THE EFFECTS OF MECHANICAL LOADING ON THE LOCAL MYOFIBROGENIC DIFFERENTIATION OF AORTIC VALVE INTERSTITAL CELLS

by

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Abstract
Calcific aortic valve sclerosis is characterized by focal lesions in the valve leaflet. These lesions are rich in myofibroblasts that express α-SMA and cause fibrosis. Lesions tend to occur in regions of the leaflet that are subjected to large bending loads, suggesting a mechanobiological basis for myofibrogenic differentiation and valve pathogenesis. In this thesis, a bioreactor was developed to study the effect of physiological loading on myofibrogenic differentiation of valve interstitial cells. Cyclic loading of native porcine aortic valve leaflets ex vivo resulted in increased α-SMA expression, predominantly in the fibrosa and spongiosa (similar to sclerotic leaflets). Cofilin, an actin-binding protein, was also upregulated by loading, suggesting it plays a role in mechanically-induced myofibrogenesis. Similarly, loading of a tissue engineered aortic valve leaflet model resulted in increased α-SMA transcript and protein expression. These data support an integral role for mechanical stimuli in myofibrogenic differentiation and sclerosis in the aortic valve.
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1. Introduction

1.1. Motivation

One of the leading causes of cardiac related deaths throughout the world is aortic valve disease. In the Western world, calcific aortic valve sclerosis is the most prevalent valve disease. Aortic valve sclerosis refers to a condition in which bone-like calcified deposits form in the valve leaflets, coinciding with leaflet thickening that leads to reduced valve function, reduced blood flow, and decreased cardiac output. Ultimately this can lead to congestive heart failure. These areas of fibrotic thickening and calcification are referred to as sclerotic lesions.

Sclerotic lesions tend to occur in leaflet regions that qualitatively are subjected to the highest bending stresses, stresses that result in both tensile and compressive strains. The spatial correlation between high stress regions and the sites at which sclerotic lesions form was previously attributed to mechanical inflammation, cell damage and tissue degeneration. More recent observations point to a more complex, active cell-mediated pathology with a mechanobiological basis for disease initiation and proliferation. However, the mechanisms by which mechanical forces regulate cell function to cause disease are unknown.

The majority of cells found throughout the interstitium of a healthy aortic valve leaflet are fibroblast-like cells. In contrast, sclerotic aortic valve leaflets contain both fibroblasts and myofibroblasts. Myofibroblasts have characteristics similar to both smooth muscle cells and fibroblasts, and are thought to be the product of a myofibrogenic differentiation process that occurs in fibroblasts. The fibroblast and myofibroblast phenotypes are easily distinguished from one another by their expression of various proteins. Commonly, myofibroblasts are distinguished from fibroblasts by their expression of $\alpha$-smooth muscle actin ($\alpha$-SMA), which is contained in cytoskeletal stress fibers. While the fibroblast and myofibroblast phenotypes are well defined, it is the process by which the fibroblast differentiates into the myofibroblast that is of interest. Previous research has identified various regulators of this differentiation process, including the mechanical environment and inflammatory cytokines. However, it is generally unknown what factors regulate the differentiation of valve interstitial fibroblasts to the pathogenic myofibroblast phenotype. The spatial correlation between sites of high stress and focal lesion formation suggest a mechanobiological basis for myofibrogenesis in valve interstitial cells. This is the focus of this thesis.
1.2. Objectives

The overall objective of this research was to study how mechanical loading affects the myofibrogenic differentiation of aortic valve interstitial cells in physiologically-relevant systems. This research can be broken down into four main objectives:

- Objective #1: To establish and characterize a system for the application of physiological loads to native aortic valve leaflets and a tissue engineered aortic valve leaflet model.
- Objective #2: To determine the effects of applied physiological loading on myofibrogenic differentiation of valve interstitial cells in native aortic valve leaflets.
- Objective #3: To establish and characterize a tissue engineered aortic valve leaflet model containing aortic valve interstitial cells.
- Objective #4: To determine the effects of applied physiological loading on myofibrogenic differentiation of valve interstitial cells in a tissue engineered aortic valve leaflet model.

1.3. Thesis Organization

The purpose of this first chapter was to introduce the reader to the motivation behind this research as well as the objectives that were addressed in this thesis. Chapter 2 introduces and thoroughly examines various topics specifically related to this research, including aortic valve anatomy, disease pathology, interstitial cell differentiation, and valve tissue engineering. Chapter 3 describes in detail the experimental setup and characterization of the bioreactor system, including the design criteria and experimental testing. Chapter 4 covers the application of physiological strain to native aortic valve leaflets, including experimental methods, results and discussion. Chapter 5 details the development of a tissue engineered aortic valve leaflet model for the study of aortic valve interstitial cell mechanobiology and the application of physiological strain to said model. Chapter 6 summarizes all novel results and conclusions, while also discussing recommendations for future studies related to this research, while Chapters 7 and 8 list references and contain appended material, respectively.
2. Background & Literature Review

2.1. The Aortic Valve

2.1.1. Aortic Valve Function
The aortic valve is located between the left ventricle and the aorta, through which blood flows from the heart into the systemic circulation. As the ventricles contract and ventricular pressure increases, oxygenated blood is forced from the left ventricle through the aortic valve into the aorta. As the ventricles relax and ventricular pressure decreases, the aortic valve closes in order to prevent blood from flowing back into the left ventricle.

2.1.2. Aortic Valve Anatomy
The aortic valve consists of three leaflets, and is referred to as a tricuspid valve or a semilunar valve due to the leaflet’s crescent moon shape (Figure 1). While closing, the valve is designed as such to allow for all three leaflets to simultaneously meet in the middle of the blood flow channel. This process is referred to as coaptation. Surrounding the aortic valve is a ring of dense fibrous tissue called the valve annulus, which provides an attachment point for the bases of all leaflets. The juncture where two leaflets come together is referred to as the commissure.

A normal aortic valve leaflet is thin and opaque. At the structural level, an aortic valve leaflet consists of three layers: the ventricularis, the fibrosa and the spongiosa (Figure 2). The ventricularis is located on the ventricular side of the valve leaflet, and consists of radially-aligned elastin fibers. The fibrosa is located on the aortic side of the valve leaflet, and consists of circumferentially aligned collagen fibers. Finally, the spongiosa is located between the ventricularis and fibrosa. While primarily composed of water, the spongiosa also contains

Figure 1: Healthy human aortic valve [Freeman et al., 2005].
glycosaminoglycans and small amounts of collagen and elastin that hold the ventricularis and fibrosa together.

![Aortic valve leaflet cross-section](http://heartlab.robarts.ca/)

At the cellular level, the aortic valve consists of two cell types: interstitial and endothelial cells. Endothelial cells line the outer edges of the valve leaflet, while a heterogeneous population of interstitial cells can be found on the inner leaflet portions (Figure 3). More specifically, interstitial cells reside within the matrix of collagen, elastin and glycosaminoglycans that make up the non-cellular portion of the leaflet [Taylor et al., 2003].

![Aortic side of valve leaflet at the cellular level](http://heartlab.robarts.ca/)

As stated, the interstitial cell population is a heterogeneous one composed of cells with three distinct phenotypes: fibroblasts, smooth muscle cells and myofibroblasts. Fibroblasts, which make up the majority of the interstitial cell population in normal and healthy aortic valve leaflets, are mostly considered to be quiescent. Smooth muscles cells were identified with electron microscopy as occurring both singly and arranged in thin bundles throughout the leaflet, but do not make up a large percentage of the overall interstitial cell population [Taylor et al., 2003]. Recent data indicates that the fibroblasts act as a precursor for the third type of interstitial...
cell, the myofibroblast [Walker et al., 2004]. Myofibroblasts are unique mesenchymal cells sometimes referred to as “hyperactivated” fibroblasts, and can have characteristics ranging from those of smooth muscle cells to fibroblasts, and are typically identified by their expression of α-smooth muscle actin (α-SMA), which is incorporated in cytoskeletal stress fibers [Taylor et al., 2000]. The activation of myofibroblasts is regulated by mechanical tension and cytokines that control cellular differentiation, proliferation, contraction, ECM secretion, cell migration, wound healing and tissue remodeling [Tomasek et al., 2002]. In fact, studies have suggested that myofibroblasts play a vital role in the remodeling and maintenance of the aortic leaflet extracellular matrix (ECM), including the production of collagen, elastin, proteoglycans, fibronectin, cytokines, chemokines and a variety of growth factors [Walker et al., 2004]. Furthermore, there is a significant increase in the number of myofibroblasts found in remodeling or diseased valve leaflets in comparison to healthy leaflets, providing evidence of interstitial cell plasticity and the role these myofibroblasts may play in an inflammatory response. Myofibroblasts are thought to be eliminated from the interstitial cell population by apoptosis following completion of their remodeling and wound healing activities, but more recently it has been suggested that the interstitial cell population is dynamic and reversible, allowing for myofibroblasts to revert back to the quiescent fibroblast and vice-versa [Rabkin-Aikawa et al., 2004]. Further discussion of the myofibroblast and its connection to calcific aortic valve disease will be discussed in the next section.

2.2. Calcific Aortic Valve Disease

2.2.1. Background

One of the leading causes of cardiac related deaths throughout the world is calcific aortic valve disease. This is a slowly progressive disorder with effects ranging from mild thickening and calcification of aortic valve leaflets with no impairment of blood flow, referred to as aortic valve sclerosis, to severe calcification and thickening of valve leaflets causing serious impairment of leaflet function and resultant blood flow obstruction, referred to as aortic valve stenosis. In the past, aortic valve sclerosis and stenosis were considered different pathological conditions, but recent evidence suggests they are in fact representative of different stages of the same disease process [Stewart et al., 1997].

The disease progression can be broken down into two phases. The thickening and formation of calcium deposits occurring over many decades is referred to as the asymptomatic,
or sclerotic, phase, while the onset of disrupted blood flow is referred to as the symptomatic, or stenotic, phase. Major health problems arise when patients reach the symptomatic phase, when aortic valve thickening and calcification has progressed to the point to where it is affecting blood flow and heart function. With the onset of symptoms, the two year survival rate without treatment is approximately 50% [Cowell et al., 2004]. Once the stenotic phase of calcific aortic valve disease has been reached and surpassed, treatment methods, including aortic valve replacement, become more invasive and dangerous.

Furthermore, it is important to note that while all patients suffering from aortic valve sclerosis may not have the disease progress to blood flow impairment, many patients suffer fatal cardiovascular events without reaching the symptomatic phase. Studies have demonstrated that patients suffering from aortic valve sclerosis had an increased risk of 50% for cardiovascular death, including increased risk of cardiac infarction and stroke, even in the absence of impaired blood flow typical of aortic valve stenosis [Otto et al., 1997].

Sclerosis of the aortic valve is common among the elderly, being found in approximately 29% of individuals 65 years of age or older, and in approximately 50% of individuals 80 years of age or older [Otto et al., 1999]. With such a large portion of the population affected by aortic valve sclerosis, and with increased mortality rates seen in patients regardless of whether the disease progresses to aortic valve stenosis, it is important to be able to identify patients in the sclerotic phase. By achieving a greater understanding of the mechanisms involved in the onset and progression of aortic valve sclerosis, it may be possible to develop new treatment methods that can disrupt or stop the progression of leaflet calcification and thickening before heart function is detrimentally affected.

2.2.2. Pathogenesis of Aortic Valve Sclerosis
Aortic valve sclerosis is identified by the thickening and calcification of aortic valve leaflets (Figure 4). In the past, aortic valve sclerosis was considered to be a degenerative and non-modifiable process, caused by age associated valvular wear and tear and passive calcium deposition. In contrast, recent studies have suggested that aortic valve sclerosis is, in fact, an active disease process, complete with chronic inflammation, lipoprotein deposition, renin-angiotensin activation [O’Brien, 2006], and most importantly, active leaflet calcification [Rajamannam et al., 2003]. These processes have been observed in both normal tricuspid aortic valves and bicuspid aortic valves originating from congenital defects.
Figure 4: Aortic valve showing leaflet thickening and calcium deposition (arrow) typical of aortic valve sclerosis [Freeman et al., 2005].

The disease process of aortic valve sclerosis begins with the formation of aortic valve lesions, or more specifically, focal subendothelial plaque-like lesions originating on the aortic side of the valve leaflet and extending into the adjacent fibrosa. It is at these lesion sites where sclerotic activity is observed: accumulation of “atherogenic” lipoproteins, active inflammatory response and calcification. These aortic valve lesions are often initiated by or allowed to progress due to mechanical loading and inflammatory cytokines, often leading to valvular interstitial cell differentiation to pathogenic phenotypes.

2.2.3. Interstitial Cell Differentiation in Aortic Valve Sclerosis

On the cellular level, interstitial cells located within aortic valve lesions show phenotypic differentiation into cells having myofibrogenic and osteogenic characteristics. As stated, myofibroblasts can arise from fibroblasts located in the aortic leaflet interstitium, and their activation is tightly regulated by cytokines that have a variety of functions. Evidence suggests that myofibroblasts play an important role in valvular wound healing, as there is very little positive expression of α-SMA throughout the interstitial cell population of a normal, healthy aortic valve leaflet, yet an increased number of α-SMA positive myofibroblasts appear in aortic valve leaflets that were wounded surgically to induce an injury repair response [Walker et al., 2004]. Regardless of whether myofibroblasts are eliminated by apoptosis or revert back to the quiescent fibroblast phenotype, if their life cycle is not regulated properly, myofibroblasts will persist with continued force generation and ECM secretion, leading to aortic valve thickening and fibrosis [Desmouliere et al., 1996].

In the long term, interstitial cells located within aortic valve lesions also show differentiation into cells having osteogenic phenotypes that lead to valvular calcification. As
stated, the process of calcification has been shown to be an active process rather than a passive, degenerative process. Calcium deposits found on aortic valve lesions have been found to obtain both calcium and phosphate as hydroxyapatite, a mineral found predominately in bone [Mohler et al., 1999]. As well, many proteins involved in the regulation of bone deposition and resorption have been detected in calcified aortic valve lesions [Mohler et al., 2001], giving evidence that cells of an osteogenic phenotype are involved. Recently, a subset of aortic valve interstitial cells were isolated and found to express osteogenic markers. This subset was also found to spontaneously produce hydroxyapatite-containing calcific nodules in vitro [Mohler et al., 1999].

In studying the pathogenesis of aortic valve sclerosis, it is apparent that the myofibrogenic differentiation of resident aortic valve interstitial fibroblasts, as identified by the positive expression of α-SMA, is a very crucial marker for disease progression. Pho et al. [2007] were able to show that porcine aortic valve leaflets showed an approximately 30% increase in α-SMA positive interstitial cells between healthy and sclerotic valve leaflets. Overall, it is apparent that increasing α-SMA expression, as a function of myofibrogenic differentiation, is a key to the pathogenesis of aortic valve sclerosis.

2.2.4. Effects of Loading on Aortic Valve Leaflets
Throughout the cardiac cycle, aortic valve leaflets are subjected to varying degrees of pressure and flow loading which results in compressive and tensile mechanical loading. On the cellular level, interstitial cells residing in one localized area are subjected to different loading conditions when compared to interstitial cells in other localized areas, that is, one interstitial cell experiences different loading than the next. This complex environment defines how interstitial cells organize and behave within the aortic valve leaflet. Attempts to elucidate the load-dependent response of aortic valve leaflets have been made by looking at each type of loading individually.

In vivo, aortic valve leaflets are subjected to an average pressure load of approximately 100 mmHg during diastole, but can reach as much as 200 mmHg in patients suffering from hypertension. This pressure load on the closed aortic valve generates both tensile and compressive forces on the aortic valve leaflet. In an attempt to observe the effects pressure loading has on aortic valve leaflets, Xing et al. [2004] established a pressure chamber device in which static pressure loads could be applied to unconstrained free-floating porcine aortic valve leaflets. Results showed that aortic valve leaflet collagen synthesis increased with applied
pressure, but that no difference in \( \alpha \)-SMA expression was observed between pressure-loaded leaflets and controls. To gain a more accurate picture of pressure effects, aortic valve leaflets were subjected to cyclic pressure in an attempt to better mimic the \textit{in vivo} environment. Similar to the previous study, unconstrained free-floating porcine aortic valve leaflets were placed in a pressure chamber and subjected to cyclic pressure of varying magnitude at varying frequencies [Xing \textit{et al.}, 2004]. Similar to the previous study, leaflet collagen synthesis was positively correlated and proportional to pressure magnitude. Although the increase in collagen synthesis was marginally greater for leaflets subjected to cyclic pressure when compared to static pressure, the predominant factor appears to be pressure magnitude. Furthermore, the expression of \( \alpha \)-SMA was once again observed to have no correlation to pressure loading, whether it is static or cyclic. These studies suggest that pressure loading alone bears no effect on the myofibrogenic differentiation of fibroblasts found within the leaflet interstitium.

