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Michael P. Nilo

EFFECTS OF CYCLIC STRAIN ON TGF-β1 ACTIVATION AND PHENOTYPE MODULATION THE AORTIC VALVE INTERSTITIAL CELLS

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A THESIS

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EFFECTS OF CYCLIC STRAIN ON TGF-β1 ACTIVATION AND PHENOTYPE MODULATION OF THE AORTIC VALVE INTERSTITIAL CELLS

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BIOMEDICAL ENGINEERING

ABSTRACT

Aortic valve interstitial cells (AVICs) are responsible for maintaining tissue structure and function within the highly dynamic mechanical environment of the aortic valve leaflets. AVICs have a phenotypic plasticity and become highly active myofibroblasts that remodel the extracellular matrix during times of development, repair, and disease. Furthermore, AVICs have demonstrated a synergistic biosynthetic response to the combination of cyclic strain and active TGF-β1; however, the response of TGF-β1 signaling to levels of cyclic strain has not been examined. Therefore, we hypothesized that mechanical strain of AVICs regulates activation of TGF-β1. To test this hypothesis, monolayer porcine AVIC cultures were exposed to either physiologic (10-15%) or pathologic (20-30%) cyclic strain (0.75 Hz) using a Flexcell® system for 6 and 24 hours. Active and total TGF-β1 were quantified using a PAI-1 luciferase assay and all other protein levels were quantified using indirect or sandwich ELISA. We report that pathologic strain increases TGF-β1 activation and αSMA expression. Pathologic strain also increased levels of proteases known to activate TGF-β1, MMP-9 and plasmin. Inhibition of these proteases eliminated strain-dependent activation of TGF-β1, and

reduced the mechano-sensitive increase in αSMA. Our findings demonstrate that TGF-β1 activation by AVIC is strain-dependent and that this activation is a potential key initiator of degenerative aortic valve disease.

DEDICATION

I would like to dedicate this thesis to my parents, Jeannie and Joseph, for supporting all of my work thus far; my brother, Nick, for reminding me what is important; and to my dog, Bock, for being a dog.

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ACRONYMS

AV	Aortic Valve
AVEC	Aortic Valve Endothelial Cell
AVIC	Aortic Valve Interstitial Cell
CICP	Collagen I C-terminal Propeptide
DAVD	Degenerative Aortic Valve Disease
ECM	Extracellular Matrix
Hsp47	Heat Shock Protein 47
ICAM	Intercellular Adhesion Molecule
LAP	Latency Associated Protein
LLC	Large Latent Complex
LTBP-1	Latent Transforming Growth Factor Binding Protein – 1
MMP	Matrix Metalloproteinases
SLC	Small Latent Complex
TGF-β1	Transforming Growth Factor β1
TIMP	Tissue Inhibitor of Metalloproteinases
TSP-1	Thrombospondin 1
VCAM	Vascular Cell Adhesion Molecule
αSMA	Alpha Smooth Muscle Actin

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1.0 INTRODUCTION

1.1 The Mammalian Heart

The mammalian heart is responsible for delivering oxygenated blood to the body. Its four chambers alternately fill to first oxygenate blood via pulmonary circulation, then deliver this oxygenated blood to the systemic circulation. This blood delivers oxygen and nutrients to the muscles and organs, after which the deoxygenated blood returns to the heart, and the cycle is repeated. In normal circulation (**Figure 1)**, deoxygenated blood from the body enters the right atrium. It then passes through the tricuspid valve, into the right ventricle to be pumped through the pulmonary valve into the pulmonary system and becomes oxygenated in the lungs. The newly oxygenated blood returns to the heart in the left atrium, where it then passes through the mitral valve into the left ventricle. From here the blood is pumped out through the aortic valve (**AV**) into the aorta, where it travels to the rest of the body. In an average human being, this cycle takes place continuously at 70 cycles per minute and pumping approximately 2,000 gallons of blood through the body each day.

