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Aortic Valve Endothelial Cells and Adhesion Molecules: Implications for a Tissue Engineered Heart Valve

Chelsea Tiller McIntosh

AORTIC VALVE ENDOTHELIAL CELLS AND ADHESION MOLECULES:

IMPLICATIONS FOR A TISSUE ENGINEERED HEART VALVE

By

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Children with congenital heart defects and patients with faulty or failing valves have the need for a suitable aortic heart valve replacement. Current treatment options have several downfalls and heavy investigation is being done into the design of an engineered valve to find an alternative that would alleviate many of these issues. Understanding the physiology of how cells interact *in vivo* is crucial to the construction of such valve. This study investigates the effect of cyclic strain in aortic valve endothelial cells on the adhesion molecules, PECAM-1, β_1-Integrin, VE-Cadherin and Vinculin. Experiments found that cyclic strain plays a role in the development of cell/cell and cell/extracellular matrix adhesions and junctions and is extremely important in the pre-conditioning of a tissue engineered construct. Without this strain the new valve would be more susceptible to inflammation, injury or possible failure after being implanted into the patient.

DEDICATION

This research is dedicated to my parents, Todd and Lisa Mauldin. Thank you for the love, encouragement, support, and inspiration you have provided me. For your constant sacrifice and generosity, I am truly grateful. You instilled in me the belief that through hard work, anything is possible.

To Alex, you are my rock. Thank you for having the answers to all my questions. Going through this process with you made it that much more enjoyable. I am so proud to call you my husband.

To all my friends and family, thank you for helping me become the person I am today. I appreciate and love each and every one of you.

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LIST OF ABBREVIATIONS

AV=Aortic Valve

MV= Mitral Valve

PV= Pulmonary Valve

TV= Tricuspid Valve

CVD= Cardiovascular Disease

AVEC=Aortic Valve Endothelial Cell

PAVEC=Porcine Aortic Valve Endothelial Cell

VIC=Valve Interstitial Cell

VEC=Valve Endothelial Cell

EC=Endothelial Cell

FEC=Fibrosa Endothelial Cell

VEC=Ventricularis Endothelial Cell

ECM=Extracellular Matrix

PECAM--1=Platelet Endothelial Cell Adhesion Molecule

TVP=Transvalvular Pressure

TE=Tissue Engineered

LSM=Laser Scanning Microscopy

CHAPTER I

INTRODUCTION

Physiology of the Heart

The heart is responsible for pumping blood through the body by a series of repeated contractions. The heart is divided into four chambers, two atria and two ventricles, along with a system of valves that assist transfer of blood through these chambers. Deoxygenated blood enters the heart at the right atrium and then passes to the right ventricle through the tricuspid valve. From the ventricle deoxygenated blood is passed to the lungs through the pulmonary valve and arteries where it is oxygenated. Oxygen nourished blood is then transported from the left atrium through the mitral valve into the left ventricle. Finally, the left ventricle blood into the aorta and on into systemic circulation. A diagram of the human heart is shown in Figure 1.1.

Flow and oxygenation of blood in the heart is made possible by heart valves that force a unidirectional flow. Heart valves are fibrous structures that passively move in response to the mechanical stimuli of blood flow and pressure. The four heart valves are classified as atrioventricular or semilunar values. The artioventricular valves, named for their position between artia and ventricles, are the mitral value (MV) and tricuspid valve (TV). The semilunar valves are composed of three half moon shaped cusps and consist of the aortic valve (AV) and the pulmonary valve (PV). These valves are responsible for transfer of blood from the ventricles into the aorta or pulmonary artery.

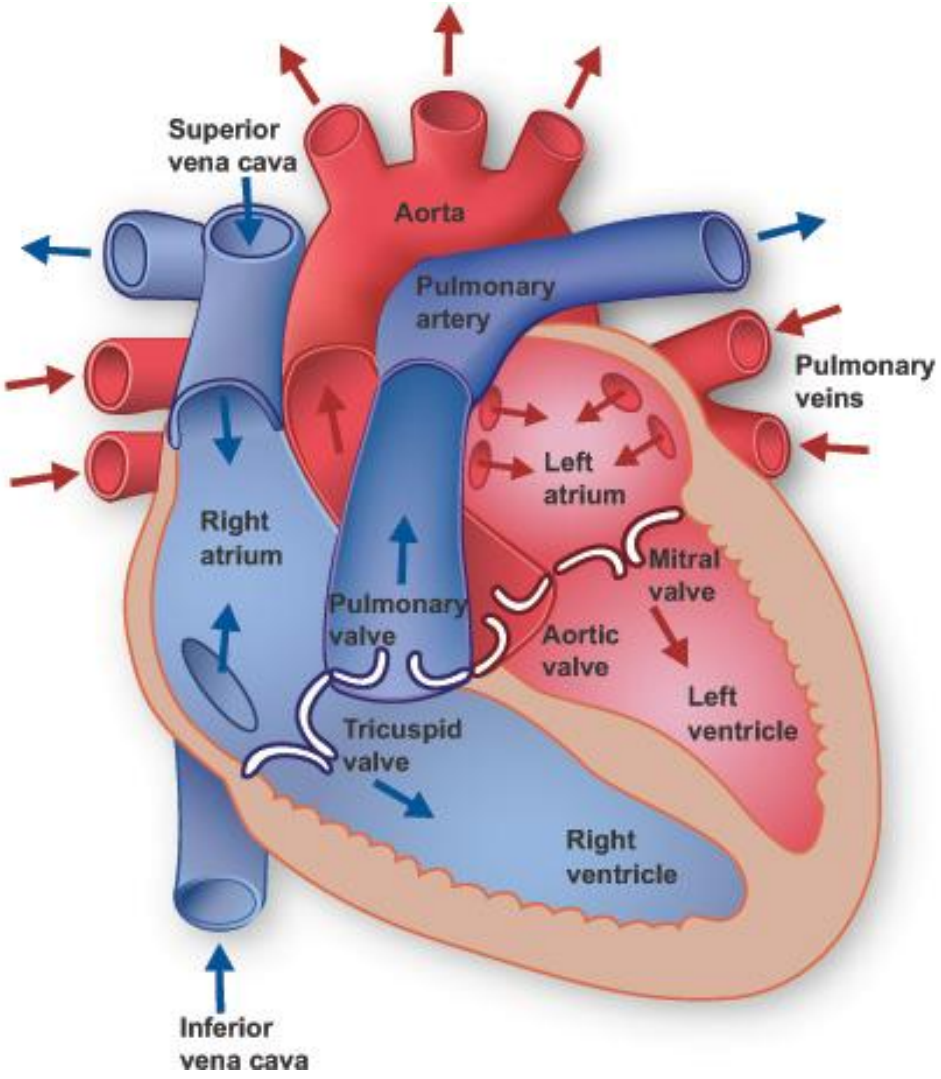

Figure 1.1 The anatomy and blood flow of the human heart. Right side of the heart is shown in blue and left side in red. Form (texasheartinstitute.com).

The cardiac cycle is the name given to the events of blood flow and pressure that occur from the beginning of one heartbeat to the beginning of the next. The cycle is broken up into two phases, systole and diastole. During systole the ventricles contract and blood is ejected. This occurs while the artrioventricular valves are closed and blood passes freely through the semilunar values and out the aorta or pulmonary artery (Figure 1.2). These valves then snap shut and prevent blood flow back into the ventricles, also known as regurgitation. Once these values close the process of diastole begins where the

ventricles are filled. During this time the artrioventricular valves are open to allow blood to flow from the atrium into the ventricles (Figure 1.2).

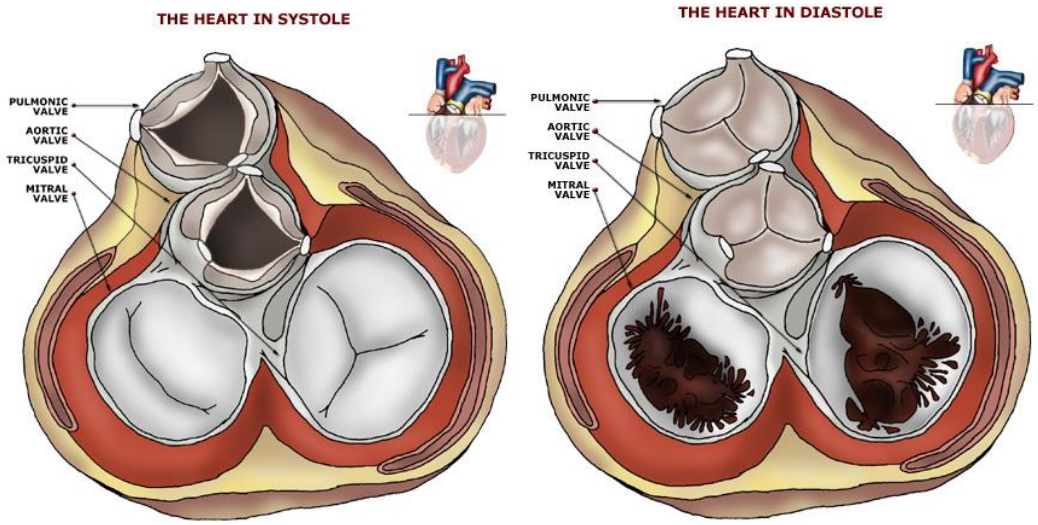

Figure 1.2 The heart in systole and diastole. From (prenhall.com).

Aortic Valve Structure

The aortic valve allows ejection of blood flow out of the left ventricle during systole and prevents regurgitation of blood back into the ventricle during diastole. The AV functions in combination with the aortic root, which connects the heart to the circulatory system. The aortic root has multiple parts that function in relationship with one another; these include the annulus, inter-leaflet triangles, sinotubular junctions, sinuses, and the aortic valve leaflets (Figure 1.3). The AV is tricuspid, having three cusps or leaflets that are semilunar. Each leaflet has four structural areas; the hinge region that joins the leaflet to the root, the belly that composes the center suspended region, the coapting surface which seals the three leaflets together, and the lanula which contains a small section of tissue named the nodule of Arantii. These sections work along side each other for the health and function of the valve.

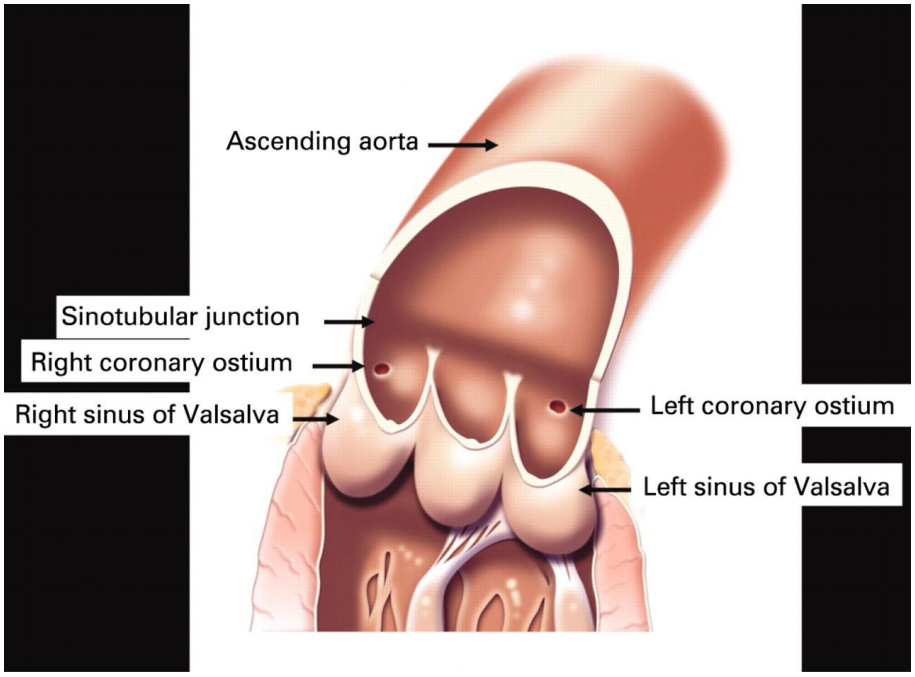

Figure 1.3 Aortic root and leaflet structure. From (1).

The aortic valve leaflets function together to allow blood to pass through and enter the body's circulation while also preventing back flow of blood. The aortic valve's fibrous structure gives it extremely flexibility along with intense mechanical strength. These leaflets tri-layered structure contributes to its remarkable properties (Figure 1.4). This makeup consists of the aorta facing fibrosa, middle spongiosa region and the ventricle facing ventricularis (2). The fibrosa layer constitutes 45% of the total thickness of the valve and is subjected to extreme amounts of shear stress due to flow of blood coming from the ventricle during systole. The fibrosa is comprised of collagen fibers oriented in the circumferential direction that help bear most of this mechanical stress (2,3,4,5). The internal layer, the spongiosa is a gelatinous region that comprises 35% of total valve thickness and contains a high concentration of glycosaminoglycans. This section functions to connect the fibrosa and ventricualris and has some stress dissipating properties (6). The ventricle facing layer, ventricularis composes 20% of total valve

thickness and is made up of a dense network of collagen and elastin. These fibers are oriented in the radial direction (5). Figure 1.4 illustrates the layers of the AV. The AV is in a continuous preloaded state where the fibrosa is under constant compression and the ventricularis under tension (5). This state can contribute to the properties of the collagen and elastin that makeup each layer of the leaflet.