As blood flows through the aortic valve, this flow subjects aortic valve leaflets to shear stress along their surfaces. Shear stress has been shown to affect transcript and protein levels in many cell lines as well as matrix production and organization. A flow chamber device was established by Weston \textit{et al.} [2001] which would allow for the application of steady and pulsatile flow at different flow rates to the surface of aortic valve leaflets, creating different shear stress environments. Results showed that both steady and pulsatile flow creating different shear stresses had no effect on the expression of \( \alpha \)-SMA in the valve leaflets, suggesting that shear stress does not affect myofibrogenic differentiation. Furthermore, Warnock \textit{et al.} [2005] created a custom device that would allow for the application of both cyclic pressure and pulsatile flow shear stresses to aortic valve leaflets. Porcine aortic valve leaflets were subsequently loaded into this device and subjected to pulsatile physiological pressure and flow [Konduri \textit{et al.}, 2005]. As seen in the individual application of cyclic pressure and pulsatile flow, the combination of both pressure and shear stress to aortic valve leaflets showed no effect on the expression of \( \alpha \)-SMA, suggesting that they not only do not affect myofibrogenic differentiation individually, they also have no effect in combination.

These studies were able to show the effects that flow and pressure loading have on aortic valve leaflets both individually and in concert by subjecting these loads to unconstrained, free-floating leaflets. \textit{In vivo}, leaflets are constrained by attachment to the aortic valve annulus, and thus are subjected to both tensile and compressive mechanical loading as a result of flow and
pressure. In contrast to flow and pressure loading, mechanical loading has been shown extensively to have a major effect on the myofibrogenic differentiation of interstitial fibroblasts and by extension the pathogenesis of aortic valve sclerosis. There is evidence to support the theory that a strong correlation exists between the pattern of mechanical strain on the aortic valve leaflet and the pattern of calcium deposition on the aortic valve leaflet. Qualitatively, the research suggests that areas of the leaflet subjected to the greatest amount of mechanical strain were the areas most likely to form aortic valve lesions [Thubrikar, 1990]. Further, tissue-level studies observed aortic valve sclerosis in patients with normal, tricuspid valves and patients with a congenital defect leaving them with a bicuspid aortic valve. Due to their shape, bicuspid valves are subjected to higher mechanical bending loads in situ in comparison to tricuspid aortic valves [Robicsek et al., 2004]. The results of this study show that patients with bicuspid valves tend to present aortic valve lesions, thickening and calcification typical of aortic valve sclerosis at ages, on average, two decades younger than those with tricuspid valves [Beppu et al., 1993]. Using a device that allowed for the application of cyclic circumferential stretching to porcine aortic valve leaflets, Balachandran et al. [2006] were able to show an almost 55% increase in whole leaflet α-SMA expression in stretched leaflets as compared to those freshly excised. As well, an almost 35% decrease in whole leaflet α-SMA expression was observed in statically incubated leaflets when compared to those freshly excised, indicating the removal of all mechanical loading may result in the disappearance of the myofibroblast phenotype from the leaflet interstitium. While much research has been focused on whole leaflet protein expression in mechanically loaded leaflets, little is known about local expression within mechanically loaded leaflets, although valve disease and cell phenotypic changes occur focally. It is obvious that the mechanical environment plays an important role in aortic valve interstitial myofibrogenesis, and by extension it is possible that it plays a role in the pathogenesis of aortic valve sclerosis.

2.3. Myofibrogenic Differentiation of Fibroblasts

2.3.1. A Proposed Pathway for Myofibrogenesis
While little is known about the process by which valve interstitial fibroblasts differentiate to myofibroblasts, numerous studies on myofibrogenesis in other tissues have resulted in a proposed general pathway for the myofibrogenic differentiation of fibroblasts. Although it can be considered more of an educated guess than exact science, the pathway seeks to provide the answer of how the quiescent fibroblast becomes the activated, would-healing myofibroblast. It
has been suggested that, along the pathway from the fibroblast and myofibroblast, a distinct cell phenotype, referred to as the proto-myofibroblast, exists [Tomasek et al., 2002]. These proto-myofibroblasts show early contractile properties, and are characterized by the formation of cytoplasmic actin-containing stress fibers, focal adhesions, and the expression of cellular fibronectin at the cell surface. Evidence points to mechanical tension as the driving force behind the formation of the proto-myofibroblast. When cultured on rigid planar substrates in vitro, fibroblasts will become proto-myofibroblasts, forming stress fibers, focal adhesions and producing extra-cellular matrix containing fibronectin prior to forming the α-SMA containing stress fibers typical of myofibroblasts [Burridge et al., 1996]. When cultured in vitro on compliant planar substrates, fibroblasts show none of these characteristics [Pelham et al., 1997]. The same characteristic change from fibroblast to proto-myofibroblast can also be seen in three-dimensional culture on stiff substrates as opposed to compliant substrates [Mochitate et al., 1991].

The final step of this suggested myofibrogenic differentiation pathway is the formation of the activated myofibroblast from the proto-myofibroblast. The most easily identifiable difference between the proto-myofibroblast and the myofibroblast is the latter’s incorporation of α-SMA into stress fibers. A recent study suggests that, during this move from proto-myofibroblast to myofibroblast, certain “rod-like structures” exist (Figure 5). These structures, connected to the plasma membrane, appear to act as a reservoir for pre-polymerized α-SMA, providing an intermediate step in the incorporation of α-SMA into stress fibers [Clement et al., 2005]. The driving force behind the proto-myofibroblast to myofibroblast change, as evidence suggests, is the isoform of transforming growth factor beta termed TGF-β1. Studies have shown the ability of TGF-β1 to cause myofibrogenic differentiation within the valve leaflet interstitium, as well as to regulate many functions of the myofibroblast phenotype including activation of α-SMA and collagen secretion [Roberts et al., 1996]. TGF-β1, in its biologically active form, has been shown in vitro to induce the expression of α-SMA, and has been shown to increase the expression of α-SMA in a dose-dependent manner [Cushing et al., 2005].
Figure 5: Proposed pathway for α-SMA incorporation in myofibrogenic differentiation: (a) α-SMA is recruited into “rod-like structures” and then incorporated into cytoplasmic actin-containing stress fibers; (b) α-SMA is completely incorporated into stress fibers completing transition to myofibroblast [Clement et al., 2005].

It appears that TGF-β1 is not the only factor involved in this final change of proto-myofibroblast to activated myofibroblast, and that mechanical tension works in concert with TGF-β1 in a synergistic manner. The synergistic effects these two variables have on aortic valve interstitial cell phenotype were studied using interstitial cell-seeded collagen matrices [Walker et al., 2004]. Collagen matrices were seeded with aortic valve interstitial cells under two different mechanical conditions: matrices were seeded free-floating in a tissue culture well or were adhered to the well for 120h prior to release, giving matrices from stressed and non-stressed environments. As well, some matrices were subjected to TGF-β1 and others not. Contraction of matrices was measured over time as an indicator for the activation of contractile myofibroblasts. The research showed a significant increase in matrix contraction by matrices subjected to both mechanical stress and TGF-β1 in comparison to matrices subjected to either mechanical stress or TGF-β1 alone or neither of the two. This enhanced contractile response correlated strongly with a 230% increase in α-SMA expression, giving an idea as to the extent of interstitial cell differentiation. These results were confirmed ex vivo through the use of native porcine aortic valve leaflets. Leaflets subjected to a treatment of both unidirectional cyclic tensile stretch and TGF-β1 simultaneously showed a significant increase in whole leaflet α-SMA expression over time in comparison to leaflets subjected to no treatment, stress treatment or TGF-β1 treatment alone [Merryman et al., 2007]. Overall, the differentiation of the fibroblast to the proto-myofibroblast, and then finally to the myofibroblast, appears to be controlled heavily by the mechanical environment and the presence and levels of TGF-β1 (Figure 6).
Many studies have reported on the whole leaflet response to mechanical loading. However, aortic valve leaflets are subjected to a heterogeneous mechanical environment in vivo, and thus aortic valve interstitial fibroblasts in one region of the leaflet could be subjected to loading that is significantly different than that in other regions of the leaflets. Due to this heterogeneous mechanical environment, the local expression of myofibrogenic proteins such as \( \alpha \)-SMA within a leaflet should be investigated to determine how varying loads within one leaflet affect cells spatially. Furthermore, the intracellular factors regulating \( \alpha \)-SMA expression have not been investigated or identified in the previous mechanical loading studies.

Figure 6: Proposed pathway for myofibrogenic differentiation of fibroblasts [Tomasek et al., 2002].

### 2.3.2. The Role of Cofilin in Myofibrogenesis

In recent studies, a proteomics approach using isotope-coded affinity tags (ICAT) was employed with cultured fibroblast and myofibroblast populations to screen for differential protein expression. Cofilin, or more specifically the non-muscle isoform of cofilin referred to as cofilin 1 (CFL1), was identified as differentially expressed between the two cell populations [Pho et al., 2007], and is of interest to this research due to its involvement in cytoskeletal remodeling.
Actin filament assembly and disassembly, as well as spatial and temporal organization, is tightly regulated within eukaryotic cells in order to allow for necessary processes such as cell locomotion and division to occur. Cofilin, a member of the actin-depolymerization factor and cofilin (ADF/cofilin) family of proteins, is a crucial regulator of actin cytoskeleton dynamics and functions to accelerate the actin filament turnover rate [Theriot, 1997]. Specifically, cofilin functions in the disassembly of actin filaments through depolymerization, consequently making actin monomers available for reorganization and remodeling of the cytoskeleton.

Actin filaments (F actin) are formed as actin monomers (G actin) polymerize in a “head to tail” manner giving the filament a helical structure with two distinct ends: the barbed end and the pointed end. In general terms, actin monomers add to the filament at the barbed end and disassociate at the pointed end. The rate of monomer addition at the barbed end is generally much faster than the rate of monomer dissociation at the pointed end, thus the dissociation rate is the limiting factor in actin turnover. The rate of actin turnover in living cells is much faster and cannot be attributed to the dissociation rate alone [Theriot 1997]. Studies have shown quantitatively that cofilin functions to accelerate the dissociation rate of actin monomers at the pointed end of actin filaments [Carlier et al., 1997]. Cofilin achieves this by binding actin filaments through the bridging of two longitudinally adjacent actin monomers within the helical structure. Studies have shown that, through this binding mechanism, cofilin reduces the crossover length of actin filaments by inducing filament twist [McGough et al., 1997]. This twist increases strain within the filament, reducing the strength of monomer-monomer bonds within the structure and allowing for filament fragmentation. The combination of increased monomer dissociation, weakened monomer-monomer bonds, as well as the increased number of filament pointed ends due to fragmentation all contribute to the role cofilin plays in actin turnover and dynamics.

It has become apparent over recent years that cofilin is involved in the myofibrogenic differentiation of fibroblasts. As stated earlier, cofilin was identified as differentially expressed between porcine aortic valve interstitial cell populations of fibroblasts (or pre-myofibroblasts) and myofibroblasts. These results were obtained through the use of ICAT, and were subsequently authenticated through the use of western blotting. This analysis showed a greater than 3-fold increase in cofilin protein expression between the fibroblast and myofibroblast population [Pho et al., 2007], comparable to the increase observed in α-SMA expression between
the two populations. More specifically, cofilin appears to be involved in the mechanism by which α-SMA is incorporated into the stress fibers commonly observed in the myofibroblast phenotype. Research studying the incorporation of α-SMA into these stress fibers throughout the process of myofibrogenesis found that cofilin was expressed in what was termed “rod-like structures”, suggested to be the precursor to the incorporation of α-SMA in myofibroblast stress fibers [Clement et al., 2005]. Furthermore, a small interfering RNA (siRNA) was employed to knockdown cofilin expression in the myofibroblast population [Pho et al., 2007]. This siRNA was shown to knockdown cofilin expression by over 80% as compared to an irrelevant siRNA through western blotting, while α-SMA levels remained unchanged. Myofibroblasts treated with the cofilin siRNA and the irrelevant siRNA were stained for α-SMA and observed under fluorescent microscopy. Observations showed that the knockdown of cofilin prevented the formation of α-SMA-containing stress fibers (Figure 7) that are commonly associated with the myofibroblast phenotype (Figure 8) [Pho et al., 2007].

Figure 7: α-SMA expression in myofibroblasts with cofilin knockdown through siRNA, as observed with fluorescent microscope [Pho et al., 2007].
While various studies have shown the role cofilin plays in actin dynamics in vitro, it is of interest to study what role cofilin plays in actin dynamics in aortic valve leaflets ex vivo, and more so how cofilin expression is related to myofibrogenic differentiation in the aortic valve interstitium as a function of cell mechanics and the disease process.

2.4. Heart Valve Tissue Engineering

2.4.1. Background

In the past half-century engineering has played a major role in the treatment of calcific aortic valve disease. With the development of cardiopulmonary bypass in the early 1950’s came the ability for physicians to perform open heart surgery. With this ability came the realization that the complete replacement of calcific aortic valves was surgically possible. Through the interdisciplinary work of physicians and engineers came the idea for a replacement aortic valve that functioned mechanically on the “ball-in-cage” principle. This valve, termed the Starr-Edwards prosthetic heart valve (Figure 9) after its inventors, physician Albert Starr and mechanical engineer Lowell Edwards, was a major achievement in the treatment of calcific aortic valve disease. It was not without its problems though, with many recipients suffering from massive hemorrhaging, thromboembolisms and infection often leading to death [Lifton 2007].
To combat the inherent problems with the mechanical “ball-in-cage” replacement valve, focus turned to the development of replacement valves composed of biological materials. This began with the complete replacement of a patient’s aortic valve with that of another species, more specifically a porcine aortic valve. Many problems arose with this replacement technique, including rejection due to the antigenicity of the porcine valve and valve degradation [Lifton 2007]. Further research showed that cross-linking with gluteraldehyde eliminated this antigenicity and increased durability [Carpentier et al., 1969]. As well, the addition of a prosthetic stent reduced inflammation as well as allowing for ease of implantation. However, these bioprosthetic valves were not as durable as the mechanical “ball-in-cage” model and showed a tendency for increased rates of calcification [Carpentier et al., 1974]. In the many years since, advancements have been made in the development and production of bioprosthetic aortic valves that have reduced the complications involved. Unfortunately, these complications are still a major problem and have become an intrinsic part of receiving an aortic valve replacement. While still in its infancy, the engineering of living tissue has shown promise as a way to study, and possibly in the future to treat, calcific aortic valve disease.

With advances in science and technology, the idea that living tissue, and possibly entire organs, can be engineered and developed outside of the human body in the laboratory setting is becoming a real possibility. While the engineering of an entire human heart for transplant is more dream than reality at this time, the development of functioning cardiac valves is currently showing great promise. Reproducing the anatomical shape of heart valves is one of the major hurdles currently facing the successful engineering of functioning valves, and is directly related to the scaffold material used for cell attachment and proliferation. As such, previous attempts at heart valve tissue engineering can be classified into two categories: heart valve engineering using
biological acellular tissue scaffolds and heart valve engineering using synthetic scaffolds. Examples of both are discussed below.

Many synthetic materials have been used as the scaffold material in the cultivation of heart valve replacements, including but not limited to polyglycolic acid, poly-L-lactic acid and polyglactin. Using a scaffold composed of a polyglactin woven mesh surrounded by two polyglycolic acid mesh sheets, Shinoka et al. [1995] created an engineered heart valve leaflet by seeding ovine arterial interstitial cells onto the scaffold every day for two weeks followed by the addition of ovine arterial endothelial cells to create an outer monolayer. Autologous and allogenic tissue engineered leaflets were then implanted into lambs to replace the right posterior pulmonary leaflet. Allogenic leaflet recipients contracted serious infectious complications resulting in death, with implanted leaflets showing significant shrinkage and deterioration. In contrast, autologous leaflet recipients survived without complications, with implanted leaflets maintaining size, shape and functionality. Implanted leaflets, removed for histological analysis, showed appropriate cellular architecture and resembled the native valve tissue. Sutherland et al. [2005] used mesenchymal stem cells to create a functioning semilunar heart valve for implantation. Mesenchymal stem cells were sourced from ovine bone marrow and seeded onto custom, semilunar valve-shaped polyglycolic and poly-L-lactic acid scaffolds which were cultured in vitro for four weeks. Valves were then implanted at the pulmonary position in sheep. These tissue engineered valves showed no evidence of infection or thrombosis in vivo, and many sheep survived over four months at which time the implanted valve had lost little, if any, structural integrity or function. Upon explant, tissue engineered valves showed a striking resemblance to native valves in terms of extracellular matrix distribution, although some structural differences did exist. Overall, these are just two examples of the possibilities that tissue engineering is creating in the treatment of human pathologies.