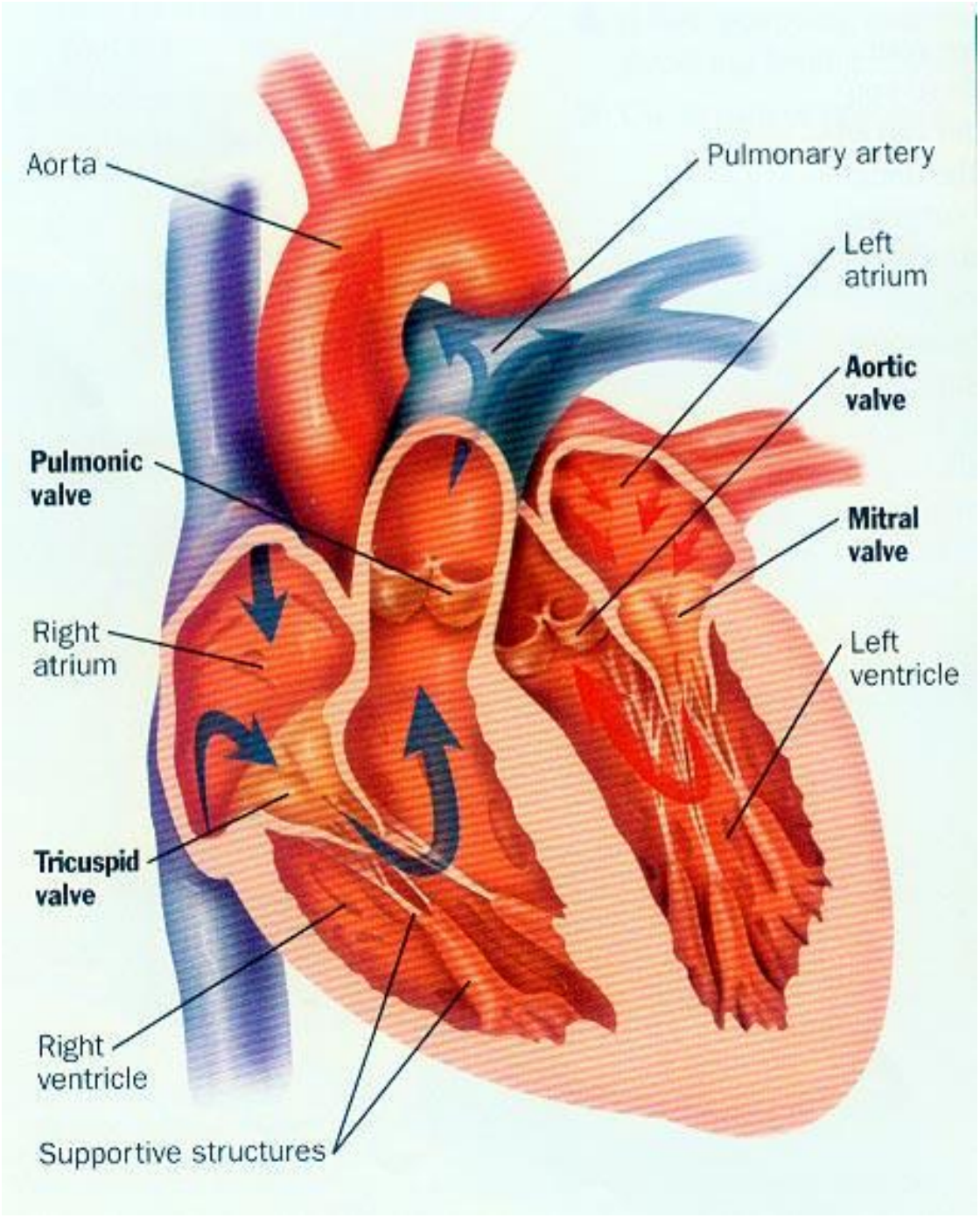

Figure 13: Anatomy and circulatory flow of the mammalian heart [faculty.ksu.edu]

1.2 The Aortic Valve

The aortic valve separates the left ventricle from the aorta, and its sole responsibility is to maintain the unidirectional blood flow from the ventricle to the body. The AV is composed of a trileaflet structure, where each leaflet is a thin, supple, membranous tissue. The valve is structured as such to passively allow blood to flow out, yet extend and coapt to prevent regurgitation into the ventricle. The leaflets are tri-layered structures composed of a network of extracellular matrix (**ECM**) layers. The ventricularis, located on the left ventricle side of the valve is composed primarily of elastin. The fibrosa, on the aortic side, is composed of collagen fibers, while the middle layer, rich in glycoaminoglycans, is called the spongiosa. The surface of the leaflets are covered by endothelial cells, known as the aortic valve epithelial cells (**AVECs**), which shield the interstitium from blood flow. Aortic valve interstitial cells (**AVICs**), the major cellular component of the AV, are spread throughout the ECM of the valve and are responsible for maintaining the structure and function of the AV ECM.

1.3 Aortic Valve Disease and Pathology

Degenerative AV disease (DAVD) is characterized by thickening and stiffening of the valve tissue, which impairs the leaflet's ability to coapt and sustain the necessary unidirectional blood flow. Loss of compliance in the leaflets leads to valve obstruction and increased systolic pressure needed to bypass the valve, as well as an inability to seal properly during diastole [1]. Furthermore, progression of the disease frequently leads to significant stenosis, fibrotic lesions and calcification of the valves (**Figure 2**), increasing the relative risk of death from cardiovascular causes 1.66-fold, comparable to diabetes or

prevalent cardiovascular disease [2]. In a recent study, it was found that patients with diseased valves have twice the number of serious cardiovascular events (death, stroke, and myocardial infarction), which is comparable to the risk associated with diabetes. After adjusting for typical cardiovascular risk factors, DAVD independently increases the cardiovascular risk by 50% [3].

