity, respectively); autofluoresence was not utilized. FACS was run using a BD Biosciences FASCAria (BD Biosciences, Franklin Lakes, NJ). Post-sort analysis of at least 10,000 recorded events was run following the sort to ensure accurate results.

Ficoll sorting was attempted using various percentages of Ficoll (Fisher Scientific, Pittsburgh, PA). Initial attempts were made using a discontinuous gradient with 11, 22, and 31 % w/v solutions of Ficoll. Refinements were made based on initial results, and subsequent sorts using discontinuous Ficoll gradients were made using layered 3% and 6% w/v solutions. The gradients were made by slowly and carefully pipetting enough of the filtered Ficoll solution so as to be approximately one inch (~3mL

in a 15mL conical tube). The higher percentage (more dense) layers were pipetted first, and the lower percentage (less dense) layers were added on top. The cells, suspended in 3mL of media, were placed on top of the uppermost Ficoll layer, and the gradients were centrifuged at 8000g for 45 minutes.

Cell sorting based on filtering was accomplished using a custom designed filter holder that allowed easy transfer from one 50mL conical tube to another to ensure minimal contamination and loss of cells. Cells harvested from three rat tails were pooled and passed through a 70 μm filter to eliminate debris. Cells were then passed through a 10μm filter. The filter was then flipped over into a new conical tube and rinsed thoroughly with media to remove cells larger than 10μm. Based on examination of previous cell filtering trials, cells larger than 10μm were considered notochordal, while cells smaller were considered chondrocyte-like.

Culture and gene expression analysis of notochordal and chondrocyte-like cells

Notochordal and chondrocyte-like cells were cultured together as a mixed population or separately as individual populations on type II collagen films to observe differences in gene expression and potential effects of the presence or absence of notochordal cells on chondrocyte-like cells. Nucleus pulposus cells were harvested from nine rat tails as previously described and pooled into three groups of three tails. Cells were sorted using the cell filtering method to separate notochordal and chondrocyte-like cells. Type II collagen films were prepared according to the previously described procedure. Following isolation of nucleus pulposus cells and re-suspension in media, the cells were passed through a 70 μm filter. The remaining volume was divided, with half plated immediately as a mixed population. The remaining mixed cell suspension was

passed through a 10 μm filter, and sorted notochordal cells and chondrocyte-like cells were plated individually. All cells were cultured for 72 hours at 37°C in alpha-MEM containing 2% FBS and 1% penicillin-streptomycin. Images were taken following 72 hours using a Hamamatsu Orca-ER camera attached to an Olympus IX81 microscope. Cells were then rinsed with DPBS. The cells that were cultured as a mixed population were then lifted from the surface using trypsin (Sigma-Aldrich, St. Louis, MO) and centrifuged at 360g for 10 minutes. The trypsin was carefully removed and the cells were resuspended in media and passed through a 10μm filter to separate notochordal and chondrocyte-like cells. All cells were then lysed, RNA was harvested and gene expression was analyzed as previously described.

Results and statistical analysis

PCR results were analyzed using the ΔΔct method, which uses the threshold values reported by the PCR software. Comparison between the internal control gene (GAPDH) and each experimental gene are first made (Δct). This value is then compared to the same value for the experimental condition used as a control (ΔΔct). Statistical analysis was performed using ANOVA with Tukey's post-hoc analysis to determine significant differences, specifically by comparing the Δct values for each gene and experimental condition. Two data sets were generated and compared: 1) chondrocyte-like cell gene expression normalized to notochordal gene expression, for each culture condition, and 2) unsorted cultured normalized to sorted cultured for each cell type.

Results

Separation by size of notochordal and chondrocyte-like cells resulted in the optimal results for dividing the two populations of cells (Figure 23). Sorting using

filtration generated the most consistent results with regard to actual separation of each population. Likewise, retention of cells was high.