While the use of synthetic materials as scaffolds have shown much promise, the use of native, decellularized tissue as a scaffold material would presumably give much improved results. Native tissue, whether animal or donor derived, can be decellularized and act as the base scaffold for the development of tissue engineered heart valve replacements. Through the use of various techniques, such as enzymatic digestion [Zeltinger et al., 2001] and freeze-drying [Curtill et al., 1997], resident cells can be removed leaving an acellular tissue with reduced immunogenicity consisting almost entirely of extracellular matrix proteins that can act as a great
template for the attachment of living cells [Mol et al., 2004]. As well, entire heart valves can be
decellularized, leaving a heart valve-shaped scaffold that can help address the problems faced in
creating a functioning, engineered heart valve. Curtil et al. [1997] showed an ability to
decellularize porcine heart valves and to repopulate said valves with both human fibroblasts and
endothelial cells. Unfortunately, through the use of static culturing, physiological cellularity of
the tissue engineered heart valves was not obtained. Zeltinger et al. [2001], using acellular
porcine aortic valves, were able to successfully seed human neonatal dermal fibroblasts. Through
dynamic culture utilizing a pulsatile fluid flow bioreactor, repopulated heart valves were
subjected to culture conditions that simulated the in vivo opening and closing of heart valves.
Although, as with static culture, physiological cellularity was not obtained, seeded cells showed
an ability to remodel the acellular scaffold through the production of extracellular matrix
proteins. Bertipaglia et al. [2003] seeded acellular porcine aortic valve leaflets with aortic valve
interstitial cells and showed that through static culture, the repopulated engineered leaflets
demonstrated all of the cell phenotypes observed in native aortic valve leaflets, including
fibroblasts, smooth muscle cells and myofibroblasts. Even more interesting, a lining of
endothelial cells on the tissue engineered leaflets was also identified, suggesting heterogeneous
differentiation potential of valvular interstitial cells [Taylor et al., 2000] or perhaps
contamination of the isolated interstitial cell population with endothelial cells. A relatively
successful heart valve engineering attempt was documented by Schenke-Layland et al. [2003],
using acellular porcine pulmonary valves. Decellularized valves were seeded with ovine arterial
myofibroblasts and cultured in a dynamic pulsatile flow bioreactor. After sixteen days, ovine
arterial endothelial cells were seeded to the scaffold and constructs were cultured statically for a
further two days. These tissue engineered heart valves were observed to have mechanical
properties that were comparable to both ovine and porcine tissues. Furthermore, constructs
showed a tissue organization and morphology that closely resembled the native heart valve
tissue. In the same way, Taylor et al. [2002] seeded acellular bovine type I collagen matrix
sponge scaffolds with human heart valve interstitial cells in order to analyze the cells behavior in
a biological, three-dimensional matrix for possible tissue engineering applications. Interstitial
cells residing in the collagen scaffold were observed to maintain viability and phenotype as well
as to proliferate. These finding indicate that type I collagen sponge scaffolds may be used to
simulate the in vivo environment in which valvular interstitial cells reside and furthermore may
be used in the engineering of heart valve replacements. As well, by simulating the \textit{in vivo} environment, type I collagen sponge scaffolds may allow for further studies into the behavior and response of interstitial cells to various stimuli without any of the confounding effects present \textit{in vivo}. Overall, various tissue engineering techniques using acellular native tissue as the scaffold material have shown great promise in the cultivation of functioning heart valve replacements.

In addition to direct treatment applications, tissue engineering has become a very useful tool in the study of biological systems and disease. As observed by Taylor \textit{et al.} [2002], a cell type of interest can be isolated and grown \textit{in vitro} in a three-dimensional scaffold to create tissue that can very closely mimic the \textit{in vivo} environment. These cells can then be analyzed and subjected to various stimuli in order to elucidate important information such as what functions they perform or what role they play in disease progression. One example of this technique involved the study of glycosaminoglycan (GAG) synthesis by mitral valve interstitial cells [Gupta \textit{et al.}, 2007]. Using collagen gel scaffolds, interstitial cells were seeded and cultured \textit{in vitro} to create a three-dimensional tissue. This tissue was then subjected to static tensile strain, resulting in a significant increase in the total synthesis of GAGs as compared to non-strained controls. Experiments such as this can lead to a greater understanding of how the human body functions, but can also lead to important discoveries in disease pathology and treatment regimens.

\subsection*{2.4.2. Methods for Tissue Engineering}

The first step towards creating an engineered tissue is the selection of a cell and/or tissue source. Generally, human cells and/or tissues are difficult to come by, especially in the large amount needed for culturing engineered tissue and for experimentation. In many cases, including this research, porcine cardiovascular tissue are used as a model for human cardiovascular tissue due to anatomical, functional and organizational similarities, as well as their availability. Other species, including bovine, rabbit, canine and rat, have also been utilized as cell sources for tissue engineering. Upon choosing a cell source, a protocol must be established for the isolation of said cells from their native tissue. After isolation, isolated cells generally have to be allowed to proliferate by serial passaging \textit{in vitro} in order to obtain a sufficient amount of cells for successful seeding.
Once a cell source has been established, a three-dimensional scaffold material upon which cells can adhere and function must be chosen. General guidelines for choosing an appropriate scaffold material include choosing a material that is biocompatible, biodegradable, has surface chemical properties that allow for cell adherence, and are very porous to allow unrestricted and spatially uniform cell distribution as well as cell growth, nutrient supply and removal of metabolic waste [Vunjak-Novakovic et al., 2003]. Referring back to biodegradation, the rate of scaffold degradation should be proportional to the rate of tissue formation in order for the scaffold to maintain mechanical integrity throughout culturing [Mol, 2005]. Many different scaffold materials have been used for tissue engineering, including but not limited to, non-biologically derived materials such as polyglycolic acid and biologically derived materials such as acellular collagen matrices. No matter what scaffold material is chosen, this material must be hydrated prior to seeding. Commonly, scaffolds are hydrated in the culture media used when culturing the cells of interest in vitro. The goal of this hydration is to displace all air trapped within the scaffold, leaving an environment more hospitable for cell adherence at the time of seeding. In order to obtain thorough hydration, it is often enough to just immerse the scaffold in culture media. Depending on scaffold material, different techniques may need to be employed such as applying pressure manually to the scaffold or subjecting scaffolds to a vacuum while immersed in culture media to effectively displace all air [Vunjak-Novakovic et al., 2003].

Once a cell source and scaffold material has been chosen, a method by which cells will be delivered to the scaffold must be established. Cell seeding generally starts with the uniform application of a large amount of cells to the scaffold. Uniform application is important in achieving a scaffold with a spatially uniform distribution of attached cells, which provides a base for uniform tissue generation. The initial number of cells, the method for cell application, and the conditions and duration of cell seeding must be thoroughly considered, as it is generally difficult to compensate for any deficiencies in the method once seeding is complete and culturing has begun [Vunjak-Novakovic et al., 2003]. Many methods for cell delivery to the scaffold have been attempted. In the seeding of collagen sponge scaffolds, Taylor et al. [2002] placed scaffolds in the wells of a tissue culture plate along with a high density cell suspension containing heart valve interstitial cells. One finding, which underlines the importance of initial cell density for application, was that as the initial seeding density was increased, the amount of cell proliferation in the scaffold also increased. Overall though, these tissue engineering efforts resulted in
constructs with non-uniform tissue growth and cellularity. This method of cell seeding is commonly referred to as “static” seeding. Generally, static seeding involves placing the scaffold into a static, high density cell suspension for a pre-set amount of time to allow for cell attachment. This method is useful for thin scaffolds, but often results in a loss of cells and an overall cellular distribution on the scaffold that is non-uniform. For example, the static seeding of bone marrow stromal cells onto estherified hyaluronic acid sponge meshes by Qi et al. [2004] resulted in scaffolds showing very uneven cellular distribution characterized by cell aggregation on the surfaces with very little internal cellularity. Results such as this can often be improved by manually applying the high density cell suspension directly and evenly to the scaffold surface.

In an attempt to improve on the static seeding methods, with the aim being to create tissue engineered constructs with a spatially uniform distribution of cells, various dynamic seeding protocols were introduced. In general terms, dynamic seeding involves the delivery of cells to the scaffold by means of flow. A very simple example of dynamic seeding was performed by Engelmayr et al. [2005] in which vascular smooth muscle cells were seeded onto 50:50 polyglycolic acid and poly-L-lactic acid scaffolds. Scaffolds were placed free-floating in a high density cell suspension in a 50 mL conical tube whose lid had been modified to allow gas exchange. These conical tubes were immediately placed on a rotisserie rotating device inside an incubator and rotated at 8 RPM. The principle behind this concept was that the rotational motion would provide mixing and flow of the cell suspension that would generate convective motion of cells into the interior of the scaffold [Vunjak-Novakovic et al., 2003], improving the internal cellularity of the engineered tissue. As well, in contrast to static seeding, cells that fail to encounter the scaffold for attachment are not lost to the bottom of the container but in fact remain in flow and will have many opportunities for successful attachment. There are many variations of this simple dynamic seeding, including the use of orbital shakers or magnetic stir bars to create flow. In seeding estherified hyaluronic acid sponge meshes with bone marrow stromal cells dynamically through the use of magnetic stir bars, Qi et al. [2004] were able to show a vast improvement in cell distribution uniformity and overall scaffold cellularity when compared to statically seeded scaffolds. These simple dynamic seeding methods work well with small scaffolds, but more complex dynamic seeding methods have also shown much promise and improvement. One such system, developed by the National Aeronautics and Space Administration (NASA), involves placing the scaffold in the annular space of rotating wall
vessel (RWV) between the outer wall and central core gas exchange membrane. In principle, the
vessel spins a cell suspension containing the scaffold slowly in order to neutralize the effects of
gravity, encouraging cells to grow in a natural manner. When comparing this system to the use of
rotating conical tubes, Sutherland et al. [2002] found that tissue engineered constructs seeded in
the RWV showed a more evenly distributed and larger cell population. While these dynamic
seeding methods were a definite improvement on the static seeding methods, uniform cellularity
through the internal regions of scaffolds, especially those larger in size, continued to be a
problem. In an attempt to rectify this problem, the concept of dynamic perfusion cell seeding
came about. A scaffold would be placed in a sealed cassette that would not allow flow around the
scaffold but instead would force flow through it. In this way, the cell suspension would be forced
through the thickness of the scaffold allowing internal cell attachment. One such method,
developed by Alvarez-Barreto et al. [2007], involved forcing a cell suspension containing
mesenchymal stem cells (MSCs) back and forth in an oscillatory manner through a cassette
containing a poly-L-lactic acid scaffold. This method showed much improvement in seeding
efficiency and the spatial distribution of cells when compared to static seeding. Overall, dynamic
seeding of scaffolds generally increases cell attachment and improves the spatial distribution of
attached cells. Depending on application, one seeding method may be more appropriate than
another.

Once cells have been delivered and seeded onto the scaffold, the engineered tissue must
be allowed to cultivate. In the same way as seeding, tissue engineered constructs can be
cultivated both statically and dynamically. Static cultivation involves placing the construct in
static culture media in an incubated environment. This can prove successful with thin scaffolds,
but a lack of media perfusion to the innermost sections of a thick scaffold will result in an
uneven cell growth and viability. For this reason, dynamic cultivation is generally preferred.
Through rotational motion, the use of magnetic stir bars, and especially through the use of
perfusion cassettes, culture media can better reach the internal sections of a construct via
convective means. This will allow for cells in the innermost regions of the construct to remain
viable and proliferate by receiving a steady supply of nutrients and the removal of cellular waste.
Overall, the construct will have a much more uniform cell distribution which is required for
uniform tissue formation.
3. Bioreactor Design & Characterization

3.1. Introduction
In order to study the effects of mechanical strain on tissue and cells, a custom bioreactor was designed and manufactured, coordinated by Joel Rebello and Waleed Nasar, based on a design by Engelmayr et al. [2003]. This bioreactor device allowed for the application of mechanical strain to native porcine aortic valve leaflets as well as to the proposed tissue engineered aortic valve leaflet model (a PAVIC-seeded collagen sponge scaffold). The bioreactor was constructed of stainless steel and polycarbonate to allow for ease of sterilization, and a lid was designed similar to that of a 6-well plate lid in order to allow gas exchange (Figure 10). For the design of the bioreactor, the following design criteria were considered: the accommodation of native and tissue engineered aortic valve leaflets, the application of physiological loads within an incubated environment, and the maintenance of sterility and cell viability.

![Figure 10: Bioreactor (A) without gas exchange lid and (B) with gas exchange lid.](image)

3.2. Accommodation of Native and Tissue Engineered Aortic Valve Leaflets
The bioreactor consists of six culture wells, and within each well, on either side, are a variety of posts to allow for the fixation of either native porcine aortic valve leaflets or the tissue
engineered aortic valve leaflet model. Two dynamic posts are located in the middle of each well and are commonly attached to a central piston that can provide biaxial three-point flexure.

In order to accommodate the tissue engineered leaflet model, four 2 mm diameter stainless steel posts are used, two on either side of the well. At each side of the well, the leaflet model can be fixed by bringing the adjustable post together with the stationary post and tightening the screw to hold the adjustable post in place (Figure 11). Tygon PVC tubing (McMaster, 5347K21) with an outer diameter of 3 mm was placed over the posts to provide a no-slip surface when fixing the leaflet model in place. Posts on either side are 20 mm apart, and therefore leaflet models must be slightly longer in length in order to be fixed in the bioreactor. Once fixed in the bioreactor, the dynamic central posts was dropped into place on either side of the leaflet model and commonly attached to the driving piston for the application of strain.

Figure 11: Bioreactor well setup for tissue engineered aortic valve leaflet model showing (A) leaflet model being fixed between outer posts and (B) introduction of central dynamic posts.

For the application of strain to native porcine aortic valve leaflets, the general dimensions of porcine leaflets were considered in order to allow for proper fixation within the well. Porcine aortic valve leaflets were excised and measured, and were found to have an average length of approximately 20 mm. To accommodate for the leaflets, two stationary posts, 1 mm in diameter, were placed 15 mm apart and 3 mm from the plane created by the 2 mm diameter stationary posts. This was completed after original manufacture by this investigator. Tygon PVC tubing (McMaster, 5347K21) with an outer diameter of 2 mm was placed over the posts to provide a no-slip surface. In order to fix the leaflets in place, a suture needle (Syneture, GS-833) was pushed through either edge of the leaflet, and was fixed in the well by placing the leaflet between the outer posts and the suture needles outside of the posts (Figure 12). Once the leaflet was fixed in
the well, the dynamic central post was dropped into place and commonly attached to the driving piston for the application of strain.

![Image](image1.png)

Figure 12: Bioreactor well setup for native porcine aortic valve leaflets showing (A) leaflets being fixed between outer posts and (B) introduction of central dynamic post.

3.3. Application of Physiological Loads

3.3.1. Bioreactor System Setup

To apply strain to either the native porcine aortic valve leaflet or the tissue engineered leaflet model, an actuating device was needed to interface with the bioreactor piston in order to drive the dynamic central posts. A stepper motor actuator (Ultra Motion, HT17-075) was utilized for this purpose. The stepper motor actuator (Figure 13) was powered by a high torque NEMA-17 size stepper motor with an Acme nut 0.0833 inches/revolution lead screw and a total stroke length of 2 inches. The actuator was equipped with one normally-open externally adjustable position switch and had a 1/4-28 UNF threaded hole nose fitting. The motor housing was coated with a rubber polymer to keep moisture out, allowing it to function in an incubated environment, and the actuator came equipped with a bracket mount tube fitting to allow it to be fixed to a base.

![Image](image2.png)

Figure 13: Stepper motor actuator utilized to interface with bioreactor piston and drive dynamic posts.
After the actuator was chosen, a custom interfacing joint was designed in order for the actuator to attach to the bioreactor piston. This interfacing joint was designed such that the actuating motion could be transferred to the bioreactor piston without incurring any potentially damaging side loads at either the actuator or bioreactor end. As well, the actuator end of the joint had to be able to be screwed into the nose fitting, while the bioreactor end had to allow for a screw to be placed through it for attachment to the piston. A nut was added to allow for adjustment of length from the actuator. After design, the interfacing joint was fabricated from stainless steel by machinists at the University of Toronto Department of Mechanical and Industrial Engineering Machine Shop (Figure 14).