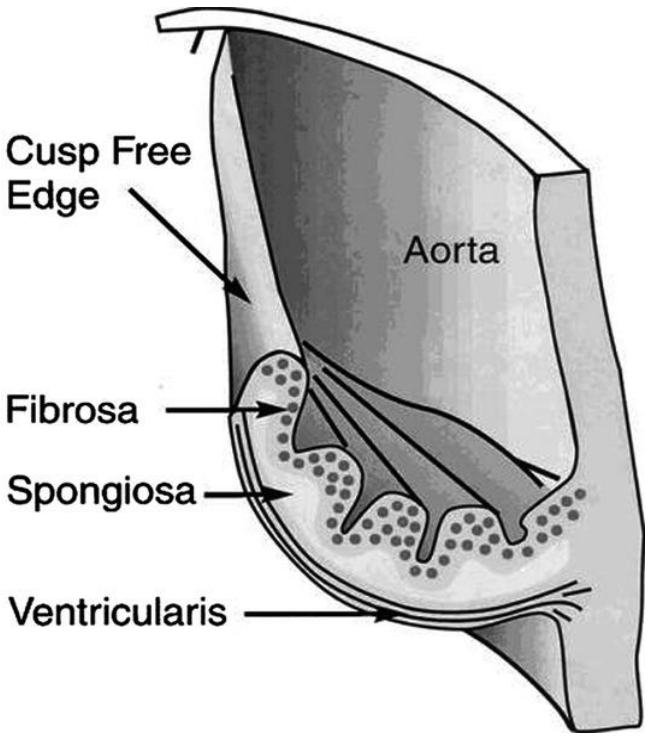

Figure 1.4 Layers of the aortic valve. From (7).

Aortic Valve Cells

The aortic valve consists of two cell types, the endothelial cells (AVECs), which line the surface, and the interstitial cells (AVICs) that populate the interstitum of the valve. The endothelium is a thin layer of cells that lines the interior surface of blood vessels and provides an interface between circulating blood and the rest of the vessel wall. These cells are endothelial cells (ECs) and they line the entire circulatory system

and any surface that comes in direct contact with blood. These cells work to provide a non-thrombogenic surface and to sense and respond to circulating stimuli in the blood stream. These cells are extremely sensitive to alterations in their hemodynamic and mechanical environments. The endothelial cells of the AV line both the fibrosa and ventricularis surfaces of the valve. The ECs are maintained on a layer of fibronectin and collage type IV (8). This sub-endothelial layers have been shown to be similar on both side of the valve with respect to height, pore diameter, pore depth and fiber diameter. AVECs have been shown to be incredibly responsive to fluid shear stress, cyclic pressure and cyclic strain (9). AVECs behave differently than various other ECs along the vasculature. When tested against laminar flow, aortic ECs, like most endothelial cells, were seen to align with the direction of flow. However valvular ECs were shown to align perpendicular to the flow, indicating a distinct phenotype (10). In addition, aortic ECs have a distinct phenotype and gene expression profile when compared to vascular ECs (11, 12). Several studies have also shown that ECs isolated from different sides of the valve behave differently and have different transcription profiles (13). These findings launched studies to investigate the response of valvular ECs to mechanical forces.

Mechanical Environment

The aortic valve exists in an intense and physically demanding mechanical environment. It is constantly under a variety of complex and dynamic forces. The AV must fully open and close in excess of 3 billion times over the span of an average lifetime. The blood volume that passes through the aortic valve varies from 1-20L per minute (14). Average blood volume through the AV at rest is 5L per minute. The total stress on a leaflet during systole is approximately 50kPa and near 500kPa during diastole

(15). The fibrosa surface bears more diastolic load while the ventricularis takes on the systolic load. Left ventricle ejection bears a fluid shear stress on the AV due to blood flow at a peak rate of 1.35 +/- .35 m/s (16). Wall shear stress on the surface of the leaflet has been reported varying from 20 dynes cm^{-2} to 1000 dynes cm^{-2}. (17-19). The AV also experiences high transvalvular pressure (TVP) where a TVP of 80 mmHg represents physiological conditions and 100-120mmHg represent diastolic blood pressure for stage I and stage II hypertension. The TVP dictates the strain of the aortic valve. This strain can occur in both the radial and circumferential direction and can vary greatly in magnitude.

Aortic Valve Disease

Cardiovascular Disease (CVD) represents a variety of conditions and pathologies that include heart failure, hypertension, stroke, myocardial infarction, congenital heart defects, angina pectoria, valve defects and arthrosclerosis. 1 in 3 Americans have been diagnosed with some type of CVD making CVD the most common cause of death in the United States. The number of deaths from CVD is greater than the combined types of cancer. One of the most common CVD related surgeries is heart valve replacement and the most common replacement is the aortic valve (20). In 2005, there were 43,900 deaths nationwide from valvular heart disease with 27,390 attributed to aortic valve disease. Additionally of 93,000 valve disease related hospital discharges, over half, 49,000 were from aortic valve disease issues and complications (21). The risk for myocardial infarction and death from a cardiovascular related disease is increased 50% when aortic valve sclerosis is present (22).

The aortic valve is responsible for allowing oxygenated blood to leave the heart and enter the body's circulation. Any obstruction or impeding force of this valve can

have severe consequences for not only heart function but entire body function. Abnormalities of the aortic valve can fall into two categories, aortic stenosis and aortic regurgitation. Aortic valve stenosis is characterized by the significant obstruction of blood flow due to a constricted valve. Stenosis can be caused by a build up of calcium or scar tissue on the valve or be present at birth as a congenital defect. A precursor to stenosis is aortic valve sclerosis, which is defined as a thickening and calcification of a normal aortic valve but causes no impedance of blood flow. The second type of AV disease is aortic regurgitation, which occurs when retrograde blood is allowed to dump back into the ventricle during ventricle filing following valve closure. Regurgitation reduces net blood flow to the circulatory system. Both types of AV disease can occur simultaneously or individually. Improper functioning of the aortic valve can have severe consequences; the heart is required to work harder to supply the body's circulation with oxygen and nutrients. This causes thickening of the ventricular wall, which eventually leads to ventricular hypertrophy and congestive heart failure.

Congenital heart defects sre the most common birth defect in newborns and occur in approximately 1 percent of births. In the United States more than 1 million adults are living with a congenital heart defect. The defect can involve the walls or the valves of the heart and include mitral value prolapse, aortic dilation, ventricular septal defect, pulmonary stenosis, right ventricular hypertrophy and an overriding aorta. Many congenital heart defects require extensive treatment, which currently requires surgical intervention.

Current Treatment Options

With the high instance of aortic valve disease there is a strong need for innovative solutions to degenerative valves. More than 100,000 US patients require a total valve replacement every year (23). Currently there are several options for valve treatment or replacement but none are completely ideal. Among these treatments are mechanical or tissue valves, which include bioprosthetic valves (from porcine or bovine source) or a allograft from a human source. Choice of valve type is dependent on the needs of the patient. Figure 1.5 illustrates the different types of valve replacements.

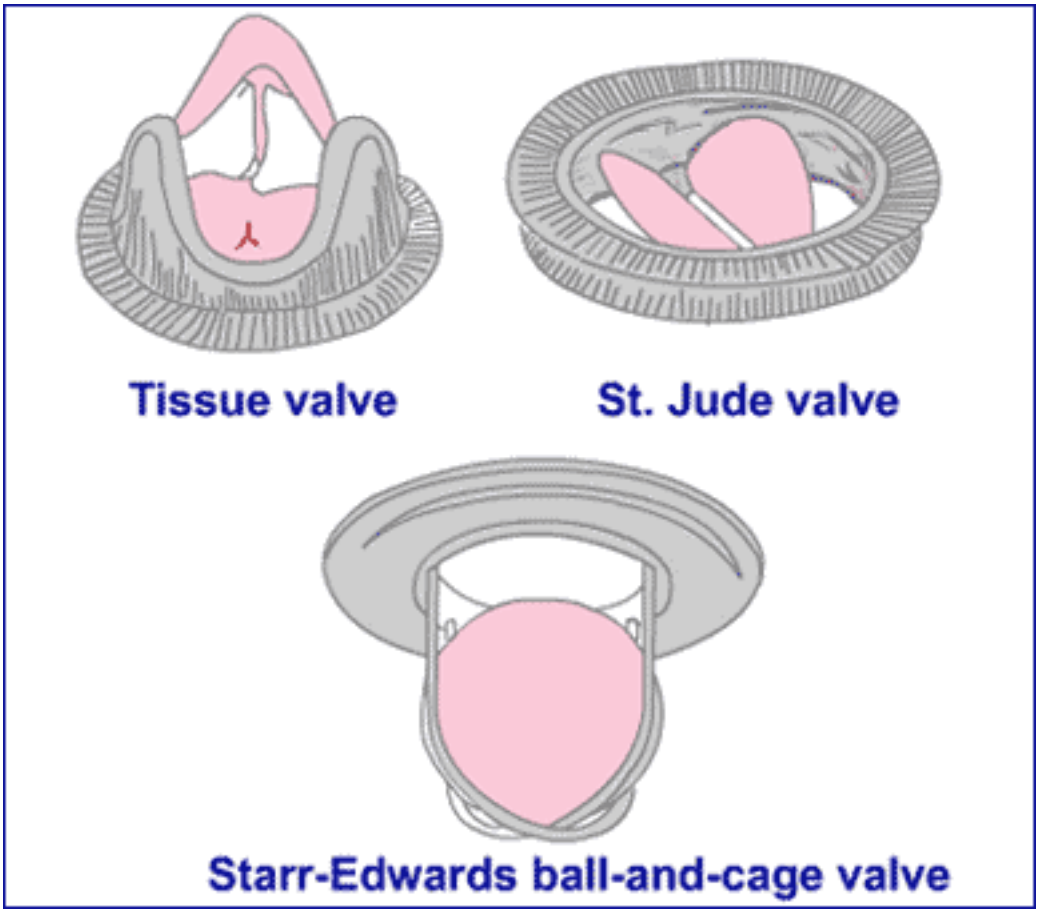

Figure 1.5 Different options for aortic valve replacement. From (medindia.com).

The term tissue valve is used to describe two types of valves, an allograft or a xenograft replacement. Tissue heart valves are used in more than half of all valve replacements (24). Allografts or homografts are valves taken from human cadavers or from diseased hearts after removal for transplant. These valves are in theory a more ideal choice because of the valves extreme structural similarity. These valves are preserved without chemical cross-linking before transplant into the new patient. Allograft valves have good hemodynamic properties, a low infection rate and low incidence of thromboembolic complication (2). The defeat of these valves is their progressive degeneration that limits their long time success. This problem usually leads to failure of the bioprosthesis, cuspal malfunction and noncalcific structural damage (25). These valves are similar to bioprosthetic valves in that they both face possible damage from the preservation process and handling but these valves are also void of cells. In both valve types the collagenous network is initially present but is incapable of renewing (2). Therefore, implanted cyropreserved allograft valves exhibit a void of cells, loss of distinct structural features and extensive collagen degradation (26).

Bioprosthetic valves or xenografts are taken from either of bovine or porcine source and implanted in place of a patient's native valve. Porcine valves are chosen for their large availability and structural and hemodynamic similarities. Bovine valves are fabricated from 3 cut pieces of the animal's pericardium and attached to a supporting stent. These valves are treated with gluteraldehyde that kills the cells in the valve to reduce and prevent antigenicity and proteolytic degradation following implantation. The resulting cell death can lead to calcification and stiffening of the valve after implantation, leading to the need for yet another replacement. Similarly to allograft valves, these valves are incapable of repair and any damage to the extracellular matrix is cumulative. The

clinical success, failure modes and mechanisms of deterioration are extremely dependent on course, preservation and handling of the tissue prior to implantation. The method of tissue attachment can also have an effect due to the method determining the stress state of the tissue during the cardiac cycle (2).

A final tissue solution is a Ross procedure also known as a PV-to-AV autograft. This procedure consists of transplanting the individual's PV to replace their ineffective AV and then the PV is replaced by an artificial valve. This procedure generally has good results with patient's hemodyanamic performance, can allow for remodeling and injury response and can allow for growth with a child or young adult. This technique also reduces the need for anticoagulation therapy that is needed for mechanical replacement and can allow the patient to lead an active lifestyle.

Mechanical valves are another alternative in valve replacement. Mechanical valves exist in several forms but are generally made from pyrolytic carbon coasted metal surfaces (27). This material is used for its excellent biocompatibility and thromboresistence, high fatigue strength and wear resistance. These mechanical valves can be composed of a mobile occluder in a metallic cage or be a set of tilting disks that are attached to a carbon ring. These valves open and close passively from the changes in pressure and blood flow during the cardiac cycle. These valves are favorable for their structural durability, but they suffer from two limitations. They contain hinged designs and components that must remain inside the flow zone which results in intense physiological shear stresses that can induce platelet lysis and protein aggregation on the valve surface. Even with over 40 years of research and development no mechanical valve can overcome this hurdle (28). Secondly, all mechanical valve recipients must go through anticoagulation treatment for the rest of their lives. This drug therapy has serious

occupational and lifestyle restrictions. Even with life alerting drug treatments mechanical valve patients have a 2-5% annually cumulative risk of suffering a serious bleeding event such as hemorrhage, stroke or infarction (29).

Tissue Engineering

Tissue engineering is an emerging area of science that formed out of a relationship between engineering and biology to create a new tissue or organ to replace a dysfunctional tissue. This area of science has the potential to offer many solutions to medical and biological problems. Simmons defined tissue engineering as a set of tools at the interface of biomedical and engineering sciences that uses living cells or attracts endogenous cells to assist tissue formation or regeneration and creates a therapeutic or diagnostic benefit (13). The basic characteristics of a heart valve replacement were given in 1962 by Dr. Dwight Harken and extended by Sacks and Schoen for tissue engineered valves (30). 1) It must be non-obstructive, 2) closure must be prompt and complete, 3) it must be non-thrombogenic and non-immunogenic, 4) it must accommodate the somatic growth of the recipient, and 5) must last a lifetime of the patient in an environment that requires it to be durable enough to endure millions of load cycles and be capable of ongoing remodeling. The goal of generating a living heart valve replacement would be a valve that has healthy cells, can repair ongoing extracellular matrix (ECM) damage, adapt to a changing environment and grow with a growing recipient (2). The ability of this heart valve will depend, on its living cellular component (VECs and VICs) to function normally, maintain homeostasis, and repair structural injury to the ECM (23, 31). Generally, cells are seeded onto a synthetic, porous, biocompatible and biodegradable polymer scaffold in the shape of a trileaflet valve and given time to grow and develop in

a bioreactor prior to implantation (32). The scaffold will then be constructed to degrade and be replaced by the newly formed ECM.