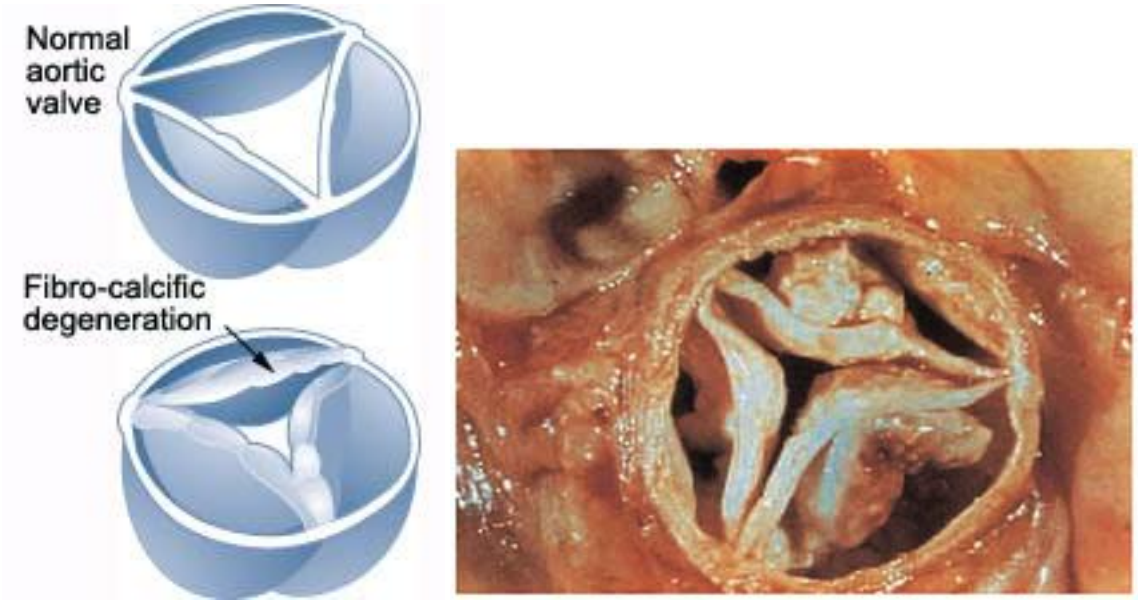

Figure 14: Healthy AV and diseased AV with significant calcification and stenosis [www.heart-valve-surgery.com]

The risk factors for DAVD are similar to the traditional risk factors for atherosclerosis and coronary artery disease: age, male gender, hypertension, diabetes, obesity, and smoking [4-6]. These similarities in risk factors, as well as the seemingly fibrotic, inflammatory response have led some to believe that DAVD is just a variation of atherosclerosis [7]. However, recent studies have shifted away from this thinking and focused on biochemical and biomechanical factors as potential effectors of AV disease [8, 9].

AV disease is a significant problem that is associated with cardiovascular morbidity and mortality. In 2003, it directly resulted in 12,471 deaths and was a contributing cause of death in 26,336 cases in the United States[10]. In fact, recent clinical evidence has shown that AV sclerosis affects over 25% of people over 65 years of age, and almost 40% of those over 75 years old [10]. Current treatments are restricted to total valve replacement. With more than 12,000 deaths per year in the U.S. alone attributed to AV disease [10] and a lack of understanding of the mechanisms leading to it, a better understanding of the pathogenesis of AV disease is needed.

1.4 Aortic Valve Biomechanics

The biomechanical properties of the AV leaflets are dictated by the blood flow that controls its opening and closing. The AV is responsible for allowing unidirectional blood flow while minimizing the resistance to flow. During systole the AV opens in response to the ventricle contraction and blood flow into the aorta. Blood rushes by the ventricular side of the leaflets, accelerating to a peak value of 1.35 +/- 0.35 m/s [11], causing shear strains that are primarily felt by the AVECs. As the ventricle relaxes and flow decelerates during diastole, the valve quickly closes to prevent retrograde flow. With each diastolic cycle the AV must support a transvalvular pressure of 80 mmHg[12].