![Custom designed actuator to bioreactor interfacing joint.](image)

A custom base was designed and built from acrylic glass. This base allowed for the bioreactor and actuator to be mounted on one singular base, interfaced using the custom designed joint, and was the proper size to be placed in an incubator. As well, the acrylic glass provided a base material that could easily be cleaned and sterilized. The design of the base allowed for the bioreactor to be detached from the actuator and removed from the base when needed. The actuator was fixed to the base and could only be detached through removal of screws. The design also ensured that the actuator was completely in line with the bioreactor piston in order to avoid any potentially damaging side loads (Figure 15).
Once the base was setup and the actuator had been interfaced with the bioreactor, a controller system was needed to program the actuator motion. A separate, external programmable step motor drive (Applied Motion Products, Si2035) was utilized, and with the aid of the supplied software, allowed for the actuator to be programmed to displace the bioreactor piston to different positions at different speeds (Figure 16). The first thing the motor drive needed was a power source supplying 110 V AC, and thus a regular wall plug was wired to supply this power. The actuator motor had eight leads and was parallel connected to the motor drive (Figure 17). The actuator position switch was also hardwired into the motor drive for input as well as an external 24 V computer power supply for power. The position switch has two leads: one red lead and one black lead. The red lead was connected to the I/O serial plug of the motor drive at input 4 (pin 6), the black lead was connected to the ground/common lead of the computer power supply, and a connection was made from the +24 V lead of the computer power supply to a common input (pin 8) on the motor drive. As well, the motor drive was supplied with a computer connection cord for programming which would attach at the PC/MMI input. For ease of transport, a custom acrylic glass base was built and the motor drive and computer power supply were attached to it.

Figure 15: Custom acrylic glass base for actuator and bioreactor.

Figure 16: Si2035 motor drive used to control actuator motor and receive input from position switch.
3.3.2. Bioreactor System Programming

In order to program and control the actuator motion, the supplied software, SiProgrammer, was used. The software was installed on a personal computer and was required to be running on the computer prior to the connection of the motor drive. The supplied computer connection cord was then plugged into the computer serial input and into the motor drive. Following this, the computer power supply was always plugged in first to provide power to the position switch, and finally the motor drive was plugged into a wall outlet. The software would recognize the motor drive model number, and allow for a series of steps to be entered and downloaded into the motor drive. The first step in programming the motor drive was to input the base distance rate, which was computed from the lead screw specifications to be 2.108 mm/revolution and further to 9488 steps/mm (Figure 18).
To enter the first step in the program, and any subsequent steps, the down arrow next to the corresponding line was clicked, which would bring up a menu with many movement choices (Figure 19).

Figure 18: Home screen of SiProgrammer after entering base distance rate.

Figure 19: Movement choices menu accessed by clicking down arrow next to step number.
The first step of the program was to “Seek Home”. This step allowed for the actuator to move to a home position as dictated by the position switch. After clicking the corresponding button, a further menu would appear. The motor direction was selected as CCW as this recedes the actuator, and the speed was set as desired. As well, the input was selected as IN4 (the position switch was hardwired to input 4) and the condition was selected as “Low”, as the position switch is a normally open switch and thus low is considered closed (Figure 20).

Figure 20: “Seek Home” menu: first step of program is to seek home.

After input of the first seek home step, the second step of the program was entered. From the movement choices, the “Feed to Length” option was chosen. The motor direction was selected as CW to push the actuator out, and the speed was set as desired. As well, the distance that the actuator would move out from the home position was entered (Figure 21).

Figure 21: “Feed to Length” menu: second step of program is for actuator to push out a defined distance at a defined speed.
The third step involved bringing the actuator back the same distance that it pushed out in the second step. Once again, the “Feed to Length” option was chosen, and all options remained the same as the last step except for the motor direction, which was selected to be CCW to bring the actuator back (Figure 22).

![Feed to Length menu](image)

Figure 22: “Feed to Length” menu: third step of program is for actuator to pull back same distance at same speed as second step.

The final step for input was a looping step to allow the program to continue running without interruption. The “Go To” option was selected, and required an input of a step number to revert back to, which in this case was step 2 (Figure 23).

![Go To menu](image)

Figure 23: “Go To” menu: final step of program is to loop back to second step.

After entering these steps, they appeared on the home screen in order. The “Download” button was selected and the program was subsequently transferred into the motor drive internal memory. Disregarding speed and distance specification, this was the basic program used for applying strain to both native porcine aortic valve leaflets and the tissue engineered leaflet model. Once the program was downloaded to the motor drive, it was unplugged from the wall.
and computer, and the computer power supply running the position switch was also unplugged. For future use, the computer power supply was always plugged in first and once running, the motor drive was plugged in. At this time, the motor drive would perform the program standing alone. Overall, the program functions as such: the actuator would pull in until reaching the position switch; the actuator would then push out at the defined speed to the defined distance; the actuator would then pull back at the same speed over the same distance as the previous step; finally the program would loop back and push out once again (Figure 24).

![SiProgrammer home screen with steps programmed and ready to be downloaded to motor drive.](image)

**Figure 24**: SiProgrammer home screen with steps programmed and ready to be downloaded to motor drive.

### 3.3.3. Dynamic Pin Displacement and Physiological Bending

In order to replicate the physiological degree of bending for both the native porcine aortic valve leaflets and the tissue engineered leaflet model, the displacement of the central dynamic pin in the bioreactor was carefully chosen such that the resultant degree of bending applied by the dynamic pin (and by extension the actuator) was physiologically relevant. Using aortic valve dimension data acquired by Swanson et al. [1974], two important angles of deflection observed in coapted aortic valve leaflets were determined (Figure 25). The first angle related to the leaflet deflection observed when viewing the leaflet from the side (identified in blue as angle A). With the provided data, the angle of deflection was calculated to be 112°. The second angle related to the leaflet deflection observed when viewing the leaflet from the aortic wall (identified in red as...
angle B). Once again, with the provided data, the angle of deflection was calculated to be $116^\circ$. Thus, to provide a physiological relevant loading to either the native aortic valve leaflets or the tissue engineered leaflet model, the angle of deflection as applied by the central dynamic pins of the bioreactor should be reasonably close to the above stated angles.

![Figure 25: Aortic valve with relevant physiological angles identified [Swanson et al., 1974].](image)

For the application of strain to the native porcine aortic valve leaflets, the dynamic pin displacement, as entered into the SiProgrammer “Feed to Length” commands, was chosen to be 6 mm. At this displacement, the angle of deflection of the leaflet in the bioreactor was approximately $110^\circ$. This is very close to the physiological deflection angles observed in aortic valve leaflets in situ, and thus the bioreactor provided physiologically relevant loading to the porcine leaflets.

When applying strain to the tissue engineered leaflet model, the dimensions for fixation in the bioreactor were slightly different, thus a different amount of dynamic pin displacement was required. The displacement, as entered into the SiProgrammer “Feed to Length” commands,
was chosen to be 8 mm. At this displacement, the angle of deflection of the leaflet model in the bioreactor was approximately 103°. This is reasonably close in value to the physiological deflection angles observed of aortic valve leaflets in situ, and thus the bioreactor also provided physiologically relevant loading to the tissue engineered leaflet model.

![Native Leaflets: Pin Displacement = 6 mm, θ = 110°](image1)

![Leaflet Model: Pin Displacement = 8 mm, θ = 103°](image2)

Figure 26: Dynamic pin displacement and resultant deflection angles.

### 3.4. Maintaining Sterility and Cell Viability

As stated, the bioreactor was designed to allow for the application of strain while also providing an environment in which living cells could maintain normal function, remain viable and proliferate. While it was designed to allow for these things, an important first step was to ensure that the bioreactor actually could maintain a population of cells in a sterile environment. Using PAVICs, a proof or principle experiment was performed to show that the bioreactor could support a population of cells while maintaining sterility.

#### 3.4.1. Materials & Methods

**3.4.1.1 PAVIC Isolation Protocol**

PAVICs were isolated from porcine hearts obtained from a local abattoir and transported to the laboratory on ice in phosphate buffered saline (PBS) with calcium and magnesium (Invitrogen, P1504). Under sterile conditions, an incision was made down the aortic wall and through the aortic valve at one of the three commissures. The three leaflets of the aortic valve were then dissected and placed in a series of three washes consisting of PBS with calcium and magnesium with 1% penicillin/streptomycin antibiotic solution (Sigma, P4333) and 1% amphotericin B (Sigma, A2942). Following these three washes, the intact leaflets were placed
into 5 mL of a collagenase solution consisting of type I collagenase (Sigma, P0130) with a concentration of 150 U/mL (diluted in PBS without calcium and magnesium (Sigma, P3813)) and incubated at 37°C and 5% CO₂ for twenty minutes. Following this incubation, the leaflets were agitated by vortex vigorously for one minute to remove endothelial cells from the leaflets. The leaflets were then transferred into a Petri dish where they were minced into very small pieces using scissors. These small pieces were then placed into another 5 mL of 150 U/mL collagenase solution and incubated at 37°C and 5% CO₂ for two hours. The small leaflet pieces were then agitated by vortex again for one minute to release interstitial cells. Following this, the collagenase solution was filtered using a 70 µm cell strainer (BD Falcon, 352350) to remove the small leaflet pieces. The strainer was rinsed twice with 5 mL of supplemented culture media, which contained Dulbecco’s modified Eagle’s medium (Sigma, D7777) with 10% fetal bovine serum, 1% penicillin/streptomycin antibiotic solution and 1% amphotericin B. The resulting cell solution was centrifuged into a cell pellet and resuspended in supplemented culture media.

### 3.4.1.2 PAVIC Preparation

Following isolation, cells were counted using a Beckman Coulter Vi-CELL XR cell viability analyzer unit. Glass coverslips measuring 18 mm in diameter were placed in the wells of three 6-well plates and isolated cells were applied to the coverslips at a density of 10000 cells/cm² and incubated at 37°C and 5% CO₂ for 24h in 5 mL of supplemented culture media. Three coverslips were removed at this time and cells were removed from the coverslips by applying 0.25% trypsin with EDTA for ten minutes. Cells were then counted using the Vi-CELL to get an initial cell count. As well, six coverslips were removed and transferred to the wells of the bioreactor, while a further six coverslips were transferred into the wells of a new 6-well plate for incubation in 5 mL of media. At time points of both 72h and 168h three coverslips were removed from each environment (bioreactor and 6-well plate), and cells were removed from the coverslips and counted for comparison.

### 3.4.1.3 Statistical Analysis

In order to identify significant differences in the amount of PAVICs in each environment, a method supplemental to analysis of variance (ANOVA), called Tukey’s honestly significantly different (HSD) test, was utilized. This allowed for comparisons to be made between time points for each different environment. Results are reported as mean ± standard deviation, and the
3.4.2. Results & Discussion

Cell proliferation in the bioreactor was comparable to that in the 6-well tissue culture plate (Figure 27), with no significant differences observed between environments at any time point. In both the bioreactor and 6-well plate, the number of cells increased significantly with time in culture between initial and 168h counts (p<0.02 using Tukey’s test), as well as between 72h and 168h counts (p<0.02 using Tukey’s test). As well, culture media in the wells remained clear and cell viability remained well over 90% at all time points, indicating the bioreactor maintained sterility without contamination.

![Graph showing cell proliferation](image)

Figure 27: PAVIC population in bioreactor and 6-well plate is comparable at all time points, and a significant increase in number of PAVICs is observed with time (* p<0.02 relative to initial and 72h cell counts using Tukey’s test). Data are presented as mean ± standard deviation (n=3).

3.5. Conclusions

In review, the design criteria for the bioreactor were: the accommodation of native and tissue engineered aortic valve leaflets, the application of physiological loads within an incubated
environment, and the maintenance of sterility and cell viability. Utilizing a series of fixed pins, the bioreactor was shown to accommodate the dimensions of both native porcine aortic valve leaflets as well as the tissue engineered aortic valve leaflet model while keeping them fixed for the application of strain. The bioreactor, actuator and motor drive permit physiological loads to be applied to native porcine aortic valve leaflets and tissue engineered aortic valve leaflet models for biomechanics-related experimentation. Finally, PAVICs in the bioreactor proliferated normally and remained sterile, indicating that the bioreactor provides appropriate conditions for tissue culture.
4. Application of Physiological Loading to Native Porcine Aortic Valve Leaflets

4.1. Introduction

As outlined in section 2, mechanical loading appears to play a major role in the pathogenesis of aortic valve sclerosis. Mechanical loading has been shown to promote the differentiation of aortic valve interstitial cells to the myofibroblast phenotype as identified by the enhanced expression of α-SMA [Balachandran et al. 2006], typical of diseased aortic valve leaflets. In addition, interstitial cells undergoing myofibroblast differentiation show an upregulation of cofilin [Pho et al., 2007], a protein theorized to be involved in the formation of α-SMA-containing stress fibers [Clement et al., 2005]. As well, it has been shown that areas of the leaflet subjected qualitatively to the greatest amount of mechanical strain in vivo were the areas most likely to form aortic valve lesions [Thubrikar, 1990]. The development, setup and characterization of the bioreactor system brought about the ability for native aortic valve leaflets to be subjected to mechanical loading ex vivo. Through the use of the bioreactor system, the degree of mechanical loading could be controlled to mimic physiological conditions while the potentially confounding effects of the in vivo environment, such as the effects of inflammatory cytokines, could be minimized. In this way, the effects of physiological loading on native aortic valve leaflets could be studied.

Due to their availability and their structural and anatomical similarities to human aortic valve leaflets, porcine aortic valve leaflets were utilized for this study. Leaflets were subjected to applied physiological strain, and protein and transcript levels of α-SMA and cofilin were observed as an indication of myofibrogenic differentiation and by extension its correlation to the pathogenesis of aortic valve disease.

4.2. Materials & Methods

4.2.1. Aortic Valve Leaflet Preparation and Application of Physiological Loading

As with the PAVIC isolation protocol, porcine hearts were obtained from a local abattoir and transported to the laboratory on ice in phosphate buffered saline (PBS) with calcium and magnesium (Invitrogen, P1504). Under sterile conditions, an incision was made down the aortic wall and through the aortic valve at one of the three commissures. The three leaflets of the aortic valve were then dissected and placed in a series of three washes consisting of PBS with calcium and magnesium with 1% penicillin/streptomycin antibiotic solution (Sigma, P4333) and 1%
amphotericin B (Sigma, A2942). After washing, leaflets were either loaded in the bioreactor for the application of physiological strain as outlined in sections 3.2 and 3.3.3, or were placed in the wells of a 6-well plate for static culture. For the application of strain, leaflets were loaded such that the fibrosa rested against and was pushed by the central dynamic pin, mimicking the in vivo flexure conditions (Figure 28). The central dynamic pin was set to displace 6 mm at a frequency of 1 Hz continuously for 96h. In both cases 10 mL of supplemented culture media was added to each well. Leaflets remained in their respective environments for four days. This decision was based on a finding by Cimini et al. [2002] that explanted porcine aortic valve leaflets will maintain cell viability up to 96h in culture.

![Figure 28](image)

**Figure 28:** Native porcine aortic valve leaflet orientation in bioreactor for application of physiological strain, with dynamic pin in full displacement.

### 4.2.2. Paraffin Embedding, Histochemical and Immunohistochemical Staining

To assess protein expression in leaflets, samples were prepared for (immuno)histochemical staining by fixation in 3.7% formaldehyde and taken to the Histology and Pathology Laboratory located at Mt. Sinai Hospital in Toronto for processing. Sections were embedded in paraffin and 5 µm thick sections were cut and mounted. Some sections were stained for the expression of various proteins, including monoclonal mouse anti-human α-SMA (clone 1A4; Sigma, A5691), monoclonal mouse anti-human vimentin (clone VIM3B4; American Research Products Incorporated, 03-65013) and monoclonal mouse anti-human desmin (clone D33; Dako, M0760). When staining for α-SMA, 0.5% bovine serum albumin (Sigma, A8531) was used for blocking, while 1% horse serum (Sigma, H1270) was used for blocking when staining for vimentin or desmin. For all proteins, the ABC-HRP detection system was used with appropriate secondary antibodies and diaminobenzidine tetrahydrochloride (brown) or alkaline
phosphatase (red) as the chromagen. As well, some sections were stained with hematoxylin and eosin.

Once sectioning and staining was completed, slides were viewed using a Leitz Aristoplan Variophot light microscope and photos were obtained using a QImaging MicroPublisher 5.0 RTV camera along with OpenLab 4.0.4.

4.2.3. Histomorphometric Quantification
To assess the spatial expression of \( \alpha \)-SMA in strained leaflets, a series of images were taken over the length of the leaflet (images were taken as described in section 4.2.2). A template was cut from a piece of paper leaving a small rectangular opening, and this template was placed over the images in the same position for each image. The total number of cells, as well as the number of \( \alpha \)-SMA-positive cells, was counted within each rectangular opening for the ventricularis, spongiosa and fibrosa regions of the leaflet. This allowed for the percentage of \( \alpha \)-SMA-positive cells to be calculated for each region of the leaflet without bias.