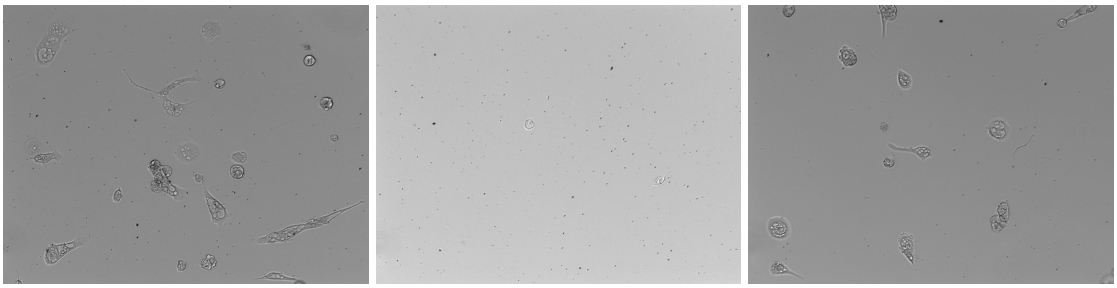


Figure 23: Mixed population (left), chondrocyte-like cells (center) and notochordal cell (right) as sorted by filtration. Note the larger number of retained notochordal cells.

Qualitatively, Ficoll sorts resulted in a decrease in accuracy in comparison to cell filtration, despite fine-tuning of the discrete density layers. The gating procedure and the post-sort analysis for the FACS procedure lost large amounts of cells, thereby decreasing yield. Both Ficoll sorts and FACS involved the cells being in a media-free/non-temperature controlled environment for extended periods of time.

Gene expression of chondrocyte-like and notochordal cells was not observed to be significantly different for either culture condition. However, non-significant differences were observed, as illustrated in Figure 22, which shows the gene expression of chondrocyte-like cells normalized to the gene expression of notochordal cells. This includes higher expressions levels of type I collagen, CD24, and keratin 8. Chondrocyte-like cells also had higher expression of type II collagen than notochordal cells when cultured together, though that difference was minimized when cultured apart. Aggrecan expression of chondrocyte-like cells was similar to notochordal cells.

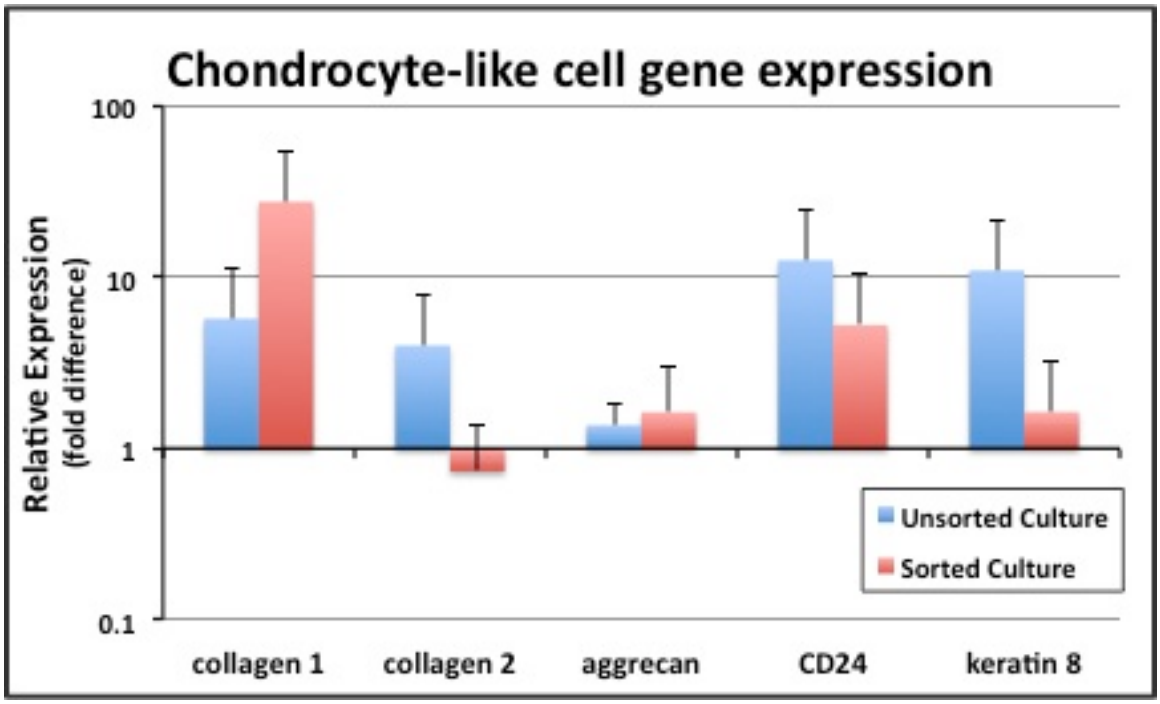


Figure 24: Comparison of chondrocyte-like cell gene expression in unsorted and sorted culture on type II collagen films. Graph shows chondrocyte-like cell gene expression normalized to notochordal cell gene expression for each culture condition. No significant differences were found.