To withstand this cyclic pressure over 3 billion times in an average lifetime, the AV has an organized fiber network. The collagen architecture responsible for withstanding the diastolic pressure imposed on the valve is aligned primarily in the circumferential direction of the leaflet [13]. Biaxial testing of valve leaflets demonstrates distinct responses in the circumferential and radial directions **(Figure 3)**. The

circumferentially aligned collagen fibers, exhibit an immediate tension response to the applied strain as the fibers are quickly straightened and pulled taut to support the load. Conversely, there is a gradual stress response in the radial direction, normal to the aligned collagen fibers. This gradual response is depicted by the long toe region where strain increases without a tension response before reaching the maximum strain. This extensibility in the radial direction is necessary for the leaflet to stretch and coapt with the other leaflets during diastole, whereas the stiffness in the circumferential direction allows the tissue to support the 80 mmHg pressure. It is estimated 10-15% strain is experienced in the circumferential direction with each cardiac cycle due to appliead diastolic pressure[14]. This mechanical response of the leaflet tissue is essential for proper function of the valve and the underlying stain mechanics are the basis for our dynamic system.

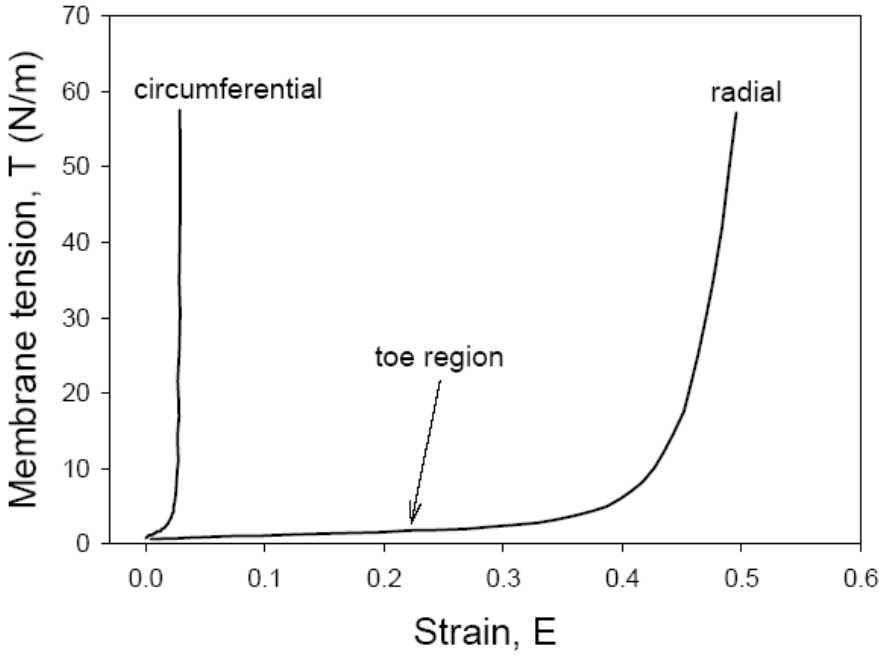

Figure 15: Membrane tension-strain response to biaxial mechanical loading of a native porcine aortic leaflet. Note distinct responses in the circumferential and radial directions. 60 N/m tension corresponds to *in vivo* diastolic pressure of 80 mmHg.

1.5 The Aortic Valve Interstitial Cells

Interstitial cells, present throughout the leaflet tissue, are responsible for the maintenance of the organized ECM network of the AV[14-16]. These AVICs are phenotypically dynamic cells that are functionally fibroblasts in their quiescent state. However, in times of development, disease and tissue remodeling, these cells become "activated" myofibroblasts, producing and secreting large amounts of tissue remodeling molecules such as transforming growth factor β1 (**TGF-β1**), matrix metalloproteinases (**MMPs**), their inhibitors (**TIMPs**), ECM proteins (e.g. type I collagen) and intracellular contractile proteins, such as α smooth muscle actin (**αSMA**) [17-22]. The complete biochemical pathways that control this phenotype are unknown; however, it has been

shown *in vitro* that addition of active TGF-β1 causes a phenotypic transformation in a dose dependent manner [21]. Recently, the phenotype of the AVIC has been identified as a key regulator and indicator of AV pathology [17, 23, 24]. Studies of myofibroblasts have shown that in healthy tissue they become apoptotic when there is no longer a need to remodel[25]; however, this process breaks down in pathological states and activated myofibroblast cells accumulate[22]. Without the elimination of myofibroblast AVICs, excessive remodeling processes may lead to the onset of AV disease.

The affect of dynamic strain on the AVIC phenotype is yet uninvestigated; however, strains described as affecting the AV leaflets have been shown to be transduced to the AVICs as well[19]. It is unclear what the precise underlying mechanism is, but AVICs are known to be aligned in the direction of the circumferential collagen. With this alignment AVICs also experience the same 10-15% cyclical strain that is imposed on the leaflets during diastole[13]. These constant repeated strains on the AVICs are likely key mechanotransductive signals that are utilized by the cells to maintain a continuous remodeling process [26], and if these strains were to increase, this could potentially contribute to DAVD due to enhanced mechanotransductive signaling.

