The Response of Annulus Fibrosus Cells to Fibronectin-Coated Nanofibrous Polyurethanes-Carbonate Anionic Dihydroxyoligomer Scaffolds

by

Menat Attia

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Department of Laboratory Medicine and Pathobiology
University of Toronto

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Department of Laboratory Medicine and Pathobiology
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2011

Abstract

Tissue engineering of the annulus fibrosus (AF) is challenging due to its complex lamellar structure. Polyurethane scaffolds have shown promise in AF tissue engineering. The current study examines whether matrix protein coatings (collagen type I, fibronectin, or vitronectin) would enhance cell attachment and promote cell and collagen orientation that more closely mimics native AF. The results demonstrate that the greatest cell attachment occurred with fibronectin (Fn)-coated scaffolds. Cells on Fn-coated scaffolds were also aligned parallel to scaffold fibers, a process that involved α5β1 integrin, determined by integrin-specific blocking antibodies. The inhibition of this integrin reduced AF cell spreading and alignment and the changes in cell shape were regulated by the actin cytoskeleton, demonstrated using cytochalasin D inhibitor. Cells on Fn-coated scaffolds formed fibrillar Fn, synthesized significantly more collagen, and showed alignment of type I collagen that more closely mimics native AF therefore facilitating the development of the tissue in vitro.
Acknowledgments

First and foremost, I would like to thank my supervisors, Doctor Rita Kandel and Professor Paul Santerre for their mentorship and encouragement over the course of this study. I would also like to thank my committee members, Professor Jonathan Rocheleau and Professor Marc Grynpas for their scientific input, time and efforts.

In addition, I would like to thank and acknowledge Dr. Meilin Yang for his dedication and countless hours spent producing the polymeric scaffolds, as well as Doug Holmyard and Robert Temkin for their help with scanning electron microscopy and Dr. John Georgiou for his help and contributions with confocal microscopy analysis.

A big thank you to Shu Qiu Li. Your support, patience and kindness is felt by all of us.

Thank you to all the members of the Kandel lab for their scientific input, but more so for all the great memories, laughs and endless support. You guys are amazing.

To my parents and brothers: I can’t thank you enough.

To my sister Shaimaa: I couldn’t have done this without you.
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<tr>
<td>ADO</td>
<td>Anionic dihydroxyloligomer</td>
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<tr>
<td>AF</td>
<td>Annulus fibrosus</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CF</td>
<td>Type I collagen + fibronectin</td>
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<tr>
<td>Coll</td>
<td>Type I collagen</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DDD</td>
<td>Degenerative disc disease</td>
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<tr>
<td>DMAC</td>
<td>N, N-dimethyl acetamide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EP</td>
<td>Cartilaginous endplate</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Fn</td>
<td>Fibronectin</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
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<tr>
<td>FA</td>
<td>Focal adhesions</td>
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<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>FB</td>
<td>Fibrillar adhesions</td>
</tr>
<tr>
<td>FX</td>
<td>Focal complexes</td>
</tr>
<tr>
<td>HEMA</td>
<td>Hydroxyethylmethacrylate</td>
</tr>
<tr>
<td>hMSC</td>
<td>Human Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>hTM</td>
<td>Human trabecular meshwork</td>
</tr>
<tr>
<td>IVD</td>
<td>Intervertebral disc</td>
</tr>
<tr>
<td>LBP</td>
<td>Low back pain</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>NC</td>
<td>Non-coated control</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleus pulposus</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycan</td>
</tr>
<tr>
<td>PU</td>
<td>Poly-carbonate urethane</td>
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<tr>
<td>PU-ADO</td>
<td>Poly-carbonate urethane + anionic dihydroxyoligomer</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartic acid peptide</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>S.E.M</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SFK</td>
<td>Src Family Kinase</td>
</tr>
<tr>
<td>Vn</td>
<td>Vitronectin</td>
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CHAPTER ONE: INTRODUCTION

1.1 Introduction

Low back pain (LBP) is a widespread and costly health care issue and accounts for a substantial number of work-related disabilities and significant losses in productivity in Western societies [1]. Although many factors are believed to cause LBP, symptomatic degenerative disc disease (DDD) appears to be the primary cause [2]. The limitations and lack of effectiveness associated with current treatment methods emphasize the need for the development of entirely new strategies for DDD treatment. Tissue engineering of the intervertebral disc (IVD) may serve as a viable alternative [2, 3].

1.1.1 The Intervertebral Disc

The human spine contains 23 intervertebral discs (IVDs). Bound axially to the vertebral bodies through the cartilaginous endplates, these structures provide flexibility to the spine and allow for various types of movement such as flexion, extension and rotation [4, 5].

The IVD is a heterogeneous structure with regional variations in extracellular matrix (ECM) composition, cell morphology and phenotypes. It is composed of three distinct tissues; the nucleus pulposus (NP), the annulus fibrosis (AF) and the cartilaginous endplates (EP) [3-5]. The development of the embryonic spinal column and the IVDs arise from the central notochord and the surrounding mesenchyme. The notochord, originating from the endoderm, ultimately gives rise to the NP, whereas the mesenchyme, of mesodermal origin, develops into the AF, the cartilaginous EPs and the vertebrae [4, 5]. During development, the notochord undergoes segmentation and disappears above and below the developing IVDs and is replaced by mesenchymal cells that ultimately form into the bone. The AF surrounds and encloses the NP while maintaining a distinct demarcation between its own collagen-rich structure and the gelatinous proteoglycan-rich NP. The cartilaginous endplates and the vertebral bodies
also develop from the mesenchyme [4]. Soon after birth, however, the abundance of notochordal cells in the NP decreases and is replaced by phenotypically “chondrocyte-like” cells of mesenchymal origin. Notochordal cell abundance continues to decrease with age and these cells are thought to be gone from the human disc by 10 years of age [4]. This loss of notochordal cells, in addition to an increase in proteoglycan content in the inner AF, results in a less pronounced NP-AF boundary [6]. By adulthood, all the cells of the human IVD are of mesenchymal origin, yet interestingly, are able to produce and maintain distinct ECMs capable of imparting different functions in the IVD [4] (Figure 1.1)

**Figure 1.1** The intervertebral disc unites adjacent vertebral bodies and serves two main functions: 1. To dissipate mechanical loads experienced by the spine and 2. To impart flexibility and allow for movement. Illustration adapted from http://neuro-chirurgie.org

As the IVD grows and an abundant ECM is synthesized, secreted and organized by the cells, the density of cells in the IVD also decreases overall, with cells eventually making up approximately 1% of the total volume of the disc [3, 5], thus making the mature IVD one of the most acellular tissues inside the body [4]. The production and maintenance of ECMs having different biochemical compositions and organization within the IVD is critical in that they promote structurally and functionally distinct regions within its overall structure, which allows them to work in concert to provide flexibility as well as to meet the demands of the mechanical loads constantly experienced by the spine [3].
The NP is made up of round, chondrocyte-like cells and is comprised of a random network of mostly type II collagen fibrils embedded in a proteoglycan- (PG)-rich hydrogel-like gelatinous matrix [2, 4, 7]. The NP is approximately 20% collagen and 50% PG by dry weight, with aggrecan being by far the most abundant PG on a weight basis [4, 8, 9]. In the mature disc, aggrecan is substituted with both chondroitin sulfate and keratin sulfate side chains. These side chains play a crucial role in NP function in that their associated negative charges contribute to the NP’s ability to imbibe water and consequently, contribute to the swelling pressure of the NP which is necessary for the resisting of compressive loads on the spine [4, 10]. In addition to aggrecan, other PGs found in the disc include versican, decorin, biglycan, fibromodulin, lumican and perlecan [4].

Conversely, the AF is a fibrous structure consisting of fibroblast-like cells in a highly organized structure comprised of concentric layers, or lamellae, of highly oriented collagen fibrils [4, 5, 11, 12]. Many AF lamellae wrap around and enclose the NP. Thus, although the NP can support approximately 70% of the compressive load exerted on the spine due to its water content and swelling pressure, it is the confinement of the NP within the AF that ultimately sustains this swelling pressure and balances the applied loads [13]. Therefore, the structure and composition of the AF gives it tensile-force bearing capabilities that allow it to resist and withstand the compressive forces that transmit outwards from the NP [3]. Degeneration of the disc changes the patterns of these applied loads and thus, alters its mechanobiology [14]. NP degeneration is characterized by a loss of water, making the NP smaller, decompressed, more fibrotic and less able to withstand compressive loads [15]. This results in more forces transferred away from the NP and towards the periphery, mainly to the AF [14, 15]. An increase in direct load to the AF, amongst other factors, causes the formation of AF fissures and tears [15]. These changes in loading pattern results in asymmetry of stress distribution which perpetuates the degenerative state and reduces overall load-bearing capabilities [14]. Furthermore, a recent study by Zhang et al. showed that AF cells may require tensile mechanical forces to survive, as it was observed that growing AF cells on softer substrates resulted in cellular apoptosis and accelerated disc degeneration [16]. In the In addition to this, alterations in disc kinematics also take place with a reduction
in range of motion, including extension, rotation and flexibility, for severe stages of disc degeneration [14, 17].

The cartilaginous EPs interface 90% of the NP and AF structure with the vertebral body [5]. They consist mostly of hyaline cartilage [18]. At birth, the EPs make up approximately 50% of the intervertebral disc and contain large vascular channels which provide the IVD with nutrition. By adulthood, the EP size is reduced so that it accounts for only 5% of the intervertebral space [11]. Moreover, the vascular channels within the EPs become filled with ECM shortly after birth and are essentially absent by the end of the first decade of life [5]. The relative avascularity and acellularity of the IVD [5, 19, 20] in conjunction with mechanical loading [21] and various other factors (discussed below) are believed to be the main contributors to the degeneration of the IVD and in the development of DDD [4].

1.1.2 Degenerative Disc Disease

An autopsy study showed that 97% of individuals 50 years of age or older had some form of DDD, involving both the NP and/or AF [22]. The onset of this degeneration is believed to begin as early as the second decade of life [23]. Although the definitive causes of DDD are unknown, several factors are believed to contribute to this degenerative process.

One contributing factor to the development of DDD is the lack of vascular supply in the adult IVD. A study of cadaveric human vertebrae demonstrated that the number of vascular channels perforating the osseous vertebral plate diminishes drastically between six and thirty months of age [24]. As a consequence of this avascularity, the IVD must receive nutrition via diffusion through the central permeable region of the EP, and to a lesser extent, from the blood vessels that surround the lateral edges of the AF [25, 26]. This feature contributes to the disc's limited capacity for self-repair [5, 27].

Excessive mechanical stress can also damage the IVD. Although mechanical loading can regulate matrix remodeling and compressive forces have been shown to favourably influence IVD cell metabolism and cell viability, the positive effects stemming from these
forces are ultimately dependent on the frequency and magnitude of the load [28, 29]. Intermittent loading within physiological ranges shows beneficial effects and this is believed to be partly due to enhanced nutritional supply and waste removal via fluid flow [30]. This results in increased proteoglycan content and reduction in metalloproteinase production. In contrast, proteoglycan content decreases and metalloproteinase activity increases under conditions of static compression outside the physiological range [30-36]. As such, adverse loading events may contribute to disc ECM degeneration.

Genetics also predispose to developing DDD [4, 15] and it is believed to be the strongest risk factor. This has been demonstrated by studying DDD in identical twins, which revealed that genetic inheritance accounts for 50% – 70% of the disc degeneration [37].

Cells at the centre of the IVD are 6mm to 8mm away from the nearest blood supply [1]. Being the largest avascular organ in the body [1, 33], cells rely on simple diffusion through the cartilaginous endplates for nutrients and waste exchange [10, 15]. With aging and degeneration, the cartilaginous endplates thicken and calcify [1]. As a consequence, oxygen tension at the centre of the disc is lower compared to cells at the periphery, and cells switch predominantly to glycolysis to meet energy demands, resulting in a high concentration of lactic acid and a low pH [8, 15], therefore perpetuating the degenerative process [38-41].

In some cases, the degenerative process is believed to begin with a decline in cellularity and proteoglycan loss in the NP resulting in the tissue’s diminished ability to retain water [1]. In the young healthy disc, the fibrous AF acts primarily like a tensile skin to contain the swelling of the hydrated NP. However, as the disc ages, loss of water content in the NP causes the AF to act as a fibrous solid to resist compressive forces directly [15]. Moreover, the remaining cells of the NP begin to fail to maintain the matrix and catabolic events supersede and contribute to the structural disorganization of the adjacent AF. This breakdown of AF architecture results in the formation of internal fissures and annular tears, which spread outward and further compromise disc function [42]. There are three types of tears found in the annulus: circumferential tears, peripheral rim tears and radial fissures. Both circumferential tears and peripheral rim
tears are related to disc trauma. Radial fissures, however, are associated with DDD and the degeneration of the NP [43, 44]. Whether degeneration resulting from this process begins within the NP is not known and it has been argued that the degeneration of the AF precedes that of the NP [43, 44]. The development of radial fissures may result in the migration of the NP and in such cases, the disc is considered to be herniated or prolapsed if it extends beyond the AF. Disc prolapse has been stimulated via the bending and compression of cadaveric discs with loading above physiological limits or with repetitive loading [45-48] and it occurs most readily in discs of individuals 30 - 40 years of age and thus is believed to be due to the onset of a weakened AF by this age [46]. Other factors contributing to DDD include loss of notochordal cells [4], repetitive heavy lifting and environmental factors such as smoking [15].