Likwise, no significant changes in gene expression of chondrocyte-like cells were observed between culture conditions. However, non-significant differences were observed between culture conditions. Trends show that chondrocyte-like cells were observed to produce more CD24 when cultured with notochordal cells than without. Though lacking statistical significance, trends were also observed for type I collagen, type II collagen, and keratin 8. Specifically, chondrcocyte-like cells produced less type I collagen while producing more type II collagen and keratin 8 when cultured with notochordal cells. In contrast, notochordal cells produced more type I collagen and less type II collagen when cultured with chondrocyte-like cells.

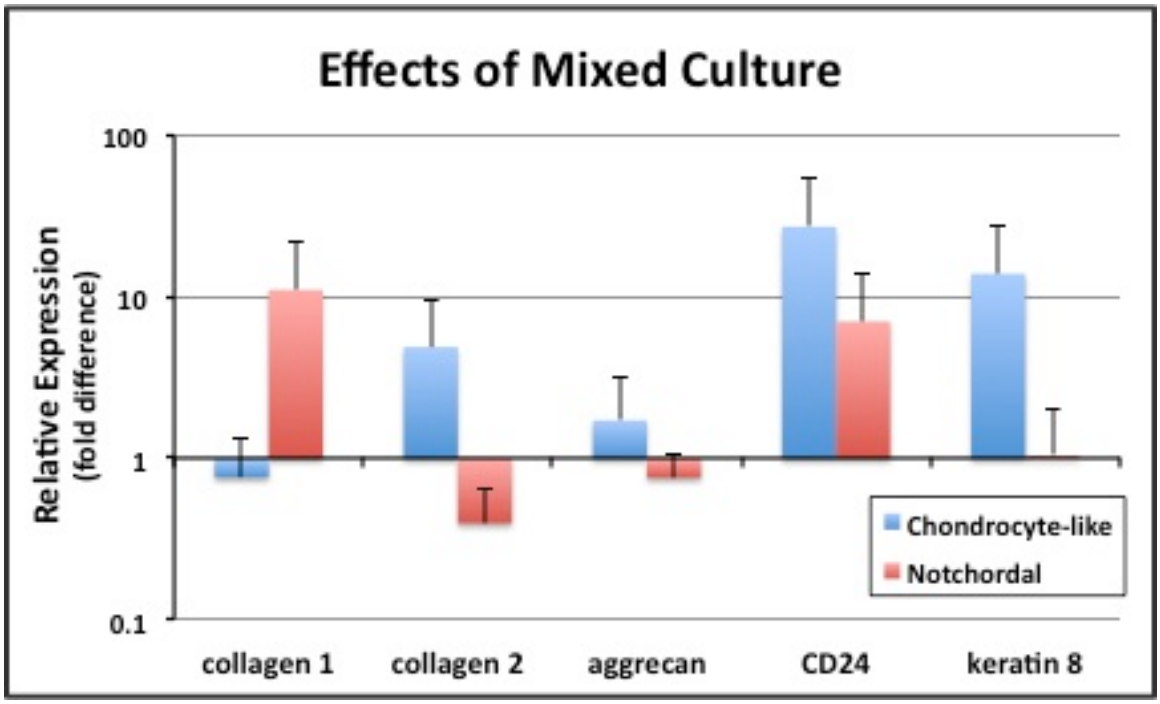


Figure 25: Effects of mixed culture on chondrocyte-like and notochordal cells, showing changes in gene expression of each cell type cultured together relative to expression levels when cultured individually on type II collagen films. Changes were not statistically significant. Graph shows gene expression of each cell type when cultured as a mixed population relative to the gene expression of that cell type when cultured individually.