1.6 Strain, TGF-β1, and DAVD

Conditions such as hypertension and age that correlate with increased AV disease risk may also contribute to increased strain on the AVICs. Robicsek et al. theorized that a likely initiator of DAVD is an inhibited stress transfer in the AV leaflets due to diminished compliance in the aortic wall [27]. As compliance decreases in the aortic wall, vessel dilation decreases, inhibiting stress transfer from the collagen fibers in the

circumferential direction to the highly extensible radial direction [28, 29]. A 60% decrease in aortic wall compliance after the age of 60 will likely result in increased AV strains in the circumferential direction and, concurrently, on the AVICs which are oriented and anchored to the collagen fibers [13] leading to the speculation that the AVICs in the elderly experience higher levels of strain [9]. This increased strain may result in modified cellular signaling, which could, through some unknown mechanism, lead to persistent activation of AVICs resulting in DAVD.

TGF-β1, as mentioned before, has the ability to cause convert AVICs to the diseased, myofibroblast phenotype. In a previous study, an 11 – fold increase of total TGF-β1 was observed within AV tissue exposed to active TGF-β1 and cyclic strain [8]. In this study, markers for AVIC myofibroblast phenotype (αSMA), collagen biosynthesis (HSP47 and CICP), and total TGF-β1 were examined after 14 days of isolated or combined treatment of active TGF-β1 and cyclic stretch. Results showed a significant increase of all markers exposed to the combined treatment (**Figure 4).**

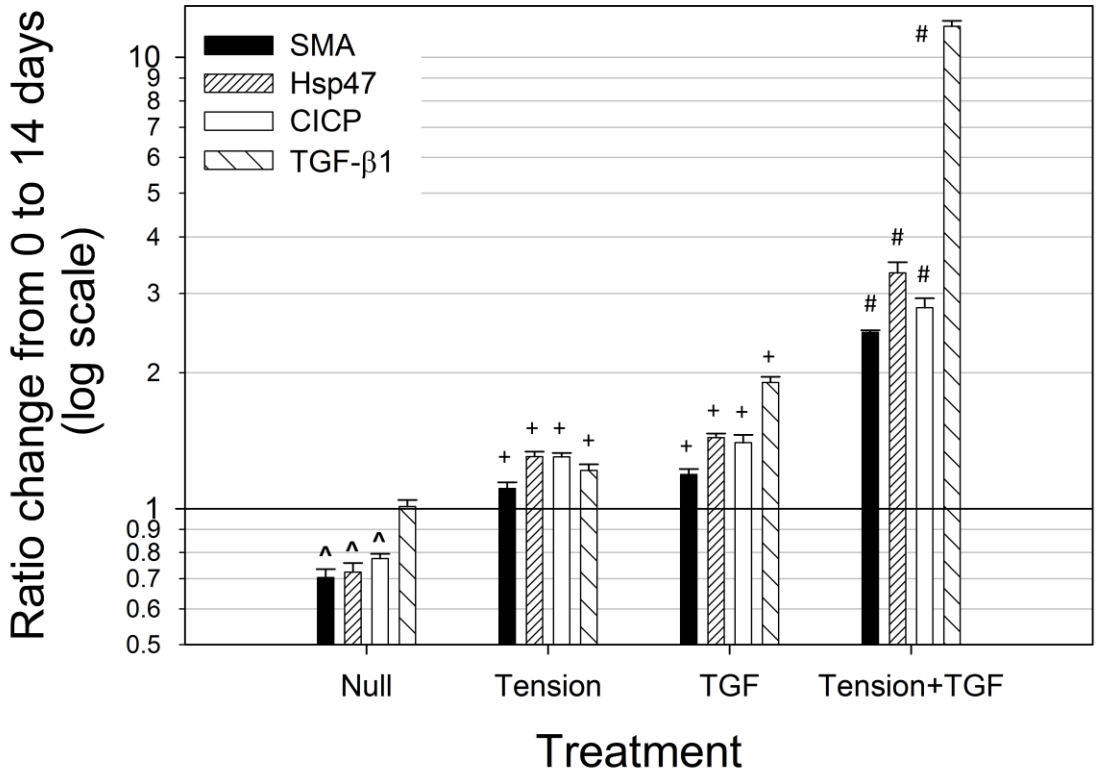

Figure 16: Porcine AV leaflets exposed to treatments of tension and/or active TGF-β1 showed marked increases in markers for myofibroblast phenotype, collagen I synthesis and total TGF-β1. Note log-scale and 11-fold increase in total TGF-β1 when both leaflets are exposed to tension and TGF-β1[8].