Recellularization of a decellurized allograft valve does occur but its focus is limited to the regions of the arterial wall and the cusp base (33). The process of *in vitro* cell seeding prior to implantation is more attractive for introducing a cell population. The key phases that have to occur during *in vitvo* and *in vivo* tissue formation and maturation are cell proliferation, sorting, and differentiation, ECM production and organization and degradation of polymer scaffold and remodeling and possibly growth of the tissue along side the growth rate of the individual. Figure 1.6 illustrates the tissue engineering paradigm.

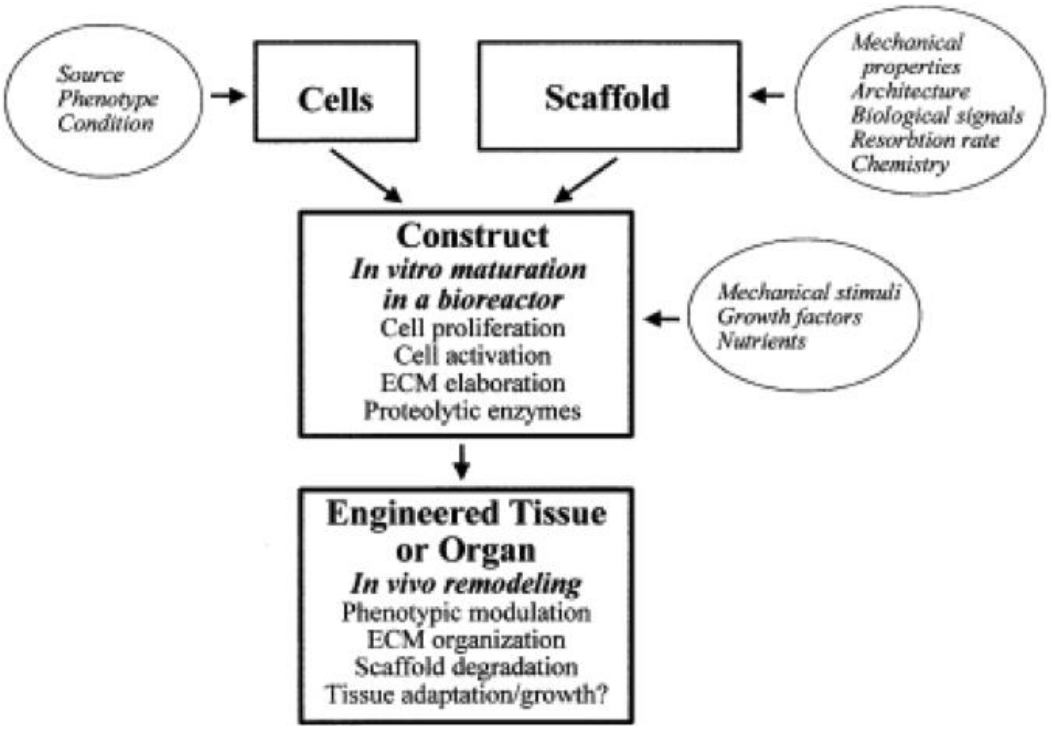

Figure 1.6 The tissue engineering paradigm is the logical progression from cell course to implantation. The paradigm starts with a decision regarding cell course and scaffold material. Then the seeded construct needs to mature *in vitvo* . Finally, the tissue is implanted into the patient and the construct undergoes *in vivo* remodeling to produce a functional replacement tissue or organ (34).

Proteins of Interest

Heart Valves experience a plethora of forces, fluid shear stress and cyclic strain act directly on the endothelial cells that line the valve. AVECs experience contact guidance where cell morphology is influenced by the stability of the ECM, focal adhesions and cytoskeletal elements (35). As forces deform the cells, active cytoskeletal rearrangements produce a variety of signaling cascades from monocyte recruitment to alignment alterations. Alterations are a result of phenotypic expressions of various adhesion molecules and the location of these molecules. Cell adhesion molecules are proteins located on the cell surface involved with the binding of other cells or with the

ECM in the process of cell adhesion. These proteins are typically transmembrance receptors that are composed of three domains: an intracellular domain that works with the cytoksksleton, a transmembrane domain and an extracellular domain that interacts with other adhesion molecules or the ECM. The adhesion molecules considered crucial for proper endothelial cell function and proteins of interest for this study are Platelet Endothelial Cell Adhesion Molecule (PECAM-1) also known as cluster of differentiation 31 (CD31), Cadherin 5, type2 or Vascular Endothelial (VE) Cadherin, Vinculin, and Intergin $\alpha 5\beta 1$ (β_1-Integrin). PECAM-1 modulates recruitment of monocytes and senses mechanical stimuli (36). VE-Cadherin is a cell/cell adhesion molecule considered crucial to cell bonding and motility. It gives cells the ability to adhere in a homophilic manner and may play an important role in endothelial cells biology through the control of adhesion and organization of intercellular junctions. The integrity of intercellular junctions is a major determinant of the permeability of the endothelium. Cadherin is required to maintain a restrictive endothelial barrier. VE-Cadherin is localized on cells/cell junctions (37). It's function is dependent on another adhesion molecule associated with binding to the ECM, β_1-Integrin. β_1-Integrin is often investigated due to it's predominate interaction with collagen. It mediates attachment between a cell and another cell or the ECM. β_1-Integrin also plays a role in cell signaling and therefore can define cellular shape, mobility and regulate the cell cycle. Vinculin is a vital accessory molecule involved in force transduction and mediating cellular response. It binds intregrins to F-actin networks at the intracellular face of the plasma membrane and also mediates cytoksletal mechanics (38). Vinculin appears to play a key role in shape control based on its ability to modulate focal adhesion structure and function.

Objective and Scope of Study

Aortic valve disease is an overwhelming and severe health issue that poses problems for millions of Americans today. There is currently no ideal treatment option. The development of a successful aortic valve replacement would have life changing affects for millions of people and families. The most promising solution for the problem of AV disease is the development of a tissue engineered valve that can be implanted into the patient and last their lifetime thus depleting the need for further surgery or medical therapy. There is still a long journey of research before a viable valve can be constructed but every piece of the puzzle is useful in the design of a successful tissue.

The ideal tissue engineered heart valve should elicit no adverse or out of the ordinary response. The aim of this study is to further characterize the *in vitro* response of VECs to mechanical forces to ensure that preconditioning is necessary for a successful tissue engineered valve. A thorough understanding of cell behavior is needed to predict and prevent adverse reactions or failure of the valve following implantation. The new valve will face a harsh and demanding environment once it is implanted into the body. There have been several studies that have shown the extreme environment is necessary in maintaining homeostasis in the AV and without mechanical forces the valve can have structural and mechanical changes (39-42) The practice of mechanical preconditioning has been shown to improve microstructure and mechanical properties and formation of the extracellular matrix (43, 44).

The objective of this study is to investigate the affect of cyclic strain on several cell matrix and cell-cell interaction proteins in AVECs. The understanding of how these proteins interact with and without strain will give us understanding into what factors and

preconditioning techniques are needed for a heart valve construct that is as close to identical to a native valve as possible.

CHAPTER II

METHODS

Cell Isolation and Culture

Porcine hearts were obtained directly following slaughter from a local abattoir (Sansing Meat Service Maben, MS). Female Yorkshire/Hampshire pigs were slaughtered before 6 months of age with post-slaughter weight of no more than 120 lbs. Valves were transported to the laboratory in ice-cold Dulbecco's Phosphate Buffered Saline (PBS; Sigma, St. Louis, MO). Cell culture and isolation were conducted as previously cited (45,46). Freshly excised AV leaflets were pinned fibrosa (concave up) or ventricularis (concave down) side up on sterile rubber mat. Endothelial cells were swabbed with sterile cotton swabs following collagenase type II digestion (~600 U/ml; Invitrogen, Carlsbad, CA) for 10 minutes at 37° C and 5% CO_2. The resulting cell solution was centrifuged at 1000 rpm for 5 minutes and the pelleted cells were plated on T 12.5 cm^2 tissue culture flasks. Porcine Aortic Valve Endothelial Cells (PAVEC) fibrosa and ventricularis cells were cultured separately in Dulbecco's Modified Eagle Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS; Invitrogen, Carlsbad, CA) and 1% Anti-biotic/Anti-mycotic solution (ABAM; Invitrogen, Carlsbad, CA), using standard tissue culture methods. Cells were split at 75-85 % confluency from a T12.5 cm^2 flask into a T25 cm^2 flask and eventually into a T75 cm^2 flask. Cells were then maintained in T75 cm^2 flasks. Cells were frozen for long-term storage and recovered as needed for experiments. Cobblestone morphology, contact inhibition and the presence of

PECAM -1 confirmed EC phenotype characterization. Fibrosa endothelial cells (FEC) and ventricularis endothelial cells (VEC) used for experiments were between passage 4 and 6.

Application of Cyclic Strain/ FX-4000T™ Flexercell® Tension Plus™

There have been several studies on the response of vascular endothelial cells to cyclic stain. The role of oxidative stress and nitric oxide synthase expression have been related to cyclic strain in arterial cells (47,48,49). The Flexcell system is the most commonly used device to apply strain to cultured cells. The Flexcell systems works by applying negative pressure to custom culture plates with a flexible silicone membrane. A schematic diagram of the system is shown in Figure 2.1.

Cells were centrally seeded (~$5x10^5$/well) into 6-well BioFlex™ culture plates pre-coasted with collage type I (Flexcell International, Hillsborough, NC) and grown for 24 hours in 3 ml DMEM supplemented with 10% FBS and 1%ABAM. BioFlex™ plates were then placed in the Flexcell® FX4000-T™ Tension Plus System (Flexcell International, Hillsborough, NC).

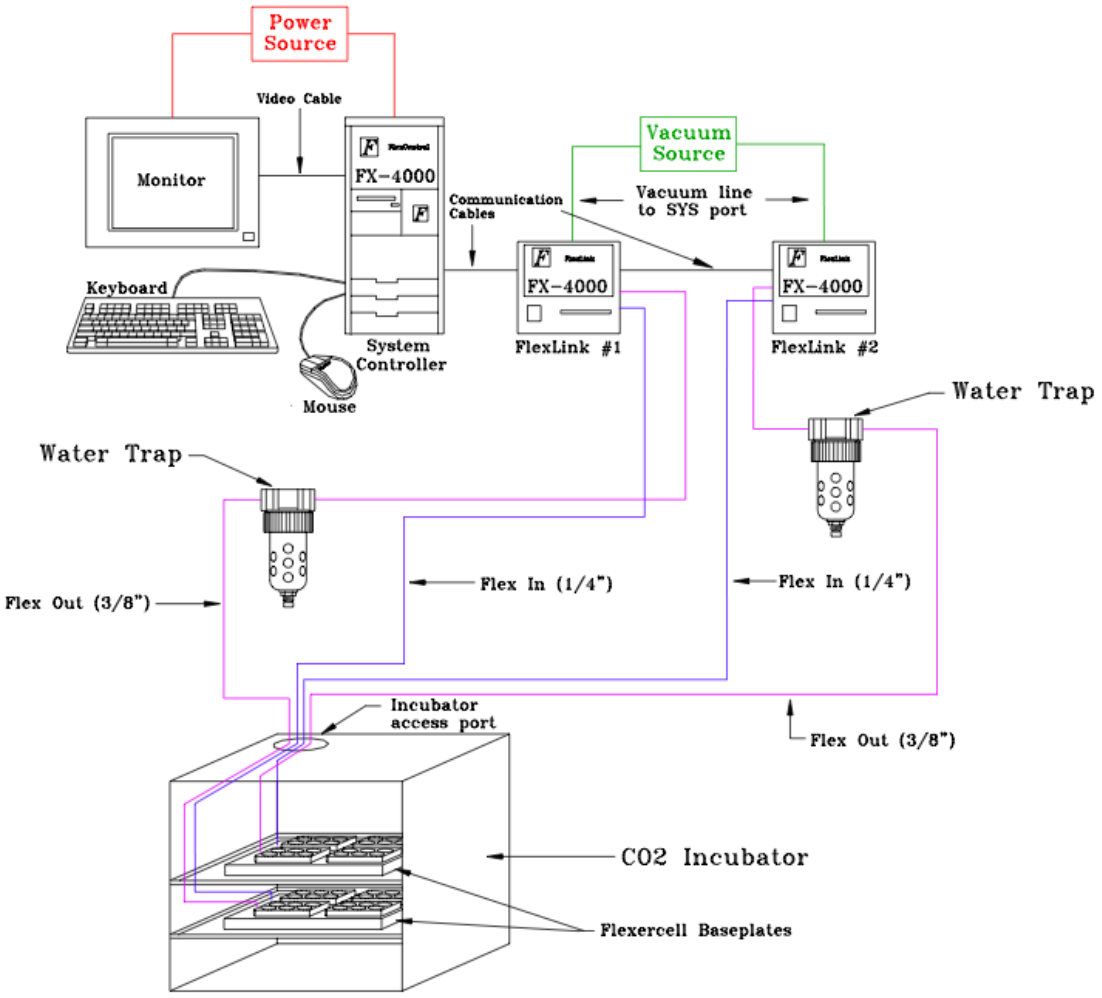

Figure 2.1 Schematic of the FX-4000™ Flexercell® Tension Plus™ system. Collagen Type I Bioflex™ plates are seeded with cells and stretched in an incubator via application of cyclic negative vaccum pressure.