Discussion

Filtration was chosen as the sorting method due to the many different factors, including the accuracy of the sort and precision from one sort to the next. The procedure also took the least amount of time, kept the cells in media, and exposed them to minimal physical stress. The entire process could also be performed in a sterile environment. Ficoll and FACS sorting methods, on the other hand, kept cells outside of a 37°C, controlled, sterile environment for long periods of time without the nutrients provided by supplemented cell culture media. The yield for the filtration was also the highest, and the procedure was the simplest. Initial results showed an ability to separate the larger notochordal cells, but an inability to capture the smaller cells intact. This was determined to be from applying too much pressure to the solution with the pipette to force it through

the filter. By allowing gravity and gentle perturbations of the filter holder to bring the cell solution through the filter instead of the pipette, cell retention improved to acceptable levels. Nonetheless, further refinements are still possible. By using multiple filters of different sizes, one could increase the purity of each cell population, though at the cost of yield. Experimental demands would dictate the balance between accuracy and yield, though as it stands the procedure produces two distinct cell populations using a single size discrimination. Errors possible in this procedure could result from a loss of cell stuck to the filter or from less than 100% pure notochordal or chondrocyte-like cell populations, a possibility by only using a single filter to separate the two populations.

Using cell filtration to separate the two cell populations of the nucleus pulposus, observations could be made about their comparative gene expression and their affects on each other. While no gene was found to produce a statistically significant result, increasing the number of trials could result in significance. The current experimental conditions were established to recreate the cellular environment of a healthy disc (mixed culture) and a degenerate disc (sorted culture). This was done to observe how chondrocyte-like cells react to the loss of the notochordal cell population. The trends in gene expression indicate a decrease in anabolic genes collagen II and aggrecan, and a decrease in expression of genes typically considered 'notochordal'. The increase in aggrecan production concurs with studies by Erwin and Inman, and by Aguiar *et al.* (Erwin and Inman 2006) (Aguiar, Johnson and Oegema 1999). This is notable in the context of developing cell-based therapies for disc degeneration, as it demonstrates that chondrocyte-like cell gene expression changes as a result of notochordal cells loss, and any implanted cell should replace or re-stimulate the expression of these genes in

chondrocyte-like cells, in addition to replacing the extracellular matrix production of notochordal cells. This study also shows that chondrocyte-like cells express CD24, adding to the evidence that chondrocyte-like cells may be of notochordal, rather than mesenchymal, origin. However, the comparative gene expression of notochordal and chondrocyte-like cells indicates that, if they are both of notochordal origin, they still may be two distinct phenotypes. The results here concur with Kim *et al.*, who observed similar aggrecan and collagen II expression levels for notochordal and chondrocyte-like cells (Kim, et al. 2009), as well as with Chen *et al.*, who observed that notochordal cells have lower expression levels of type I collagen (Chen, Yan and Setton 2006).

While this work showed that chondrocyte-like cell gene expression is not significantly changed by the presence of notochordal cells in culture, other studies have demonstrated that notochordal cells can have measureable effects on other cell types. Use of media conditioned by a nucleus pulposus cell population containing notochordal cells has been used in multiple studies with success. Korecki *et al.* used conditioned media to observe stimulation of mesenchymal stem cells towards a nucleus pulposus-like phenotypes, as judged by gene expression and glycosaminoglycan production (Korecki, et al. 2010). Likewise, increases in proteoglycan production and cell proliferation were observed in chondrocyte-like cells stimulated by notochordal cell-conditioned media in a study by Erwin *et al.* (Erwin and Inman 2006). These studies suggest that soluble factors secreted by notochordal cells may influence other cell types, including the chondrocyte-like cells present in the nucleus pulposus. While this present study did not observe significant changes in chondrocyte-like cell gene expression as a result of co-culture with

notochordal cells, differences observed suggest that further trials may in fact yield significant results.

Future work should continue to explore these experimental conditions, probing more catabolic (ECM breakdown) and anabolic (ECM buildup) genes to determine how the balance between the two is affected by notochordal cell loss. Examination of other ADAMTSs, MMPs, and collagens may elucidate what exactly implanted therapeutic cells need to express in order to reestablish healthy nucleus pulposus ECM. Additionally, cultured sorted cells on different substrates, such as chondrocyte-like cells on hybrid or type I collagen thin films, might more accurately recreate the environment of the degenerative nucleus pulposus.

CHAPTER 7: CONCLUSION

The objectives of this study were to develop an *in vitro* culture substrate that would allow for preservation and observation of the *in vivo* phenotypes of intervertebral disc cells and to use this substrate to study the interactions of the intervertebral disc cells. The goal was to potentially elucidate what a potential cell-based disc degeneration therapy must accomplish to repair the disc. This goal was approached by examining the specific phenotypes of each cell of the intervertebral disc and by examining how chondrocyte-like cell expression is affected by notochordal cells.