Current treatment options for DDD include conservative management (such as medication and physiotherapy to alleviate pain) or surgical intervention which include interbody fusion or total disc replacement. Interbody fusion induces ossification of vertebral bodies and may alleviate pain, however, long-term observation shows degeneration of adjacent spinal segments and altered biomechanics in humans [49] and has also been confirmed in animal models [50]. The damaged disc can also be removed and replaced with a synthetic prosthesis. However, wear debris and loosening with time are known to occur and pose other challenges especially given the location near major blood vessels and nerves [51]. The focus of these treatments is symptomatic relief by removing the degenerated tissue but none of them aim to prevent or treat the underlying problems that lead to DDD. Therefore, developing a regenerative medicine approach to treating IVD degeneration would be ideal as it shows much promise in that it functions to promote the repair and regeneration of the disc. Also the tissue can remodel in response to mechanical loading [2, 3]. Successful tissue engineering of degenerated NP is ultimately dependent upon the presence of healthy AF tissue capable of withstanding the swelling pressure exerted by the restored NP. Conversely, if the AF itself is compromised, tissue engineering of an in vitro NP-AF composite is necessary [3]. It is the proper functioning of AF tissue that allows the disc to withstand normal loads experienced by the spine and is thus critical to IVD function [3, 52].
1.1.3  *Annulus Fibrosus*

The outer AF is a highly fibrous tissue that encloses the NP and acts as a link between vertebral bodies [53, 54]. It is comprised of 15-25 lamellae primarily made up of type I collagen fibrils [3] (Figure 1.2). The collagen fibrils that comprise the lamellae are at a 60° angle to the vertical and alternate at this same angle between adjacent lamellae. This allows them to slide past one another and imparts some degree of deformation to the overall structure of the AF [53]. The AF is also a radially non-uniform tissue, having very little proteoglycan content and consisting predominantly of type I collagen, the content of which is greatest in the outermost lamellae and decreases as it proceeds toward the innermost lamellae adjacent to the NP. In contrast, type II collagen is the main collagen found in the NP and gradually decreases in abundance from the NP outwards, and is lowest in the outermost AF lamellae. The AF is approximately 60% water and 60-70% collagen by dry weight, and the relative proportions of type I collagen to type II vary from 70:30 for the innermost lamellae to 85:15 for the outermost lamellae [4]. Other collagens present in the AF include collagens type V and XI which form hybrid fibrils with collagen types I and II [4, 55]. Collagens XII and XIV have also been found to reside on the surfaces of type I collagen [4]. Proteoglycans of the AF typically make up less than 15% by dry weight, with aggrecan being the most predominant proteoglycan in the IVD [2]. The high type I collagen content of the AF is typical of tension resisting tissues whereas the type II collagen found in the NP is characteristic of compression resisting tissues [54]. The inner AF experiences both types of forces and thus has structural and compositional characteristics of both tissues [56]. The structure of the AF is however more intricate than is typically described. For example, the interlamellar septae of the inner annulus contain proteoglycan aggregates capable of imbibing water in much the same way as the NP. In contrast to this, the proteoglycans found in the outer lamellar layers are comprised of proteoglycan monomers which are believed to interact with and modulate the behaviour of the collagen fibril network [57]. As mentioned previously, aggrecan is the most abundant proteoglycan in both the NP and AF [4]. Furthermore, veriscan is found in all regions of the IVD throughout life, and increases in the AF in adults [4]. Lastly, the disc also contains several members of the leucine-rich repeat family of proteoglycans which are characterized by their ability to
interact with collagen in the AF; the most important of which are decorin and biglycan [4, 58, 59]. Moreover, elastin in the form of elastic fibres, is also found in the AF running parallel to collagen fibrils in the lamellae. Although it is present in low concentrations, elastin’s specialized location allows the AF to recoil and helps restore its shape following deformation. This is in contrast to elastin’s association as a loose network with type II collagen in the NP [4].

**Figure 1.2** The annulus fibrosis surrounds the nucleus pulposus. The collagen-rich and highly organized structure of this tissue gives it the tensile strength capable of withstanding the forces brought on by the swelling of the nucleus pulposus. Illustration adapted from http://bonesubstitute.org.

In addition to variations in AF ECM components and amounts, different cell phenotypes have also been identified in various regions of the AF, specifically the inner and outer AF [60]. In a histological study by Bruehlmann et al., cells having a fusiform shape within the lamellae of the 20% outermost region of the AF had cellular processes that extended along the collagen fibres as well as made contact with adjacent cell processes. These processes are believed to be characteristic of tensile bearing tissues capable of mechanical sensing of the surrounding environment [61]. The inner AF, which accounted for 50% of the radial thickness of the annulus in this study, contained cells that were spherical in shape having few short processes. Interspersed amongst
these cells were large bodied cells having long extensive processes that assumed no
directional preference and made little contact with adjacent cells; these cells were
mostly confined in the region next to the NP. The boundary in between the inner and
outer AF contained both fusiform and spherical cells. The third cellular phenotype
characterized in the AF was identified in the interlamellar space (or interlamellar
septae). These cells assumed a rounded, spherical morphology and had distinct broad,
flat processes with multiple branching sites and formed a lace-like pattern [60]. The
variation in cellular phenotypes, in addition to the AF’s highly organized and fibrous
structure, endows it with great tensile strength.

1.2 Tissue Engineering of the Annulus Fibrosis

Due to the limitations and challenges associated with current DDD treatments, there is a
growing consensus that the current methods are not effective and that there is a need to
develop an entirely new approach for treating DDD. Tissue engineering appears to be a
good alternative for treating this disease as it promotes tissue regeneration. This was
demonstrated for example, by studies showing that injection of NP cells or stem cells,
showed a reduction in the rate of degeneration in experimental models of disc
degeneration [62, 63]. In light of this, however, the transplantation of NP tissue, in
contrast to NP cells, was better able to delay the development of DDD and suggests
that treatment using tissues may be more effective than a cell-based approach to tissue
regeneration [64]. This is highlighted further via studies in which the transplantation of
IVD grafts has been successful in animal models [65, 66] and in humans [67]. Since the
use of autographs is not possible and allografts are limited by their availability and their
associated risk of transmitting disease, the application of tissue engineering techniques
for the development of an entire IVD construct in vitro is particularly desirable.

Tissue engineering of a functional IVD is dependent on three fundamental components:
the cell source, the scaffold, and molecular, biological or mechanical signals [3].
Choosing an appropriate scaffold is of particular importance to IVD tissue engineering in
that it may help to provide mechanical properties such as weight-bearing capabilities, as
well as present cells with biochemical, molecular and topographical cues to help guide
and modulate the growth of in vitro tissue [68]. Although attempts at tissue engineering of NP have been successful [69], tissue engineering of AF has been proven to be more challenging due to the highly oriented and complex nature of the tissue. To date, various scaffolds have been evaluated for AF tissue engineering such as polyglycolic acid / polylactic acid based materials [70], collagen-hyaluronan [71], PDLLA/45S5 Bioglass ® composite films [72] and alginate materials [73] and limited success has been shown with all of them. Polylactides and polyglycolides for example form acidic degradation products, decreasing the local pH which has been shown to alter cell metabolism and slow the rate of ECM synthesis [74]. To overcome this, a poly-carbonate urethane scaffold containing an anionic dihydroxyloligomer (PU-ADO) that possesses several properties that render it suitable for AF tissue engineering has been developed [75].

1.2.1  **Poly-Carbone Urethane Scaffold**

The biocompatible and biodegradable characteristics of polyurethanes have made them desirable materials for the fabrication of implant components that directly contact biological systems, such as pacemaker insulators, vascular grafts and most recently, soft tissue engineering scaffolds [76-79]. Briefly, polyurethanes are segmented polymers composed of a repeating urethane linkage and alternating hard (crystalline) and soft (elastic) segments and as such, the ratios of these segments can be varied in such a way that many different polyurethane polymers can be made and tailored for their intended uses. This chemistry can be tailored to yield degradable polymers with well defined degeneration products [80].

In the Santerre lab, an elastomeric poly-carbonate urethane scaffold (PU), which incorporates carbonate bonds, has been developed [81]. The incorporation of carbonate bonds has the advantage of producing, non-toxic degradation products, primarily soluble diols and CO₂, upon in vivo degradation. Furthermore, the polar character of PUs can be modified by incorporating oligomeric additives into the polymer which contain polar chemical groups (i.e. hydroxyl, ionic groups, etc.) and that are able to migrate and populate the surface of the biomaterial [82]. This is particularly important in
that it is the surface of a material that determines its initial biocompatibility since it is able to direct protein adsorption and consequently affect cell attachment and the metabolic response of cells following their attachment [75]. It is commonly accepted that cell attachment is the single most important aspect of cell interaction with a biomaterial and that this process begins with the adsorption of proteins on the material, followed by cell attachment, spreading, proliferation and tissue formation [83]. Furthermore, cellular behaviour and shape can be modulated according to the specific types of proteins that are adsorbed to the scaffold [84, 85]. The Kandel-Santerre labs have developed a modified PU containing an anionic dihydroxyoligomeric (ADO) additive to form PU-ADO and this polymer can be electrospun [79] in order to fabricate aligned nanofibrous PU-ADO scaffolds having a highly oriented structure resembling the ECM of the native AF lamellae. Previous studies using aligned PU-ADO, have demonstrated that at an optimal ADO concentration of 0.5 wt/wt%, AF cell attachment was enhanced and the cells produced more collagen. It was demonstrated that these observations were directly related to the adsorption of proteins both from the serum and from newly synthesized proteins by AF cells [75]. In light of these findings, the work contained herein was undertaken in order to investigate the influence of adhesion protein(s) (specifically type I collagen, fibronectin and vitronectin) on AF cells when the former proteins were pre-coated onto PU-ADO. The nature of this interaction will be evaluated with the ultimate goal of defining optimal conditions for the generation of aligned AF tissue in vitro.

1.2.2 Adhesion Proteins for Annulus Fibrosus Tissue Engineering on Polyurethane Anionic Dihydroxyoligomer Scaffold

Extracellular matrix proteins mediate initial cell attachment and spreading and are well known to provide biological cues and modulate the behaviour of adherent cells. In most cases, ECM proteins bind to the biomaterial surfaces in a non-specific manner when these materials are exposed to in vivo biological environments. In contrast, many tissue engineering strategies utilize a biomimetic approach for design, in which biomaterial surfaces are modified in a controlled manner through the immobilization of intact biomacromolecules, such as collagen, fibronectin or vitronectin, or short peptide
sequences, such as the signature integrin-binding RGD peptide sequences, in order to control/modulate cell behaviour [84]. There are various methods of incorporating these molecules onto biomaterials, however, surface adsorption is the simplest approach to forming favourable adhesive interfaces between cells and tissue engineering scaffolds [85].

1.2.3 **Type I Collagen**

Collagens are synthesized by cells in the form of procollagen monomers having a triple helical structure and contain the characteristic hydroxyproline amino acid resulting from post-translational modifications of the protein [86]. The formation of a fibril collagen network provides the basis of the ECM found in various soft and hard connective tissues such as bone, skin, and muscle. It is a highly organized, three-dimensional network surrounding cells, and thus plays an important role in maintaining the structural and biological integrity of these tissues [87]. To date, 28 collagens have been identified with collagens I, II, III and V constituting the majority of the ECM of bone, cartilage, AF, tendon, skin and muscle [87]. Furthermore, of all the collagens, type I collagen (ColI) is the most fibrous form and makes up approximately 85% of the collagen synthesized by fibroblasts [88]. It plays important roles in embryogenesis, wound healing, tumour invasion and cardiovascular disease [89]. Although the mechanism of Coll fibrillogenesis has yet to be fully elucidated, it is generally understood that the mechanism is highly dependent on an essential step involving the removal of C and N terminal peptides from procollagen bundles resulting in a large decrease in the solubility of their helical region, allowing for the spontaneous self-assembly of Coll fibrils [90]. Fibrillar Coll is also amongst the most important proteins capable of withstanding mechanical stress and stain in the body [91]. Of particular importance however is the organization of parallel and highly oriented Coll fibrils within lamellar structures of which there exists two great examples of this in the body; the cornea and the outer AF of the IVD [53]. It has been shown by others that the modification of various nanofibrous polymeric and gel-based scaffolds using collagen is able to promote cell differentiation and guide tissue formation for various cell types [86, 92, 93].
1.2.4  Fibronectin

Fibronectin (Fn) is a multifunctional glycoprotein found in plasma and ECM, and is synthesized by many different cell types as a protein dimer consisting of similar or identical subunits of 220-250 kDa joined by a disulphide bond [94, 95]. Furthermore, it can be present in the soluble form in the circulation or as an insoluble, multimeric fibrillar form located in the ECM [96]. The fibrillar form of Fn is assembled into an extensive Fn fibrillar matrix which plays a crucial role in cell adhesion, embryogenesis, migration, and wound healing [97-101]. The assembly of this fibrillar structure is a dynamic, cell-driven process which is dependent on the interaction of Fn with integrins on the cell surface, the actin cytoskeleton (discussed below) and the forces generated from cell contraction [96]. Briefly, the process of Fn matrix assembly, known as Fn fibrillogenesis, begins with the α5β1 integrin interacting with Fn dimers in the ECM through the RGD recognition sequence on the protein. This integrin-ligand engagement allows for the subsequent unfolding of soluble Fn dimers allowing for the exposure of cryptic binding sites necessary for self-association and Fn fibre assembly. The forces necessary for the unfolding of Fn dimers is brought on by sufficient tension generated through cell contractility and the actin cytoskeleton [89, 96, 102, 103]. This unfolding also exposes several other cryptic sites on Fn, including collagen-binding sites allowing for anchorage of the Fn matrix to the underlying ECM [104] making it an essential component in tissue function, and in many cases is known to play a key role in matrix dynamics during embryonic development [105].

In light of its known in vivo functions, many tissue engineering techniques have modified scaffold surfaces by coating with Fn in order to influence cell interactions with biomaterials, typically for the enhancement of cell attachment, and this has been done for various cell types and scaffolds [106-111]. Interestingly, Hayes et al., showed that the embryonic development of the rat AF was closely associated with the organization of Fn found on the cell surface at the E16 time-point. This assembly of Fn fibrils in the developing rat AF lamellae coincided with the re-organization of the actin cytoskeleton
and the α5β1 integrins and these events preceded the oriented deposition of type I collagen in the lamellae [53].

1.2.5 *Vitronectin*

Vitronectin (Vn) is a multifunctional adhesive glycoprotein present in both blood and the ECM [112]. It has a molecular weight of 75kDa and exists as either a single chain protein or as an endogenously clipped form with both chains held together by a disulfide bond [83]. It is capable of binding several molecules in the blood and the ECM, including collagen, and is able to bind to cells via the αvβ3 integrin receptor and as such, has been shown to play important roles in cell attachment, spreading and migration [112]. Although no studies thus far describe specific roles for Vn in the AF, other tissue engineering applications have modified scaffolds by incorporating Vn to promote cell attachment and drive cellular differentiation [113-115]. For example, in comparison to Coll and Fn, Vn resulted in greater adherence of human osteoblast-like (HOS) cells under mechanical strain [113]. The presence of Vn also directed the constitutive expression of osteopontin suggesting that it may promote survival under strain in these cells [113].

1.3 **Annulus Fibrosus Cell Interaction with Adhesion Proteins**

Cell adhesion to the ECM is a fundamental requirement for normal embryonic development, adult homeostasis and immune function. Depending on the cell type and the specific tissue, the multi-component cellular structures that mediate cellular attachment can take on a number of different forms and their protein composition, localization and proteolytic function allow for their classification [116]. Approximately 150 proteins are known to participate in cell adhesion providing local signaling environments, mechanical stability and structural support for tractional movement [117]. In particular, the integrins are one of the best characterized cell surface receptors and participate in a number of physiological roles within the body [118].
1.3.1 **Integrins and Adhesion Complexes**

Integrins are the main family of transmembrane cellular receptors that mediate direct contact with the ECM and participate in a number of biological processes such as cell proliferation, suppression of apoptosis, cell migration, embryogenesis and homeostasis [119]. A functional integrin is composed of non-covalently bound α and β subunits. At present, 18 α and 8 β subunits have been characterized in humans, existing in 24 unique heterodimeric combinations [120]. Each subunit has a large extracellular domain (>700 residues) at the N-terminal, a transmembrane region comprised of a single helix of variable lengths and a cytoplasmic domain (13 to 70 residues) [121]. It is understood that prior to making contact with the ECM, both subunits are in an inactive conformation, with the extracellular domain of the integrin assuming a bent conformation, and both cytoplasmic domains bound to each other. Upon ligand binding, a conformational change takes place resulting in extracellular domain extension and separation of the cytoplasmic domains allowing for recruitment of intracellular structural and signaling proteins resulting in the formation of stable contacts between the external environment and the cell interior [116, 119].