4.2.4. RNA Extraction
After being subjected to applied strain in the bioreactor or static culture for four days, native porcine aortic valve leaflets were removed for RNA extraction. Using the RNeasy Fibrous Tissue Mini Kit (Qiagen, 74704), leaflet samples were first homogenized in the supplied lysis buffer in order to thoroughly disrupt and break up the tissue. Digestion of the homogenized tissue was also carried out, as per the manufacturer’s directions, using proteinase K which degrades the structural proteins that keep the tissue intact. The extraction of RNA was carried out by following the manufacturer’s instructions supplied with the RNeasy Fibrous Tissue Mini Kit. After RNA extraction, the concentration and quality of the extracted RNA was assessed using a NanoDrop ND-1000 spectrophotometer and the supplied software. From each sample, 1.5 \( \mu \)L of extracted RNA solution was placed on the platen, and the RNA concentration (in ng/\( \mu \)L) and RNA quality were assessed by absorbance measurement. Since nucleic acids are detected at 260nm, and contaminants such as proteins and salts are detected at 280nm, a large 260/280 ratio for a sample is desired. Typically, good quality RNA will have a 260/280 ratio of approximately 2.
4.2.5. cDNA Synthesis
Following RNA extraction and testing using the spectrophotometer, the resultant RNA strands were used to synthesize complementary single strands of DNA, referred to as cDNA. RNA samples, in 50 µL tubes, were diluted with nuclease-free water to a total volume of 10 µL such that each sample contained the same amount of RNA. Following this, 1 µL each of oligo dT (Invitrogen, 58862) and 10 mM dNTP mix (Invitrogen, 18427-013) was added to each sample. The tubes were then loaded into an Eppendorf Realplex² Mastercycler for cDNA synthesis. The Mastercycler brought tubes to 65°C for five minutes and then to 42°C for thirty seconds before pausing. At this time, tubes were removed and the following reagents were added to each sample: 4 µL of 5X first strand buffer (Invitrogen, Y00146), and 1 µL each of 0.1 M DTT (Invitrogen, Y00147), reverse transcriptase superscript (Invitrogen, 18064-014) and RNaseOut (Invitrogen, 10777-019). Tubes were then returned to the Mastercycler, which brought tubes to 42°C for one hour, 72°C for fifteen minutes and finally down to 4°C until removed from the machine. At this point, the quantity and quality of the synthesized cDNA was assessed using the spectrophotometer as before. cDNA concentration was given in ng/µL based on the absorbance at 260 nm, while quality was assessed once again through the 260/280 ratio.

4.2.6. Regular Polymerase Chain Reaction (PCR)
The resultant cDNA was used for polymerase chain reaction (PCR) to amplify sections of cDNA selected through the use of custom forward and reverse primers. In new 50 µL tubes, cDNA samples were diluted with nuclease-free water to a total volume of 1 µL such that each sample contained the same amount of cDNA. To each 1 µL sample of cDNA, the following reagents were added: 5 µL of ThermoPol buffer (New England BioLabs, M0267L), 1 µL of 10 mM dNTP mix, 0.5 µL of Taq DNA polymerase (New England BioLabs, M0267L) and 40.5 µL of nuclease-free water. As well, 1 µL each of a forward and reverse primer were added to each sample (Table 1).

Once all reagents had been placed in the tubes with the cDNA samples, the tubes were placed in the Mastercycler and brought to 95°C for two minutes. Following this, the following steps were cycled thirty times: 95°C for fifteen seconds, 60°C for fifteen seconds, and then 72°C for twenty seconds. After thirty cycles, the tubes remained at 72°C for seven minutes before being brought down to 4°C until removal, leaving the PCR products for further analysis.
### Table 1: PCR primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Manufacturer</th>
<th>Accession #</th>
<th>Amplicon Size</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Invitrogen, 7040511</td>
<td>AF017079</td>
<td>122 bp</td>
<td>5′-TGTACCACCAACTGCTTGCCG-3’</td>
<td>5′-GGCATGGACCTGTTGGCATCGAG-3’</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Invitrogen, 45450458</td>
<td>See Simmons et al., 2004</td>
<td>156 bp</td>
<td>5′-CGATGAAGGAGGGCTTGAAACAGGG-3’</td>
<td>5′-CGTGACCACTGCGAGCGTGAGAT-3’</td>
</tr>
<tr>
<td>Cofilin</td>
<td>Invitrogen, 7008265</td>
<td>NM_001004043</td>
<td>145 bp</td>
<td>5′-GAAGGAGGACCTGTTGTTCA-3’</td>
<td>5′-GACCTCCTCGTAGCAGTTCG-3’</td>
</tr>
</tbody>
</table>

### 4.2.7. Gel Electrophoresis

In order to quantify the amount of GAPDH, α-SMA or cofilin found at the transcript level, gel electrophoresis using the PCR products was performed. Prior to electrophoresis, a gel was made. Two grams of agarose powder (BioShop, AGA002) was mixed into 50 mL of 1X TAE buffer as diluted from 40X TAE buffer (Promega, V4281) with deionized water. The mixture was heated until the agarose powder was dissolved at which point 3 μL of ethidium bromide (Sigma, E1510) was added. The mixture was agitated by hand to ensure disbursement of the ethidium bromide and was then poured into a mould that created a flat, thin 4% gel with wells for addition of the PCR products.

Once the gel solidified, it was placed into a Labnet Gel XL Ultra V-2 gel electrophoresis machine containing enough 1% TAE buffer to completely immerse the gel. At this point, 10 μL of each sample was mixed thoroughly with 2 μL of 6X orange loading dye solution (Fermentas, R0631), and each total 12 μL mixture was placed into a well on the gel. The voltage was set to 100 V and was run for fifteen minutes.
4.2.8. **Gel Imaging and Semi-Quantification**  
Immediately after running the gel for fifteen minutes at 100 V, the gel was taken for imaging. The gel was placed in a BioDoc-It Imaging System with an M-20 Transilluminator in order to observe and take digital images. Photos taken using the BioDoc-It system were analyzed using ImageJ software. Using the cursor, a box was drawn on the image around the amplicon size where positive expression would appear on the gel (Figure 29A). Following this, the ImageJ analysis tool “Plot Profile” was used to generate a graph of light intensity correlating to position in the box (Figure 29B). From this graph, the numerical value of light intensity for each well was recorded. For one sample, the numerical light intensity reading taken for either coflin or α-SMA was divided by the reading taken for GAPDH, which was used as a baseline or housekeeping transcript due to its constant expression level in all cells. These light intensity ratios were used to describe the transcript expression in that sample. For example, the larger the ratio of coflin light intensity to GAPDH light intensity as taken from the gel, the more coflin transcript there is expected to be present in the sample. Thus all results are reported as either coflin or α-SMA expression relative to GAPDH expression.

![Figure 29: Method for semi-quantification of PCR results by (A) drawing a box around positive expression and (B) running a plot profile, both in ImageJ.](image)

4.2.9. **Statistical Analysis**  
For each PCR sample, two technical replicates were made. This means that one sample of cDNA resulted in two sets of PCR products for each of GAPDH, α-SMA and coflin. The mean transcript expression, for each sample, was taken as the average numerical light intensity between technical replicates. Results were reported as mean α-SMA or coflin expression relative to GAPDH expression, ± standard deviation. The studentized t-test was used to identify
significant differences in transcript expression between samples from different environments. The sample size and p-value are identified in the figure caption.

For histomorphometric analysis, the percentage of $\alpha$-SMA-positive cells for the ventricularis, spongiosa and fibrosa regions of the strained leaflets was calculated, and results were reported as mean percentage of $\alpha$-SMA-positive cells ± standard deviation. Analysis of variance (ANOVA) and Tukey’s honestly significantly different (HSD) test were utilized to identify statistical differences in the percentage of $\alpha$-SMA-positive cells between leaflet regions. The studentized t-test was used to identify significant differences in the whole leaflet percentage of $\alpha$-SMA-positive cells.

4.3. Results

4.3.1. Application of Physiological Strain and Static Culture with Supplemented Culture Media Containing 10% Fetal Bovine Serum

Native porcine aortic valve leaflets were either loaded into the bioreactor for the application of strain or cultured statically. In both cases the leaflets were cultured using Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin antibiotic solution and 1% amphotericin B. Using regular polymerase chain reaction (PCR) and ImageJ to quantify gel results, a significant increase in the expression of the $\alpha$-SMA transcript was measured in the porcine aortic valve leaflets subjected to applied strain (Figure 30) for four days as compared to the leaflets that were cultured statically (p<0.05 using t-test). As well, a significant increase in the expression of the cofilin transcript (Figure 31) was also measured in the leaflets subjected to applied strain for four days as compared to those statically cultured (p<0.03 using t-test). The upregulation of the $\alpha$-SMA transcript indicates that mechanical loading of leaflets may be affecting the phenotype of resident cells and promoting myofibrogenesis. Upregulation of the cofilin transcript may indicate an increase in turnover of actin filaments or an increase in the formation of $\alpha$-SMA-containing stress fibers, consistent with the upregulation of the $\alpha$-SMA transcript.
Figure 30: α-SMA transcript expression relative to GAPDH expression for strained and statically cultured leaflets in 10% FBS (* p<0.05 relative to static culture condition using t-test). Data are presented as mean ± standard deviation (n=3). Similar results were observed in preliminary experiments.

Figure 31: Cofilin transcript expression relative to GAPDH expression for strained and statically cultured leaflets in 10% FBS (* p<0.03 relative to static culture condition using t-test). Data are presented as mean ± standard deviation (n=3). Similar results were observed in preliminary experiments.
Leaflets subjected to applied strain in the bioreactor also showed positive expression of α-SMA at the protein level as indicated by immunohistochemical staining. In contrast, statically cultured leaflets showed very little, if any, positive expression. In terms of the percentage of α-SMA-positive cells, the mechanically loaded leaflets had a significantly larger percentage than those statically cultured (p<0.001 using t-test). The staining showed focal areas of α-SMA positive cells located in the fibrosa and spongiosa regions of the leaflets (Figures 32 and 33, respectively). This pattern of α-SMA expression is comparable to that observed in sclerotic porcine aortic valve leaflets [Pho et al., 2007]. These results are consistent with the upregulation of α-SMA at the transcript level, and indicate a shift in cell phenotype from the quiescent fibroblast to the myofibroblast.

Figure 32: α-SMA staining showing positive protein expression in the fibrosa region of a strained leaflet in 10% FBS, original magnification 40X.

Figure 33: α-SMA staining showing positive protein expression in the spongiosa region of a strained leaflet in 10% FBS, original magnification 40X.
The spatial position of α-SMA-positive cells within strained leaflets was assessed through histomorphometric analysis. The percentage of α-SMA-positive cells was calculated for the ventricularis, spongiosa and fibrosa regions of the leaflet. α-SMA-positive cells were absent from the ventricularis in all strained leaflets, but appeared as approximately 9% of the total spongiosa cell population and as approximately 15% of the total fibrosa cell population (Figure 34). The percentage of α-SMA-positive cells in the spongiosa region was not significantly different to that of the fibrosa region, but both regions showed a significantly larger percentage of α-SMA-positive cells when compared to the ventricularis region (p<0.05 using Tukey’s test). These results indicate that myofibrogenic differentiation, as indicated by the percentage of α-SMA-positive cells, is more prevalent in the spongiosa and fibrosa regions of the aortic valve leaflet when subjected to applied strain.

Figure 34: The percentage of α-SMA-positive cells in various regions of the mechanically loaded leaflet in 10% FBS. The percentage of α-SMA-positive cells in the spongiosa and fibrosa are statistically similar, but are both significantly larger (p<0.05 using Tukey’s test) than the percentage observed in the ventricularis (0%, not shown).
4.3.2. Application of Physiological Strain and Static Culture with Supplemented Culture Media Containing 1% Fetal Bovine Serum

FBS is a natural extract taken from bovine fetuses, and contains many growth factors possibly including transforming growth factor β1 (TGF-β1). As discussed in section 2.3.1, TGF-β1 has been observed to not only play a role in the appearance of α-SMA-positive aortic valve interstitial myofibroblasts, but acts, along with mechanical tension, to synergistically cause myofibrogenic differentiation [Merryman et al., 2007]. Thus, after observing the upregulation of α-SMA using supplemented culture media containing 10% FBS, 1% FBS was used in an attempt to reduce the amount of TGF-β1 in culture and by extension eliminate any confounding effects in order to elucidate the effects of mechanical loading individually. Native porcine aortic valve leaflets were either loaded into the bioreactor for the application of strain or cultured statically. In both cases, leaflets were cultured using Dulbecco’s modified Eagle’s medium supplemented with 1% fetal bovine serum (FBS), 1% penicillin/streptomycin antibiotic solution and 1% amphotericin B. Leaflets subjected to applied strain in the bioreactor showed positive expression of α-SMA at the protein level as indicated through immunohistochemical staining. Statically cultured leaflets showed very little, if any, positive expression. In terms of the percentage of α-SMA-positive cells, the mechanically loaded leaflets had a significantly larger percentage than those statically cultured (p<0.001 using t-test). The staining showed focal areas of α-SMA positive cells located in the fibrosa and spongiosa regions of the leaflets (Figures 35 and 36 respectively), consistent with results observed when using supplemented media containing 10% FBS. As before, this pattern of α-SMA expression is comparable to that observed in sclerotic porcine aortic valve leaflets [Pho et al., 2007].
As previously, the spatial position of α-SMA-positive cells within strained leaflets was assessed through histomorphometric analysis. The percentage of α-SMA-positive cells was calculated for the ventricularis, spongiosa and fibrosa regions of the leaflet. α-SMA-positive cells were virtually absent from the ventricularis in all strained leaflets (approximately 0.3% of total ventricularis cell population), but appeared as approximately 7% of the total spongiosa cell population and as approximately 9% of the total fibrosa cell population (Figure 37). The percentage of α-SMA-positive cells in the spongiosa region was not significantly different from that in the fibrosa region, but both regions showed a significantly larger percentage of α-SMA-
positive cells when compared to the ventricularis region (p<0.01 using Tukey’s test). These results indicate that myofibrogenic differentiation, as indicated by the percentage of α-SMA-positive cells, is more prevalent in the spongiosa and fibrosa regions of the aortic valve leaflet when subjected to applied strain. These results are consistent with the spatial expression of α-SMA observed when using supplemented media containing 10% FBS.

Figure 37: The percentage of α-SMA-positive cells in various regions of the mechanically loaded leaflet in 1% FBS. The percentage of α-SMA-positive cells in the spongiosa and fibrosa are statistically similar, but are both significantly larger (*p<0.01 using Tukey’s test) than the percentage observed in the ventricularis.

4.4. Discussion

When comparing aortic valve interstitial fibroblasts to myofibroblasts, cultured in vitro, myofibroblasts have been observed to upregulate both α-SMA and coflin expression. Thus, it has been speculated that coflin is a marker of myofibrogenic differentiation [Pho et al., 2007]. Analysis using PCR showed a greater than 35% increase in the expression of the α-SMA transcript in physiologically strained porcine aortic valve leaflets when compared to those cultured statically, as well as a greater than 60% increase in the expression of the coflin transcript. Thus, the correlation between α-SMA and coflin expression also appears to hold true.
in whole aortic valve leaflets \textit{ex vivo}. Furthermore, while the correlation between mechanical loading and whole leaflet $\alpha$-SMA expression has been made previously [Balachandran \textit{et al.}, 2006], we now report that there is a correlation between mechanical loading and whole leaflet cofilin expression. Cofilin has been shown to play a vital role in the assembly, disassembly and organization of actin filaments within eukaryotic cells [Carlier \textit{et al.}, 1997, McGough \textit{et al.}, 1997, Theriot 1997]. As well, it has been suggested that cofilin plays a direct role in the incorporation of $\alpha$-SMA filaments into cytoskeletal stress fibers [Clement \textit{et al.}, 2005]. Through siRNA knockdown of cofilin \textit{in vitro}, Pho \textit{et al.} [2007] observed that aortic valve interstitial cells were unable to form the $\alpha$-SMA-containing stress fibers that are typically observed in interstitial cells cultured on stiff tissue culture plastic. It is speculated then that the application of mechanical loading to aortic valve leaflets \textit{ex vivo} causes an upregulation of cofilin in order to increase the rate of actin turnover, and possibly through increased intercellular actin concentrations or by direct $\alpha$-SMA incorporation into stress fibers, leads to the increased expression of $\alpha$-SMA.