This device applies negative pressure beneath the BioFlex™ plate wells via a vacuum pump that is monitored by a pressure transducer allowing precisely defined equibiaxial stretch up to 30% (Figure 2.2). 25mm loading posts were used to give a uniform equibiaxial strain, as previously described (50). Stretch can be applied with any waveform and frequency; a sinusoidal waveform at 1Hz was chosen to most accurately mimic the cardiac cycle. Cells were flexed for 24 hours at cyclic strains of 0-10% and 0-20%. The duration matches previous studies where ECs elicited a biological response to

cyclic strain over a 24 hour time frame (51). The chosen strains represent a varied range that would mimic biological and pathological strain conditions (52). Another group of plated cells were also statically cultured for 24 hours to serve as a comparison control.

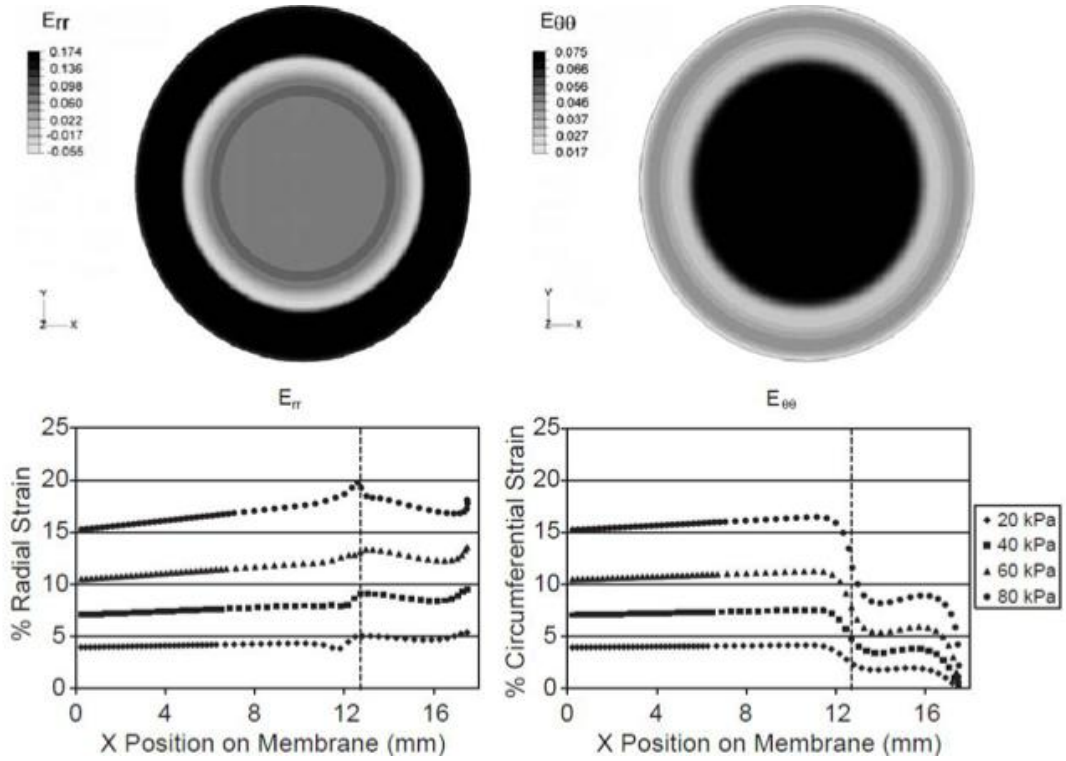

Figure 2.2 Stress distribution in the Flexcell membranes being stretched by negative vacuum pressure over a loading post. Endothelial cells are centrally seeded for uniform radial and circumferential profiles. From (50).

Laser Scanning Confocal Microscopy

Immediately following strain or static regimens, cells were rinsed with sterile PBS (all steps used just enough reagent to completely cover the cells, approximately 2mL) in BioFlex™ plates and fixed with 4% paraformaldehyde (Electron Microscopy Sciences. Hatfield, PA) in PBS for 30 minutes in the dark at room temperature under sterile conditions. Cells were then rinsed in 0.1% Triton X-100 (Sigma, St. Louis, MO) and 0.01M glycine (Sigma; St. Louis, MO) in PBS for 30 minutes to permeabilize membranes

and remove traces of fixative. Cells were rinsed with 5% Bovine Serum Albumin (BSA; Sigma, St. Louis, MO) in PBS and then again with 5% BSA in PBS containing 5% Normal Goat Serum (NGS; Sigma, St. Louis, MO). Primary antibodies (mouse anti-human, IgG_1; β_1-Integrin, PECAM-1, Vinculin, IgG_2; VE-Cadherin, Millipore, Temecula, CA, 1:250 in PBS with 1%BSA) were added to cells with gentle agitation for overnight conjugation. Cells were rinsed in 1%BSA/PBS and again in 5%BSA/5%NGS/PBS. Secondary antibodies (AlexaFluor 488 rabbit anti mouse IgG_1 and IgG_2, Invitrogen, 1:100 in PBS with 1%BSA) were added to cells and allowed to incubate for 2 hours in the dark followed by two washes with 1%BSA/PBS and a rinse in PBS. F-actin staining utilized AlexaFluor 635 Phalloidin (3 µg /ml, Invitrogen, 1:100 in PBS) for 30 minutes at 4°C. After two rinses in PBS cells were stained with DAPI (1:2500 in PBS, Invitrogen, Carlsbad, CA) for 15 minutes followed by 2 rinses in PBS.

Collagen membranes were cut out of the BioFlex™ plates using a scalpel and laid on a microscope slide and coated with Flurogel with Tris Buffer (Electron Microscopy Sciences, Hatfield, PA). A coverslip was adhered to the slide with clear nail polish. A Zeiss LSM 510 (Carl Zeiss, Thornwood, NY) confocal microscope was used with a 10 X or 20X objective. DAPI was visualized with a 420-480 bandpass filter. 505-530nm bandpass and 650nm longpass filters were used to see the fluorescent conjugated antibodies, Alexaflour 488 and 635 respectively. 12 bit 1024x1024 images were captured using plane scanning mode to acquire each image at a single focal plane.

Carl Zeiss LSM Image Software Version 3.5.0.223 was used for image processing during image capture. Following image acquisition, Adobe Photoshop CS4 (Adobe Systems Incorporated, San Jose, CA) was used for post processing analyzing. All image processing was performed according to the guidelines from The Microscopy Association

of American (Personal Communication- John Mackenzie). This method utilized histogram stretching and gamma adjustment to avoid data manipulation that that can occur from conventional brightness/contrast methods. Light and dark levels are set using a histogram of pixel values for each fluorescent channel. Histogram stretching spreads data from each channel across a 4095 level (for 12 bit images) spectrum that allows for contrast of the data to be enhanced. This method takes care not to over represent bright sports or have the dark side of the pixel spectrum artificially set to black. Dark levels are set at the level where color is first seen, hence the first relevant pixels are set in the histogram and all background (area not representing a stain) is set to a histogram level of zero or true black. Light levels, or saturation point, are set to the first detectible pixels in each individual channel. This assures that the brightest pixel level is now set to the saturation point. Following histogram stretching, gamma levels are set sequentially across all channels. Identical gamma levels are used for all green channels to give accurate comparison between images. Images are saved in TIFF format to avoid any loss of data or image quality due to compression.

Protein Quantification

Immediately following Flexcell testing wells were washed 2 times with PBS. Cells were solubilized with 1ml of lysis buffer (buffer dependent on ELISA kit used) and allowed to sit on ice for 15 minutes. After brief and gentle scraping with a cell lifter (Sigma, St. Louis, MO) lysates were collected and frozen at -80° C until further testing. Before use, samples were centrifuged at 2000 x g for 5 minutes and supernatant was transferred to a clean test tube. Protein concentrations were quantified using the Bicinchoninic Acid Protein Assay Kit (Sigma, St. Louis, MO). Sample absorbance was

measured on a spectrophotometer and compared to a standard curve to determine total protein concentration. Protein concentrations were examined and a final working concentration of 130 μg/ml was determined based on majority sample concentration. All samples were diluted in diluents specified by each ELISA. An ELISA for each molecule of interest (VE-Cadherin: DuoSet IC, R&D Systems, Minneapolis, MN. Integrin α5β1: DuoSet IC, R&D Systems. Minneapolis, MN. PECAM-1: Human ELISA Kit, Abcam, Cambridge, MA. Vinculin: Human VCL ELISA Kit, TSZ ELISA, Framingham, MA.) was performed according to included protocols. Protein concentration was determined using a standard curve and then graphed for FEC and VEC for each protein.

Statistical Analysis

Samples were run with 3 biological replicates and 2 technical replicates. One-way analysis of variance (ANOVA) was performed using SAS analysis software to determine significant differences ($p \leq 0.05$) between FEC and VEC in each condition. An unpaired t-test was used to determine significant differences ($p \leq 0.05$) between strains for FEC and VEC.

CHAPTER III

RESULTS

Protein Quantification

Protein quantification by ELISA demonstrated the presence and quantity of cell matrix and cell-cell adhesion molecules at cyclic strains of 0-10 and 0-20% for 24 hours and a static condition for the same time period. Levels of PECAM-1 expression were not significantly different in comparison to static control samples for both FECs and VECs. Similarly, there was no notable difference when comparing cell types to one another (Figure 3.1). When analyzing protein expression for VE-Cadherin and β_1-Integrin there were noticeable similarities when cells were strained at 20%. Both molecules had a significant increase in expression when comparing cell types at the highest strain condition. Interestingly, FECs demonstrated a significant increase of β_1-Integrin in comparison to VECs while VECs experienced higher quantities of VE-Cadherin in contrast to FECs. FECs also experienced a significant increase of β_1-Integrin compared to static conditions along with VECs that experienced higher levels of VE-Cadherin against static cells (Figure 3.2, 3.3). In studies with Vinculin any presence of strain caused a significant up regulation of the molecule in VECs while FECs all behaved uniformly. The only side specific differences were observed at the static condition (Figure 3.4).

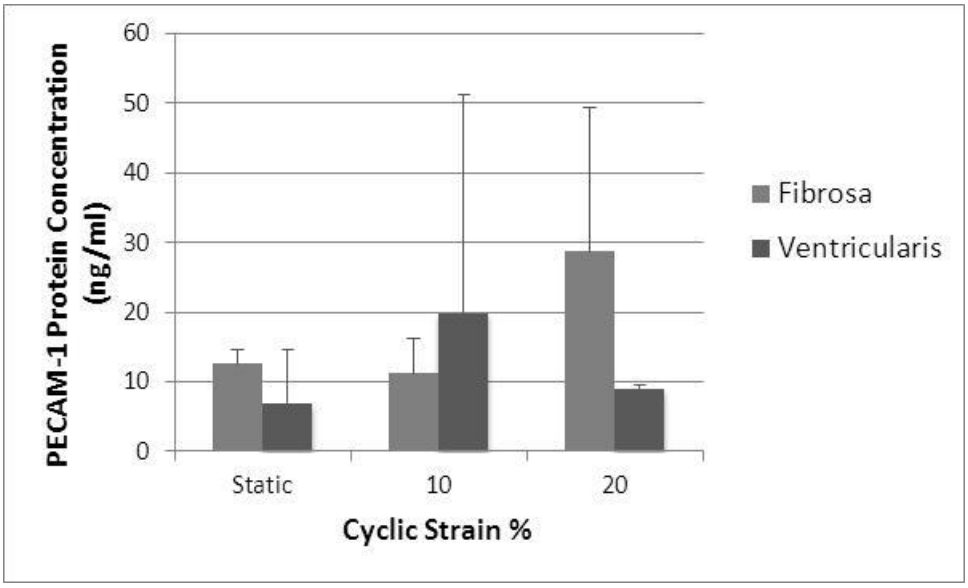

Figure 3.1 Level of PECAM-1 protein concentration in cell lysates isolated from FECs and VECs exposed to cyclic strain for 24h. Bars represent mean values. Error bars represent standard deviation (n=3).

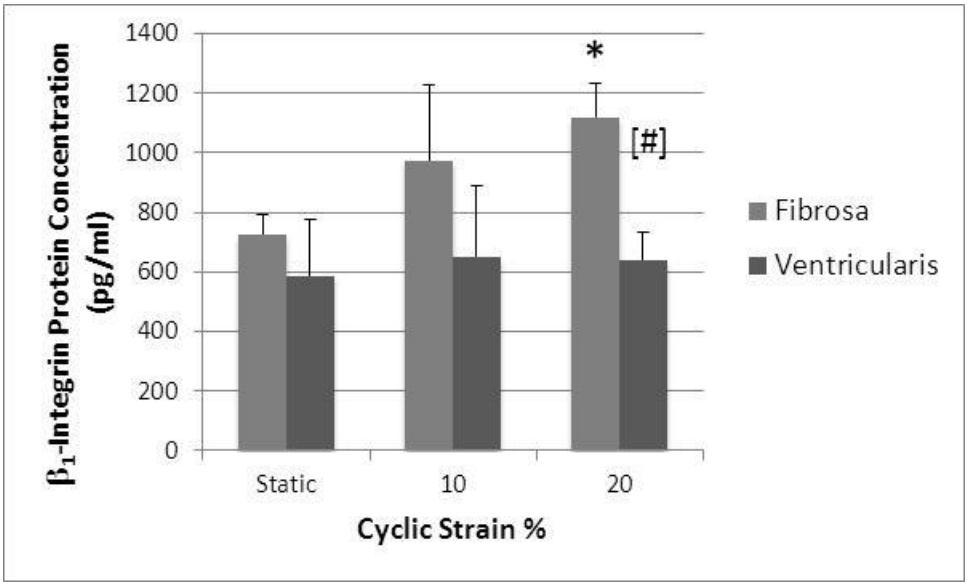

Figure 3.2 Level of β_1-Integrin protein concentration in FECs and VECs exposed to cyclic strain for 24h. Bars represent mean values. Error bars represent standard deviation (n=3). * denotes statistically significant up-regulation when compared to static culture ($p \leq 0.05$). # denotes significant difference between cell types for that strain condition ($p \leq 0.05$).