1.7 Transforming Growth Factor β-1

TGF-β1 is a ubiquitous growth factor that is responsible for regulating cellular proliferation and differentiation, embryonic development, wound healing, apoptosis and angiogenesis [30]. In the AVICs, it is secreted as part of a large latent complex (**LLC**), which consists of latency associated protein (**LAP**), latent TGF-β binding protein 1 (**LTBP-1**), and mature TGF-β1. The LAP and TGF-β1 are non-covalently bound to form the small latent complex (**SLC**). The LAP is then covalently linked by disulfide bonds to LTBP-1. The LLC allows for correct secretion, folding, and storage of TGF-β1 in the

ECM. Activation of TGF-β1 requires its dissociation from the LAP in the ECM. Activation can be the result of changes in ionic strength, pH changes, heat, various soluble factors, ECM composition, integrins-mediated factors and possibly mechanical factors [30-33]. Once activated, TGF-β1 binds to the cell via three high-affinity receptors. TGF-β1 first binds to TβRII or TβRIII which then recruits the other as well as TβRI [30]. In the canonical signaling pathway, this binding leads to phosphorylation of signaling proteins Smad2 and Smad3. Smad2/3 is joined by Smad4 and these proteins then translocate to the nucleus, which leads to gene transcription and the associated cellular effects (**Figure 5**). Since active TGF-β1 and cellular strain has been shown to cause a phenotypic change in AVICs, it is possible that strain accounts for the activation of TGF-β1 and increased TGF-β1 signaling.

As mentioned, TGF-β1 can be activated via several different mechanisms, but only a few of these have been extensively studied. Plasmin is a serine protease that is generated by the proteolysis of plasminogen. It was the first protease to have documented TGF-β activating capacity, and it does so by proteolytic cleavage of the LAP [34]. Plasmin may cooperate with thrombospondin-1 (**TSP-1**) to activate TGF-β1 [35]; however TSP-1 has been shown to be able to work independently of plasmin [36]. TSP-1 is an ECM protein that binds and potentially induces a conformational change in the LAP. This disrupts the electrostatic interactions between the LAP and active TGF-β1 which, in turn, allows active TGF-β1 to be released or to come into contact with its receptors on the cell [36]. TSP-1 is present in several areas of the body, including the heart. In healthy cells, TGF-β1 signaling increases the production of plasminogen activation inhibitor – 1 **(PAI-1)**,

which inhibits the production of plasmin, but this schematic may be hindered in pathologic conditions.

Another known activator is matrix metalloproteinase-9 (**MMP-9**). MMP-9 is a matrix degrading enzyme that has been shown to activate TGF-β as well as be over expressed in diseased heart valves [37-39]. More importantly, MMP-9 has been shown to be up-regulated in AV tissue exposed to elevated cyclic stretch[40]. It localizes to a cell and, once there, it digests the LAP and releases the active TGF-β1. However, its activation ability is CD44 dependent, and it preferentially cleaves TGF-β2 and TGF-β3[39]. Also, mice with mutated MMP-9 production did not depict any TGF-β1 deficient characteristics [30]. Because MMP-9 can activate TGF-β through proteolytic degradation of the latent TGF beta complex, α_v containing integrins activate TGF-β1 by creating a close connection between the latent TGF-β complex and MMPs. Integrins $\alpha_v\beta_6$ and $\alpha_V\beta_3$ are suggested to bind the latent TGF-β1 complex and proteinases, simultaneously inducing conformation changes of the LAP and sequestering proteases in close proximity of the cell surface[41]. Despite the fact $\alpha_v\beta_6$ is an integrin associated only with epithelial cells, the α_v family of integrins may be responsible for activation in a similar manner within the AVICs.