By immunohistochemical staining, an increase in the percentage of $\alpha$-SMA-positive cells in mechanically loaded leaflets when compared to those statically cultured was observed in the whole leaflet, consistent with the increase observed at the transcript level and with previous research. Using an \textit{ex vivo} tensile circumferential stretch bioreactor, Balachandran \textit{et al.} [2006] observed qualitatively that $\alpha$-SMA-positive cells appeared predominantly in the ventricularis region of stretched aortic valve leaflets, with little or no $\alpha$-SMA expression in the fibrosa. While the statistical significance of these findings was not reported, it is not consistent with what was observed in this research. In both the 1% and 10% FBS conditions, mechanically loaded leaflets showed a statistically greater percentage of $\alpha$-SMA-positive cells in both the fibrosa and spongiosa regions as compared to the ventricularis region. This inconsistency may be due to the differences in the experimental conditions or in the application of mechanical loading to the leaflets.

Native porcine aortic valve leaflets in this study were subjected to uniaxial three-point flexure at a physiologically relevant angle along the circumferential axis. Through mechanical testing of native aortic valve leaflets, Vesely \textit{et al.} [1992] found that the loose connections that pass through the spongiosa between the ventricularis and fibrosa regions of the leaflet allowed them to slide relative to each other during circumferential flexure, thus distributing localized
strain at the point of flexure over a greater length of the leaflet. This anatomy allows the leaflet to have a high tensile strength while remaining pliable and flexible. Thus, it can be argued that uniaxial three-point flexure more accurately reflects the \textit{in vivo} mechanical loading of aortic valve leaflets. The tensile stretch bioreactor, by design, cannot allow for the fibrosa and ventricularis regions to slide relative to each other due to samples being fixed at either end. Furthermore, Sugimoto \textit{et al.} [1999] identified bending flexure as the major mode of leaflet deformation during the cardiac cycle \textit{in vivo}. This may help to explain the fact that, through use of the three-point flexure bioreactor, the spatial distribution of $\alpha$-SMA-positive cells closely resembles that observed in sclerotic aortic valve leaflets, with $\alpha$-SMA-positive cells located in both the fibrosa and spongiosa, but not in the ventricularis [Pho \textit{et al.}, 2007]. This is in contrast to Balachandran \textit{et al.} [2006], who observed qualitatively that $\alpha$-SMA-positive cells appeared predominantly in the ventricularis region of stretched aortic valve leaflets. The absence of $\alpha$-SMA-positive cells in the ventricularis may be explained by flexural experiments reported by Merryman \textit{et al.} [2006]. Native aortic valve leaflets subjected to three-point flexure similar to that in this experiment showed no evidence of interstitial cellular contraction in the ventricularis typically correlated with $\alpha$-SMA-positive myofibroblasts. The fibrosa region of the aortic valve leaflet has been measured to have an elastic modulus that is almost twice that of the ventricularis in the circumferential direction [Vesely \textit{et al.} 1992], most likely due to the presence of compliant elastin in the ventricularis, and thus the fibrosa would act as the main load bearing region and be subjected to the greatest resultant internal stress. These findings further validate the observations made in this study, and also provide more evidence that mechanical loading plays an integral role in the myofibrogenic differentiation of aortic valve interstitial fibroblasts.

When comparing strained leaflets between samples that were cultured in supplemented media containing 1% FBS and 10% FBS, the mean percentage of $\alpha$-SMA-positive cells in the entire leaflet was statistically larger in the 10% FBS group than those in the 1% FBS group ($p<0.002$ using t-test). When comparing the mean percentage of $\alpha$-SMA-positive cells in the fibrosa regions between the two culture conditions, there was a significantly larger percentage of $\alpha$-SMA-positive cells in the fibrosa region of the 10% FBS condition as compared to the 1% FBS condition ($p<0.02$ using t-test). The mean percentage of $\alpha$-SMA-positive cells was statistically similar in the spongiosa and ventricularis regions between the 10% FBS and 1% FBS conditions. As stated previously, FBS is a natural extract taken from bovine fetuses, and contains
many growth factors possibly including transforming growth factor β1 (TGF-β1). It is speculated that the increase observed in the percentage of α-SMA-positive cells between strained leaflets cultured in 1% FBS and 10% FBS can be attributed to the increased presence of TGF-β1. As discussed in section 2.3.1, TGF-β1 has been observed to not only play a role in the appearance of α-SMA-positive aortic valve interstitial myofibroblasts, but acts, along with mechanical tension, to synergistically cause myofibrogenic differentiation [Merryman et al., 2007]. Thus, by increasing the amount of FBS in the culture media and by extension the amount of TGF-β1, the combined effects of three-point flexure and TGF-β1 may have led to the statistically significant increase in the mean percentage of α-SMA-positive myofibroblasts found in the fibrosa as well as the whole leaflet. No significant change in the mean percentage of α-SMA-positive myofibroblasts between the spongiosa regions of leaflets loaded in 10% and 1% FBS was observed. This may be attributed to diffusional limitations, whereas TGF-β1 was not able to diffuse into the spongiosa, or possibly due to a lack of statistical power. In order to further elucidate the singular effects that mechanical loading has on aortic valve leaflets, the confounding effects that TGF-β1 has on interstitial cell phenotype could be eliminated by using culture media without FBS or through the use of a TGF-β1-receptor blocking agent.

### 4.5. Conclusions

Applying physiological strain to native porcine aortic valve leaflets allowed for the investigation of how leaflets respond to mechanical loading. Through semi-quantitative PCR, strained leaflets were observed to have increased expression of α-SMA and cofilin transcripts when compared to statically cultured leaflets. In addition, expression of α-SMA at the protein level was observed primarily in the spongiosa and fibrosa regions of strained leaflets. This pattern of α-SMA protein expression very closely resembles the α-SMA expression observed in sclerotic aortic valve leaflets. Furthermore, leaflets loaded in supplemented culture media containing 10% FBS showed a greater percentage of α-SMA-positive interstitial myofibroblasts when compared to leaflets loaded in 1% FBS.
5. Development of a Tissue Engineered Aortic Valve Leaflet Model System to Study Valve Mechanobiology

5.1. Introduction

Studying the effects that applied strain has on porcine aortic valve leaflets gave some insight as to how the interstitial cell population of these leaflets reacted to differing mechanical environments. Upregulation of $\alpha$-SMA and coflin at both the protein and transcript levels was observed in these leaflets, but it is impossible to say if these findings were the result of some interplay between the endothelial and interstitial cell populations, if these effects were caused by the interstitial cell population alone, or if other factors played a role. To address the complexities of native leaflets and to eliminate potentially confounding factors, an attempt was made to engineer a model tissue consisting only of interstitial cells in a physiologically relevant type I collagen scaffold.

Collagen sponge scaffolds manufactured by Collagen Matrix Incorporated and composed of bovine type I collagen fibers were chosen as the base scaffold material for this engineered tissue (Edgepark, CGMCS3040). The manufacture of these scaffolds involves isolating intact collagen fibers in order to retain their intrinsic intermolecular crosslinks while maintaining their native arrangement. This is achieved through sequential extraction of non-collagenous materials from bovine tendons. First, tendons are purified by water extraction to remove soluble blood proteins. Following this, a sodium chloride extraction is performed to remove non-covalently bonded proteins and polysaccharides. Alcohol extraction is then used to remove lipids, and finally acid and alkali extraction is performed to remove any acid or alkali sensitive proteins, carbohydrates and lipids [Cloft et al., 2000]. This results in an insoluble, intact collagen matrix, referred to as insoluble collagen or fibrillar collagen (Figure 38). The decision to use type I collagen sponges as a scaffold material was based on its demonstrated ability to support normal human aortic valve interstitial cell (VICs) function. It has been shown that VICs readily attach to collagen sponge scaffolds, while maintaining their viability and proliferative potential [Taylor et al., 2002]. Furthermore, VICs seeded in collagen sponge scaffolds appear to maintain the expression of their original phenotype when compared with native human aortic valve leaflets. This includes the positive expression of various interstitial cell markers such as vimentin and prolyl-4-hydroxylase, and comparable expression of $\alpha$-SMA to native aortic valve leaflets.
To establish a tissue engineered aortic valve model, PAVICs were seeded in type I collagen sponge scaffolds and assessed for viability, growth, distribution, morphology and phenotype. As described below, a variety of seeding methods were evaluated.

![Figure 38: Unseeded collagen sponge scaffold viewed with scanning electron microscope, original magnification (A) 30X and (B) 100X.](image)

**5.2. Cell Sourcing**

In order to accurately study aortic valve interstitial cells and their response to applied mechanical strain, collagen sponge scaffolds would have to be seeded with a pure population of PAVICs. When isolating PAVICs from porcine aortic valve leaflets, there is often endothelial cell (PAVEC) contamination in the isolated population. In an attempt to remove this PAVEC contamination from the isolated cell population, magnetic cell sorting was tested and characterized. In addition, the removal of PAVEC contamination through serial passaging was also characterized.

**5.2.1. Materials & Methods**

**5.2.1.1 Magnetic Cell Sorting (MACS) to Remove PAVECs**

Upon completion of the PAVIC isolation protocol outlined in section 3.4.1.1, a magnetic cell sorting procedure was carried out in order to further remove PAVEC contamination. The principle behind this procedure was that a primary mouse anti-porcine antibody would be
introduced to the suspended cells that would label any PAVECs in the population. The antibody used was CD31 (Serotec, MCA1746G). Following this, goat anti-mouse labeled magnetic beads would be introduced, attaching to the contaminating PAVECs. The suspension would then be run through a magnetic field, removing contaminating PAVECs and leaving a pure PAVIC effluent. This procedure was carried out by following the manufacturer’s instructions supplied with the materials (Miltenyi Biotec, 130-048-401).

5.2.1.2 Cytospin Preparation for PAVEC Contamination Analysis

Cell pellets of each sample, prior to and after performing the MACS procedure, were resuspended in supplemented culture media and cells were counted using a Beckman Coulter Vi-CELL XR cell viability analyzer unit. Each cell suspension was then brought to a concentration of 300000 cells/mL, and 0.4 mL of this suspension was loaded into the tube located on the back of a plastic cytospin module. Each plastic module was then loaded with a glass slide and placed into a cytospin centrifuge for nine minutes at 700 RPM. This would splatter the cell suspension on the slide for future analysis through fluorescent staining. The cell splatter was then fixed with 37% formaldehyde (Sigma, F1635) diluted to 3.7% with distilled water for ten minutes, and then dehydrated with 95% ethanol for ten minutes. Cells remaining in suspension and not subjected to the cytospin were plated on glass coverslips and incubated at 37°C and 5% CO₂ for 24h for future double immunostaining.

5.2.1.3 Fluorescent Immunostaining for PAVEC Contamination Analysis

After preparation of a cell splatter for each sample, a fluorescent immunostaining technique was carried out. Each slide was first rinsed twice with PBS for five minutes on an orbital shaker, and then permeabilized with 0.1% triton X-100 (Sigma, T8532) for another five minutes. Slides were then rinsed again, twice with PBS for five minutes, before being blocked with 3% bovine serum albumin (Sigma, A8531) diluted with PBS for twenty minutes at 37°C. A primary antibody for identification of PAVECs was then prepared, using 10% mouse anti-porcine CD31 diluted with 3% bovine serum albumin. After blocking, this primary antibody was applied and incubated overnight in a humidified chamber. Slides were washed twice again with PBS for ten minutes, and blocked with 10% goat serum diluted with PBS for thirty minutes at room temperature. The secondary antibody, a goat anti-mouse fluorophore (AlexaFluor, 568), was diluted to 1% in goat serum and applied for one hour in a humidified chamber. Slides were
washed again with PBS for ten minutes and the Hoechst nuclear stain (Sigma, H6024) was applied for five minutes at 0.1% diluted in PBS. The slide was rinsed with PBS and deionized water, and mounting medium PermaFluor (Thermo Scientific, 434990) was added. Samples were then ready for viewing under a fluorescent microscope, and showed the presence of all cells through nuclear staining (blue) and all PAVECs through CD31 staining (green). Through counting the number of CD31-positive cells and comparing that to the overall cell number, the percentage of PAVEC contamination in the isolated population was calculated.

5.2.1.4 **Confirmation of PAVIC Phenotype by Double Immunostaining**
In order to confirm that the cells isolated were, in fact, PAVICs, a double immunostaining protocol was carried out. Cells that had been cultured on glass coverslips for 24h were stained for CD31 as before, but prior to the application of the Hoechst nuclear stain, cells were stained for α-SMA expression. Slides were once again blocked with 3% bovine serum albumin for twenty minutes at 37°C, and a fluorophore-conjugated primary mouse anti-porcine antibody for α-SMA (Sigma, F3777) was diluted to 1% with 3% bovine serum albumin and applied for one hour in a humidified chamber. Slides were then rinsed and the Hoechst nuclear stain was applied as previously described. In addition to the CD31-positive PAVECs staining green, α-SMA-positive PAVICs would stain red.

5.2.1.5 **PAVEC Contamination after Serial Passaging**
It was thought that PAVEC contamination, in an isolated cell population that was not subjected to MACS, could be minimized through serial passaging. Isolated cells, after four passages, were cultured on glass coverslips for 24h and stained for the presence of PAVECs as described in section 5.2.1.3.

5.2.1.6 **Statistical Analysis**
Slides subjected to immunostaining for CD31 were viewed using a Leica DMIRE2 fluorescent microscope, and photos were taken using a Hamamatsu C4742-95 digital camera and captured using OpenLab 4.0.4 software. Three images of each splatter were taken at completely random positions on the slide to avoid bias. The total number of cells in each image was counted as identified by the Hoechst nuclear staining. The total number of PAVECs in each image was counted as identified by the CD31 staining. The studentized t-test was utilized to identify any
significant differences between cell populations subjected to MACS and not subjected to MACS. Data are presented as mean ± standard deviation, with the sample size and p-value identified in the figure caption.

5.2.2. Results & Discussion
Qualitatively, MACS treatment removed most PAVEC contamination, leaving an almost completely pure population of PAVICs (Figures 39 and 40). Contamination was quantified as the percentage of CD31-labelled PAVECs in the total cell population (Figure 41). Without MACS, the mean PAVEC contamination was found to be approximately 41%, while with MACS the mean contamination was found to be approximately 6%, a statistically significant decrease (p<0.01 using t-test).

Figure 39: Cell population recovered from porcine aortic valves, not subjected to MACS, labeled for CD31 (green) and Hoechst nuclear stain (blue), original magnification 10X.
Figure 40: Cell population recovered from porcine aortic valves, subjected to MACS, labeled for CD31 (green) and Hoechst nuclear stain (blue), original magnification 10X.

Figure 41: Quantification of PAVEC contamination in cell population (* p<0.01 relative to without MACS condition by t-test). Data are presented as mean ± standard deviation (n=3).

To confirm these results, a small number of cells from the unpurified and purified populations were plated onto glass cover slips, allowed to adhere over 24h, and double stained
for both CD31 and α-SMA, a PAVIC marker. In the population that was not subjected to MACS, both interstitial and endothelial cells were observed (Figure 42). In contrast, in the cell population that was collected as the effluent following MACS, the cells only showed positive α-SMA labeling, confirming that MACS yields a pure PAVIC population directly from isolation (Figure 43).

Figure 42: Cell population recovered from porcine aortic valves, not subjected to MACS, labeled for CD31 (green), α-SMA (red), and Hoechst nuclear stain (blue), original magnification 20X.

Figure 43: Cell population recovered from porcine aortic valves, subjected to MACS, labeled for CD31 (green), α-SMA (red), and Hoechst nuclear stain (blue), original magnification 20X.
Due to the large number of PAVICs needed for collagen sponge scaffold seeding, the isolated cell population would have to be grown on tissue culture plastic and expanded through serial passaging. It was thought that through serial passaging that all PAVEC contamination would be selectively removed through the use of PAVIC-specific supplemented culture media. Passage four cells cultured on glass coverslips for 24h were stained for CD31 expression. These passage four cells showed no positive expression, indicating that serial passaging could also be used to remove PAVEC contamination from isolated PAVICs (Figure 44).

Figure 44: Passage four cells show no positive expression of CD31, with Hoechst nuclear stain (blue), original magnification 10X.

5.2.3. Conclusions
Through the use of MACS, it has been shown that an almost pure population of PAVICs can be isolated directly from porcine aortic valve leaflets. In addition, since a very large population of PAVICs is needed, isolated cells not subjected to MACS can be grown on tissue culture plastic and expanded through serial passaging, and through using PAVIC-specific supplemented cell culture media any PAVEC contamination will be removed.

5.3. Materials & Methods

5.3.1. PAVIC and Collagen Sponge Scaffold Preparation
PAVICs were isolated following the protocol outlined in section 3.4.1.1. After isolation, PAVICs were typically expanded through four passages prior to seeding on collagen sponge scaffolds. After four passages, cells were removed from the tissue culture plastic by replacing the
supplemented culture media with 0.25% trypsin with EDTA (Invitrogen, 25200056) and incubating for ten minutes at 37°C and 5% CO₂. After removal from the tissue culture flasks, cells were then counted using a Beckman Coulter Vi-CELL XR cell viability analyzer unit and resuspended in supplemented media at the desired cell density for seeding. In all seeding experiments, unless otherwise stated, collagen sponge scaffold pieces were hydrated in supplemented culture media for at least 24h prior to seeding in order to enhance cell attachment. This was accomplished by placing scaffold pieces of the desired dimensions in 50 mL conical tubes containing 50 mL of culture media.