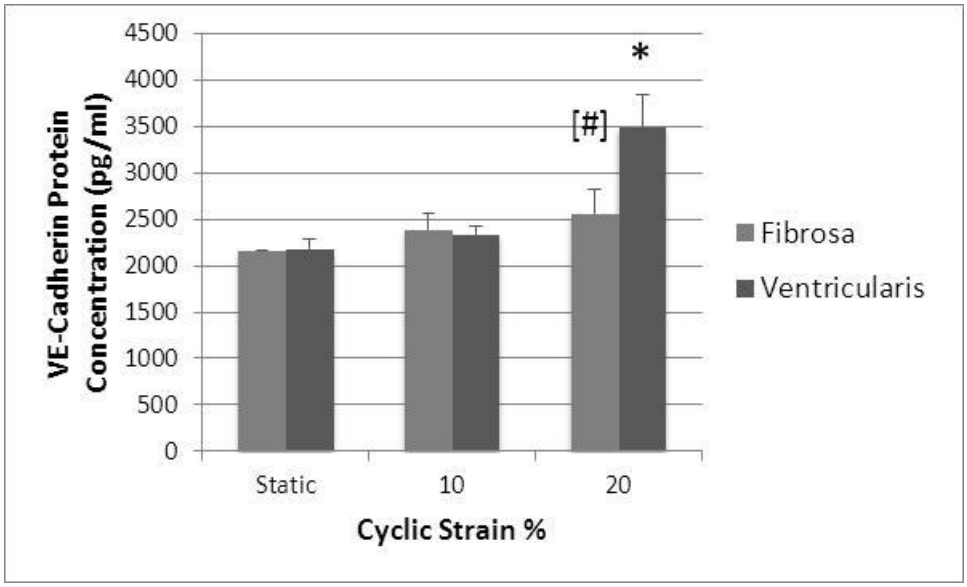

Figure 3.3 Level of VE- Cadherin protein concentration in FECs and VECs exposed to cyclic strain for 24h. Bars represent mean values. Error bars represent standard deviation (n=3). * denotes statistically significant up-regulation when compared to static culture ($p \leq 0.05$). # denotes significant difference between cell types for that strain condition ($p \leq 0.05$).

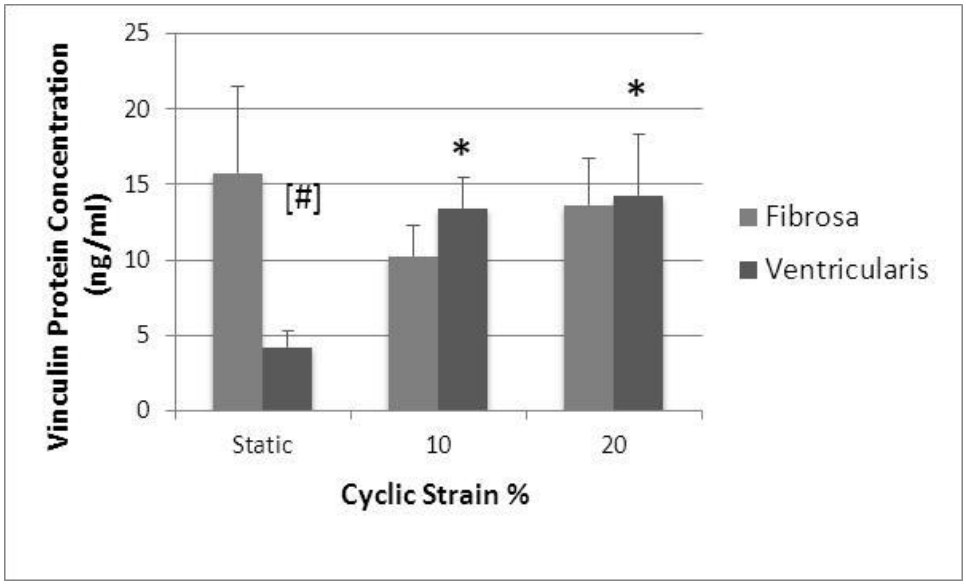

Figure 3.4 Level of Vinculin protein concentration in FECs and VECs exposed to cyclic strain for 24h. Bars represent mean values. Error bars represent standard deviation (n=3). * denotes statistically significant increase when compared to static culture ($p \leq 0.05$). # denotes significant difference between cell types for that strain condition at ($p \leq 0.05$).

Confocal Laser Scanning Microscopy

To investigate spatial location and presence of cell matrix and cell-cell adhesion molecules in AVECs, CLSM was used to visualize expression of PECAM-1, β_1-Integrin, VE-Cadherin and Vinculin. Figure 3.5 (A, C, E) demonstrates that FECs experienced an increase in PECAM-1 production when strained at 20%. These images show PECAM-1 spreading out from the cell with this increase in strain. Figure 3.5 (B, D, F) indicates that there is no significant change in protein concentration for all levels of cyclic strain in ventricularis cells. PECAM-1 on the fibrosa side appears to have a relationship to an increase in cyclic strain where ventricularis cells are not affected.

Staining for β_1-Integrin in FECs and VECs shows that this protein is changed by strain in cells from the fibrosa side of the valve. Figure 3.6 (A, C, E) show similar expression of β_1-Integrin in static and 10% conditions while cells at 20% strain show an

increase of this molecule. In Figure 3.6 (E), Integrin can be seen spreading out from the tight packed location it had in static and 10% cultures. When analyzing VECs the images show all test levels produce around equal levels of β_1-Integrin. The only side specific difference present is at 20% strain where there appears to be an increase of FEC molecule production.

Images captured for VE-Cadherin indicate that the fibrosa side of the valve has an unaffected protein regulation while VECs show a relationship to strain. An increase in cadherin production can be visualized in VECs when comparing 20% strain to control samples (Figure 3.7 (B) and Figure 3.7 (F)). A side specific difference is represented in Figure 3.7 (E) and Figure 3.7 (F) where VECs have a higher level of VE-Cadherin expression than FECs.

Analysis of Viniculin stained images provides evidence that any presence of strain produced an up regulation of VECs while FECs had no change for any test condition (Figure 3.8). Ventricularis cells appear to increase their vinculin production as they are strained. Images in Figure 3.8 (A) and Figure 3.8 (B) indicate the only side specific differences occurs when cells are under static conditions.

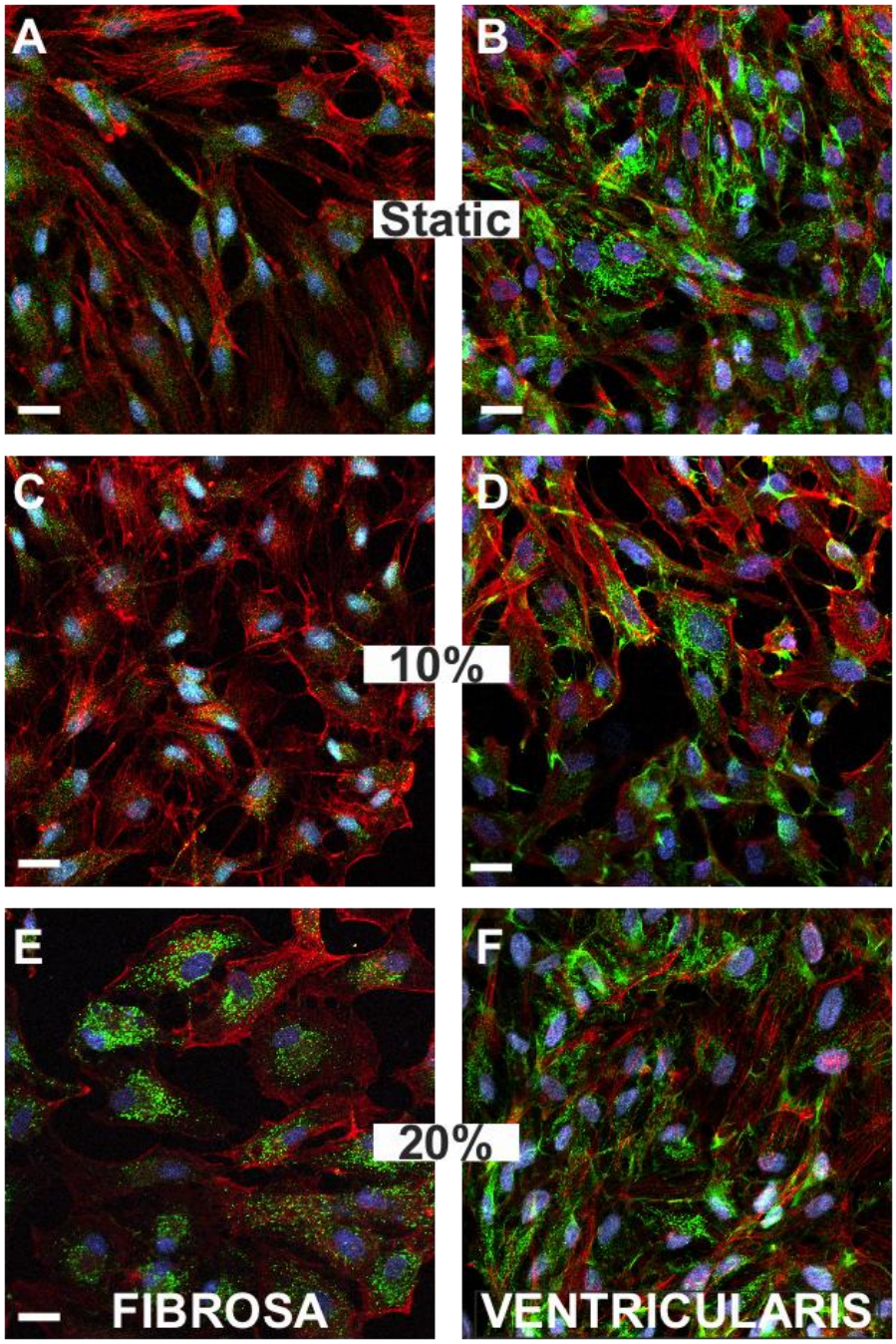

Figure 3.5 Confocal laser scanning microscopy images of FECs (A, C, E) and VECs (B, D, F) under static conditions (A, B) and exposed to 24hr of cyclic stretch at 10% (C, D), and 20% (E, F). *Blue* cell nuclei, *red* F-actin, and *green* PECAM-1. Scale bars represent 10 μm.

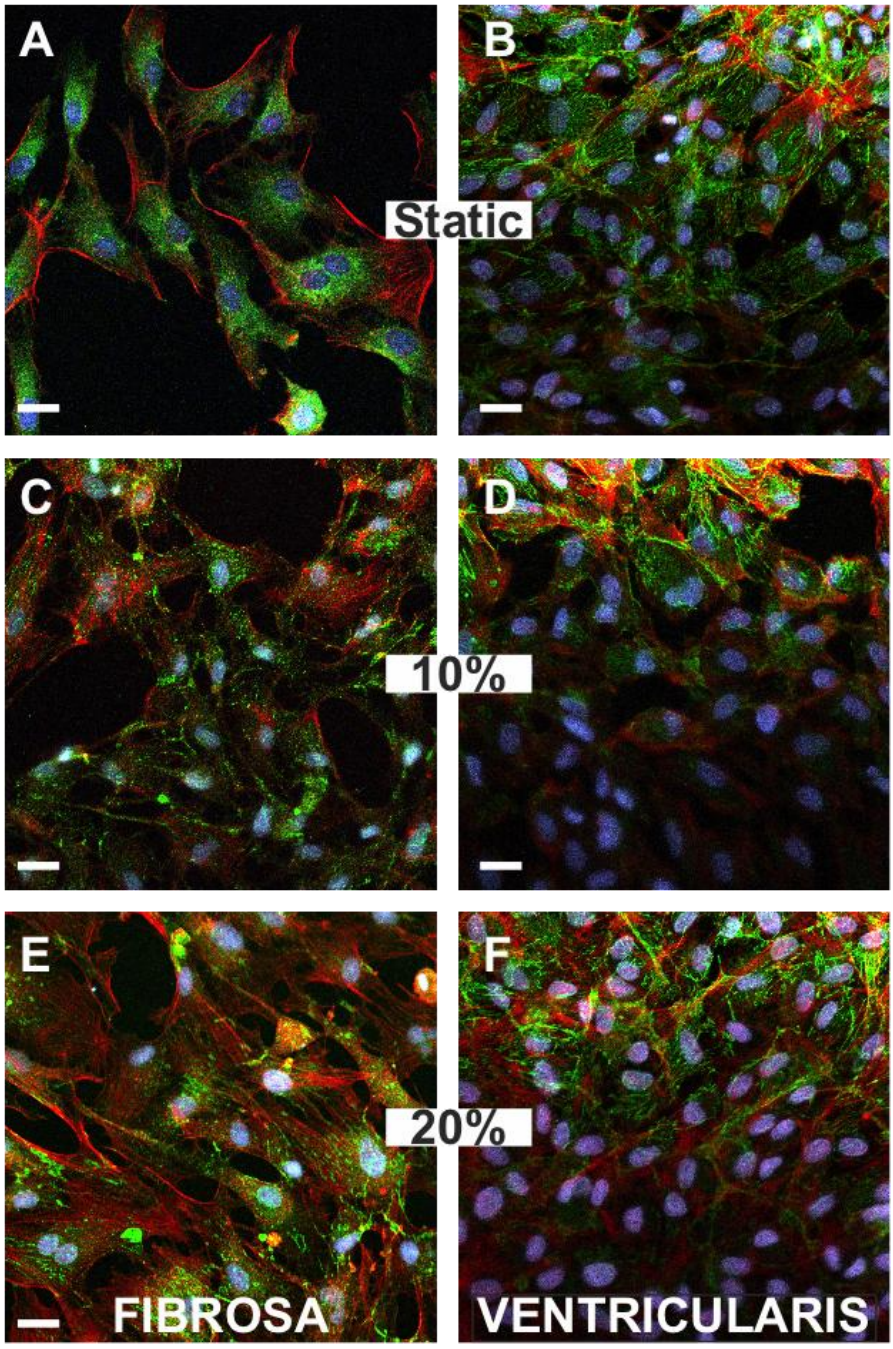

Figure 3.6 Confocal laser scanning microscopy images of FECs (A, C, E) and VECs (B, D, F) under static conditions (A, B) and exposed to 24hr of cyclic stretch at 10% (C, D), and 20% (E, F). *Blue* cell nuclei, *red* F-actin, and *green* β_1-Integrin. Scale bars represent 10 μm.