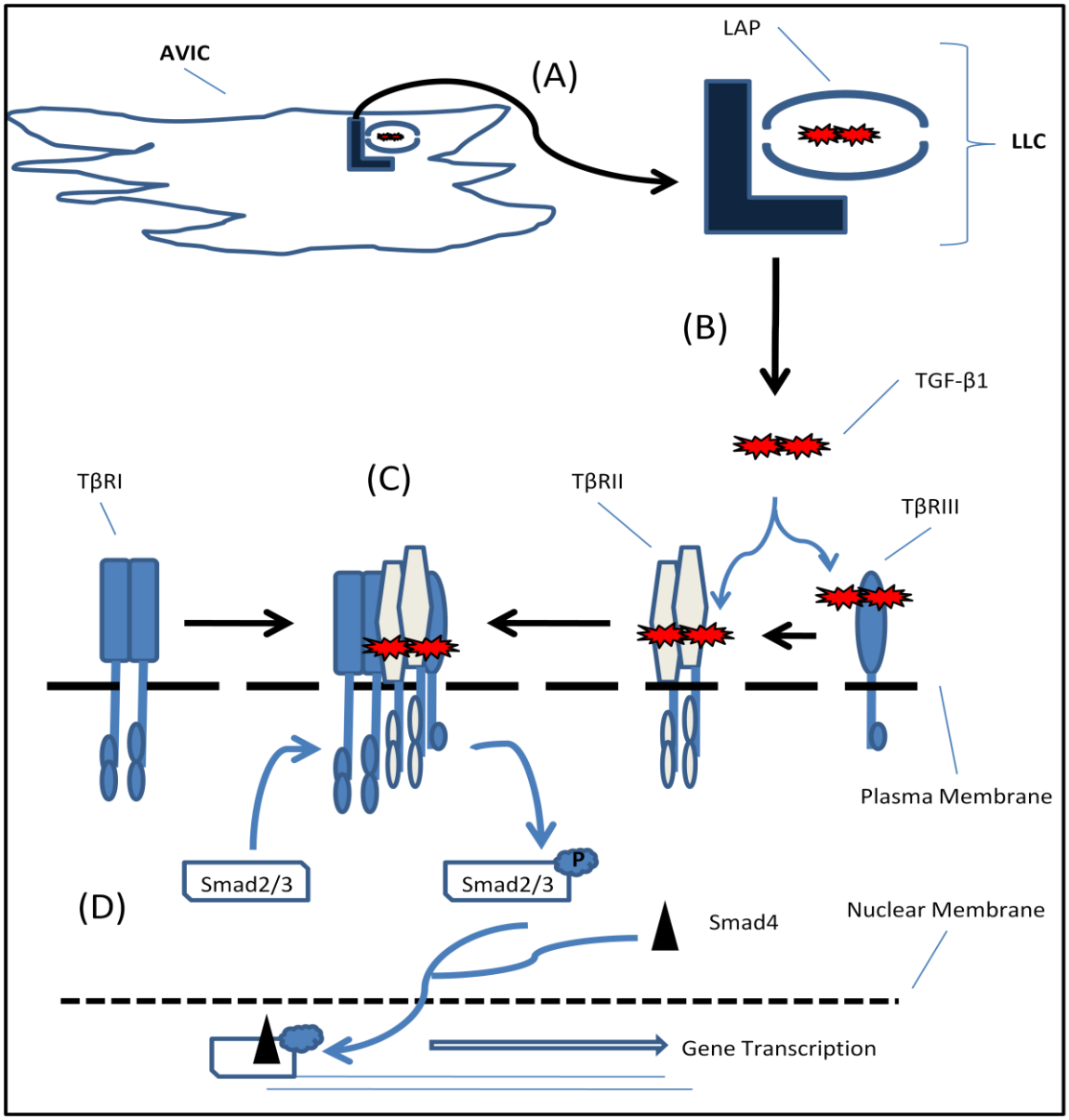

Figure 17: Mechanism of TGF-β1 synthesis, activation and signaling – **(A)** TGF-β1 is secreted as a large latent complex (LLC). This complex consists of a homodimer complex containing mature TGF-β1 non-covalently bound to its proprotein, also known as latency-associated peptide (LAP). This complex, known as the small latent complex (SLC), is then covalently linked to latent TGF-β1 binding protein. The complex is secreted as a whole and stored in the ECM. **(B)** Activation occurs when the mature TGF-β1 homodimer is released from the LTBP and LAP. This can occur via changes in pH, heat, one of several known molecular activators, integrin $\alpha_v\beta_3$, or another unknown mechanism. **(C)** Once activated TGF-β1 binds to TβRII or TβRIII which recruits the other as well as TβRI. **(D)** This activated receptor complex recruits and phosphorylates Smad2 or 3. Smad2/3 is joined by Smad4 and the complex enters the nucleus leading to gene transcription. The cellular effects of this transcription include increased TGF-β1 production and an activation of AVIC phenotype.

1.8 Dynamic Strain of Monolayer Cell Cultures

Growth and adaptation in all cells and organ systems of the human body respond to biomechanical stimuli [42-45]. As a result, alterations in the mechanical environment can also influence the progression of disease, healing, and remodeling. In fact, a recent study concentrated on the effect of cyclic mechanical strain on AVECs [46], showed that pro-inflammatory molecules, including intercellular adhesion molecules (**ICAM**), vascular cell adhesion molecules (**VCAM**) and endothelial leukocyte adhesion molecules (E-selectin) were up-regulated when monolayer cultures were exposed to strain levels (20%) above a physiologic (10%) level. This result shows the impact cellular strain has on the AV. Because the AV tissue is actually maintained by the AVICs, we believe their response to levels of cyclic strain will provide important information about the mechanobiology of the AV.