Integrins are believed to have an important role in AF tissue function and homeostasis, as the expression of various integrin subunits by porcine and human AF cells has been demonstrated by others [120, 122]. In a study by Nettles et al., histological examination revealed similar levels of expression of α5, β1, β3 and β5 subunits in both the porcine and human AF. Moreover, porcine AF expressed more α1 and β4 subunits, and human AF tissue showed positive staining for αv and β6 whereas porcine AF did not. The expression of the α5 and β1 subunits is of particular importance in that it forms the α5β1 heterodimer, the primary fibronectin integrin [122]. This integrin has also been shown to undergo reorganization on the surfaces of fibroblasts during AF development in the rat [53]. The α5β1 integrin also mediates cell-specific responses to mechanical forces from the ECM and is capable of switching from a relaxed and tension state resulting from the contraction of the actin cytoskeleton [123]. The ability of AF cells to sense and respond to mechanical forces is crucial to the tensile force-bearing capabilities of the tissue and suggests a potentially important role of the α5β1 integrin for the proper function of AF tissue. For example, it was observed that chondrocytes in cartilage were unable to
regulate their morphology and ECM synthesis in response to mechanical shear forces when the α5β1 integrin was blocked [124]. Cartilage is a specialized force-bearing tissue much like the AF of the IVD and thus, α5β1 may serve an equally important role in the AF.

Integrin-ligand binding and activation is able to induce various cell responses through the formation of stable complexes with the ECM. These complexes involve integrins, intracellular linker proteins, various kinases and the actin cytoskeleton and are formed from the maturation of initial integrin-ECM contacts. Their variations in composition allow for the tailoring of signaling activities and cellular functions in response to changes in ECM environment and composition [116]. Adhesion complexes have been characterized based on compositional and morphological differences and three main types exist: focal complexes (FX), focal adhesions (FA) and fibrillar adhesions (FB) [116]. Focal complexes are small, transient structures associated with the formation of lamellopodia and filopodia and are usually seen behind the leading edge of spreading or migrating cells and are believed to “sample” the local ECM environment prior to the formation of stable contacts [116]. Focal adhesions are distinguished as larger complexes, some arising from the maturation of FXs [116]. They are located along the base and periphery of the cell and contain many adapter proteins that bind the actin cytoskeleton allowing for mechanical stability and enabling tractional forces to be transmitted from the cell to the ECM and vice versa [116, 125]. Fibrillar adhesions are classified as a subset of FAs and are long, stable structures that run parallel to bundles of Fn fibrils in vivo [116, 125]. These structures are distinguished by being rich in tensin (a cytosolic adapter protein that binds to the actin cytoskeleton) [116, 125], and the Fn integrin α5β1, and are sites of localized matrix deposition and Fn fibrillogenesis [102, 126].

Although distinct adhesion complexes have been described, varying in composition and stability, the events leading up to the assembly and sequential recruitment of adhesion-associated proteins are relatively common amongst them. Integrin activation, following ECM ligand binding, results in a conformational change of the cytoplasmic tail and consequently, interaction with intracellular proteins. For this activation to be complete, the conformational change of the β subunit must be assisted by the binding of talin, a
cytoskeletal protein [127]. This conformational change triggers the clustering of activated integrin receptors and allows for a complex hierarchal recruitment of proteins and sets the stage for future focal adhesion assembly [128]. Constituents of a focal adhesion are not all able to bind integrins and thus a hierarchal chain is established whereby integrin-binding proteins act as linkers that bind integrins directly and link them to other proteins. The formation of these adhesion sites is thus dependent on protein conformation, binding motifs and signaling domains [116].

1.3.2 Integrins and the Actin Cytoskeleton

The actin cytoskeleton plays essential roles in cell function, controlling cell contractility necessary for sustained cell spreading and for generating and transmitting mechanical forces, as well as forms the actin structures necessary for cell migration such as lamellipodea and filopodia. To perform these functions, the actin cytoskeleton is linked to the ECM through integrins [129]. Integrins are believed to make a diverse number of connections with the actin cytoskeleton which differ for cell types as well as for different regions of the cell [119]. Many integrin-associated accessory proteins such as talin, filamin, α-actinin, vinculin and tensin have actin-binding sites; however, whether these domains primarily function to bind to actin or to other proteins remains unclear [119]. Two main mechanisms of integrin binding to actin are believed to exist: the capture of existing actin filaments and the nucleation of new actin filaments. The mechanism of actin capture is consistent with the actin-binding abilities of integrin-associated proteins and has been demonstrated in cells that develop elaborate actin cytoskeletons, such as muscle cells [130]. Integrins may also promote the synthesis of new actin filaments and this is suggested by the requirement of nucleation proteins such as Arp2/3 [131] and has been demonstrated in a study in which isolated integrin complexes were able to nucleate actin on the surface of magnetic beads [132].

As a direct link between the ECM and the actin cytoskeleton, integrins are able to transmit external forces to cells, and vice versa, cells are able to generate forces onto their external environment. The concept of force transmission being capable of altering integrin-actin associations is demonstrated by the maturation of focal complexes into
focal adhesions upon force application and mechanical stimulation [133]. Furthermore, FBs and Fn matrix assembly depends on forces generated by cells [102]. The ability of AF cells to sense and respond to mechanical forces is essential to its function and as such, underscores the importance of actin organization in this tissue. Furthermore, actin stress fibres, a specialized structure of the actin cytoskeleton, have been shown to play important roles in the orientation and elongation of fibroblasts in the developing rat AF and the subsequent deposition of the oriented collagen [53].

Actin stress fibres are typically formed from the bundling of 10-30 actin filaments [134] which are held together by various actin cross-linking proteins, most notable of which is α-actinin [135]. The staining pattern of α-actinin shows periodic band patterning alternating between non-muscle myosin and tropomyosin in arrays analogous to muscle sarcomere organization [136]. Three categories of actin stress fibres exist, based on their localization within cells: ventral stress fibres, dorsal stress fibres and traverse arcs [137]. Ventral stress fibres are the most common and are situated along the base of the cell and are connected to FAs on either end. Dorsal stress fibres are attached to FAs at only one end at the base of the cell, with the remainder of the structure rising towards the dorsal surface and diffusing into a loose network of actin filaments. In contrast, traverse arcs are short actin bundles that lie beneath the dorsal face in areas of lamelllopoidea and filopodia formation in migrating cells. Traverse are more transient structures, disassembling and reassembling during migration and do not form attachments with FAs [135]. Stress fibre assembly and dynamics is linked to Rho family of small GTPases, RhoA, RhoB and RhoC, with the majority of pathways focused on RhoA which appears to be the major regulator of stress fibre formation under many physiological conditions [138]. Furthermore, although the dynamic role of actin stress fibres in cell migration has been well documented, variations of their structure make some stress fibres less suited for motility and more for static contraction. These static stress fibres have well-defined roles in generating contractile forces in tissues and a number of specialized cells are able to do this. Static contraction such as this promotes cell polarity and orientation and plays pivotal roles in the reorganization and remodeling of the ECM during development and in wound healing [105]. It may also aid cells to withstand mechanical forces. For example, cells of the vasculature form stress fibres...
and undergo static contraction when exposed to hydrostatic pressure from fluid shear forces and cyclic stress [135].

1.4 **Hypothesis**

Due to the improved cell attachment and subsequent increase in tissue collagen synthesis resulting from serum protein adsorption to PU-ADO observed previously, the hypothesis of this study is that pre-coating nanofibrous aligned PU-ADO scaffolds with specific ECM proteins known to be involved in cell adhesion (type I collagen and/or fibronectin and/or vitronectin) will enhance annulus fibrosus cell attachment, direct cell shape toward alignment with the scaffold, and increase collagen production and orientation.

1.5 **Objectives**

1. To develop a cell culture system to study AF cell interaction with PU-ADO.

2. To determine the adhesion protein(s) that promote enhanced cell attachment and AF cell orientation and alignment.

3. To investigate the role of integrins in the interaction of AF cells with the adhesion protein pre-coated onto PU-ADO.

4. To evaluate the production and organization of collagen by AF cells on adhesion protein pre-coated PU-ADO.
1.6 References


CHAPTER TWO: MANUSCRIPT
The Response of Annulus Fibrosus Cell to Fibronectin-coated Nanofibrous Polyurethane-Anionic Dihydroxyoligomer Scaffolds

Menat Attia¹, 4, J. Paul Santerre MD, PhD³, Rita A. Kandel MD³, 4

CIHR-BioEngineering of Skeletal Tissues Team¹, Department of Pathology and Laboratory Medicine², Mount Sinai Hospital, Institute of Biomaterials and Biomedical Engineering³, and Department of Laboratory Medicine and Pathobiology⁴, University of Toronto, Ontario, Canada,

Keywords: Annulus fibrosus, nanofibrous scaffolds, polyurethane, intervertebral disc, tissue engineering

Manuscript Accepted to Biomaterials, October 2010
2.1 Abstract

Tissue engineering of the annulus fibrosus (AF), a component of the intervertebral disc, has proven to be challenging due to its complex oriented lamellar structure. Previously it was demonstrated that poly-urethane (PU) scaffolds containing an anionic dihydroxyoligomers (ADO) may be suitable to use in this application. The current study examines whether matrix protein(s) coatings (type I collagen, type I collagen and fibronectin, fibronectin, or vitronectin) would promote cell and collagen orientation that more closely mimics native AF. The greatest cell attachment occurred when scaffolds were pre-coated with fibronectin (Fn). Cells on Fn-coated scaffolds were aligned parallel to scaffold fibers, a process that involved α5β1 integrin, as determined by integrin-specific blocking antibodies, which in turn reduced AF cell spreading and alignment. Cell shape was regulated by the actin cytoskeleton as cells grown in the presence of cytochalasin D did not spread. Cells on Fn-coated PU-ADO scaffolds formed fibrillar Fn, synthesized significantly more collagen, and showed linear alignment of the secreted type I collagen when compared to cells grown on the other protein-coated scaffolds and the non-coated control. Thus Fn-coating of PU-ADO scaffolds appears to promote properly oriented AF cells and collagen, which should facilitate developing AF tissue that more closely mimics the native tissue.
2.2 Introduction

The intervertebral disc (IVDs) imparts flexibility to the spine, allowing for movement and dissipation of mechanical loads[1, 2]. It is a heterogeneous structure composed of three distinct tissues; the inner proteoglycan (PG)-rich nucleus pulposus (NP), the outer collagen-rich annulus fibrosus (AF) and the cartilage endplates (EP). The AF is a highly organized tissue comprised of layers of collagenous lamellae having a specific angle of orientation of 60° to the vertical that alternates with successive lamellae. This fibrous tissue has tensile strength and functions to withstand the forces exerted by the swelling of the NP[3]. The confinement of the NP by the AF allows the IVD to sustain the compressive loads experienced during the activities of daily living[4]. Structural breakdown of one or more of these tissues compromises IVD function and may contribute to the onset of degenerative disc disease (DDD)[2, 5, 6].

The prevalence of DDD is very high as demonstrated in an autopsy study which demonstrated that 97% of individuals 50 years of age or older have some form of the disorder[7]. Although the causes of DDD remain unknown, genetics[1, 6, 8], a decline in cellularity[1], loss of notochordal cells[2, 5], mechanical stress[9-12] and calcification of the cartilage endplate[2, 13, 14] have all been implicated in the pathogenesis of this disorder. There are various treatment options for DDD, ranging from conservative management (medication and physiotherapy), to surgical intervention such as interbody fusion or total disc replacement. However, these have limited success with the potential for side effects[15, 16]. For these reasons, there is a need to develop novel therapies for the treatment of chronic symptomatic DDD. Tissue engineering an IVD suitable to use as a disc replacement is one approach that shows much promise in that it functions to promote tissue repair and/or regeneration[3, 5, 17].
Although it is possible to generate NP tissue[18], tissue engineering of the AF has proven to be more challenging due to its highly oriented lamellar structure. Various polymeric scaffolds have been used to try to accomplish this, including polyglycolic acid / polylactic acid based materials[19], collagen-hyaluronan[20], PDLLA/45S5 Bioglass ® composite films[21], and alginate materials[22], but all show limited success. For example, polylactides and polyglycolides form acidic degradation products resulting in reduced local pH and decreased extracellular matrix synthesis[23]. More recently, others have developed aligned structures using collagen[24] and polycaprolactone[25]. Poly-carbonate urethane (PU) is also a good scaffold candidate as it is elastic, biodegradable, biocompatible, non-toxic to AF cells and can be electrospun to resemble the structure and orientation of native AF lamellae[26-28]. Modifying the PU by combining it with an anionic / dihydroxyl oligomeric additive (PU-ADO) results in a higher surface energy and enhanced cell attachment compared to standard PU scaffolds[28]. The anionic dihydroxy oligomers (ADO) promoted the adsorption of protein from serum and newly synthesized protein by the AF cells in order to enhance cell adhesion to the scaffolds and showed that this may be important in enhancing AF tissue formation[28].

Although the proteins involved in modulating AF cell attachment to PU-ADO is unknown, it has been estimated that approximately 150 proteins can participate in cell adhesion[29]. Integrins are commonly involved in mediating cell attachment and also interact with actin to direct cell shape[30-32]. Studies have shown that specific integrin subunits, such as α5, αv, and β1 and β3, are expressed by porcine and human AF cells[33, 34]. Interestingly, integrin activation and consequently changes to the actin cytoskeleton appear to be important for the deposition of oriented collagen as shown in studies of the developing rat IVD[35]. This raises questions in regards to the role of integrins in AF tissue formation. In this study, we sought to determine whether pre-coating PU-ADO with extracellular matrix proteins known to be involved in
mediating cell attachment, such as fibronectin, type I collagen, or vitronectin, will influence AF cell attachment, shape and collagen production and orientation. An understanding of these early molecular events with the PU-ADO scaffolds will facilitate developing organized AF tissue in vitro that mimics the native tissue.