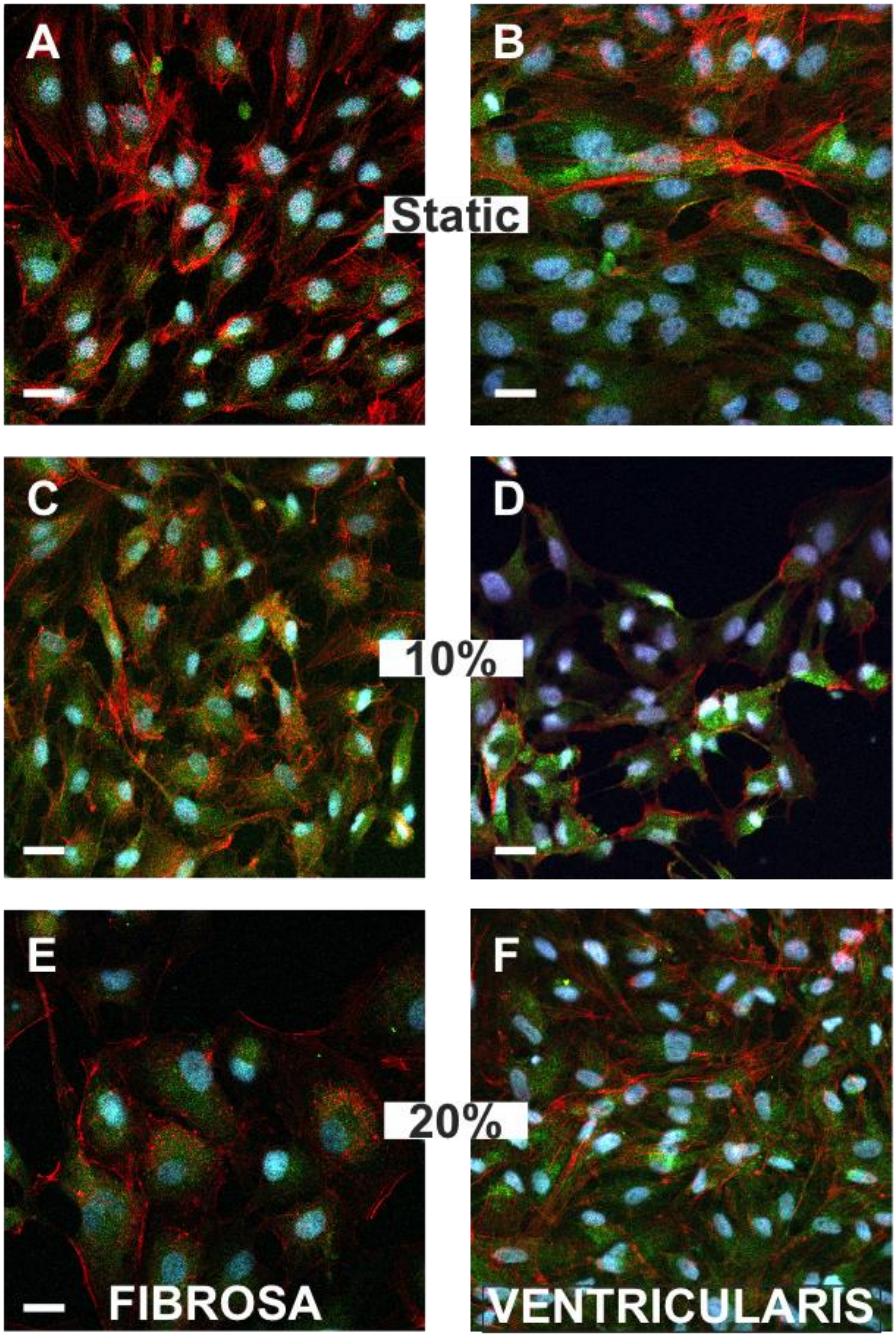

Figure 3.7 Confocal laser scanning microscopy images of FECs (A, C, E) and VECs (B, D, F) under static conditions (A, B) and exposed to 24hr of cyclic stretch at 10% (C, D), and 20% (E, F). *Blue* cell nuclei, *red* F-actin, and *green* VE-Cadherin. Scale bars represent 10 μm.

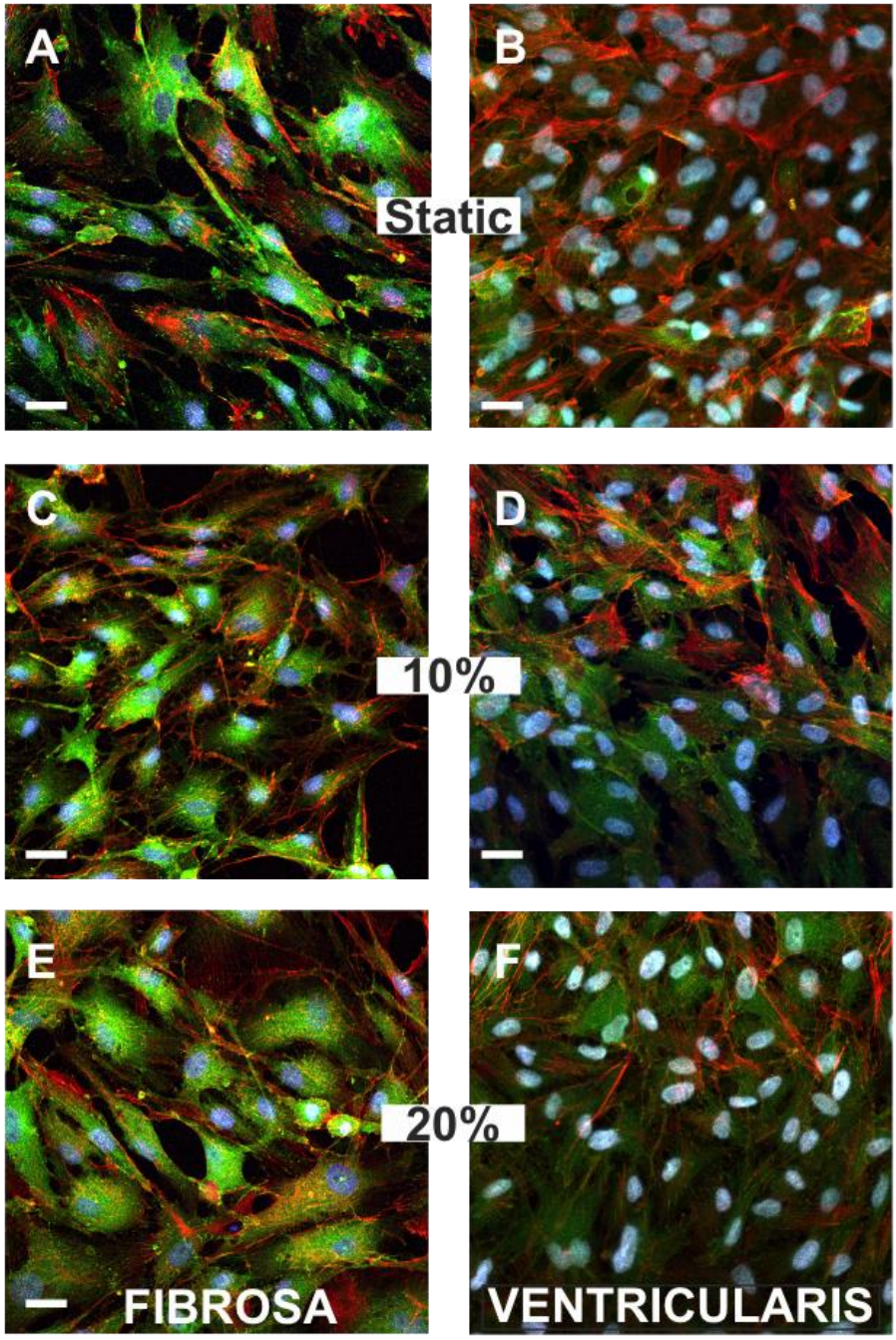

Figure 3.8 Confocal laser scanning microscopy images of FECs (A, C, E) and VECs (B, D, F) under static conditions (A, B) and exposed to 24hr of cyclic stretch at 10% (C, D), and 20% (E, F). *Blue* cell nuclei, *red* F-actin, and *green* Vinculin. Scale bars represent 10 μm.

CHAPTER IV

DISCUSSION

Tissue engineered heart valves offer great promise as a successful alternative to traditional valve replacement therapies. The development of these valves is especially important for pediatric patients where the valve grows with the individual and artificial replacements must be replaced to accommodate growth. The major advantage of a tissue-based valve is its ability to remodel and adapt to injury and a changing environment. The key to this remodeling characteristic is the presence of a viable cell population. A fundamental understanding of cell behavior is crucial to predict and prevent adverse reactions and potential failure. Once these tissue-engineered valves are implanted in a patient they must behave as close to the native valve as possible. They are immediately exposed to an extremely demanding and complex mechanical environment. One of the major hurdles of developing a useful tissue based valve replacement is insuring that the cells of the construct will perform in the correct manner. The results of this study demonstrate the need to include strain in the preconditioning of cells prior to implantation of the construct into the patient.

This study found that the adhesion molecule PECAM-1 had no response to changes in cyclic strain. This protein had the same response to all levels of strain and did not exhibit any side specific differences for any condition or comparison. Though not significant at $p \leq 0.05$, Figure 3.5 shows a small up-regulation of PECAM-1 on the fibrosa side in response to cyclic strain. This can be contributed to PECAM-1's function outside

the cell. PECAM-1 senses mechanical stimuli and helps assist an immune response. Confocal images show PECAM-1 working around cells at this increased stain state. At a cyclic strain of 20% PECAM-1 is likely reacting to the increase in mechanical stimuli and assisting with the immune response by migrating monocytes and activating integrins. The ventricularis side of the valve shows no reaction to an increase or decrease of cyclic strain. This most likely occurs as a result of the stress states on opposing sides of the valve. The ventricularis side of the valve experiences extreme strain during the cardiac cycle as blood pushes against the surface to open the aortic valve and move into the aorta. The cells on this side of the valve are likely conditioned to strain and therefore show no response in PECAM-1 production with increased stress to the cells. On the other side of the valve the cells aren't as used to strain and consequently show an increase in PECAM-1 concentration as the cells are working to produce an immune response to this stress.

Cyclic strain had an interesting contribution to β_1-Integrin protein expression. β_1-Integrin is responsible for cell signaling and cell bonding and is often investigated for its prominent interaction with collagen. The ventricularis side of the valve appears to have no expression response to changes in cyclic strain while the fibrosa side reacts to increased strains. At a p value of $p \leq 0.05$ there is a significant increased production of β_1-Integrin when cells are strained at 20% in comparison to statically cultured cells. This p value indicated there is a 95% probability that protein expression is significantly increased. If the probability of significant expression is reduced at 90% ($p \leq 0.1$) cells at 10% strain have significant protein production in comparison to static cells. With a higher number of replicates in the study it would be likely shown that any level of cyclic strain would produce a significant increase in fibrosa ECs. Even though this result isn't as significant as the first conclusion it still represents some importance and should be

considered in evaluating results. This observance is likely due to the cells making increased cell/cell and cell/ECM attachments as the strain on the cell is increased. β_1-Integrin interacts with collagen outside of the cell to create bonding. Confocal images show us that as these cells are stretched they spread out from cell boarders in an effort to interact with collagen and help the cell/ECM network maintain its shape and mobility (Figure 3.6). β_1-Integrin works not only to help maintain cell shape and cell attachments but it also works as a communication system to transduce information from the ECM to the cell and also reveal the status of the inside of the cell to the outside. This communication allows for rapid and flexible changes in the environment, for example when the cell experiences an increase strain.

There is also an intriguing relationship between fibrosa and ventricularis at the 20% strain condition. The fibrosa responds to stretch while the ventricularis has no significant change for any condition. This difference can be attributed to what is occurring on each side of the valve during the cardiac cycle. During systole when the AV opens to expel blood out of the ventricle the fibrosa experiences very little strain in comparison to the under side of the valve, the ventricularis, that is exposed to high forces as blood rushes past. The opposite sides of the valve have likely adapted to the *in vivo* environments and the ventricularis side has developed some resistance to strain where the fibrosa side of the valve has an increased response to strain because it hasn't developed the same protective properties. This idea is supported by the results found in this study. ELISA testing produced results that showed an increase in β_1-Integrin production on the fibrosa side as strain was deviated from static where the ventricularis experienced no change in production. Here strain is having no effect on the ventricularis cells because they are accustomed to a constant strain environment.