The work here shows that type II collagen films are unable to form at 300 μg/mL concentrations, but that surfaces were able to be formed with both type I and type II collagen present. This provided a unique set of cell culture surfaces that allowed examination of annulus fibrosus and nucleus pulposus cell phenotypes and observe the effect of collagen fibrils and collagen type on each cell. Inclusion of adsorbed type I and type II collagen surfaces allowed for examination of cellular dependence of collagen fibers as opposed to collagen monomer, and the use of automated quantitative microscopy allowed for insights into the cellular response and correlation of morphology with gene expression.

Using these surfaces, the morphology and gene expression of each cell type was examined. Annulus fibrosus gene expression was found to be independent of cell culture surface, though AF cell area and proliferation were surface dependent. Specifically, cell area was smaller on collagen fibrils than on surfaces made of adsorbed type I collagen monomer, but larger than on adsorbed type II monomer. The data also suggests that the

cells interact with the fibrils that are present in the films (but absent in the adsorbed surfaces) with the same mechanism, regardless of fibril type.

The results for nucleus pulposus cells show no statistical differences in area on any of the culture surfaces; however, that is most likely a result the high standard deviations associated with a mixed population. Nevertheless, examination of the values suggests a dependence of the presence of fibrils and an inability to distinguish between the types of collagen for cell area, similar to AF cells. Changes in gene expression were observed, both among culture groups and between groups and the *in vivo* controls. However, collagen 2 and aggrecan levels were able to be maintained at *in vivo* levels, setting the fibrillar collagen surfaces apart from other monolayer culture systems presented in the current literature. This presents collagen thins films as a viable culture surface for nucleus pulposus films.

Type II collagen films were used for the third objective, which evaluated cell sorting methods to separate the individual notochordal and chondrocyte-like cell populations of the nucleus pulposus. While unable to successful utilize FACS as a sorting method, use of a single filter to separate the two populations yielded results comparable with other studies. However, by culturing the chondrocyte-like cells as sorted and unsorted populations, observations of the direct effect of notochordal cells on chondrocyte-like cell gene expression could be made. As such, no significant differences in gene expression were found between cell types or as a result of notochordal cells present in culture. However, additional trials may reveal significant differences in the trends observed. Likewise, use of an additional filter to more accurately separate the two populations (though at the cost of total cell yield) may enable differences to be observed.

The system of sorting and culture detailed here allows for direct examination of the effects of notochordal cells on chondrocyte-like gene expression. This is unique compared to other studies, which are either cross-species or use conditioned media.

Overall, this study demonstrated the potential of collagen thin films for use with the cells of the intervertebral disc. It highlighted differences between nucleus pulposus cell types and the fact that they exert influence on each other. This emphasizes the fact that the cells, and the interactions between them, need to be better understood in order to develop effective cell-based therapies for disc degeneration.

Future work

Future work for this project can proceed in multiple different directions. For the hybrid films, further characterization of the type II collagen present would be beneficial. The amount of each type of collagen deposited on the surface, or the ratio between the two, would shed light on how the cells are interacting with the collagen present. Likewise, a better understanding of how the films are formed, and what the interaction between the type I collagen base-layer and the type II collagen is would aide in the formation of other type II collagen surfaces. Finally, inclusion of other molecules present in the intervertebral disc, such as aggrecan, could have a multitude of effects and should be examined. Cell attachment of nucleus pulposus cells could potentially be improved, and changes in morphology and gene expression could likewise be observed.

The results for cell sorting shown here demonstrate the viability of this unique experimental setup. Increased number of trials could result in statistically significant differences of the observed trends. Alternatively, it could confirm that there are no differences in gene expression between the two cell types when cultured in monolayer.

Use of a three-dimensional culture surface, as well as using a second filter to more accurately separate the chondrocyte-like and nucleus pulposus cells, could also provide meaningful results.