2.3 Materials and Methods

2.3.1 Poly-carbonate urethane synthesis and fabrication

PU-ADO was prepared as described previously[28]. Briefly, the base polymer was synthesized from the reaction of poly(1,6-hexyl 1,2-ethyl carbonate)diol, 1,6-hexane diisocyanate and 1,4-butane diol in N, N-dimethylacetamide solvent at a temperature between 60° and 70°C. The ADO additive was synthesized by reacting polytetramethylene oxide, hydroxyethylmethacrylate (HEMA) and lysine diisocyanate in N, N-dimethyl acetamide (DMAC) solvent overnight in a temperature range of 50°C – 60°C, followed by hydrolysis of the lysine and HEMA ester groups to form carboxylate and hydroxyl groups. ADO was added to an optimal concentration of 0.5 weight% relative to the PU[28]. Nano-fibrous scaffolds were fabricated by electrospinning the PU-ADO onto a rotating mandrel at 1200 RPM with an applied voltage of 20,000 volts. Scaffold fibre thickness and orientation was evaluated by scanning electron microscopy (SEM) prior to use[36]. To confine the cells to the scaffold (and prevent spill over), an 8mm diameter scaffold was generated using a biopsy punch. It was placed over the top of a 200μL microfuge tube which had been modified to form a culture vessel by cutting off the end of the tube. The scaffold was held in place by the rim of the cap of the microfuge tube (the inner aspect of the cap had been excised) and sterilized by gamma radiation (4MRad) for 48 hours.
2.3.2 **Annulus fibrosus cell isolation**

Bovine caudal spines from 6 - 9 months old calves were obtained from a local abattoir. Intervertebral discs (IVDs) were harvested aseptically and placed in serum-free Dulbecco’s Modified Eagle’s Medium (DMEM) containing 1% antibiotic cocktail (penicillin G, Streptomycin sulphate, and amphotericin B; Invitrogen Co., Auckland, NZ). The discs from up to two tails were combined to get significant cells for each experiment. The outer annulus fibrosus, avoiding inclusion of inner AF and NP, was harvested, placed in DMEM, and cut into pieces less than 5mm$^3$. Cells were released from the tissue by sequential enzymatic digestion, 0.5% protease (Sigma Chemical Co., Saint Louis, MO) for 1-2 hours at 37°C, followed by 0.2% collagenase (Roche Molecular Biochemicals, Indianapolis, IN) overnight at 37°C. Cells were then filtered to remove undigested tissue, pelleted by centrifugation for 8 minutes at 600 RCF (Allegra 21 Centrifuge, Beckman Co.) and cell number quantified. Primary AF cells (P0) were placed in monolayer culture (density of 10,000 cells / cm$^2$) in DMEM media containing 5% fetal bovine serum (FBS). Media was changed every two days and the cells were passaged at approximately 80% confluency. These passaged (P1) cells were used for all experiments. The cells (50µL aliquots, 50,000 cells/scaffold) were seeded on the aligned nanofibrous scaffold which had been placed in a tissue culture well (24-well plate) containing high glucose serum-free DMEM (500µL) prior to seeding. Cells were grown in serum-free DMEM under standard culture conditions.

2.3.3 **Pre-coating poly-carbonate urethane with adhesion proteins**

The PU-ADO scaffolds were coated with either collagen type I (Coll), fibronectin alone (Fn), a combination of collagen type I and fibronectin (CF), or vitronectin (Vn). Briefly, Coll was prepared by dissolving calf skin collagen (0.5mg/mL, Sigma, Saint Louis, MO) in 0.1N sterile acetic acid for two days at 4°C with shaking. 50µL of this solution was placed on the scaffold
and the acetic acid was left to evaporate overnight in a tissue culture hood with airflow maintained. The scaffold was then washed 3 times with phosphate buffered saline (PBS). Fn and Vn were prepared at a final concentration of 10µg/mL in sterile PBS and 50µL was used to coat the scaffold overnight at 4°C, as previously described[34]. Following this, the scaffold was washed three times with PBS. Coated and non-coated scaffolds were then washed with 2% sterile bovine serum albumin (BSA in PBS, Sigma, Saint Louis, MO) for 2 hours at 37°C, 5% CO₂[34]. In addition, non-coated (NC), no BSA, scaffolds served as a negative control. All scaffolds were washed 3 times in PBS with a final wash in serum-free DMEM prior to AF cell seeding.

2.3.4 Annulus fibrosus cell attachment

To evaluate cell attachment, AF cells were seeded serum-free onto the different scaffolds and after 6 hours, the scaffolds were harvested and subjected to papain digestion (Sigma, Saint Louis, MO; 40µg/L, 20 mmol/mL ammonium acetate, 1mmol/L EDTA and 2 mmol/L dithiothreitol) for 24 hours at 65°C. The DNA content of each sample was determined using the Hoechst 33258 dye binding assay (Polysciences) followed by fluorometric analysis (emission at 458 nm, excitation at 365 nm) as previously described[28]. The data was expressed as a percentage relative to the DNA content of the seeding aliquot.

2.3.5 Scanning electron microscopy (SEM)

SEM was used to visualize the scaffolds. Scaffolds, either as made or 6 hours post-seeding, were gently washed three times in PBS and fixed in 2% glutaraldehyde. Samples were then dehydrated using an ethanol gradient up to 100%, subjected to critical point drying (Bal-Tec, CPD 030) and sputter coated with gold (Denton Vaccum, Moorestown, NJ; Desk II). Cells were
visualized by secondary electron imaging under scanning electron microscopy (SEM) (Philips model FEI XL30 ESEM).

SEM micrographs were imported into Image J software, for semi-quantification of AF cell size. The software scale bar was calibrated according to the image scale bar and AF cell area was determined by tracing the cell outline and calculating the area. Approximately 50 cells in direct contact with the scaffold were measured per sample. All experimental groups were repeated in duplicate and three separate experiments were performed.

2.3.6 **Assessment of the actin cytoskeleton**

To visualize the actin cytoskeleton, the samples (6 hours after cell seeding) were harvested, washed 3 times in PBS (containing Ca$^{2+}$ and Mg$^{2+}$, PBS $+\div$) and fixed in 4% paraformdehyde (wt/vol in PBS) overnight at 4°C. Samples were then washed 3 times with PBS $+\div$, permeabilized using 1% Triton X (Biorad, in PBS) for 15 minutes at room temperature with gentle shaking. The actin cytoskeleton was stained with phalloidin-rhodamine (Alexa-fluor 568, Invitrogen, Eugene, OR; diluted 1:20 in 1% Triton X solution) in the dark at room temperature for 20 minutes. Samples were washed 3 times with 1% Triton X, mounted onto slides, coverslipped using Permafluor Mountant solution (anti-fade Immunon, Pittsburgh), and stored in the dark at 4°C until undergoing confocal imaging. Images were collected with a Leica Zeiss confocal laser-scanning microscope system (Oberkochen, Germany) at 40x and 60x objective power (HCX PL APO 63.0/1.32 oil immersion lense and 40.0/1.25 oil immersion lense, respectively). Emission was detected at a range of 560nm to 650nm and samples were collected at a single representative plane using a z-axis increment of 0.3µm at a resolution of 512 x 512 pixel units. Photomultiplier gain and black level settings were held constant between repetitions.
In selected experiments, cytochalasin D (c8273, Sigma-Aldrich, Saint Louis, MO, 10 μM final concentration) was added to the cell suspension immediately before seeding onto the scaffold in order to prevent actin filament formation.

2.3.7 Quantification of collagen synthesis

To assess collagen synthesis, $^3$H-proline (1μCi / scaffold) was added 6 hours after seeding and the scaffold harvested 24 hours later. Cultures were washed in PBS and papain digested as described above. To measure the amount of collagen released into the media, 667μL of media from each well was precipitated on ice with saturated ammonium sulphate (500μL), added drop-wise with frequent gentle mixing, followed by gentle rotation at 4°C for one hour and left static overnight at 4°C. The samples were centrifuged at 14,000 RPM for 30 minutes at 4°C, and the pellet was washed 3 times with 1mL of cold 70% ethanol, dried at room temperature for 1 hour, and then re-suspended in 100μL of 10% SDS (in dH$_2$O). The amount of newly synthesized collagen was quantified using a β-scintillation counter (Beckman). Total collagen synthesis was quantified by combining the cpm obtained from the papain digest and the media. Three constructs for each condition were pooled to achieve sufficient counts. The amount of collagen synthesized was expressed per μg DNA.

2.3.8 Analysis of integrin expression by annulus fibrosus cells

To measure the expression of integrins known to bind to the ECM proteins utilized in this study, cell-seeded scaffolds were harvested 6 hours post-seeding, washed gently in PBS +/+ and fixed in 4% paraformaldehyde at 4°C overnight. The cells were then washed 3 times in PBS +/+ , followed by 1% TritonX for 15 minutes at room temperature with gentle shaking, blocked with 5% goat serum (Sigma, Saint Louis, MO) and 3% BSA (Sigma, Saint Louis, MO) in PBS +/+ for 1 hour at 37°C and incubated with the individual integrin antibodies α5β1 (clone JBS5,
Millipore, αvβ3 (clone LM609, Chemicon) and β1 (clone 4B4, Beckman Coulter) (all 1:100 dilution in blocking solution) either overnight at 4°C or 1 hour at room temperature. Samples were washed 3 times for 5 minutes in 1% TritonX (PBS) with gentle shaking. The cells were stained with phalloidin-rhodamine as described above, washed 3 times for 5 minutes in 1% TritonX and incubated with Alexa-488 conjugated secondary anti-mouse IgG1 antibody (Invitrogen, Eugene, OR; 1:300 dilution in blocking solution) for 45 minutes at room temperature in the dark followed by 3 washes in 1% TritonX and mounted onto slides and imaged using confocal microscopy as described in section 2.3.6 with Alexa-488 signal detected at an emission range of 540nm to 600nm.

To examine the role of integrins in cell attachment to the scaffolds, AF cells were pre-incubated with integrin blocking antibodies (same antibodies to α5β1, αvβ3 and β1 as used above) or an IgG antibody control (IgG1, Beckman Coulter) for 1 hour at 4°C with gentle shaking and then seeded as described above in the presence of the antibody on either ColI or Fn coated PU-ADO scaffolds. Samples were harvested after 6 hours and cell morphology analyzed by SEM and the actin cytoskeleton visualized by confocal microscopy as described in section 2.3.6.

2.3.9 Extracellular matrix collagen and fibronectin distribution and organization

To assess the organization of collagen and fibronectin in the extracellular matrix, AF cells were seeded on scaffolds in DMEM supplemented with 5% FBS and harvested either 6 or 48 hours post-seeding. For the 48hr cultures, the media was changed at 24 hours to DMEM supplemented with 5% FBS and ascorbic acid (100µg/mL final concentration). At the time of harvest, the constructs were washed in PBS +/- and fixed in 4% paraformaldehyde overnight at 4°C. Samples were incubated with antibody reactive with collagen type I (Human anti-rabbit collagen type I, Gentaur, 1:200 dilution in blocking solution) or fibronectin (Clone Fn-15, Sigma, Saint
Louis, MO). Immunoreactivity was detected using anti-rabbit Alexa-488 (Invitrogen; 1:300 in blocking solution) for collagen type I and anti-mouse Alexa-488 (Invitrogen; 1:300 in blocking solution) for fibronectin and imaged using confocal microscopy, as described in section 2.3.6.

2.3.10 Statistical analysis

All experiments were repeated at least three times and each condition was done in either duplicate or triplicate. The data from individual experiments were combined and results expressed as the mean ± standard error of the mean (S.E.M). Differences for which more than two variables were compared were evaluated using a one-way analysis of variance (one-way ANOVA) followed by a Tukey post-hoc test. For comparing two data groups a Student’s T-test was used. Significance was assigned at p < 0.05.

2.4 Results

Annulus fibrosus cell attachment on pre-coated PU-ADO

As shown in Figure 2.1, pre-coating PU-ADO with ECM proteins (ColI, Fn, a combination of ColI and Fn (CF) and Vn) resulted in a significant increase in AF cell attachment to the scaffold after 6 hours as compared to the non-coated (NC) control (31 ± 2.7 %) (p<0.05) or the BSA-coated control (7 ± 2.0 %) (p<0.005). Amongst the various protein coatings, Fn resulted in significantly greater (p<0.05) cell attachment as compared to all other conditions (Fn: 73 ± 5.6 %; ColI: 51 ± 3.4 %; CF: 54 ± 2.5 %; Vn: 53 ± 5.7 %).

Annulus fibrosus cell shape on pre-coated scaffolds

By 6 hours after seeding marked differences in AF cell shape and orientation were observed amongst the cells on the different coated scaffolds (Figure. 2.2A). Scaffolds pre-coated with
collagen type I only (ColI) or collagen type I in combination with fibronectin (CF) resulted in similar AF morphology in which cells were spread and formed cellular processes that extended in all directions. These cells showed no orientation relative to the scaffold fibres. There appeared to be more spread cells (with fewer round cells) on the CF/PU-ADO as compared to the ColI/PU-ADO. In contrast, cells on Fn-coated scaffolds were elongated and oriented parallel to scaffold fibres. Vn-coated scaffolds showed a mixture of cells that remained round or slightly spread with some orientation parallel to the PU-ADO fibres. AF cells remained round and showed no orientation on the non-coated (NC) and bovine serum albumin (BSA) coated controls.

Cell spreading was semi-quantified by measuring AF cell area for each condition (Figure 2.2B). Cells on pre-coated scaffolds (ColI, CF, Fn and Vn) all had significantly greater AF cell size as compared to cells on the NC control (121 ± 6.9 µm²) (p<0.05) and the BSA control (93 ± 2.3 µm²) (p<0.005). Cell areas on ColI (387 ± 26.6 µm²) and CF (515 ± 41.2 µm²) coated scaffolds were not significantly different from each other. AF cells on Fn-coated scaffolds exhibited the largest cell size (834 ± 12.2 µm²) and were significantly greater as compared to all other conditions (p<0.005). Cells on Vn-coated scaffolds were significantly smaller than cells on CF (p<0.05).

**Pre-coated PU-ADO scaffolds and actin cytoskeleton organization in annulus fibrosus cells**

Due to the differences in cell morphology, the organization of the actin cytoskeleton was analyzed. Cells grown on ColI and CF-coated scaffolds showed similar actin organization (Figure 2.3). Phalloidin staining showed cortical actin as well as bundles of actin, characteristic of stress fibres throughout the cytoplasm. Cells on Fn-coated scaffolds appeared to have the
greatest amount of actin, as depicted by the strong staining seen throughout the cytoplasm as compared to cells on other pre-coated scaffolds. These cells also assumed the highest degree of cytoskeleton alignment with the orientation of stress fibres parallel to scaffold fibres. Cells on Vn-coated scaffolds had a very different cytoskeleton arrangement. Although actin was present diffusely throughout the cytoplasm, there was no stress fibre formation. Cells on scaffolds that were not coated (NC) or coated with BSA were similar in appearance, showing cortical actin distribution; no stress fibres were seen. As coating the scaffolds with Fn resulted in cellular orientation that mimics that of the in vivo tissue, cells grown on those scaffolds were examined further and compared to cells on Coll coated scaffolds which did not show this orientation.

**Actin cytoskeleton and annulus fibrosus cell shape**

To confirm the role of actin in regulating cell shape, the cells were treated with cytochalasin D, an inhibitor of actin polymerization. In the presence of this drug, cells failed to spread on both Coll and Fn pre-coated PU-ADO, and assumed a round morphology (Figure 2.4A). The actin was punctate; no cortical actin nor stress fibres were observed in these treated cells (Figure 2.4B).

**Integrins and annulus fibrosus cell orientation**

As seen in Figure 2.5, cells grown on Fn-coated PU-ADO showed relatively greater expression of α5β1 integrin as compared to AF cells on Coll-coated scaffolds as indicated by the amount of staining. Cells on both Fn and Coll-coated scaffold showed similarly low levels of αvβ3 expression.

Integrins appear to be involved in the interaction of AF cells with Coll and Fn-coated PU-ADO. Blocking α5β1 integrin when cells were seeded onto Coll-coated scaffolds (Figure 2.6A) resulted
in slightly less spreading as cells appeared rounded or if spread had fewer processes when compared to the IgG-treated control. The cells showed less orientation of the actin cytoskeleton. Blocking αvβ3 integrin resulted in more spreading as cells were more flattened and had fewer cellular processes in contrast to IgG and α5β1 treated samples. There was less actin alignment. Blocking both α5β1 and αvβ3 resulted in the least amount of cell spreading with loss of actin cytoskeleton organization. Blocking the β1 subunit also eliminated cell spreading and actin orientation for these cells.