VE-Caderin is responsible for cell/cell adhesions and cell motility. More importantly it is responsible for control of intercellular junctions and their integrity. The state of VE-Cadherin outside the cell is directly proportional to the permeability of the endothelium. Cadherin is required to maintain a restrictive endothelial barrier (53). VE-Cadherin shows a correlation to increased strain with cells isolated from the ventricularis side of the valve. In these studies, 20% strain is used to represent a pathological strain. ELISA results show that cells from the ventricularis have significantly increased VE-Cadherin molecules at 20% strain indicating an increase of cell/cell attachments in these cells. An increase in VE-Cadherin production also shows that *in vivo* Cadherins are responsible for restricting the endothelial barrier and not letting anything pass into the cell. Vestweber explains that VE-Cadherin is the major adhesive mechanism for the integrity of the endothelial cell contacts and that it needs to be locally down regulated to allow anything to pass through the barrier (53). Opposite of this behavior are fibrosa cells that show the same level of protein expression for all levels of strain. This indicates that fibrosa cells don't have as strong cell/cell attachments and are more vulnerable to cell invasion. This may be a contributor to the propensity for lesions to form there. Inflammatory molecules migrate to these cell junctions to enter the cell and a smaller VE-Cadherin population allows them to penetrate the cell. AV lesions have been found to preferentially develop of the fibrosa side of the valve (54,55). An aortic valve subject to a chronic inflammatory response may develop calcific lesions, similar to atherosclerotic plaques. Non-sclerotic post-mortem valves fail to express the adhesion molecules ICAM-1, VCAM-1 and E-Selectin, while the pro-inflammatory proteins are present on the endothelium of excised non-rheumatic generative diseased valves (56,57). This idea is supported by work of Metzler et al. that found an increased level of inflammatory

molecules in VECs with a 20% cyclic strain. This study looked at ICAM, VCAM-1 and E-Selectin in VECs under varying levels of cyclic stretch using a Flexcell stretch system like the one used in this study. They discovered statistically significant up-regulation ($p \leq 0.05$) of these inflammatory molecules at 20% when compared to 10% cultures (58).

Vinculin is a vital accessory molecule involved in force transduction and mediating cellular response. It binds intregrins to F-actin networks at the intracellular face of the plasma membrane and also mediates cytoskeletal mechanics (37). Vinculin appears to play a key role in shape control based on its ability to modulate focal adhesion structure and function (59). On the ventricularis side of the valve we observe protein production to have a statistically significant increase when cells experience stress. This supports the finding that cells respond to mechanical stress applied to ligand-bound integrins by enlarging focal adhesions (60-63). This is associated with increased recruitment of vinculin to focal adhesions (64). Evidence of this migration can be seen in B, D and F of Figure 3.8 where vinculin has increased expression and recruitment around cells. There is also evidence that vinculin recruitment has a role in strengthening cell adhesions (65). The observation of increased vinculin production with increased cell stress can also be attributed to an increase and strengthening of cell adhesions in order to help the tissue withstand these strains. When cells are tested without strain their protein production drops off because without the presence of strain they aren't stimulated to produce vinculin.

We see a different result on the fibrosa side of the valve where vinculin production doesn't have a significant change with any change of strain condition. This could be explained by the same mechanism that cells responded with integrin production. On the ventricularis side of the valve cells are conditioned to respond to high levels of

strain and protein production is regulated accordingly. On the opposite side of the valve, the fibrosa, the cells are not used to high levels of strain and therefore have not developed a protection mechanism for cell stress. Therefore, the cells on this side of the valve don't have a change in vinculin production because they haven't developed the adaptive mechanism that cells from the opposite side have.

These findings support the work of previous studies that demonstrated the importance of the demanding environment that valves exist in. They found that this environment is important in maintaining homeostasis in the AV and the absence of these forces can cause structural and phenotypical changes in the tissue (38-41). The results of this study demonstrate the need for physiological strain for cells and their adhesion molecules to function properly. Without strain these proteins don't have any stimulation for production. Especially in the case of vinculin and integrin where the need for strain is crucial. Tissue engineered valves need the presence of strain during their preconditioning treatments in order for these proteins to be expressed and for them to work within the tissue. Without strain tissue engineered constructs won't have the same cell/cell junctions as native cells. After implantation this would leave a patient more susceptible to inflammation, valve injury or failure.

It is also important to assess the side-specific differences when using AVECs for a tissue engineered heart valve. When looking at the effect of cyclic strain on these cells side specific behavior is observed for each protein studied. With PECAM-1 VECs don't experience any change in protein behavior while FECs have an increased production with an increased strain of 20%. β_1-Integrin concentrations are effected on the fibrosa side of the valve and experience an increase as strain percentages are increased. On the opposite side of the valve no change in integrin production is observed. Results from VE-Cadherin

studies showed that FECs were not affected at all by strain where VECs had an increase in VE-Cadherin concentration when strained at 20%. Finally, vinculin results indicated that the fibrosa side of the cell did not respond to strain with an increase in production. However, the opposing side of the valve experienced a significant increase in vinculin concentration with increased strain percentages. With the observance of all these opposing behaviors on different sides of the valve it makes an increasingly important argument for AVECs to be separated and treated differently when being used for seeding onto a tissue engineered construct.

A major limitation to this study was that mechanosensitive protein expression was evaluated in isolated cells. *In vivo* cells exist in a ECM formed from collagen, elastin and glycosaminoglycans; this network provides structural and mechanical support to the tissue and cells. It can also help transmit mechanical signals to the network of cells. In engineered valves a scaffold material provides this structure. The scaffold can be comprised of synthetic polymers (PGLA or PLA) or natural materials (collagen, fibrin or decellurized valves). Since the mechanical response of cells can be dependent on the ECM environment, the isolated and cultured cells may not be an accurate representation of an *in vivo* reaction. This preliminary data provides great justification for the continuing exploration into how cyclic strain can influence and model the *in vitvo* development and pre-conditioning of tissue engineered heart valves.

CHAPTER V

CONCLUSION AND FUTURE STUDIES

Conclusion

This study found that cyclic strain has an effect on the adhesion molecules of AVECs and that strain should be considered when seeding a scaffold or valve with ECs in the construction of an engineered valve. This work demonstrated that strain is needed for proper protein expression. It is very possible that strain might stimulate these proteins to expression. This supports the need for pre treating a tissue engineered construct with strain prior to implantation to allow for proper valve development. If the construct isn't exposed to strain the same cell/cell and cell/ECM junctions may not form and could leave the patient more susceptible to inflammation after implantation. This study also showed that too much strain can cause adverse effects but without strain adhesion proteins aren't properly expressed and cell/cell and cell/ECM junctions aren't allowed to properly form and function. These findings are vitally important for the progression of finding a fully successful aortic valve replacement for patients with faulty or insufficient valves. When a construct is implanted into a patient it is immediately exposed to a demanding mechanical environment so it makes logical sense that the valve would need to be pre-treated with such stresses. The quantitative results from this work provide support for this idea and give ammunition to continue studying the effects of mechanical stresses on AVECs

Future Studies

This study focused on looking at adhesion molecule expression in a population of isolated and cultured AVECs. Even though this is a sufficient starting place for initial research in a specific area it would be beneficial to study the activity of these proteins in cells that are attached to an ECM and functioning in their native environment. It would be interesting to see if cells being removed from their original 3D habitat would have an effect on these proteins. Ideally, a valve would be removed from the host and then stretched on a device and protein expression evaluated. This work showed that strain was essential for proper protein expression but how do these proteins interact with a potential substrate? A decellularized valve would like behave like the membranes used in this study. If a polymer scaffold was used would it provide the same signals for expression and support the same response? It would also be interesting to use a bigger population of samples and see if this has a significant effect on the statistical results. Determining protein concentration using another method might also provide some insight and comparison data to explore these proteins with. Finally, looking into classifying active and inactive adhesion molecules in theses cell populations could be beneficial in more validation for this study. There are several more areas that need to be investigated but this study provides a good basis and demonstrates the need for further research in this area. Extensive research in this area could help provide essential clues into the best design for the perfect tissue engineered valve.

REFERENCES

(1) Vargas HA, Hoey ETD, Gopalan D, Agrawal SKB, Screaton NJ, Gulati GS. Congenital and acquired conditions of the aortic root: multidetector computed tomography features. Postgraduate Medical Journal 2009; 85: 383-391.

(2) Schoen FJ. Evolving concepts of cardiac valve dynamics: the continuum of development, functional structure, pathobiology, and tissue engineering. Circulation 2008 Oct 28;118(18):1864-80.

(3) Doehring TC, Kahelin M, Vesely I. Mesostructures of the aortic valve. Journal of Heart Valve Disease 2005 Sep;14(5):679-86.

(4) Sacks MS, Smith DB, Hiester ED. The aortic valve microstructure: effects of transvalvular pressure J Biomed Mater Res 1998 Jul;41(1):131-41.

(5) Vesely I. The role of elastin in aortic valve mechanics. J Biomech 1998 Feb;31(2):115-23.

(6) Stephens EH, Chu CK, Grande--Allen KJ. Valve proteoglycan content and and age: Relevance to an age--specific tissue--engineered heart valve. Acta Biomater 2008 Sep;4(5):1148-60.

(7) Croft LR, Mofrad MRK. Computational Modeling of Aortic Heart Valves. Modeling in Biomechanics 2010 Jan; 221-248

(8) Brody S, Anilkumar T, Liliensiek S, Last JA, Murphy CJ, Pandit A. Characterizing nanoscale topography of the aortic heart valve basement membrane for tissue engineering heart valve scaffold design. Tissue Eng 2006 Feb;12(2):413-21.

(9) Butcher JT, Nerem RM. Valvular endothelial cells and the mechanoregulation of valvular pathology. Philos Trans R Soc Lond B Biol Sci 2007 Aug 29;362(1484):1445-57.

(10) Butcher JT, Penrod AM, Garcia AJ, Nerem RM. Unique morphology and focal adhesion development of valvular endothelial cells in static and fluid flow environments. Arterioscler Thromb Vasc Biol 2004 Aug;24(8):1429-34.

(11) Butcher JT, Simmons CA, Warnock JN. Mechanobiology of the aortic heart valve. J Heart Valve Dis 2008 Jan;17(1):62-73.

(12) O.C. Jaffee. The development of the arterial outflow tract in the chick embryo heart. Anat. Rec. 1967; 158(1): 35-42.

(13) Simmons CA, Grant GR, Manduchi E, Davies PF. Spatial heterogeneity of endothelial phenotypes correlates with side-specific vulnerability to calcification in normal porcine aortic valves. Circ Res 2005 Apr 15;96(7):792-9.

(14) Misfeld M, Sievers HH. Heart valve macro-- and microstructure. Philos Trans R Soc Lond B Biol Sci 2007 Aug 29;362(1484):1421-36.

(15) M. Thubrikar, et al. Strrsses of natural versus prosthetic aortic valve leadlets in vivo. Ann Thorac. Surg. 1980; 30(3): 230-239.

(16) Yoganathan AP, He Z, Casey JS. Fluid mechanics of heart valves. Annu Rev Biomed Eng 2004;6:331-62.

(17) Dwyer HA, Matthews PB, Azadani A, Jaussaud N, Ge L, Guy TS, et al. Computational fluid dynamics simulation of transcatheter aortic valve degeneration. Interact Cardiovasc Thorac Surg 2009 May 4.

(18) Nakamura M, Wada S, Yamaguchi T. Quantitative evaluation of intra--aortic flow disturbance by the fluid momentum index: Effect of the left ventricular systolic function on the hemodynamics in the aorta. Technol Health Care 2007;15(2):111--20.

(19) Nandy S, Tarbell JM. Measurement of wall shear stress distal to a tri--leaflet valve in a rigid model of the aortic arch with branch flows. J Biomech Eng 1988 Aug;;110(3):172--9.

(20) Rajamannan NM, Bonow RO, Rahimtoola SH. Calcific aortic stenosis: an update. Nat Clin Pract Cardiovasc Med 2007 May;4(5):254-62.

(21) Lloyd--Jones D, Adams R, Carnethon M, De SG, Ferguson TB, Flegal K, et al. Heart disease and stroke statistics----2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 2009 Jan 27;119(3):480-6.

(22) Otto CM, Lind BK, Kitzman DW, Gersh BJ, Siscovick DS. Association of aortic-- valve sclerosis with cardiovascular mortality and morbidity in the elderly. N Engl J Med 1999 Jul 15;341(3):142-7.

(23) Vesely I. Heart valve tissue engineering. Circ Res 2005 Oct 14;97(8):743-55.

(24) El Oakley R, Kleine P, Bach DS. Choice of prosthetic heart valve in today's practice. Curculation. 2008. 11, 253-256.

(25) Schoen FJ, Levy RJ. Calcification of tissue heart valve substitutes: progress toward understanding and prevention. Ann Thorac Sur 2005; 79: 1072-1080.

(26) Mitchell RN, Jonas RA, Schoen FJ. Pathology of explanted cyropreserved allograft heart valves: comparison with aortic valves from orthotropic heart transplants. J Thorac Cardiovasc Surg 1998;115:118-127.

(27) Butcher JT, et al. Aortic valve disease and treatment: The need for naturally engineered soltions. Adv Drug Deliv 2011.

(28) Zilla P, et al. Prosthetic heart valves.:catering for the few. Biomaterials 2008;29(4):285-406.

(29) Grunkemeier GL, et al. Long-term performance of heart valve prostheses. Curr Probl Cardiol. 2000;25(2):73-154.

(30) Sacks MS, Schoen FJ, Mayer JE. Bioengineering challenges for heart valve tissue engineering. Annu Biomed Eng 2009;11:289-313.