APPENDIX

Annulus Fibrosus Cell area distributions

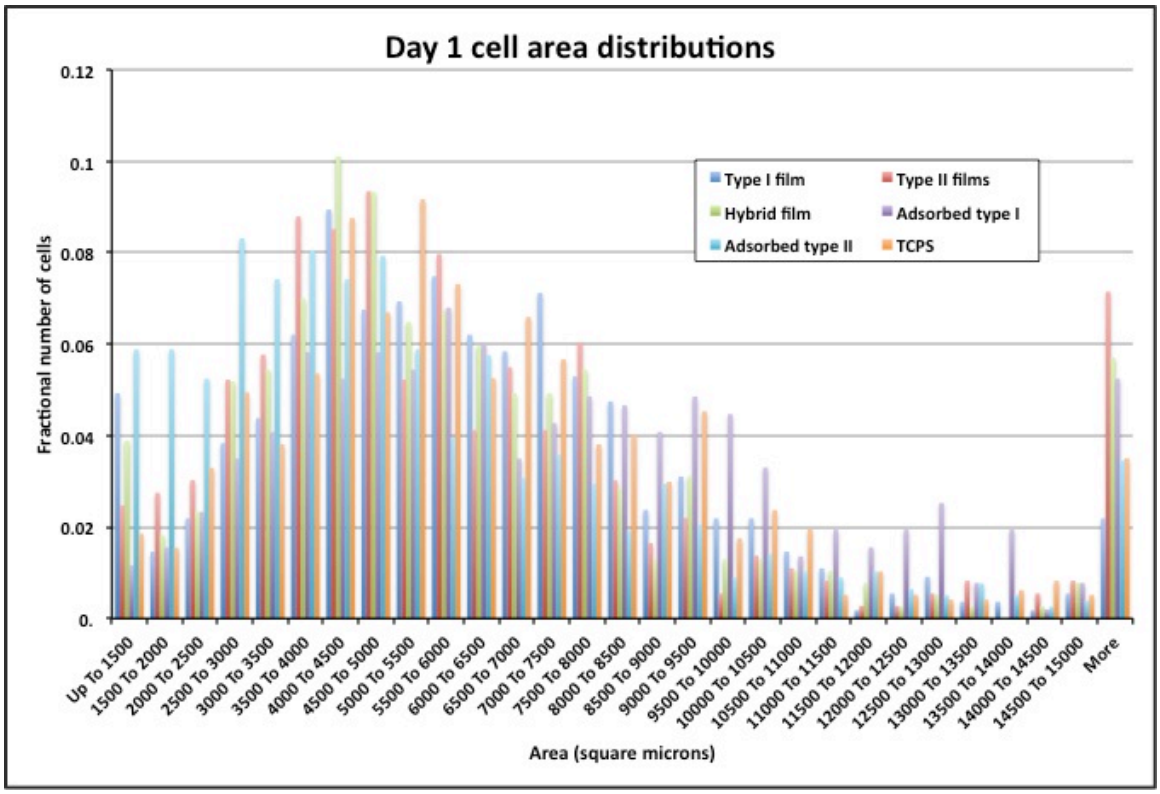


Figure 26: AF cell Day 1 area distributions

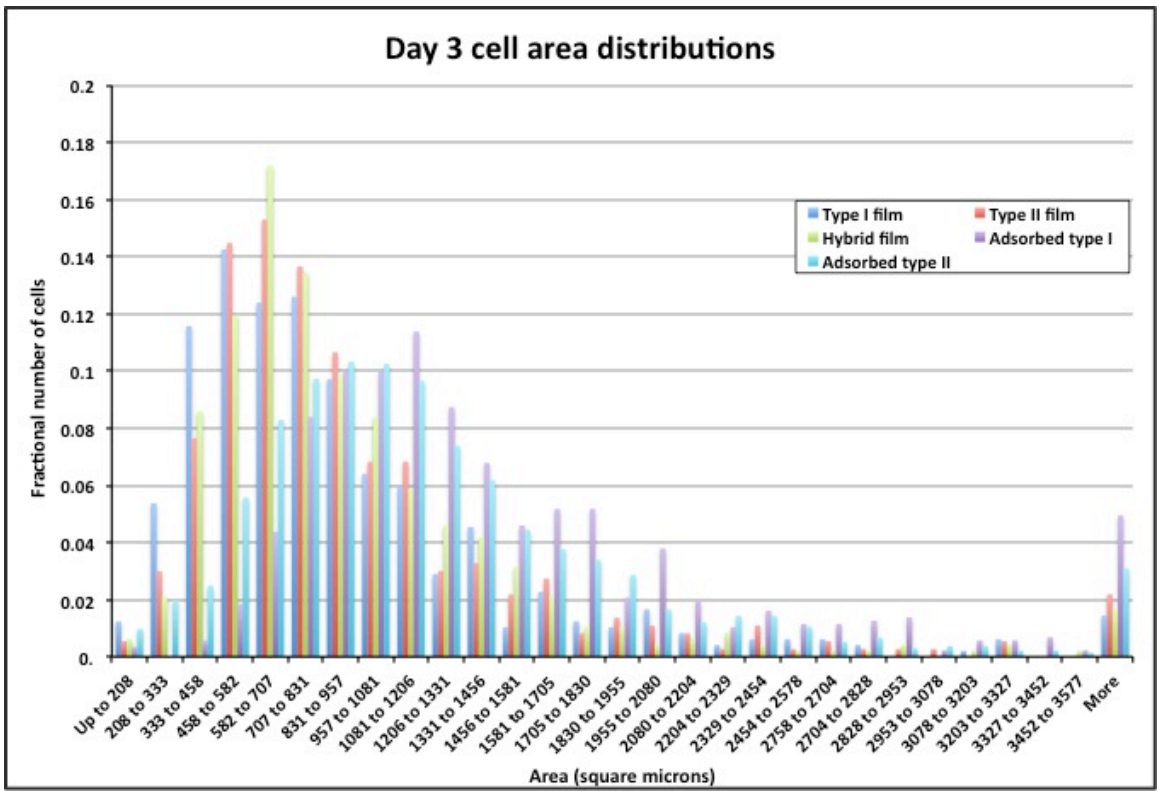