For Fn-coated scaffolds (Figure 2.6B), blocking the α5β1 integrin resulted in loss of cell alignment and more cells appeared round. In the spread cells actin alignment was seen, but in the less spread and round cells, this was lost. Blocking αvβ3 integrin had the least effect, with cells remaining spread (although appearing slightly flattened as compared to the IgG control) and the actin network was oriented and aligned. Blocking both α5β1 and αvβ3 appeared to be more effective in preventing cell spreading as more cells were round as compared to blocking α5β1 alone. There was a loss of actin orientation as well in these treated cells. When the β1 integrin subunit only was blocked, the cells appeared similar to blocking α5β1, although more cells appeared round under the latter condition. The actin cytoskeleton showed a cortical distribution in the round cells and stress fibers in the spread cells.

**Collagen and fibronectin synthesis and organization by annulus fibrosus cells**

In light of the distinct morphologies and actin organization of cells on ColI and Fn-coated scaffolds, the synthesis of collagen by these cells was evaluated. Cells on Fn-coated scaffolds accumulated significantly more newly synthesized collagen (p<0.005) (Fn = 5787 ± 250 cpm /µg of DNA) as compared to cells on the other scaffolds (ColI = 2667 ± 112 cpm /µg of DNA; NC = 3757 ± 414 cpm /µg of DNA; BSA = 3521 ± 234 cpm /µg of DNA) (Figure 2.7A). As well these
cells synthesized significantly (p<0.01) more total collagen (Figure 2.7B) (Fn = 6287 ± 213 cpm/µg DNA; Coll = 3009 ± 114 cpm/µg DNA; NC = 4175 ± 470 cpm/µg DNA; BSA = 4087 ± 338 cpm/µg DNA).

Confocal microscopy confirmed the increased accumulation of type I collagen by 6 hours on Fn-coated PU-ADO compared to Coll-coated PU-ADO (Figure 2.8). This was also evident at the 48 hour time point. The collagen was predominately intracellular at the 6 hr early time point. By 48 hours, the collagen remained localized inside AF cells on Coll-coated scaffolds in contrast to cells on Fn-coated scaffolds in which extracellular deposition of collagen type I oriented parallel to the AF cells was observed (Figure 2.8).

As fibronectin matrix assembly has been shown to precede the organized deposition of collagen in embryonic rat AF[35], the cultures were examined for the presence of Fn. Cells on Fn-coated scaffolds showed both punctuate and fibrillar distribution of Fn as early as 6 hours post seeding. In some cells the linear Fn connected with the actin cytoskeleton as indicated by yellow staining (indicating overlap of stain). When the Fn-coated scaffold (without cells) was stained with the antibody reactive with Fn no punctuate/linear staining was observed (data not shown). The cells on the Coll-coated scaffolds demonstrated predominantly punctuate distribution of Fn (Figure 2.9).

2.5 Discussion

The current study used extracellular protein(s) such as collagen type I, alone or in combination with fibronectin, fibronectin alone, or vitronectin (all of which are known to influence the adhesion of different cell types) to examine the regulation of AF cell and collagen orientation on
PU-ADO scaffolds. The data suggests that Fn plays a pivotal role in influencing the AF cell under the culture conditions studied. The greatest cell attachment occurred when the scaffolds were pre-coated with Fn. Fn also influenced cell spreading and alignment, as cells on these coated scaffolds were aligned parallel to scaffold fibers. Cell orientation involved β1 integrins and in particular the α5β1 integrin, as blocking these integrins, using specific antibodies, reduced AF cell spreading and alignment but not cell attachment. This response was specific to α5β1, as blocking the ανβ3 integrin had no effect on cell spreading. Cell shape was also regulated by the actin cytoskeleton as growing the cells in the presence of cytochalasin D prevented cell spreading on Fn-coated scaffolds. In addition, cells on the Fn-coated scaffolds formed fibrillar Fn, synthesized significantly more collagen, and showed linear alignment of the secreted type I collagen. Thus, specific adhesion onto PU-ADO scaffolds appears to be critical to facilitating properly oriented AF cells and both collagen synthesis and its structural orientation.

Fibronectin is a multifunctional glycoprotein present in plasma in a soluble form and in the ECM. It is expressed by many cell types[28, 37, 38] and contributes to cell adhesion, migration, proliferation and tissue development[39-43]. In light of its known in vivo functions, many investigators have developed tissue engineering techniques which involve coating scaffold surfaces with Fn or RGD peptides in order to influence cell interactions with biomaterials[44-49]. For example Meinel et al. showed that coating a silk fibroin scaffold with Fn resulted in rapid cell spreading of human Mesenchymal Stem Cells (hMSCs) on electrospun random and aligned scaffolds, with cell orientation along silk fibres achieved using only the aligned scaffold[45]. There is little known about the effect of Fn-coating of scaffolds on AF cells. However the effects of Fn on AF cell shape and orientation observed in the current study are in line with its role during the development of the rat AF lamellae in vivo[35, 50]. In a study by Hayes et al.[35], the growth of the outer AF and the elongation of fibroblast sheets at the E16
time-point and beyond was shown to coincide with the formation of actin stress fibres and presence of α5β1 integrin (the primary Fn integrin). The stress fibres appeared to direct the assembly of cell-surface Fn into a fibrillar Fn matrix, characterized by long streaks of Fn which co-localized with sub-membranous actin stress fibres. Interestingly these events preceded the organized deposition of collagen type I in the lamellae, similar to that observed in the current study. Fn fibrillogenesis is a cell-driven process dependent on capturing and moving soluble Fn on the cell surface through the α5β1 integrin and the subsequent unfolding of soluble Fn dimers in order to expose cryptic binding sites necessary for self-association and Fn fibre assembly. The unfolding of dimers is brought on by sufficient tension generated through cell contractility [51-54]. Fn fibrillogenesis has been documented in many cell types and is a key player in matrix remodeling during development[55]. It is yet to be elucidated whether the process of Fn fibrillogenesis in this PU-ADO tissue engineering system involves newly synthesized Fn or the reassembly of the Fn used to pre-coat the scaffold or a combination of both.

Bovine annulus fibrosus cells express α5β1 integrins and a small number of αvβ3 integrins when cultured on PU-ADO scaffolds. These integrins have been detected in AF cells within native discs by others [33, 34]. Human and porcine AF cells both express α1, α5, β1, β3, β4, and β5 integrin subunits; although human AF cells expressed lower levels of α1 and β4 than porcine AF. As well, there are species differences as human AF cells expressed αv and β6 whereas porcine AF did not. It is not known whether bovine AF cells in vivo express these integrins or whether the integrin expression was influenced by the scaffold on which they were grown relative to native AF tissue. Observed differences in the types of integrins expressed, specifically, the higher expression of α5β1 on Fn-coated PU-ADO raises the possibility that there has been up-regulation of this integrin in the presence of its preferred primary ligand, fibronectin[34]. Integrin expression profiles have been shown to vary depending on the ligand present in other
cell types[56, 57] and this in part may have contributed to increased cell attachment on Fn-coated scaffolds.

Blocking integrins α5β1 and αvβ3 (also capable of binding Fn)[33] and β1 did not appear to inhibit cell attachment. These findings are consistent with that reported by Gilchrist et al. for αvβ3 integrin, but contrary to those for β1. In their study, blocking αvβ3 did not affect porcine AF cell attachment to the Fn-coated tissue culture plates, in contrast, β1 blocking resulted in complete inhibition of AF attachment to the plates[34]. These differences may be due to several factors. Firstly, the longer cell culture time used for this study (6 hours) versus that used by Gilchrist et al. (1 hour), could have provided AF cells with sufficient time to express integrins on their surface and compensate for the blocking. Secondly, differences in the expression profiles for integrins on human and porcine cells[33] suggest that species differences may account for the discrepancy. Thirdly, it has been demonstrated that surface chemistry of the PU-ADO alone is capable of mediating AF cell attachment independent of integrin-adhesion protein interactions[28]. It is also possible that AF cell attachment to PU-ADO, when integrins are blocked, may occur via attachments through molecules other than integrins. CD44, for example, a major receptor for hyaluronan which is present during embryonic development[58] may be one such mechanism. This concept of establishing “soft contacts” as initial adhesions is utilized by other cells such as chondrocytes[29] and A6 epithelial cells[59]. This attachment occurs within seconds [59]. As such, the greater cell attachment observed for AF cells on Fn-coated PU-ADO and the ability of AF cells to attach despite integrin blocking suggests that AF cells may use a mixture of adhesion molecules and various mechanisms for attachment to the PU-ADO scaffold, but that the α5β1 integrin is important for cell and collagen alignment.
A greater reduction in cell spreading was observed when both α5β1 and αvβ3 integrins were blocked as compared to blocking α5β1 engagement only for cells on Fn-coated scaffolds. This is consistent with findings by Charo et. al., who showed that α5β1 and αvβ3 work in concert to mediate spreading of melanoma cells on Fn[60]. However, it must be noted that some AF cells were not affected by the blocking antibody(s). There are several possible explanations. It may be that some degree of cell spreading on Fn pre-coated PU-ADO in the presence of a single blocking antibody may be due to integrin-independent mechanisms, which have been shown to occur in other cell types[61-63]. For example, Zhang et al. showed that depletion of talin (by deleting the talin1 gene), an integrin-actin linking protein[64], resulted in incomplete integrin activation upon Fn binding and consequently inhibited actomyosin contractility and force-dependent activation events[65] dependent on stress fibre formation and contraction[66]. However, these cells showed some initial spreading which was regulated by Src Focal Kinase (SFK)-linked signaling[65]. Alternatively, it has been shown that not only ligand specificity, but also ligand density and spatial organization on the substrate impacts cell behaviour and may be responsible for the relative insensitivity of some cells to the integrin blocking antibody(s)[67-69]. Specifically, in a study by Cavalcanti-Adam et al., inter-ligand spacing ≥73nm resulted in reduced cell spreading and a failure to assemble focal adhesions and stress fibres[70]. As such, the observations in the current study could be attributed to the expression of new, unblocked integrins on the surface of AF cells and the spacing of relatively fewer integrins capable of binding Fn on the scaffold, consequently affecting cell behaviour and spreading. Finally, it may not be possible to block all integrin engagement under the conditions used. Further studies will be undertaken to differentiate between these possibilities.

Growing AF cells on Fn-coated PU-ADO resulted in significantly greater collagen synthesis and accumulation than those grown on Coll-coated scaffolds. There are several possible explanations
for this. It may be due in part to AF cell spreading and orientation as cells on Coll-coated scaffolds did not exhibit this alignment and accumulated less collagen. Previous studies from our lab[71] and others[72, 73] indicate that cell shape appears to be correlated to matrix production. Spiteri et al showed that chondrocyte spreading can regulate matrix synthesis and retention. Furthermore, this effect has been shown to be mediated through α5β1 integrin[74]. Alternatively, it may be that the formation of fibrillar Fn by the AF cells may have contributed to this enhanced collagen accumulation. Fibrillar Fn has been shown to be a site of localized matrix deposition[30] and contributes to collagen type I fibril assembly[75, 76]. The organization and maintenance of collagen type I fibrils have been shown to require the presence of a fibrillar Fn matrix[77, 78].

2.6 Conclusion

In summary pre-coating aligned PU-ADO scaffolds with Fn provides molecular and topographical cues that allow AF cells to orient themselves parallel to scaffold fibres. This is mediated in part by α5β1 integrins and results in the formation of Fn fibrils and the subsequent deposition of aligned collagen type I. These findings support that Fn-coated PU-ADO aligned nanofibers provide an optimal scaffold construct to engineer AF tissue, in vitro. Additional long term studies are being planned to further evaluate tissue formation.

2.7 Acknowledgements

This research was supported by CIHR (M0P8672). MA was supported by an NSERC CGS scholarship. The authors would also like to thank Harry Bojarski and Ryding-Regency Meat Packers for providing bovine tissues and Dr. Meilin Yang for the synthesis of the scaffolds.
2.8 Figure images and captions

**Figure 2.1** Annulus fibrosus cells were seeded on scaffolds either non-coated (NC) or coated with bovine serum albumin (BSA) or the adhesion proteins (Collagen Type I, Coll; Collagen Type + Fibronectin, CF; Fibronectin, Fn; Vitronectin, Vn) in serum-free media. Cells were harvested after 6 hours and cell attachment quantified. DNA are reported as a percentage of initially seeded cells. * Statistically significant (p<0.01) as compared to BSA samples, # significant (p<0.05) as compared to NC samples and ‡ significant (p<0.05) as compared to all conditions. Results are expressed as mean ± SEM (n=3 experiments, each done in triplicate).

**Figure 2.2** Annulus fibrosus cells were seeded on scaffolds either non-coated (NC) or coated with bovine serum albumin (BSA) or different adhesion proteins (Collagen type I, Coll; Collagen Type I + Fibronectin, CF; Fibronectin, Fn; Vitronectin, Vn) in serum-free media. After 6 hours, cell morphology was analyzed by SEM (A). Cell size was semi-quantified from SEM micrographs (B). * Denotes significance (p<0.005) as compared to NC and BSA, # denotes significance (p<0.05) as compared to Coll and Vn and ‡ denotes significance (p<0.005) as compared to all conditions. Results are expressed as mean ± SEM (n=3 separate experiments, each condition done in duplicate).

**Figure 2.3** Annulus fibrosus cells were seeded on either non-coated (NC) or pre-coated scaffolds (bovine serum albumin, BSA); Collagen type I, Coll; Collagen Type I + Fibronectin, CF; Fibronectin, Fn; Vitronectin, Vn in serum-free media. After 6 hours, cells were stained for actin (red) and analyzed by confocal microscopy. Images were taken at a single representative plane. Scale bars represent 50µm; inset represents 2X magnification.

**Figure 2.4** Annulus fibrosus cells in the presence or absence of cytochalasin D were seeded on scaffolds coated with Collagen Type I (Coll) or Fibronectin (Fn) under serum-free conditions. Cells were harvested after 6 hours and either stained for actin (red) and analyzed by confocal microscopy (A) or processed for SEM (B). Confocal images were taken at a single representative plane. Scale bars represent 50µm.

**Figure 2.5** Annulus fibrosus cells were seeded on scaffolds pre-coated with Collagen Type I (Coll) or Fibronectin (Fn) under serum-free conditions. After 6 hours, cells were co-stained for integrins α5β1, αvβ3 or β1 (green) and actin (red) and analyzed by confocal microscopy. Yellow signal represents co-localization of components. Images were taken at a single representative plane. Scale bars represent 10µm.
**Figure 2.6**  Annulus fibrosus cells were seeded in the presence of integrin blocking antibodies α5β1, αvβ3, α5β1 + αvβ3 or β1 or IgG as a negative control on PU-ADO scaffolds either pre-coated with Collagen Type I (Coll) (A) or Fibronectin (Fn) (B) in serum-free media. Cells were harvested after 6 hours and stained for actin (red) and analyzed by confocal microscopy (top panels) or processed and analyzed by SEM (bottom panels). Confocal images were taken at a single representative plane. Scale bars represent 50 µm.