5.3.2. Static Seeding of Collagen Sponge Scaffolds
Initially, collagen sponge scaffolds were seeded statically. Scaffolds were placed free-floating in a 50 mL conical tube containing the appropriate number of cells required to achieve the desired cell density suspended in 15 mL of supplemented culture media. In order to allow proper gas exchange, small holes were drilled through the conical tube lids, and adhesive gas exchange film (Excel Scientific Incorporated, BS-25) was placed over the holes. By allowing the collagen sponges to remain free-floating in suspension, any mechanical stress placed on the PAVICs is absent. Studies have shown that without any mechanical stress, fibroblasts within collagen matrices fail to acquire the myofibroblast phenotype [Tomasek et al., 2002], consistent with the interstitial cell phenotype typical of a normal, healthy aortic valve leaflet. The scaffold-containing conical tubes were then incubated at 37°C and 5% CO₂ for as long as desired, with fresh media being added at regular intervals of 24h to 48h.

5.3.3. Dynamic Seeding of Collagen Sponge Scaffolds
In an attempt to improve on the static seeding protocol, a dynamic seeding approach was devised. This approach allowed for improved culture media perfusion into the scaffold to support cell migration and proliferation in the inner portions of the sponge. While the majority of the protocol remained the same as the static seeding protocol, one major change was instituted. In an attempt to ensure complete and thorough seeding of the scaffolds, a rotator was built based on the design of Nasseri et al. [2003] (Figure 45). The purpose of this rotator was to agitate the cell suspensions containing the scaffolds to assist cell attachment, culture media perfusion and cell migration into the scaffolds. Immediately after the scaffolds were placed in the cell suspension,
the conical tubes were placed on the rotating device inside the incubator and incubated at 37°C and 5% CO₂ for as long as desired.

Figure 45: Rotating device used for seeding and culturing PAVICs on collagen sponge scaffolds.

### 5.3.4. Manual Seeding of Collagen Sponge Scaffolds

Additionally, a manual seeding technique was employed. Hydrated scaffolds were placed on non-tissue culture treated plastic and half of the cell suspension, at the desired concentration, was applied to one side of the scaffold in 0.3 mL of supplemented culture media. Scaffolds were then incubated at 37°C and 5% CO₂ for three hours to allow for cell attachment. The scaffolds were then flipped and the remaining half of the cell suspension was applied as before. Once again, the scaffold was incubated for three hours, after which enough supplemented culture media was added to immerse the scaffolds completely. Scaffolds remained in the incubator for another 24 h, after which they were individually placed in 50 mL conical tubes containing 15 mL of supplemented culture media. The tubes were then placed on the rotator and incubated for as long as desired.

In one set of experiments, the manual seeding technique was modified to improve the hydration of scaffolds prior to seeding. Scaffolds were hydrated in a dessicator prior to manual seeding. To do so, scaffolds were placed in a Petri dish containing enough supplemented media such that scaffolds were covered. The Petri dish was then covered with a lid and placed inside a dessicator to remove any remaining air trapped in the scaffolds. The dessicator was hooked up to a vacuum pump and the pump was run for 48h.
5.3.5. **Paraffin Embedding, Histochemical and Immunohistochemical Staining**

To assess PAVIC protein expression in tissue engineered collagen sponge scaffold constructs, scaffolds were processed for (immuno)histochemical staining and analyzed as previously outlined in section 4.2.2.

5.3.6. **Live/Dead Assay and Confocal Microscopy Imaging**

To assess cell viability throughout the scaffold thickness, the Live/Dead Viability/Cytotoxicity Kit for mammalian cells (Molecular Probes, L3224) was used. As detailed in the supplied manual, the principle of the assay is that living cells are distinguished by the presence of intracellular esterase activity, while dead cells have damaged cellular membranes. Living cells are identified by the enzymatic conversion of the non-fluorescent cell-permeant calcein AM to the intensely green fluorescent calcein. Conversely, EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells.

Scaffolds for Live/Dead staining were rinsed with PBS and placed in a Petri dish. A working solution containing 2 \(\mu\)M calcein AM and 4 \(\mu\)M EthD-1 was prepared using PBS with calcium and magnesium to dilute. The working solution was added to the scaffolds such that the scaffolds were completely immersed. The scaffolds were then incubated at room temperature for thirty minutes prior to imaging. After thirty minutes, the scaffolds were removed from the working solution and fixed to the bottom of a Petri dish using industrial glue for ease of imaging. The scaffolds were then imaged using either a Zeiss LSM510 META NLO two-photon and confocal microscope with spectral imaging located at Princess Margaret Hospital in Toronto or a Zeiss LSM510 confocal microscope located in the Medical Sciences Building at the University of Toronto. Images were taken at various magnifications, and when moving into the scaffold (in the z-direction), images were taken at 10 \(\mu\)m intervals.

5.3.7. **Scanning Electron Microscopy Imaging**

Observations of VIC interactions with the scaffolds were made using scanning electron microscopy (SEM). Scaffold pieces were fixed in formalin, and then immersed serially in ethanol with concentrations of 30%, 50%, 70%, 95%, 100% and again 100% for thirty minutes each in order to dehydrate the scaffold. Scaffolds were further processed and imaged in the Faculty of Dentistry at the University of Toronto. Processing included critical point drying to
remove any remaining liquid in the samples and sputter coating the samples with a thin layer of gold particles which act as a “stain” for viewing with SEM. Scaffolds were imaged with a Hitachi S-2500 scanning electron microscope.

5.3.8. **Measuring Collagen Sponge Scaffold Contraction**
Collagen sponge scaffold contraction was monitored through photo analysis. At a given time point, collagen sponge scaffolds were placed in a Petri dish with 20 mm molded-in grids (BD Falcon, 351013). Photos of the scaffolds in the Petri dish were taken using a Sony Cyber-shot 6.0 megapixel digital camera and analyzed using ImageJ software. Due to the difficulty in measuring scaffold thickness, the scaffold dimensions were measured in terms of projected scaffold area only. Results were reported as scaffold area relative to the initial dimensions of the scaffolds prior to hydration.

5.3.9. **Statistical Analysis Methods**
Analysis of variance (ANOVA) and Tukey’s honestly significantly different (HSD) test were used to identify significant differences in scaffold contraction at different time points. Data are reported as mean ± standard deviation, and the number of samples (n) is identified in brackets in the figure caption. As well, p-values are listed in the text as well as the figure caption.

5.4. **Results**

5.4.1. **Static Seeding of Collagen Sponge Scaffolds**
Collagen sponge scaffolds, cut as circular pieces measuring 18 mm in diameter and 3 mm thick, were pre-wetted in supplemented culture media for 24h and placed in 50 mL conical tubes containing a cell suspension with a cell density of 10X10^6 cells/cm³ of scaffold. After seven days in culture, scaffolds remained intact to the touch. H&E and α-SMA staining was completed on scaffold sections (Figures 46 and 47, respectively). The scaffolds remained quite empty, with small focal areas of cell attachment along the scaffold edges and very little cell presence within the inner parts of the scaffold. There was no positive expression of α-SMA observed on any scaffolds, suggesting that the few cells present on the scaffolds were quiescent fibroblasts.
Figure 46: H&E staining of static PAVIC seeded collagen sponge scaffold after seven days culture, original magnification (A) 4X and (B) 40X.

Figure 47: α-SMA staining of static PAVIC seeded collagen sponge scaffold after seven days culture, original magnification (A) 4X and (B) 40X.

Contraction of scaffolds was monitored throughout the seeding and culturing process (Figure 48). There was no significant change in the relative scaffold area at any time point as compared to the original scaffold size (p>0.05 using Tukey’s test; original scaffold size is identified on the plot at day -1). The observation that no significant contraction occurred is consistent with the H&E staining that showed few cells present in the scaffold.
5.4.2. Dynamic Seeding of Collagen Sponge Scaffolds

In order to improve on the previous seeding technique, the cell density used for seeding was doubled to 20X10^6 cells/cm^3 of scaffold and scaffolds were seeded and cultured dynamically in 50 mL conical tubes that were rotated. As before, circular scaffold pieces measuring 18 mm in diameter and 3 mm thick were used. After seven days in culture, scaffolds remained intact to the touch. H&E staining was performed for scaffolds seeded for 24h, three days and seven days (Figures 49, 50 and 51, respectively). As the amount of seeding time increased, the distance of cell migration from the scaffold edge also increased, and there appeared to be a more homogenous PAVIC distribution throughout the scaffold as compared to the statically seeded scaffolds. However, there were fewer cells in the center of the scaffold.
Figure 49: H&E staining of dynamic PAVIC seeded collagen sponge scaffold after 24h culture, original magnification (A) 4X and (B) 40X.

Figure 50: H&E staining of dynamic PAVIC seeded collagen sponge scaffold after three days culture, original magnification (A) 4X and (B) 40X.

Figure 51: H&E staining of dynamic PAVIC seeded collagen sponge scaffold after seven days culture, original magnification (A) 4X and (B) 40X.

α-SMA staining was also completed on scaffolds seeded for 24h, three days and seven days (Figures 52 and 53). At all three time points, there is no positive expression of α-SMA.
observed in any of the scaffold sections, once again showing that the interstitial cell population within the scaffold was predominantly of the quiescent fibroblast phenotype.

Figure 52: $\alpha$-SMA staining of dynamic PAVIC seeded collagen sponge scaffold after (A) 24h culture and (B) three days culture, original magnification 4X.

Figure 53: $\alpha$-SMA staining of dynamic PAVIC seeded collagen sponge scaffold after seven days culture, original magnification (A) 4X and (B) 40X.

After seven days in culture, the Live/Dead fluorescence assay was performed on one scaffold which was then observed with confocal microscopy (Figures 54 and 55). Living cells are identified as green, while dead cells are identified as red. Images taken at a depth of 100 µm from the surface of the scaffold indicate that the majority of cells within the scaffold were alive. Images taken deeper into the scaffold showed decreased cellularity (images not shown). These findings were consistent with the heterogeneous cell distribution observed by H&E staining.
To further improve the dynamic seeding results, the culture time for the scaffolds was doubled from seven to fourteen days. As well, scaffolds were cut in rectangular pieces measuring 35 mm X 10 mm X 3 mm to fit the bioreactor. After fourteen days in culture, scaffolds appeared very delicate and did not remain intact to the touch. H&E and α-SMA staining was completed on scaffold sections. H&E staining showed the presence of cells throughout the cross-section of the scaffold, with cells appearing to be evenly distributed both in the edges and the central regions of
the scaffold (Figure 56). α-SMA staining showed no positive expression throughout the scaffold cross-section (Figure 57).

Figure 56: H&E staining of dynamic PAVIC seeded collagen sponge scaffold after fourteen days culture, original magnification (A) 4X, (B) 10X and (C) 40X.
After fourteen days in culture, the Live/Dead fluorescence assay was performed on one scaffold which was then observed by confocal microscopy (Figures 58 and 59). At a depth of 100 µm from the surface of the scaffold, the majority of cells within the scaffold were alive. Images taken further into the scaffold at a depth of 300 µm showed only a slight decrease in cellularity, consistent with what was observed with H&E.
After fourteen days of culture, scaffolds were also observed using SEM. Images taken at various magnifications (Figures 60, 61 and 62) show cells layered and spread out on the surface of the scaffold at high densities.
Figure 60: Dynamic seeded scaffold cultured for fourteen days as observed with SEM, original magnification 100X.

Figure 61: Dynamic seeded scaffold cultured for fourteen days as observed with SEM, original magnification 250X.
Contraction of scaffolds was monitored throughout the seeding and culturing process (Figure 63). There was a significant decrease in the relative scaffold area at all time points as compared to the original scaffold size (p<0.01; original scaffold size is identified on the plot at day -1). The extent of contraction was consistent with the large number of cells that were observed by H&E staining, confocal microscopy and SEM to have attached to the scaffold.
Figure 63: Dynamic PAVIC seeded collagen sponge scaffold relative contraction (* p<0.001 relative to all time points by Tukey’s test). Data are presented as mean ± standard deviation (n=6).

The dynamic seeding protocol was repeated once again starting with a larger initial scaffold size, measuring 120 mm X 25 mm X 3 mm. This larger initial scaffold size was employed in an attempt to duplicate the homogeneous cell distribution achieved previously while allowing for the scaffold to remain intact and maintain mechanical integrity. By maintaining this mechanical integrity, scaffolds could then be placed in the bioreactor and subjected to applied strain. After fourteen days of culture, scaffolds remained intact to the touch and were placed in the bioreactor for a further seven days, with some scaffolds subjected to applied strain (see section 5.5 for details) while others acted as non-strained stationary controls. H&E staining of stationary controls (Figure 64) showed a heterogeneous cell distribution with cells located focally along the edges of the scaffold with very little internal cellularity. This was a consistent result observed in all samples. As observed previously, α-SMA staining of stationary controls (Figure 65) showed no positive expression.
Figure 64: H&E staining of dynamic PAVIC seeded collagen sponge scaffold after fourteen days culture and seven days as stationary control in bioreactor, original magnification (A) 4X and (B) 40X.

Figure 65: α-SMA staining of dynamic PAVIC seeded collagen sponge scaffold after fourteen days culture and seven days as stationary control in bioreactor, original magnification 4X.

5.4.3. Manual Seeding of Collagen Sponge Scaffolds

It became apparent that there was a delicate balance between homogeneous cell distribution with scaffold degradation and heterogeneous cell distribution without scaffold degradation. In an attempt to find a balance where scaffold degradation could be avoided while achieving a homogeneous cell distribution throughout the scaffold, a manual seeding approach was developed. First, the cell density used for seeding was doubled to $40 \times 10^6$ cells/cm$^3$ of scaffold. Cells were applied manually to the scaffold surface via pipette in order to ensure cell attachment. Scaffolds with dimensions of 120 mm X 25 mm X 3 mm were used. After fourteen
days in culture, scaffolds remained intact to the touch. Upon completion of the manual seeding protocol, prior to dynamic culture, a large population of cells was observed attached to the tissue culture treated bottom of the Petri dish, indicating that some of the initial cell population used for seeding was lost, possibly due to migration from the scaffold. H&E, α-SMA, desmin and vimentin staining was completed on sections taken from scaffolds that had been cultured for fourteen days. H&E staining performed on various scaffold samples showed inconsistent seeding (Figure 66), with some scaffolds showing side-specific cell attachment, some showing cell presence only along the scaffold edge and others showing cell presence throughout the cross-section. The combination of the positive expression of vimentin (Figure 67) along with the negative expression of α-SMA (not shown) and desmin (Figure 68) was consistent with the presence of PAVICs and not smooth muscle cells.

Figure 66: H&E staining of manual PAVIC seeded collagen sponge scaffolds after fourteen days culture showing (A) cell presence along scaffold edges, (B) cell presence throughout scaffold and (C) side specific cell presence, original magnification 4X.
Scaffolds were removed from culture and observed using SEM after 24h, three day and ten days. Images taken after 24h of culture (Figures 69 and 70) showed concentrated areas of cells, and very initial cell attachment to the scaffold. After three day in culture (Figure 71), cells appeared more evenly distributed over the surface of the scaffold. Cells also appeared to have spread out within a high density layer along the scaffold edge. After ten days of culture (Figure 72), cells were evenly distributed over the scaffold surface and the surface layer appeared less dense indicating possible cell migration into the inner sections of the scaffold or collagen degradation.
Figure 69: Manually seeded scaffold cultured for 24h as observed with SEM, original magnification 500X.

Figure 70: Manually seeded scaffold cultured for 24h as observed with SEM, original magnification 2500X.
Figure 71: Manually seeded scaffold cultured for three days as observed with SEM, original magnification (A) 500X and (B) 1000X.

Figure 72: Manually seeded scaffold cultured for ten days as observed with SEM, original magnification 250X.
While the larger scaffold size appeared to have allowed for the maintenance of scaffold mechanical integrity, the cell distribution observed throughout the scaffolds was inconsistent. One problem that was identified was the lack of success observed in the pre-wetting process. Scaffolds were placed in supplemented culture media for up to a week in advance in some cases and would still not become entirely saturated. Pockets of air could be observed and scaffolds would float in the culture media. To combat this, scaffolds were placed in a Petri dish containing culture media and then placed inside a dessicator. The dessicator was then hooked up to a vacuum pump and a vacuum was applied for approximately two days. After two days, the scaffolds no longer floated and no air pockets were observed, suggesting that the scaffolds had become completely saturated with culture media. Another problem was the loss of cells due to adhesion to the Petri dish during seeding. To address the loss of cells during seeding a modified manual seeding protocol was carried out. In contrast to the previous seeding attempt, scaffolds were initially seeded on non-tissue culture treated plastic to avoid cell adhesion. As well, the initial scaffold size was reduced to 75 mm X 18 mm X 3mm in an attempt to achieve a homogeneous cell distribution. After seven days of culture, scaffolds appeared very delicate and did not remain intact to the touch. Seeding of the scaffolds was much more consistent between samples than previously, with cells distributed homogeneously throughout the scaffold (Figure 73).