(31) Mendelson K, Schoen FL. Heart valve tissue engineering: concepts, approaches, progress and challenges. Ann Biomed Eng 2006;34:1799-1819

(32) Sacks MS, Engekmayr GC, Hildebrand D. Bioreactors for heart valve tissue engineering. In: Chaduri J, Al-Rubeai, M, eds. Bioreactors for tissue engineering. Dordrecht, Neatherlands: Springer 2005:235-267

(33) Hilbert SL, Yanagida R, Souza J, Wolfinbarger L, Jones AL, Krueger P, Stearns G, Bert A, Hopkins RA (2004) Prototype anionic detergent technique used to decellularize allograft valve conduits evaluated in the right ventricular outflow tract in sheep. J Heart Valve Dis 13:831–840

(34) Rabkin E, Schoen FJ. Cardiovascular tissue engineering. Cardiovasc Pathol 2002 Nov;11(6):305-17.

(35) Davies PF, Passerini AG, Simmons CA. Aortic valve: turning over a new leaf(let) in endothelial phenotypic heterogeneity. Arterioscler Thromb Vasc Biol 2004;24:1331-1333.

(36) Kaufman DA, Albelda SM, Sun J, Davies PF. Role of lateral cell-cell border location and extracellular/trans-membrane domains in PECAM/CD31 mechanosensation. Biochem Biophys Res Commun 2004;320:1076-1081.

(37) Albuquerque ML, Flozak AS. Wound closure in sheared endothelial cells is enhanced by modulation of vascular endothelial-cadherin expression and localization. Exp Biol Med (Maywood) 2002;227:1006-1016.

(38) Critchley, D.R., *Genetic, biochemical and structural approaches to talin function.* Biochem Soc Trans, 2005. 33(Pt 6): p. 1308-12.

(39) Balachandran K, Konduri S, Sucosky P, Jo H, Yoganathan AP. An ex vivo study of the biological properties of porcine aortic valves in response to circumferential cyclic stretch. Ann Biomed Eng 2006; 34:1655–1665

(40) Konduri S, Xing Y, Warnock JN, He Z, Yoganathan AP. Normal physiological conditions maintain the biological characteristics of porcine aortic heart valves: an ex vivo organ culture study. Ann Biomed Eng 2005;33:1158–1166

(41) Weston MW, Yoganathan AP. Biosynthetic activity in heart valve leaflets in response to in vitro flow environments. Ann Bio- med Eng 2001;29:752–763

(42) Xing Y, Warnock JN, He Z, Hilbert SL, Yoganathan AP. Cyclic pressure affects the biological properties of porcine aortic valve leaflets in a magnitude and frequency dependent manner. Ann Bio- med Eng 2004;32:1461–1470.

(43) Hoerstrup SP, Sodian R, Daebritz S, Wang J, Bacha EA, Martin DP, Moran AM, Guleserian KJ, Sperling JS, Kaushal S, Vacanti JP, Schoen FJ, Mayer JE Jr . Functional living trileaflet heart valves grown in vitro. Circulation 102 2000:III44–III49.

(44) Ku CH, Johnson PH, Batten P , Sarathchandra P , Chambers RC, Taylor PM, Yacoub MH, Chester AH. Collagen synthesis by mes- enchymal stem cells and aortic valve interstitial cells in response to mechanical stretch. Cardiovasc Res 2006; 71:548–556

(45) Awolesi MA, Sessa WC, Sumpio BE. Cyclic strain upregulates nitric oxide synthase in cultured bovine aortic endothelial cells. J Clin Invest 1995 Sep;;96(3):1449--54.

(46) Awolesi MA, Widmann MD, Sessa WC, Sumpio BE. Cyclic strain increases endothelial nitric oxide synthase activity. Surgery 1994 Aug;;116(2):439--44.

(47) Balachandran K, Konduri S, Sucosky P, Jo H and Yoganathan AP. An ex vivo study of the biological properties of porcine aortic valves in response to circumferential cyclic stretch. *Ann Biomed Eng* 34: 1655-1665, 2006.

(48) Butcher JT, Penrod AM, Garcia AJ and Nerem RM. Unique morphology and focal adhesion development of valvular endothelial cells in static and fluid flow environments. *Arterioscler Thromb Vasc Biol* 24: 1429-1434, 2004.

(49) Howard AB, Alexander RW, Nerem RM, Griendling KK, Taylor WR. Cyclic strain induces an oxidative stress in endothelial cells. Am J Physiol 1997 Feb;;272(2 Pt 1):C421--C427.

(50) Metzler SA, Pregonero CA, Butcher JT, Burgess SC and Warnock JN. Cyclic strain regulates pro-inflammatory protein expression in porcine aortic valve endothelial cells. *J Heart Valve Dis* 17: 571-577, 2008.

(51) Sung HJ, Yee A, Eskin SG, McIntire LV. Cyclic strain and motion control produce opposite oxidative responses in two human endothelial cell types. Am J Physiol Cell Physiol 2007 Jul;;293(1):C87--C94.

(52) Vande Geest JP, Di Martino ES, Vorp DA. An analysis of the complete strain field within Flexercell membranes. J Biomech 2004 Dec;37(12):1923--8.

(53) Vestweber D. VE-Cadherin: The major endhthelial adhesion molecule controlling cellular junctions and blood vessel formation. Arterioscler Thromb Vasc Biol 2008;28:223-232.

(54) Otto CM, Kuusisto J, Reichenbach DD, Gown AM, O'Brien KD. Characterization of the early lesion of 'degenerative' valvular aortic stenosis. Histological and immunohistochemical studies. Circulation 1994 Aug;90(2):844-53.

(55) O'Brien KD, Reichenbach DD, Marcovina SM, Kuusisto J, Alpers CE, Otto CM. Apolipoproteins B, (a), and E accumulate in the morphologically early lesion of 'degenerative' valvular aortic stenosis. Arterioscler Thromb Vasc Biol 1996 Apr;16(4):523-32.

(56) Ghaisas NK, Shahi CN, Foley B, Goggins M, Crean P, Kelly A, et al. Elevated levels of circulating soluble adhesion molecules in peripheral blood of patients with unstable angina. Am J Cardiol 1997 Sep 1;80(5):617-9.

(57) Ghaisas NK, Foley JB, O'Briain DS, Crean P, Kelleher D, Walsh M. Adhesion molecules in nonrheumatic aortic valve disease: endothelial expression, serum levels and effects of valve replacement. J Am Coll Cardiol 2000 Dec;36(7):2257- 62.

(58) Metzler SA, Pregonero CA, Butcher JT, Burgess SC, Warnock JN. Cyclic strain regultes pro-inflammatory protein expression in porcine aortic valve endothelial cells. J Heart Valve Dis 2008 Sep 17;17(5):571-577.

(59) Ziegler WH, Liddington RC, Critchley DR. The structure and regulation of vinculin. Trends in Cell Biolou 2006 Aug 8;16(9):453-460.

(60) Ginnone, G. et al. (2003) Talin1 is critical for force-dependent reinforcement of initial integrin-cytoskeleton bonds but not tyrosine kinase activation. J. Cell Biol. 163, 409–419

(61) Balaban, N.Q. et al. (2001) Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. Nat Cell Biol 2001;3:466–472.

(62) Delanoe-Ayari, H. et al. Membrane and acto-myosin tension promote clustering of adhesion proteins. Proc. Natl. Acad. Sci. U. S. A. 2004;101:2229–2234.

(63) Rottner,K.etal.(1999)Interplay between Rac and Rho in the control of substrate contact dynamics. Curr Biol 1999;9:640–648.

(64) Zaidel-Bar, R. et al. Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. J Cell Sci 2003;116:460-4613.

(65) Gallant,N.D.etal.(2005)Cell adhesion strengthening: contributions of adhesive area, integrin binding, and focal adhesion assembly. Mol Biol Cell 2005;16:4329–4340.

APPENDIX A

PROTOCOLS

Confocal Microscope of Collagen Membranes

1. Carefully remove Flexcell plates from the Flexercell and aspirate media.
2. Rinse 2X for 5 min in Sterile PBS.
3. Fix for 30 min in the dark in fresh 4% PFA with slight agitation at room temperature
4. Rinse for 30 min at 4°C with PBS/0.01M Glycine/0.1%Triton--X. (4° has been cited for all steps, gentle agitation is preferred on belly dancer)
5. Rinse for 15 min PBS/5%BSA.
6. Block non-specific binding for 15 min with PBS/5%NGS/5%BSA
7. Incubate overnight(12--8hr) at 4°C with gentle agitation in primary antibody solution in PBS/1%BSA.
8. Rinse for 2X 5min with PBS/1%BSA
9. Incubate 15 min PBS/5%BSA/5%NGS
10. Incubate 2hr w/ secondary antibody in PBS/1%BSA. Protect from light from here on out
11. Rinse 15min PBS/1%BSA
12. Rinse 15min PBS
13. Incubate for 30min in (1:20--1:200 has worked well, w/varied strength of staining) AlexaFluor 635 phalloidin or Rhodamine phalloidin.
14. Rinse 2X 5min in PBS
15. Incubate for 15 minutes with DAPI in PBS
16. Rinse twice in PBS

17. Cut collagen membranes from plates using a scalpel.

18. Place membrances on a microscope slide and coat with Flurogel with Tris Buffer

19. Adhere a coverslip to the slide with cleat nail polish and allow to dry

Table A.1 Reagents for Fluorescent Staining

Reagent	**Company**	**Catalog Number**
Paraformaldehyde	Electron Microscopy Sciences	157- 4
Triton-X	Sigma	234729
Glycine	Sigma	410225
Phosphate Buffered Saline	Sigma	D5652
Normal Goat Serum	Invitrogen	PCN5000
Bovine Serum Albumin	Sigma	A7906
Mouse Anti Human CD 29 (β_1-Integrin)	Millipore	MAB2247
Mouse Anti Vinculin	Millipore	MAB3574
Mouse Anti Rat PECAM-1	Millipore	MAB1393
Anti VE-Cadherin	Millipore	MAB1989
AlexaFluor 488 rabbit anti mouse IgG_1	Invitrogen	A21121
AlexaFluor 488 rabbit anti mouse IgG_2	Invitrogen	A21131
DAPI	Invitrogen	D3571
Alexafluor 635 Phalloidin	Invitrogen	A34054
Flurogel with Tris Buffer	Electron Microscopy Scineces	17985-10

Seeding Flexercell Plates

1. Aspirate media from flask.
2. Rinse with PBS.
3. Add enough Trypsin to submerge the bottom surface of the container and incubate 5 minutes
4. Add an equal amount of DMEM/ 10% FBS /%1 ABAM and place solution in a centrifuge tube. Centrifuge for 5 min at 1000 rpm and aspirate supernatant.
5. Re-suspend in enough media to allow for 500,000 cells/well.
6. Gently pipette 500 μL of solution into the center of each well.
7. Let incubate for 1 hour, then add 2.5 ml DMEM/10% FBS/%1 ABAM to each well.

Cyropreservation

Freezing

1. Remove old culture medium and rinse with PBS
2. Add Trypsin to the flask and incubate for 5 minutes
3. Add equal amount of culture medium and place in a centrifudge tube
4. Centrifuge 5 minutes at 1000 RPM
5. Aspirate media/trypsin solution
6. Resuspend pellet in DMEM/10% FBS/10% DMSO to give desired freezing concentration
7. Aliquot suspension into cryo vials
8. Place vials in Nalgene Mr. Frosty and place in -80° C freezer overnight
9. Remove vials and place in -80° C liquid nitrogen

Thawing and Recovery

1. Unthaw vial by swirling in a 37° C water bath
2. Uncap vial and pipette cell solution up and down
3. Put into a centrifudge tube
4. Add 9ml of DMEM/10% FBS/10% ABAM
5. Pipette up and Down
6. Centirfudge 1000 RPM for 3 minutes
7. Aspirate media
8. Resuspend in culture media and place in T 25 flask.
9. Change media within 24 hours

Table A.2 Reagents for Cell Culture and Cyropreservation

Reagent	Company	Catalog Number
Dulbecco's Modified Eagle's Medium	Sigma	D5796
Fetal Bovine Serum	Invitrogen	10437-028
Antibiotic/Antimycotic	Gibco	15240
Trypsin EDTA	Gibco	25300
Dimethyl Sulfide	Sigma	D2650
Freezer Vials	VWR	89094-810
T 12.5 Flasks	BD Falcon	353107
T 25 Flasks	BD Falcon	353109
T 75 Flasks	BD Falcon	353136
Swabs	Texwipe	TX761
Syringe Filters	VWR	28145-477
Syringe	BD Falcon	309654
Collagenase Type II	Gibco	17101-015
Filter Bottles	Corning	430769
PBS	Sigma	D5652
Nalgene Mr. Frosty	Sigma	C1562

General Lysate Protocol

1. Make lysis buffer according to ELISA directions or use general recipe
    - 150 mM Nacl
    - 1% NP-40
    - 50 mM Tris-Cl (pH 7.4)
    - 1μg/ml Leupeptin
    - 1μg/ml Aprotinin
2. Discard the culture medium and wash cells twice with ice- cold PBS
3. Place culture dishes on ice
4. Add 1 ml of lysis buffer
5. Incubate cells for 15 minutes on ice with occasional rocking of dishes
6. Gently scrape the cell surface with a cell lifter
7. Tilt the dish and allow the buffer to drain to one side; remove the lysate with a pipette ad transfer to a micro centrifuge tube
8. Freeze the lysate for storage or centrifuge the lysate at 2000 x g for 5 minutes and transfer to a clean tube

Table A.3 Reagents for Lysates

Reagent	Company	Catalog Number
Cell Lifter	Sigma	CLS 3008
NaCl	Sigma	S3014
NP-40 Alternative	Calbiochem	492016
Tris	Sigma	154563
PBS	Sigma	D5652
Aprotinin	Sigma	A6279
Leupeptin	Sigma	L8511