**Figure 2.7**  Annulus fibrosus cells were seeded on scaffolds that were either non-coated (NC) or coated with bovine serum albumin (BSA), Collagen Type I (Coll) or Fibronectin (Fn) in DMEM media containing 5% fetal bovine serum. After 6 hours, 3H-proline was added and cultures harvested 24 hours later. The amount of newly synthesized collagen accumulated (A) and total amount synthesized (B) was quantified and and normalized to DNA. * Denotes statistically significant (p<0.05) as compared to all conditions. Results are expressed as mean ± SEM (n=3 separate experiments, each condition done in duplicate).

**Figure 2.8**  Annulus fibrosus cells were seeded on scaffolds pre-coated with Collagen Type I (Coll) or Fibronectin (Fn) and harvested after 6 or 48 hours. Samples were stained for actin (red) and collagen type I (green) and analyzed by confocal microscopy. Images were taken at one representative plane. Collagen fibril formation is indicated by a white arrow. Scale bars represent 20 µm.

**Figure 2.9**  Annulus fibrosus cells were seeded on scaffold pre-coated with either Collagen Type I (Coll) or Fibronectin (Fn). Samples were harvested after 6 hours and stained for actin (red) and fibronectin (green) and analyzed by confocal microscopy. Yellow signal represents co-localization of components. Images were taken at one representative plane. Scale bars represent 10 µm.
Figure 2.1
Figure 2.2
Figure 2.3
Figure 2.4
Figure 2.5
Figure 2.6
Figure 2.7

A

B

CPM/μg DNA

Col  Fn  NC  BSA

CPM/μg DNA

Col  Fn  NC  BSA
Figure 2.8
Figure 2.9

Coll

Fn
2.9 **References**


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CHAPTER THREE: DISCUSSION

3.1 Discussion

3.1.1 Overview
Previous work from the Kandel lab showed that changes in the surface energy of a poly-carbonate urethane (PU) scaffold through the addition of a highly adhesive anionic dihydroxyl oligomer (ADO) (forming PU-ADO) was able to alter the adhesion of AF cells on the substrate [1]. Specifically, AF cell attachment was enhanced and AF cells seeded on PU-ADO formed tissue with a significantly greater collagen content after 7 days of culturing [1]. These effects were shown to be influenced by the adsorption of adhesion proteins from the serum onto PU-ADO, and from proteins synthesized by AF cells that subsequently deposited onto the scaffold, as it was shown that culturing these cells on the above substrates under either serum-free conditions or in the presence of cyclohexamide (an inhibitor of protein synthesis), significantly reduced cell attachment to modified PU-ADO [1]. Since various types of adhesion proteins are found in serum [2] the specific protein(s) that contribute to these effects were not known. As such, the work contained herein has focused on investigating some of the proteins which could be capable of enhancing AF cell attachment on to PU-ADO and to evaluate what effects these proteins could have on AF shape and matrix production and orientation. In order to accomplish this, PU-ADO scaffolds were pre-coated with ECM adhesion proteins (type I collagen (ColI), type I collagen and fibronectin (CF), fibronectin (Fn) and vitronectin (Vn)), which are proteins well known to influence cell attachment, signaling and differentiation for various cell types [3] and are either present in serum or synthesized by AF cells.

3.1.2 The Affects of Fibronectin on Annulus Fibrosus Cells

When cell attachment for the different protein coated PU-ADO surfaces were compared to the non-coated control, the surfaces coated with Fn showed the greatest number of adhered cells. More importantly, Fn influenced cell spreading and alignment and
favored AF cell orientation along PU-ADO scaffold fibres. Cellular orientation on Fn pre-coated scaffolds involved the α5β1 integrin, as the use of specific blocking antibodies to the α5β1 heterodimer or the β1 subunit, hindered AF cell spreading and. Furthermore, blocking of the αvβ3 integrin did not affect cell spreading, suggesting that α5β1, was critical to this function. As well, cell alignment and changes in AF cell morphology under these conditions depended on the actin cytoskeleton, since cell spreading was not observed upon the addition of cytochalasin D. AF cells seeded on Fn pre-coated PU-ADO were able to form fibrillar Fn on their surface and synthesized significantly more type I collagen which was oriented parallel to the PU-ADO scaffold fibres.

Due to the structural complexity of native AF, tissue engineering of an in vitro equivalent has posed its own unique challenges. In aiming to overcome some of these challenges, PU-ADO was fabricated into aligned nanofibrous scaffolds that mimic the oriented type I collagen matrix found in the in vivo AF lamellae. The motivation to do so stems from the various studies which demonstrate that cell behaviour can be modulated not only by the chemistry of the chosen biomaterial, but also by the topographical cues that the material imparts onto cells. These structural cues may thus serve to drive the development of or to help maintain the desirable phenotypic characteristics of some cells [4]. The surface modification of biomaterials using biomacromolecules or smaller biologically active protein recognition sequences may also facilitate this process by rendering cell-biomaterial interactions more favourable [5]. The findings in this study show that Fn, in conjunction with the aligned structure of PU-ADO, was able to direct AF cell behaviour so that it showed similarities to those described for the native AF. Because of the dominant recognition of Fn by various types of cells [6, 7] and the many physiological roles that this protein has [8-12], many others attempting to bioengineer other tissue types have used Fn to modify biomaterials, primarily for the purposes of enhancing cell attachment only [13-18]. However, to the best of our knowledge, there are few studies that have shed light onto whether Fn can influence AF cellular orientation in vitro. The ability of Fn to influence cell alignment in this regard has been described in one other study [14]. In the work by Meinel et al., Fn coating of silk fibroin scaffold resulted in a similar effect, as human Mesenchymal Stem Cells (hMSCs) showed rapid spreading with orientation on an electrospun aligned scaffold but failed to achieve this orientation
when random electrospun silk fibres were used. Both scaffolds were coated with Fn [14]. The findings in the current work agree with the observations made on the importance of Fn in the context of in vivo AF development as observed in rat embryos [19]. In the latter study by Hayes et al., Fn, located pericellularly with no obvious organization was redistributed and assembled into Fn fibrils. These fibrils were colocalized with sub-membranous actin and formed a lattice-like pattern indicative of a fibrillar Fn matrix. These events took place at a critical point in AF development (E16) and coincided with the formation of actin stress fibres, which function to direct the assembly of Fn fibrils via the α5β1 integrin binding. The organized deposition of type I collagen in the lamellae followed [19], and the formation of a fibrillar Fn matrix as it was described by Hayes et al. shows that AF cells utilize the mechanism of Fn fibrillogenesis in vivo [20] in order to assemble Fn throughout their matrix and underscores the important role of this fibrillar ECM structure in AF development, and potentially AF function later on.

Many recent studies have shown that cell interaction with the fibrillar form of ECM Fn can effect the cell cycle [21-24], cell migration [25] and changes in the organization of the actin cytoskeleton [23, 26] and that these effects differ from those brought on by interaction with non-fibrillar (plasma) Fn [20] which conversely binds to components of the complement system, and in coagulation, functions to bind to fibrin and fibrinogen to make up, along with activated platelets, which leads to blood clots [6]. Furthermore, the dynamics of Fn matrix assembly results in the redistribution of the α5β1 integrin into specialized structures termed fibrillar adhesions (FBs) [27, 28]. Fibrillar adhesions are a unique type of an ECM attachment complex that have been previously described in three-dimensional matrix systems and for cells plated on two-dimensional ECM and are believed to be a specific subset of mature focal contacts [29, 30]. Characterization of these structures reveal long, highly stable complexes that are enriched with tensin and active α5β1 which run parallel to bundles of fibronectin in vivo and consists of localized collagen deposition, fibronectin matrix assembly and may serve as anchor sites for actin stress fibres [27, 28]. Their identification has highlighted the essential role that Fn matrix plays in tissue remodeling during development and wound healing [10, 27, 31].
Moreover, in contrast to focal contacts, fibrillar adhesions are deficient in paxillin and vinculin [27].

The current study’s findings suggest that AF cells seeded onto Fn pre-coated PU-ADO undergo the process of Fn fibrillogenesis, as evidenced by the formation of long streaks of Fn and their co-localization with actin stress fibres observed by confocal microscopy (Figure 2.9 Chapter 2). It may be that fibrillar adhesions occur from either the assembly of soluble Fn used to coat the scaffold or as a result of AF cells synthesizing, secreting and assembling their own Fn, or a combination of the two. Furthermore, the alignment of AF cells, their formation of stress fibres and the subsequent assembly of Fn fibrils and oriented type I collagen observed for Fn pre-coated PU-ADO are analogous to the events described for the in vivo development of the rat AF lamellae [19].

It is important to note that collagen fibril formation and orientation has been shown to depend on Fn fibrils and the assembly of an insoluble Fn matrix [32]. This has been demonstrated in studies that showed that antibody binding to the collagen-binding site on Fn inhibited collagen fibrillogenesis [33], as well as the colocalization of collagen fibrils with newly synthesized Fn fibrils and the complete inhibition of collagen assembly when Fn fibril assembly is inhibited with an α5β1 antibody in vascular smooth muscle cells [34]. Interestingly, this same study showed that disruption of actin microfilament assembly using cytochalasin D resulted in almost complete inhibition of collagen fibril assembly [34].

The importance of a functional fibrillar Fn matrix in the healthy IVD is also underscored by studies which have described the accumulation of Fn fragments in the disc during degeneration [35-37]. Of importance is the observation that the introduction of a 30kDa N-terminal fragment from Fn (Fn-f) has been shown to lead to the up-regulation of metalloproteinases (MMP-9 and MMP-13) and the down-regulation of type II collagen and aggrecan genes in NP cells cultured within alginate in vitro [37] and this has also been demonstrated in vivo [36]. This response is not specific to the disc as, Fn-f has been shown to alter chondrocyte metabolism in a similar fashion and induce degeneration in cartilage, a tissue that is closely related to the IVD [38, 39]. These important roles of Fn is underscored by the findings from this study, which demonstrate
that the formation of Fn fibrils and the early formation of an ECM Fn fibrillar network play an important role in AF cell alignment and enhance the collagen formation of the in vitro AF tissue.

3.1.3  **Integrin Expression and Annulus Fibrosus Cell Attachment**

Integrins play a number of roles during development and in regulating tissue function. They serve a particularly important function in weight-bearing tissues that are capable of responding to mechanical forces [40]. For example, chondrocytes in articular cartilage express a number of different alpha and beta integrin subunits that are important for presenting biochemical and physical signals to these cells [41]. Moreover, integrin expression is increased in osteoarthritic cartilage, as compared to healthy tissue, indicating a role for integrins in regulating cartilage health [42]. Lastly, integrin expression profiles in cartilage change with age, from fetal to adult cartilage, suggesting a role for these cell-surface receptors in chondrocyte proliferation and differentiation [43, 44]. In comparison, fewer studies have been conducted into integrin function for AF tissue, however, work done by others has shown that AF cells express a variety of integrin subunits in vivo [45, 46]. In a histological study by Nettles et al., the expression of integrins α5, β1, β3, and β5 was shown in both human and porcine AF. However, human AF expressed lower levels of α1 and β4 than porcine AF, and human AF stained positive for αv and β6 whereas porcine AF did not [46]. Our findings (Figure 2.5 Chapter 2) indicate that AF cells express integrins α5β1 and αvβ3 heterodimers and the β1 subunit on Fn and Coll pre-coated PU-ADO, albeit to different extents. From these findings, it cannot be concluded as to whether AF cells naturally express these integrins or whether their expression is induced by the scaffold onto which the AF cells were seeded, as there was less expression of these integrins on the non-coated scaffold. However, the observed differences for the integrins expressed, specifically the higher expression of α5β1 on Fn-precoated PU-ADO, suggest that AF cells may up-regulate the expression of specific integrins in the presence of their ligands, in this case Fn [45]. This also appears to be the case for Coll pre-coated PU-ADO as well, for which the β1 subunit appeared to be expressed the most in comparison to the other integrins evaluated (Figure 2.5 Chapter 2). The latter observation is consistent with the fact that all of the collagen-binding integrins contain the β1 subunit, with variations in the alpha
subunit only [47]. These findings are not surprising since integrin expression profiles have been shown to vary depending on the ligands present for other cell types [48, 49]. Furthermore, the possible up-regulation of integrins (such as the α5β1 integrin) may, in part, have contributed to the increase in cell attachment observed for Fn pre-coated scaffold (Figure 2.1 Chapter 2). In order to evaluate this further, we conducted preliminary studies to assess cell attachment when integrins were blocked in the presence or absence of the protein inhibitor cyclohexamide (Figure 4.6A Chapter 4). Antibody α5β1 blocking in the presence of cyclohexamide showed a decrease in cell attachment for Fn pre-coated scaffold and may suggest that cells were unable to attach due to an inability to synthesize the necessary integrin or potentially other attachment proteins. This was further demonstrated by confocal microscopy which showed a reduction in α5β1 on the surface of AF cells when treated with cyclohexamide (Figure 4.7A Chapter 4).

To further evaluate the role of integrins in AF cell attachment to PU-ADO, we used specific blocking antibodies against α5β1 and αvβ3 (also capable of binding Fn) [46] and β1. Interestingly, cells were still capable of attaching in the presence of these integrins. These observations are only partly consistent with those made by Gilchrist et al. [45]. In their study, blocking the αvβ3 integrin did not inhibit porcine AF cell attachment, but β1 blocking resulted in the complete inhibition of attachment of these cells on Fn coated monolayer culture [45]. The differences between our findings and the findings described by Gilchrist et al. may be due to several factors. Firstly, our attachment studies were performed 6 hours post-seeding, in contrast to Gilchrist et al. who evaluated cell attachment after 1 hour. Consequently, the longer culture time used in our experiments could have provided AF cells with sufficient time to express new integrins on their cell surface and thus compensate for the blocking procedure. Furthermore, differences in integrin expression profiles exist between human and porcine AF cells [46] suggesting that species differences (bovine AF cells were used for our studies) may account for these discrepancies. The fact that integrin blocking did not inhibit AF cell attachment in this current study also suggests that AF cells may attach to PU-ADO using other integrin-independent means.
Indeed, in previous studies, the Kandel group has demonstrated that changing the surface chemistry of PU-ADO from low to high polar character is in itself sufficient for AF cells to attach to the scaffold [1]. Furthermore, other cells utilize different mechanisms for attachment for which integrins are not involved. An example of this is cell attachment via CD44. This cell surface receptor is the major receptor to matrix hyaluronan that has been identified during embryonic ECM formation and expansion [50], and is expressed in notochordal cells of the IVD [51]. Thus it may potentially function to establish early cell contacts with the scaffold. Moreover, it has been shown that chondrocytes are capable of forming initial focal contacts and attach via a cell-surface hyaluronan coat that is independent of integrin function [52]. A6 epithelial cells have also been shown to use cell surface associated hyaluronan for rapid attachment (in the order of seconds) [53]. Therefore, in light of our findings, it appears that AF cells are most likely utilizing a mixture of adhesion molecules and various mechanisms in order to attach to PU-ADO scaffold.