Figure 73: H&E staining of manual PAVIC seeded collagen sponge after seven days culture, original magnification (A) 25X and (B) 40X.
Contraction of scaffolds was monitored throughout the seeding and culturing process (Figure 74). Due to the use of the dessicator to remove excess air from the scaffolds and to allow complete hydration prior to seeding, a large initial contraction of the scaffold was observed. Tukey’s test showed that scaffolds at day -1 were significantly larger than those at days 0 and 1 (p<0.001). Similarly, scaffolds at days 0 and 1 were significantly larger than those at days 4 and 7 (p<0.02). The original scaffold size is identified on the plot at day -1. The large amount of contraction observed, where scaffolds ended up at approximately 20% of their original area, is consistent with the large cell population observed by H&E to have attached to the scaffold.

![Graph showing scaffold contraction over time](image)

Figure 74: Manual PAVIC seeded collagen sponge scaffold relative contraction (* p<0.001 relative to day 0/day 1, **p<0.02 relative to day 4/day 7 by Tukey’s test). Data are presented as mean ± standard deviation (n=4).

### 5.5. Application of Strain to Tissue Engineered Aortic Valve Leaflet Model

In order to study the response of aortic valve interstitial cells to applied, physiological strain, dynamically seeded collagen sponge scaffolds were placed in the bioreactor for seven days and subjected to applied strain.
5.5.1. Materials & Methods

Scaffolds measuring 120 mm X 25 mm X 3 mm were seeded dynamically at a density of 20X10^6 cells/cm^3 of scaffold and cultured dynamically for fourteen days before being loaded into the bioreactor. After culture, three scaffolds were loaded into the bioreactor for the application of strain as detailed in section 3.2 and 3.3.3, while another three scaffolds were loaded into the bioreactor and statically cultured by removing the central dynamic pins from their respective wells. The central dynamic pin was set to displace 8 mm at a frequency of 1 Hz continuously for seven days. Both static and strained scaffolds remained in the bioreactor for seven days with the supplemented culture media replaced daily. Upon removal, scaffolds were sectioned in half lengthwise, with half processed for α-SMA protein staining and H&E staining as outlined in section 4.2.2, and half prepared for RNA extraction and transcript level analysis as outlined in sections 4.2.4-4.2.9.

5.5.2. Results

After fourteen days of culture, scaffolds remained intact to the touch and were placed in the bioreactor for a further seven days, with some scaffolds subjected to applied strain while others acted as non-strained stationary controls. Using semi-quantitative polymerase chain reaction (PCR) and ImageJ to quantify gel results, a significant increase in the expression of the α-SMA gene was measured in scaffolds subjected to applied strain for seven days (Figure 75) as compared to scaffolds that were statically cultured (p<0.05 using t-test). This is an indication that applied strain may have promoted the myofibrogenic differentiation of PAVICs seeded onto the scaffolds. No difference in α-SMA expression was observed between central and outside pieces of strained scaffolds. Expression of the cofilin gene was also measured but showed no significant difference in expression between strained and static scaffolds.
Figure 75: α-SMA transcript expression relative to GAPDH expression in strained and statically cultured PAVIC seeded collagen sponge scaffolds (* p<0.05 relative to static culture condition using t-test). Data are presented as mean ± standard deviation (n=3).

H&E staining of strained scaffolds (Figure 76) showed a heterogeneous cell distribution with cells located focally along the edges of the scaffold with very little internal cellularity. This was a consistent result observed in all samples, both strained and statically cultured. Scaffolds subjected to applied strain in the bioreactor showed positive expression of α-SMA at the protein level as indicated through immunohistochemical staining (Figure 77), while non-flexed sponges showed little, if any, positive expression (Figure 65). Consistent with the PCR results, this is an indication that applied strain may have promoted the myofibrogenic differentiation of PAVICs seeded onto the scaffolds.
5.6. Discussion

The overall goal in creating the tissue engineered aortic valve leaflet model was to cultivate a tissue with a homogeneous distribution of PAVICs while maintaining structural integrity to allow for the application of strain through the use of the bioreactor. It quickly became apparent that these two important characteristics, structural integrity and homogeneous cellularity, were very difficult to achieve simultaneously with the collagen sponge scaffolds and culturing methods used in this study.
Static seeding of collagen sponge scaffolds resulted in tissue engineered constructs that remained mechanically stable and intact but with little overall cellularity and heterogeneous distribution. These results are consistent with what was observed in the static seeding and culturing of aortic valve interstitial cell-seeded collagen sponge scaffolds by Taylor et al. [2002]. Scaffolds were seeded at a lower cell density than in this study, but in contrast were cultured for three weeks instead of one. Regardless, Taylor et al. [2002] observed that cells did not uniformly populate the scaffold and that in most cases cells did not reach the central core of the scaffold, consistent with results from this study. Taylor et al. [2002] also noted that as the initial cell density for seeding was increased, the potential for cell proliferation greatly improved. Thus the seeding protocol evolved to utilize a larger initial cell density for seeding, and eventually an extended culture period.

A study by Sutherland et al. [2002] discussed how seeding and culturing of arterial interstitial cells in scaffolds composed of polyglycolic acid mesh and poly-4-hydrobutyrate could be improved via the use of a rotational device, observing that tissue engineered constructs were showing improved cellularity and homogeneity of cellular distribution. Thus, a custom rotational device was built to allow for dynamic seeding and culturing of collagen sponge scaffold constructs. Consistent with Sutherland et al. [2002] and Qi et al. [2004], dynamic seeding greatly improved upon static tissue engineering efforts. Dynamic seeding improved the overall cellularity of scaffolds, with homogeneous cell distribution observed in constructs cultured for fourteen days. Unfortunately, constructs originating from smaller scaffold sizes, while showing homogeneous cellularity, lost their mechanical integrity and did not remain intact to touch. In contrast, constructs originating from larger scaffold sizes, while maintaining mechanical integrity, showed very heterogeneous cell distribution. Furthermore, efforts to seed scaffolds manually prior to dynamic culture gave similar results. Constructs were either mechanically stable or had homogeneous cellularity, but did not show both characteristics simultaneously.

Through the various seeding methods, the conditions by which the delicate balance between homogeneous cellularity and mechanical stability could be satisfied were not identified. When seeded at high densities and/or on small scaffolds, PAVIC degradation and remodeling of the collagen sponge proceeded to the detriment of mechanical integrity. When seeded at lower densities and/or on larger scaffolds, the scaffolds remained intact but the resultant overall cell distribution was heterogeneous. Despite these problems, one positive finding was that in all cases
the resultant tissue engineered construct contained only cells of the quiescent fibroblast phenotype, typical of the healthy aortic valve leaflet. Thus, if the issues of mechanical integrity and homogeneous cellularity could be resolved, this tissue engineered construct may be a good model for the healthy aortic valve interstitium.

In the future, changes may be made to the materials or culture conditions used in this study to address the problems reported in these tissue engineering results. A more robust and mechanically stable scaffold material such as acellular native heart valves [Curtit et al., 1997, Zeltinger et al., 2001, Bertipaglia et al., 2003, Schenke-Layland et al., 2003] or synthetic materials such as polyglactin and polyglycolic acid mesh [Shinoka et al., 1995, Sutherland et al., 2005] could be utilized, although such a scaffold may promote myofibrogenesis of seeded PAVICs as is seen when VICs are grown on stiff, tissue culture plastic or constrained tissue engineered constructs [Tomasek et al., 1992]. Furthermore, the use of a biologically relevant acellular leaflet scaffolds has shown great promise in maintaining structural integrity as well as native leaflet organization [Bertipaglia et al., 2003, Schenke-Layland et al., 2004]. As well, through the use of dynamic culture, tissue engineered aortic valve leaflets have been shown to have improved mechanical integrity, as well as developing structural and organizational characteristics that very closely mimic the native aortic valve leaflet [Schenke-Layland et al., 2003]. Unfortunately, dynamic culture methods such as pressure application and pulsatile fluid flow often lead to tissue engineered aortic valve leaflet structures containing α-SMA-positive aortic valve interstitial myofibroblasts [Schenke-Layland et al., 2003]. Thus, while changes in scaffold material and culture conditions may solve the problems of mechanical integrity and homogeneous cellularity, the resultant tissue engineered construct may resemble a diseased aortic valve leaflet more than a healthy one.

Despite the inability to cultivate a tissue engineered aortic valve leaflet model with both homogeneous cellularity and mechanical stability, an intact collagen sponge scaffold heterogeneously seeded with PAVICs was subjected to applied, physiological strain. After seven days of strain, there was increased expression, both at the transcript and protein level, of α-SMA. This is an indication that the previously quiescent fibroblasts that populated the unstrained constructs underwent phenotypic differentiation into the activated myofibroblast as a result of an altered mechanical environment. This gives further evidence to support the theory that
mechanical loading plays a vital role in the myofibrogenic differentiation of aortic valve interstitial fibroblasts [Tomasek et al., 2002, Balachandran et al., 2006].

5.7. Conclusions

Type I collagen sponge scaffolds allowed for aortic valve interstitial cells to attach, proliferate and maintain the quiescent fibroblast phenotype typical of healthy aortic valve leaflets. As well, aortic valve interstitial fibroblasts differentiated into myofibroblasts when subjected to mechanical loading, consistent with results reported through *in vitro* cell culture studies and *ex vivo* leaflet studies. However, these collagen sponge scaffolds were unable to maintain structural and mechanical integrity while maintaining a large population of homogeneously distributed cells; thus, further attempts to improve the tissue engineered leaflet model, whether through changes to seeding and culturing conditions or through the use of a different scaffold material, are warranted.
6. Conclusions & Recommendations

Overall, the objective of this research was to investigate how mechanical loading affects the myofibrogenic differentiation of aortic valve interstitial cells in physiologically-relevant systems.

A bioreactor system to apply physiological mechanical loading to both native porcine aortic valve leaflets and a tissue engineered aortic valve leaflet model was setup and characterized. The system was tested and observed to sustain a population of PAVICs, both in viability and proliferative potential, while maintaining sterility. An actuator was setup and programmed to apply displacement to the central dynamic pins of the bioreactor, resulting in the application of three-point flexure to both native porcine aortic valve leaflets and a tissue engineered aortic valve leaflet model at a physiological angle.

This system can be employed in any number of future studies. Specifically, it can be used to examine the mechanobiological response of any tissue sample to both uniaxial and biaxial three-point mechanical loading, and the degree of mechanical loading can be adjusted quite easily to apply greater or reduced loading as required. Additionally, the system could be modified to apply uniaxial tensile stretch or other loading conditions quite easily.

Native porcine aortic valve leaflets were subjected to physiological three-point flexure in the bioreactor over the course of four days. Mechanically loaded leaflets were observed through semi-quantitative PCR to have a significant increase in both $\alpha$-SMA and cofilin transcript expression when compared to leaflets that had been statically cultured for the same period of time. Histomorphometric analysis also revealed a significant increase in the percentage of $\alpha$-SMA-positive interstitial myofibroblasts in mechanically loaded leaflets when compared to those statically cultured. Spatially, $\alpha$-SMA-positive interstitial myofibroblasts were found predominantly in the fibrosa and spongiosa regions of mechanically loaded leaflets, where the percentage of $\alpha$-SMA-positive cells was significantly greater than what was observed in the ventricularis. When the percentage of FBS in the supplemented culture media was reduced from 10% to 1%, there was a significant decrease in the percentage of $\alpha$-SMA-positive interstitial myofibroblasts between mechanically loaded whole leaflets, and spatially between the fibrosa regions of leaflets loaded in 10% FBS and 1% FBS. This evidence suggests that mechanical loading of native aortic valve leaflets is causing the differentiation of valve interstitial fibroblasts into myofibroblasts, as correlated to the increased expression of $\alpha$-SMA at the transcript and
protein level. As well, increased expression of α-SMA, and by extension myofibrogenic differentiation, appears to occur in the spatially defined regions of the fibrosa and spongiosa, mimicking the pattern of expression observed in sclerotic aortic valve leaflets. The reduction of α-SMA-positive cells, correlated to the reduction of FBS, suggests that a component of FBS, possibly TGF-β1, is also playing a role in the myofibrogenic differentiation process. Furthermore, the upregulation of cofilin at the transcript level provides further evidence that cofilin is a determinant of myofibrogenic differentiation.

In future studies, the expression of cofilin at the protein level should be investigated in order to further compliment the observed upregulation in mechanically loaded leaflets at the transcript level. The spatial expression of cofilin at the protein level should also be considered, as it is theorized that it will be expressed spatially in the same manner as α-SMA in mechanically loaded leaflets, that is, in the fibrosa and spongiosa. Furthermore, in order to elucidate the effects that mechanical loading, and only mechanical loading, has on native aortic valve leaflets, a TGF-β1 blocking agent should be employed, or alternatively culture media without FBS should be used. Finally, the degree of mechanical loading may be varied in order to investigate if there is any dependence of leaflet protein and transcript expression on the degree of loading.

A tissue engineered aortic valve leaflet model was developed and characterized to be used to study the effects that physiological mechanical loading has on aortic valve interstitial cell differentiation. Due to their ability to sustain PAVIC viability and proliferation, acellular bovine tendon-derive type I collagen sponges were used as the scaffold material. Static seeding of PAVICs onto collagen sponge scaffolds resulted in constructs that, while retaining their mechanical integrity, had poor cellularity and lacked the homogeneous cell distribution required for even tissue formation. Dynamic seeding, along with an increased cell seeding density, resulted in constructs with homogeneous cellularity that lacked mechanical integrity. Using scaffolds with larger dimensions, the resulting constructs maintained mechanical integrity but similar to static seeded constructs lacked homogeneous cellularity. Finally, a manual seeding method utilizing dynamic culture was employed, but the conditions by which mechanical integrity could be maintained while also achieving homogeneous cellularity were not achieved. Upon completion of culture in all instances, constructs showed no positive expression of α-SMA or desmin, while showing positive expression of vimentin. This indicated that the cultivated valve constructs contained a population of interstitial cells without smooth muscle cell or
myofibroblast contamination. This panel of expression very closely resembles that observed in healthy native aortic valve leaflets. Furthermore, dynamically seeded constructs that lacked homogeneous cellularity but maintained mechanical integrity were subjected to mechanical loading in the bioreactor. After seven days in the bioreactor, loaded constructs were observed to have a significant increase in the expression of \( \alpha \)-SMA at the transcript level over those that were not loaded, and expression of \( \alpha \)-SMA at the protein level was observed in loaded leaflets but was absent in those that were not loaded. These results suggest, similar to the native leaflet experiments, that mechanical loading of collagen sponge constructs containing PAVICs causes upregulation of \( \alpha \)-SMA at both the transcript and protein level, and by extension, is causing the myofibrogenic differentiation of interstitial cells.

In future work, this tissue engineering approach must be modified in order to achieve mechanically stable constructs with a homogeneous cellular distribution. This may be achieved through the use of a scaffold material, either synthetic or acellular, that is more mechanically robust and does not degrade to the degree observed in this study. Special attention must be paid to the phenotype of PAVICs cultured on such scaffold materials, as stiffer and more robust scaffolds may induce the myofibrogenic differentiation of PAVICs observed when cultured on stiff tissue culture plastic in vitro. If this were to happen, the resultant tissue engineered construct would not be a good model for the healthy aortic valve leaflet. As well, dynamic culture through the application of mechanical loading may also give tissue engineered constructs improved mechanical integrity, but as observed in this study it will most likely induce myofibrogenic differentiation. If a mechanically stable construct with homogeneous cellular distribution is achieved, a more in-depth examination of the effects that mechanical loading has on this tissue engineered aortic valve leaflet model containing PAVICs is warranted.
7. References


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8. Appended Material

8.1. Immunohistochemical Positive Controls

Figure A: Porcine coronary artery section used as positive control for the expression of α-SMA, alkaline phosphatase (red) used as chromagen, original magnification 4X.

Figure B: Porcine aortic valve leaflet used as positive control for vimentin, diaminobenzidine tetrahydrochloride (brown) used as chromagen, original magnification 40X.
Figure C: Porcine coronary artery used as positive control for desmin, diaminobenzidine tetrahydrochloride used as chromagen, original magnification 4X.