Figure 27: AF cell Day 3 area distributions

Nucleus pulposus area distributions

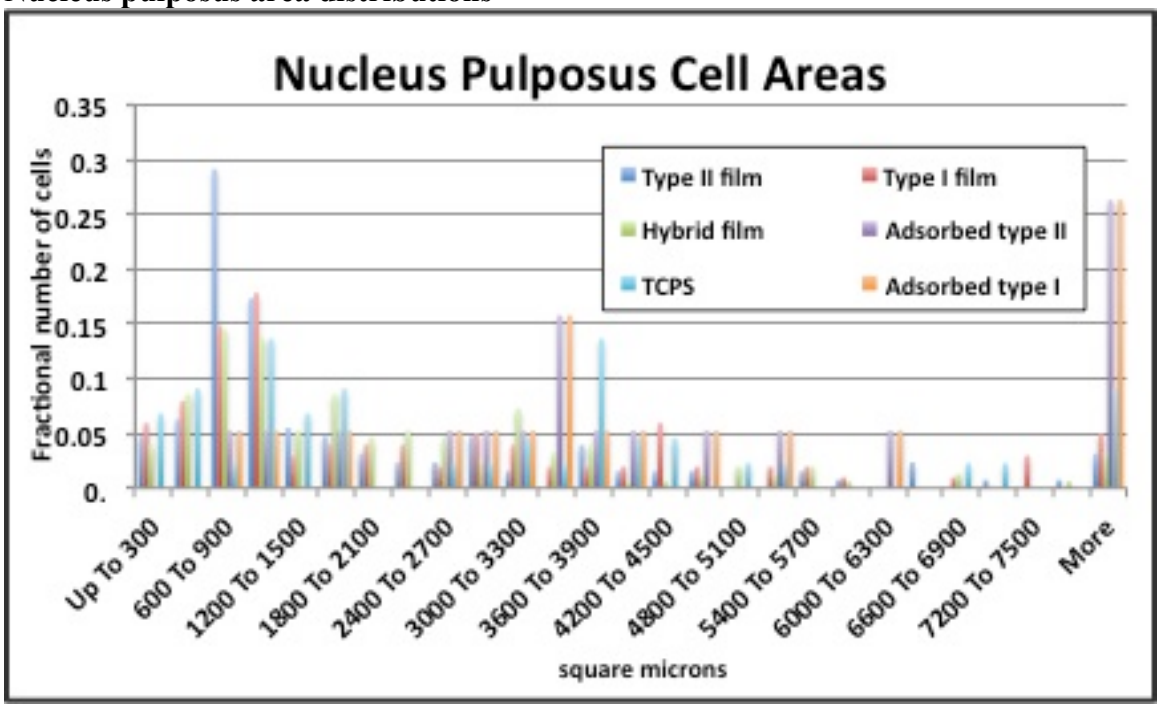


Figure 28: NP cell Day 3 area distributions

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