3.1.4 Integrins and Annulus Fibrosis Cell Attachment on PU-ADO Scaffold

Integrin blocking resulted in a reduction in cell spreading and the disorganization of the actin cytoskeleton, and this varied depending on which integrin was blocked (Figure 2.6 Chapter 2). Blocking of α5β1 integrin reduced AF cell spreading and orientation whereas blocking αvβ3 did not, suggesting that AF cells seeded on Fn pre-coated PU-ADO primarily used the α5β1 integrin to mediate cell spreading under these conditions. However, the reduction in AF cell spreading and the loss of actin cytoskeleton alignment and organization was more pronounced when both α5β1 and αvβ3 were blocked, and this is consistent with observations made by Charo et. al. which revealed that α5β1 and αvβ3 work together achieve complete spreading of melanoma cells on Fn in monolayer culture [54].

In contrast to the above findings, blocking of α5β1 and αvβ3 integrins did not greatly affect AF cell spreading on Coll pre-coated PU-ADO, however, cells failed to spread entirely with β1 blocking. This is in accordance with the fact that all known integrins
capable of binding collagen contain the β1 subunit (α1β1, α2β1, α10β1, α11β1, etc) [47] and therefore, in blocking this critical subunit, AF cells were unable to make focal contacts and spread on Coll pre-coated PU-ADO. Other studies have shown that antibody blocking of β1 inhibits collagen gel contraction by porcine AF cells indicating that this subunit is critical for cells to form mechanical integrin interactions with fibrillar type I collagen [45] and suggests that a similar interaction is inhibited for AF cells resulting in a failure to spread. Lastly, although less pronounced, a partial loss of spreading and alignment of AF cells was observed when α5β1 and ανβ3 integrins are blocked for Coll pre-coated PU-ADO, and this may have resulted due to the disruption of a secondary interaction via cell-secreted Fn under these conditions [45].

It is important to note that the ability of AF cells to show some degree of spreading and alignment despite integrin blocking may be due to integrin-independent mechanisms for cell spreading, which has been documented for other cell types as well [55-57]. Furthermore, simply binding to their ligands may not result in the complete integrin activation necessary for actin organization and sustained cell spreading and contractility [58]. Incomplete integrin activation was produced in a study by Zhang et al., and this was done by deleting the talin1 gene (an integrin-actin linking protein and an emerging key player of integrin activation) [59]. The deletion of this protein consequently inhibited actomyosin contractility and force-dependent activation events [60] that are dependent on stress fibre formation and contraction [61]; however these cells still demonstrated initial cell spreading, independent of these factors and they did so via a Src Focal Kinase (SFK)-linked signaling mechanism [60]. As such, it is plausible that the use of specific blocking antibodies (as was performed in this current study) may not have completely inhibited integrin function but, rather, resulted only its partial activation, thus affecting integrin-actin interaction and downstream signaling pathways. Our observations are analogous to those described elsewhere [60] in that AF cells were able to partially spread on Fn pre-coated PU-ADO but did not exhibit sustained cell contractility or stress fibre formation in the presence of the α5β1 antibody.

Another potential explanation as to why AF cells failed to spread with integrin blocking may be due to changes in the organization and spacing of integrin attachment points to PU-ADO. Upon blocking with integrins, it may be possible that AF cells expressed new
integrins on their surface; however, these integrins were fewer in number and thus formed attachment sites with their ligands that were more spread apart. Many studies have looked into the importance of ligand density and spatial organization on cell spreading and contractility and have suggested this as a means of modulating cell behaviour [62-65]. For example, in a study by Cavalcanti-Adam et al., inter-ligand spacing ≥73nm showed reduced cell spreading and a failure of cells to assemble focal adhesions and stress fibres. Furthermore, in a study by Lim et al. it was shown that at higher ligand densities, human fetal osteoblast cells showed an increase in cell attachment, cell spreading, selectively greater αv integrin expression, and greater FAK phosphorylation and stress fibre assembly [66]. These authors highlighted the importance of altering the nanoscale pattern of biomacromolecules on regulating cell function. Therefore, it is conceivable that the loss of AF cell spreading, orientation and stress fibre formation observed when integrins are blocked relates to integrin spacing which is dependent on the expression of new, unblocked integrins on the surface of these cells. This could have potentially signaled a low-density ligand environment to AF cells, consequently affecting their behaviour. As such, it would be of interest to study the effects of ligand density and distribution on the surface of PU-ADO on AF cell spreading and alignment and whether this results in a change in Fn formation and collagen accumulation.

3.1.5 The Actin Cytoskeleton and Annulus Fibrosus Cell Alignment on PU-ADO

Enhanced cell spreading (Figure 2.2 Chapter 2) and orientation of AF cell cytoskeleton (Figure 2.3 Chapter 2) was observed when they were seeded onto Fn pre-coated PU-ADO, relative to the other substrates. Although this may have been due to the ability of Fn to present biological cues to cells, an alternative explanation may be that the substrate’s inherent modulus [67-69] or tension on the scaffold from the sample holder affects the cells. Recent studies have described the effects of ECM or substrate rigidity on cell behaviour [31]. Specifically, cells are able to sense the modulus of the substrate via integrins and respond to this stimulus by up-regulating integrin expression and modulating downstream signaling by varying the requirement of adaptor proteins during
focal adhesion assembly [68]. Many cell types also respond to substrate modulus by generating their own tension via actomyosin contractility [31]. The mechanism underlying this response has not been fully delineated, however it is has been shown that spreading cells exert contractile forces on the ECM and surrounding substrate during the process of cell contractility and FA assembly [67]. Therefore the ECM requires sufficient resiliency in order to balance the forces generated by cells [67, 69]. An example of this was demonstrated in a study by Schlunck et al. in which the effects of substrate modulus on cells of the human Trabecular Meshwork (hTM), a mechanosensitive structure of the eye, were evaluated. The hTM cells spread most rapidly when plated on polyacrylamide gels with the greatest modulus and produced a greater number of larger FAs and contained more intensely staining stress fibres [70]. Moreover, substrate modulus resulted in increased Focal Adhesion Kinase (FAK) phosphorylation in a rigidity-dependent manner and greater amounts of α-smooth muscle actin were incorporated in filamentous actin (F-actin) bundles indicating the formation of stress fibres with enhanced contractile capabilities. Of particular importance in this work was the effect of tension on matrix fibronectin organization as it was shown in this study that hTM cells assembled elaborate fibrillar Fn networks on stiff substrates and only formed short fibrils and small nodules of Fn for low modulus materials, which also coincided with a loss of stress fibres [70]. In light of this, it would be interesting to determine specifically how changes in tension could be altered to induce changes in AF cell spreading and other behaviour.

While still a controversial topic in some circles, it is now widely accepted that actin stress fibres are not an artifact of in vitro tissue culture, and have been shown to serve many functions in vivo, such as matrix deposition and remodeling during wound healing and development and in sensing forces for mechanosensitive tissues [61]. More importantly, the formation of actin stress fibres is a crucial step in the formation of aligned AF tissue during rat embryogenesis [19]. Actin stress fibre formation was observed for AF cells seeded on PU-ADO coated with Coll, Coll + Fn (CF condition) and Fn with stress fibres showing the most alignment and orientation along PU-ADO fibres with Fn pre-coating. This same distribution of actin was observed in the histological analysis of bovine AF by Bruehlmann et al. In the latter study, outer AF cells
located within the 20% outermost region of the AF had a fusiform morphology and longitudinal cellular processes that extended along collagen fibres and contained long bundles of actin and stress fibres [71]. This actin and stress fibre organization are characteristic of tensile force bearing tissues, such as in tendon, and serve an important role in this function [72]. AF cells also experience and respond to tensile forces and therefore actin stress fibres may serve an analogous role [71]. Furthermore, in addition to cells from the outer AF described above, they observed two more phenotypically distinct cell types located in the inner AF and cells of the interlamellar septae. Interestingly, they described cells of the inner AF as being a mixture of round cells and cells having long, branching processes which assumed no directional preference and which were used to make contacts between different cells [71]. This description is similar to the observations for AF cells seeded on Coll pre-coated PU-ADO (Figures 2.2A and 2.3 Chapter 2). Moreover, in contrast to the long actin bundles and stress fibres formed in outer AF cells, Bruehlmann et al. described cells of the inner AF to have a cortical distribution of actin. Interestingly, our results also show cortical actin for AF cells in the presence of the Coll pre-coated scaffold. Cortical distribution of actin has also been described in chondrocytes of articular cartilage [73]. Although the precise function of cortical actin remains largely unknown, it may help chondrocytes to sense compressive mechanical loading [74], and may potentially serve a similar function in AF cells. Moreover, it should be noted that in endothelial cells, cortical actin and actin stress fibres serve unique functions with interplay between the two in response to physiological signals. Briefly, in the quiescent endothelial cell, cortical actin functions to provide structural support and maintain cell barrier function, but in response to appropriate biochemical signals (ie: inflammation) the cortical actin rim is typically reorganized into long stress fibres responsible for cell contraction and increases tension to promote inter-cell gap formation [75]. It is possible that the stress fibres observed in our studies represent the reorganization of cortical actin for an actively spreading cell and further studies would need to be conducted to study this.

The importance of the actin cytoskeleton for the maintenance and proper functioning of the AF is also highlighted via studies that have described differences in actin for the AF and NP; specifically, greater β-actin monomer and F-actin expression has been
described in cultured outer AF cells in comparison to NP cells [76]. However, in addition to this, microtubules and vimentin, an intermediate filament, may also be involved in regulating cell spreading. In preliminary work carried out during this thesis, a potential function of microtubules was investigated by using the inhibitor, nocodazol (Figure 4.8A Chapter 4). Although cell spreading was inhibited it was not as complete as that achieved when the actin cytoskeleton was blocked using cytochalasin D (Figure 2.4 Chapter 2). The blocking of microtubule assembly resulted in a significant decrease in the extent of cell spreading on both Coll and Fn pre-coated PU-ADO as well as the absence of stress fibres (Figure 4.8A Chapter 4). Other studies have highlighted the interplay of actin and microtubules in controlling cell contractility, stress fibre formation and their cross talk with the Rho GTPases [77, 78]. Also, recent studies have implicated microtubules as modulators of the Rho GTPases and thus affect actin cytoskeleton organization [77], including the formation of stress fibres [75]. Protein analysis reveals significantly greater β-tubulin expression in the NP than the AF [76]. In addition to microtubules, studies have also described changes in the vimentin cytoskeleton of the AF in which an extensive vimentin network was observed to traverse the entire cytoplasm of NP and AF cells from the nucleus to the cell membrane [71, 76]. In addition to this, the appearance of vimentin filaments extended into the outer AF processes [76]. Vimentin mRNA levels are significantly higher in AF than NP cells where there is an age-associated decreased in expression [76]. Although the cellular distribution of actin varies depending on the cell type in the different zonal regions in the AF, vimentin in contrast is expressed in all cells in all regions of the AF, assuming a “cage-like” appearance with filaments abundant on the intracellular aspect of the cell membrane and extending to the nucleus in the outer AF specifically [71]. This “cage-like” organization is also present in chondrocytes [79] and serves to transduce mechanical deformation from the cell membrane to the nucleus in these cells [80, 81]. Moreover, chondrocytes have been shown to maintain their actin cytoskeleton and modify their vimentin cytoskeleton in response to mechanical stimuli [73]. It would therefore be interesting to evaluate how Fn pre-coating of PU-ADO also influences the vimentin cytoskeleton in light of its proposed function in cartilage.
3.1.6 The Effects of Fibronectin on Collagen Synthesis

It was found that AF cells synthesized significantly more collagen when they were spread and elongated on Fn pre-coated PU-ADO (Figure 2.7A,B Chapter 2). Although no link has been made between AF cell morphology and matrix synthesis, previous studies from the Kandel lab [82] and others [83, 84] suggest that chondrocyte morphology and spreading can regulate cell metabolism and matrix synthesis. It is also believed that these outcomes are at least partly mediated through the engagement of the α5β1 integrin (unpublished data). As such, α5β1 integrin engagement of AF cells on Fn pre-coated PU-ADO may also act in a similar manner and alter AF cell metabolism, resulting in downstream anabolic effects and enhanced collagen synthesis. In a study by Pulai et al., apoptosis-induced cell death was observed in isolated chondrocytes incubated with α5β1 antibodies. The authors concluded that cell death occurred due to the inhibition of survival signals for chondrocytes plated on Fn, indicating that there is a mechanism by which α5β1 promotes chondrocyte survival through binding Fn [85]. This α5β1-dependent survival role has been studied in other cells such as Chinese hamster ovary cells, endothelial cells, colon carcinoma cells and neuronal cells under serum-free conditions [85]. Moreover, the role of α5β1-Fn interaction in chondrocyte survival is believed to be particularly important during early time points following cell isolation as chondrocytes have not had time to regenerate a complete matrix, as it was also found that cells were less sensitive to α5β1 blocking by day 7 [85]. The same survival signal with Fn and α5β1 may explain the metabolic advantage observed for AF cells grown on Fn coated scaffold in this study and thus an ability to produce more type I collagen at earlier time points compared to other conditions. Furthermore, in addition to producing significantly greater collagen, AF cells seeded onto PU-ADO showed initial organization and alignment of secreted type I collagen parallel to cell elongation and PU-ADO fibres (Figure 2.8 Chapter 2). This organization of the collagen coincided with the assembly of fibrillar Fn (Figure 2.9 Chapter 2). Numerous studies have highlighted the active role of a functional Fn matrix during tissue remodeling and development [20]. Specifically, it has been shown that cells synthesize and deposit collagen and other matrix proteins near Fn fibrils associated with the cell surface [28]. There is also a suggested dependence for collagen type I fibril formation on Fn fibrillogenesis [34, 86] and the
organization and maintenance of collagen type I fibrils requires the presence of a fibrillar Fn matrix [32, 87, 88]. More importantly, Fn on the surface of AF cells is organized prior to the organized deposition of collagen in the developing rat AF [19]. As such, the results contained in this study suggest that possibly pre-coating PU-ADO with Fn induces AF cells to undergo Fn fibrillogenesis (Figure 2.9 Chapter 2) and that this process may either facilitate or drive the alignment and orientation of secreted collagen type I, and that the tension resulting from the design of the culture system may provide AF cells with the forces necessary to yield the cellular contraction required to assemble Fn fibrils. The formation of Fn fibrils will be confirmed using immunohistochemistry and staining for the tensin protein that is known to associate with fibrillar adhesions and run parallel to Fn fibrils.

3.1.7 Technical Challenges

Although this study enhanced understanding of how AF cells interact with PU-ADO, it has several limitations. One limitation is the variability introduced due to differences in PU-ADO scaffold fabrication. Polymer solutions used to make the scaffold remain essentially the same, however, fabrication via electrospinning can be influenced by differences in atmospheric humidity and has been shown to affect scaffold fibre diameter and thus scaffold porosity. Although measures are taken to reduce this (ie: controlling humidity), the variability is still an inherent challenge. In light of what is known about the importance of the physical state of a biomaterial (such as porosity and modulus) on cell behaviour, this technical variability may have influenced our results, even though attempts were made to circumvent this by randomizing use of scaffolds fabricated on different days.

In addition to these technical limitations, the tension introduced by using a modified microfuge culture system may have also contributed to variability in our system since it may not have been consistent from experiment to experiment. The tension of the scaffold was not quantified and thus is purely conjectural, but it may be necessary to develop a more controlled system if tension is found to contribute significantly to the behaviour of AF cells. Nonetheless, the impact of this potential variable was considered
negligible in light of our objectives since phenotypic differences remained consistent for each protein coating condition despite potential differences in scaffold tension.

In utilizing a bovine primary cell source, biological variability in cells obtained from different calves is also possible. Moreover, AF cells used in these experiments were passaged once (P1) prior to seeding on pre-coated PU-ADO, based on data shown in Figure 4.5A Chapter 4, and this could have resulted in a cell population not entirely representative of native AF. Specific cell sub-populations in the AF have been identified [71] but their functions have yet to be fully delineated nor is it even known if the cells are different or just take on a shape in response to the forces experienced in the different locations of the AF. Furthermore, AF cells increase their integrin expression significantly after passaging in monolayer culture [45] and it was observed there was increased cell attachment occurred for passaged cells (Figure 4.5A Chapter 4). Since an understanding of different cell populations was beyond the scope of this work, cells of the same passage number (P1) were used for all the experiments. This effectively normalized any potential effects passaging may have had. Lastly, it was important to note that the method used to assess cell attachment in this current study was not suitable for distinguishing "weak" versus "strong" attachments made to PU-ADO. To do so, the application of a centrifugation assay for example, in which the applied centrifugal force is increased and cell attachment is quantified, would have to be performed. Such an assay would allow for the quantification of the relative strength of the adhesive interaction that occured between AF cells and PU-ADO, and this may provided further insight into this interaction.

3.1.8 Future Work

In light of the findings, other experiments should be conducted in the future to better understand the mechanisms involved in AF cell spreading and alignment on Fn pre-coated PU-ADO. Specifically, the role of RhoA GTPase in AF cell spreading and stress fibre assembly should be investigated. The Rho family of small GTPases is comprised of RhoA, Cdc42 and Rac. Of the three main members of this family, RhoA has been
shown to control sustained cell spreading, stress fibre assembly and contractility for the actin cytoskeleton [89]. Cdc42 and Rac also mediate changes to the actin cytoskeleton; however, they seem to be more responsible for the transient actin organization associated with cell migration and lamellopodia and filopodia formation [90]. Furthermore, Fn matrix assembly depends on cell-generated contractile forces through Rho activation [20, 27] and a recent study has demonstrated that the assembly of the Fn matrix also depends on RhoA downstream effectors, the Rho Kinases I and II (also known as ROCK I and ROCK II), with each contributing to the mechanism in a distinct way [91]. As such, it will be necessary to study the roles that RhoA and its downstream effectors ROCKs I and II play in AF cell spreading and Fn matrix assembly on PU-ADO via the use of selective inhibitors (such as C3 transferase which selectively blocks Rho but not Cdc42 or Rac, or the transfection of a dominant negative Rho [91]) and subsequent analysis of the actin cytoskeleton and ECM matrix formation and organization using techniques such as confocal analysis.

The experiments conducted in this study focused on the short-term effects on AF behaviour upon coating PU-ADO with biological molecules. In aiming to tissue engineer a viable IVD construct, it is necessary to see how the positive short-term effects described herein translate into tissue formation in the long term. Therefore, Fn pre-coated PU-ADO should be used to grow AF tissue over a longer culture period and cell alignment and Coll fibril orientation should be analyzed via histology. This will enable us to determine whether these phenotypic characteristics are maintained during tissue development and whether they are retained throughout the tissue and not only for cells in contact with Fn pre-coated PU-ADO. In parallel with the above, the effect the above parameters on the matrix molecules synthesized and accumulated needs to be determined.

The influence of mechanical stimulation on the generation of AF tissue should be undertaken. Other studies have shown that anabolic effects, increases in the synthesis of collagens type I and II and proteoglycans, and reduction in MMP activity occur in the IVD in vivo with the application of moderate compressive forces [92, 93] when IVD cells are grown in three dimensional culture in vitro [94]. Since the AF is under the influence of mainly tensile forces in vivo, the application of tensile strain should be performed on
PU-ADO, in conjunction with Fn pre-coating. Integrin receptors, in addition to transmitting external chemical signals to cells via their ligands, also function to sense and transmit mechanical forces to cells [40]. The α5β1 integrin in particular has been identified as a predominant mechanoreceptor in chondrocytes [95] and cell contractility has been shown to transition the confirmation of α5β1 from a relaxed to a tensioned state resulting in its activation and enhance the integrin’s function in mechanotransduction [96]. Also, NP cells from non-degenerated discs appear to utilize integrins for mechanosensing, whereas degenerated discs use an alternative, integrin-independent mechanism [97]. Owing to the findings for α5β1 integrin, the function of this receptor in AF mechanotransduction should be examined in response to tensile force application as well.

Lastly, it was possible to generate different cell responses for Coll and Fn pre-coated PU-ADO. It is important to note that the cell morphologies and actin organization achieved with Coll and Fn pre-coating show strong similarities with the phenotypically distinct cell types described by Bruehlmann et al. [71] for the inner and outer AF, respectively. As such, this finding highlights the potential use of applying different biomacromolecules to the PU-ADO scaffold to provide the environment that favours the development of all the cell types found in the native AF. An ability to do so would further advance the tissue engineering strategies.

3.1.9 Conclusions

Overall, this study identified that Fn in conjunction with fibre orientation of nano-spun PU-ADO plays an important role in the alignment of AF cells and orientation of type I collagen on PU-ADO. The underlying mechanisms regulating these responses need to be delineated as they will facilitate the tissue engineering of functional AF tissue in vitro for the purposes of generating an IVD construct in vitro, suitable for transplantation to treat symptomatic DDD.
3.2 References


CHAPTER FOUR: APPENDIX A

4.1 Cap Construct and Culture System

To complete the objectives of this study a culture system was developed that was amendable to adhesion protein pre-coating and able to withstand the various procedural steps required for scaffold surface modification prior to annulus fibrosus cell seeding. The system was also designed to retain the cell suspension to allow for early cell adhesion and was scaled to utilize the least amount of scaffolding material possible. Furthermore more, cell concentration for seeding the scaffold and the affects of the cell passage number for annulus fibrosus cells were determined for the system prior to the completion of the study objectives.

Figure 4.1A Gross photographic representation of poly-carbonate urethane anionic dihydroxyoligomer scaffold (A) and under Scanning Electron Microscopy visualization (B).

Figure 4.1A Gross photographic representation of poly-carbonate urethane anionic dihydroxyoligomer scaffold (A) and under Scanning Electron Microscopy visualization (B).
Figure 4.2A Development of culturing system for annulus fibrosus cells on PU-ADO. A cap construct culturing method was designed (A and B). Briefly, a 200µL microfuge tube was modified by cutting off the tip end of the tube as well as the top of the lid to allow media to flow through the construct. A circular segment of scaffold was obtained using an 8 mm biopsy punch and was placed across the top of the microfuge tube and then held in place by closing the cap of the microfuge tube. This construct was sterilized by gamma radiation (4MRad) for 48 hours prior to cell seeding. This cap construct created a well of approximately 8mm depth for the cell suspension to be retained with a surface area of 23.7 mm² once the scaffold had been placed in the microfuge tube (A). This entire construct was placed in a 24-well plate for culturing (B). An alternative method consisting of scaffold material (obtained using an 8 mm biopsy punch) and immobilized using a teflon spacer insert and used for culturing after sterilization with 4MRad of gamma radiation (C).
Figure 4.3A Optimization of annulus fibrosus cell culturing system. Cell attachment was determined using two culture methods 1. the Teflon spacer method and 2. the cap culturing method. For the Teflon spacer method, PU-ADO scaffold was cut to a uniform size using an 8mm biopsy punch and placed in a 24-well tissue culture plate with a teflon insert placed on top in order to keep the scaffold in place. The teflon spacer had a hole with an inner diameter of 6mm that was used to seed the AF cell suspension on PU-ADO. The cap culturing method utilized a modified microfuge tube with the scaffold held in place using the cap of the tube. The effect of serum proteins on cell attachment was also studied by seeding annulus fibrous cells in the absence of serum (Non-coated condition; NC) or in the presence of DMEM with 5% FBS (With serum condition; WS). The effects of seeding on dry scaffold (Dry) or scaffold that had been previously wetted and then left to dry prior to seeding (pre-wetted scaffold; Wet) was also studied. Cell attachment was determined for each condition as outlined in section 2.3.4 in this document. Results indicate that cell attachment is enhanced for the cap method upon scaffold pre-wetting and in the presence of serum proteins. * Indicates a significant difference (p<0.05) between the spacer and cap construct methods when the scaffold is pre-wetted and when AF cells are seeded in the presence of serum proteins. as compared to the spacer method and # indicates significance (p<0.05) between dry and wet conditions in the presence of adhesion proteins for the cap construct method only (n=3 experiments done in triplicate).
Figure 4.4A Determination of cell number. Annulus fibrosus cells (passaged once on monolayer culture, P1) were seeded at two densities (50,000 cells and 100,000 cells) using the cap method under serum-free conditions using DMEM media and pre-wetted, non-coated PU-ADO scaffold. Scaffolds were harvested and percent cell attachment was determined after 3, 6 and 24 hours post-seeding (as described in section 2.3.4 in this document). # Indicates significance (p<0.05) for cell attachment between 50,000 and 100,000 cells. Results indicate that at 6 hours, cell attachment is significantly higher at 50,000 cells compared to 100,000 cells. * Denotes significance (p<0.05) for both 50,000 and 100,000 cells after 24 hours of seeding as compared to 6 hours (n=3 experiments, done in triplicate).
Figure 4.5A Determination of cell passage number and the effect of cell passaging on annulus fibrosus cell attachment. Cell attachment was evaluated for primary (P0) cells and for cells that were passaged up to three times. Briefly, for P0 cells, following dissection and overnight enzymatic digestion (described in section 2.3.2 in this document), isolated P0 cells were seeded directly on to PU-ADO scaffold pre-coated with collagen type I (Coll), fibronectin (Fn) or a non-coated control (NC) (as described in section 2.3.3 in this document). To study the affects of passage number, isolated P0 cells were placed in monoculture (density of 10,000 cells / cm2) in DMEM media containing 5% FBS. Media was changed every two days and cells passaged at approximately 80% confluency. Cells used after being placed in monoculture one time were designated P1. Cells at P0 and P1-P3 were seeded on pre-coated PU-ADO using the cap culture method using a 50 µL aliquote of 50,000 cells per construct and cultured using DMEM under serum free conditions. Scaffolds were harvested after 6 hours and cell attachment determined (as described in section 2.3.4 of this document). * Denotes significance (p<0.05) between P0 and passaged (P1-P3) cells for PU-ADO pre-coated with collagen type I (Coll) or fibronectin (Fn) or the non-coated control (NC). Results indicate significantly improved cell attachment after a single passage. # Denotes significance (p<0.05) for P1 as compared to P2-P3 cells for the Fn-coated condition, (n=3 experiements, each done in triplicate).
4.2 Annulus fibrosus integrin expression and cell attachment

![Figure 4.6A Assessment of annulus fibrosus cell attachment with protein synthesis inhibition](image)

To determine if integrins are involved in AF cell attachment on Fn pre-coated PU-ADO, preliminary studies were conducted using integrin blocking antibodies against β1 and αvβ3 and an IgG control (product information and antibody blocking described in section 2.3.8 of this document). To evaluate whether the synthesis of new proteins and integrins mediated enhanced cell attachment, an inhibitor of protein synthesis, cyclohexamide, was added prior to cell seeding. Cyclohexamide was added (Cyclo) to some of the cell suspensions at a final concentration of 10µg/mL and the suspensions were inverted gently for 4 hours at 4°C then seeded using a 50µL aliquote and a total cell number of 50,000 cells. At 6 hours post seeding, scaffolds were harvested and cell attachment determined (as described in section 2.3.4 in this document). In the presence of cyclohexamide and integrin blocking, cell attachment was greatly reduced. Due to the high variability from low cell numbers and inaccuracy of cell quantification using the methods outlined with reduced cell numbers, experiments were not repeated (n=1 experiment, done in triplicate).
Figure 4.7A Integrin expression with cyclohexamide treatment. Annulus fibrosus cells were either untreated or treated with cyclohexamide (final concentration of 10µg/mL) and inverted gently for 4 hours at 4°C prior to seeding on Fn pre-coated PU-ADO in serum-free DMEM. At 6 hours post-seeding, samples were harvested and immunostained for α5β1 integrin (green) and actin (red) and analyzed by confocal microscopy (as described in section 2.3.8 in this document). Results indicate qualitatively reduced integrin expression on the surface of the AF cells suggesting that cells may up-regulate the expression of this integrin upon ligand interaction on Fn pre-coated PU-ADO, as an increased in integrin expression was not seen when protein synthesis was inhibited using cyclohexamide. These results may partly contribute to the enhanced cell attachment and spreading observed when AF cells are seeded on Fn pre-coated scaffold (n=3 experiments, done in triplicate).
Figure 4.8A Cell spreading with microtubule inhibition. The function of microtubules in annulus fibrosus cell spreading and morphology was tested using the microtubule inhibitor, nocodozal. Briefly, AF cells were either treated with nocodozal (added immediately prior to cell seeding to a final concentration of 10µM) or left untreated. A 50µL aliquot of 50,000 cells (P1) were added to each cap culture construct pre-coated with either fibronectin (Fn) or collagen type I (Coll) under serum free conditions using DMEM. Scaffold was harvested after 6 hours and stained for actin (as described previously in section 2.3.6 of this document). The results indicate that AF cells fail to spread when microtubules are inhibited and suggest that microtubules play a role in AF cell spreading and potentially alignment on PU-ADO scaffold.
Pre-coating of the PU-ADO scaffold with fibronectin (Fn) or type I collagen (Coll) was visualized using antibodies against Fn and Coll and analyzed using confocal microscopy. Briefly, PU-ADO was pre-coated with a 50µL aliquot of 10µg/mL Fn solution made in PBS and left overnight at 4°C. The scaffold was washed three times in PBS and incubated with an antibody reactive with Fn (Clone Fn-15, Sigma, Saint Louism MO, 1:200 dilution in PBS) and immunoreactivity was detected using anti-mouse Alexa-488 (Invitrogen; 1:300 dilution in PBS) and indicated Fn coating of PU-ADO (A). For pre-coating of PU-ADO using type I collagen (ColI), a 0.5mg/mL solution was made by dissolving calf skin collagen in 0.1N sterile acetic acid for two days at 4°C with shaking. A 50µL aliquot of this solution was used to coat PU-ADO and left to evaporate overnight in the tissue culture hood with airflow maintain. Samples were incubated with an antibody reactive to collagen type I (Human anti-rabbit collagen type I, Gentaur, 1:200 dilution in PBS) and immunoreactivity detected using anti-rabbit Alexa-488 (Invitrogen; 1:300 in PBS) and indicated ColI coating of PU-ADO (B). For both samples, confocal images were collected at a single representative focal plane and the gain was increased until a signal was detected. Images were taken as described in section 2.3.8 and photomultiplier gain increased 3 times above that used for samples.