1.9 Overview and Hypothesis

The AV is a dynamic environment, continuously exposed to large cyclic strains, wherein an organized ECM network, populated by AVICs constantly remodels and repairs itself to maintain physiologic function. These strains are transduced from the ECM of the leaflet to the cells. In response to these strains, AVICs maintain this structural integrity through protein synthesis and enzymatic degradation. The AVICs are phenotypically dynamic cells that shift to a myofibroblast phenotype during times of disease, development and repair. If left in this state AVICs will likely over-synthesize proteins and possibly begin to form calcific nodules. The inflammatory cytokine, TGF-β1, has been shown to initiate and perpetuate this change in monolayer cultures of

AVICs[21]. Previous studies have connected cyclic strain with increased TGF-β1 synthesis in the AV[8]. It also has been suggested that the AVICs are exposed to increased strains at the onset of DAVD due to the risk factors of hypertension and age. Currently, there lacks a connection between the increased cyclic strain, present at the onset of disease, TGF-β1 signaling and the phenotype of AVICs. In an attempt to make this link, we hypothesize that strain above physiologic levels of 10-15% will lead to an increase in TGF-β1 activation as well as an increase in the myofibroblast phenotype of AVICs. By making this connection we aim to elucidate a likely contributor to the pathologenesis of DAVD and its connection to both biophysical and biochemical factors.

1.10 Specific Aims

As average life spans increase throughout the world, valvular disease will become more significant and thus understanding the etiology of the disease more important [4]. By examining the biochemical properties of AVICs in a complex, dynamic environment, set to mimic physiologic and pathologic strains they encounter *in vivo,* we propose to examine the effect of increased cyclic strain on the activation of TGF-β1. We want to investigate the cytokine signaling and protein release that results from increased strain in hopes of finding a target for treatment of AV disease. TGF-β1 is a prime target because of its known activation of AVICs and presence in other fibrotic diseases. Identifying early markers of the disease and understanding their mechanisms could result in the development of preventative or therapeutic treatments.

- Specific Aim 1: *Demonstrate a mechano-dependent link between increased AVIC strain and increased TGF-β1 synthesis and activation.* By examining physiologic, and pathologic strains on monolayer cultures of AVICs, we aim to demonstrate the relationship between AVIC strain and TGF-β1 activity.
- Specific Aim 2: *Demonstrate that increased AVIC strain leads to increased TGF-β1 signaling and an activated AVIC phenotype.* Using the AVIC monolayer cultures from specific aim 1, we will determine if the increased extracellular TGF-β1 activity leads to escalated levels of canonical intracellular TGF-β1 signaling and a shift of the AVICs to the myofibroblast phenotype. This will show a causal relationship between increased AVIC strain and AVIC activation.
- Specific Aim 3: *Determine potential mechanisms by which increased strain leads to activation of TGF-β1.* By quantifying and strategically blocking known activators of TGF-β1, we aim to determine if the activation is strictly mechano-dependent, or if increased strain leads to a rise in activator molecule synthesis.

With our background knowledge of AV mechanobiology and the possible connection to TGF-β1, we set forth with experiments to attain our goals that are outlined in the specific aims. In the following chapters, the experimental design, materials, methods and results will be detailed. Further, these results were used to draw conclusions, make suggestions and discuss experimental limitations, all of which are written in the later chapters of this thesis.

2.0 MATERIALS AND METHODS

2.1 AVIC Isolation and Culture

Porcine aortic valves were obtained from a local abattoir (Thompson's Meat Processing, Alexandria, AL), excised onsite immediately after sacrifice, and processed as described previously [47]. Briefly, leaflets were dissected from the aorta, washed in PBS, scraped to lyse valve endothelial cells, and minced. The tissue was digested in DMEM (Invitrogen, Carlsbad, CA) with collagenase I (2 mg/ml, Sigma, St. Louis, MO) for 30 minutes to liberate the AVICs from the tissue. AVICs were cultured on T-75 tissue culture treated flasks in complete media containing 10% FBS (Atlanta Biologicals, Atlanta, GA), 1% penicillin/streptomycin (Sigma), 1% amphotericin B (Sigma) with glutamine in DMEM and grown to 70 - 90% confluence. Previous studies have shown that AVICs retain phenotypic integrity up to 10 passages after initial harvest [48]; therefore, we used cells at passages 5-8.

2.2 Flexcell® System

In order to simulate the cyclic strain experienced by AVICs *in vitro,* we employed a Flexcell® FX-5000 Tension system. The Flexcell® Tension system is a computer-regulated bioreactor that uses vacuum pressure to apply cyclic or static strain to cells cultured on flexible-bottomed culture plates. A vacuum pump with a reservoir tank provides the strain on the culture plates that are allowed to remain in the incubator. The

pump is monitored by the Flexlink control box, which is set using provided software (Figure 6). The software allows us to program procedures to strain monolayer cultures of AVICs at specific frequencies and waveforms. The flexible-bottom culture plates allow us to strain cells within the plate in a gradient nature, where the maximum strain occurs at the edges of the plate and lowers to 0% strain at the center of the well. Maximum strains are recorded throughout this thesis. The system was limited to 0.75 Hz at the pathologic strain, therefore the frequency was set there for all strains.

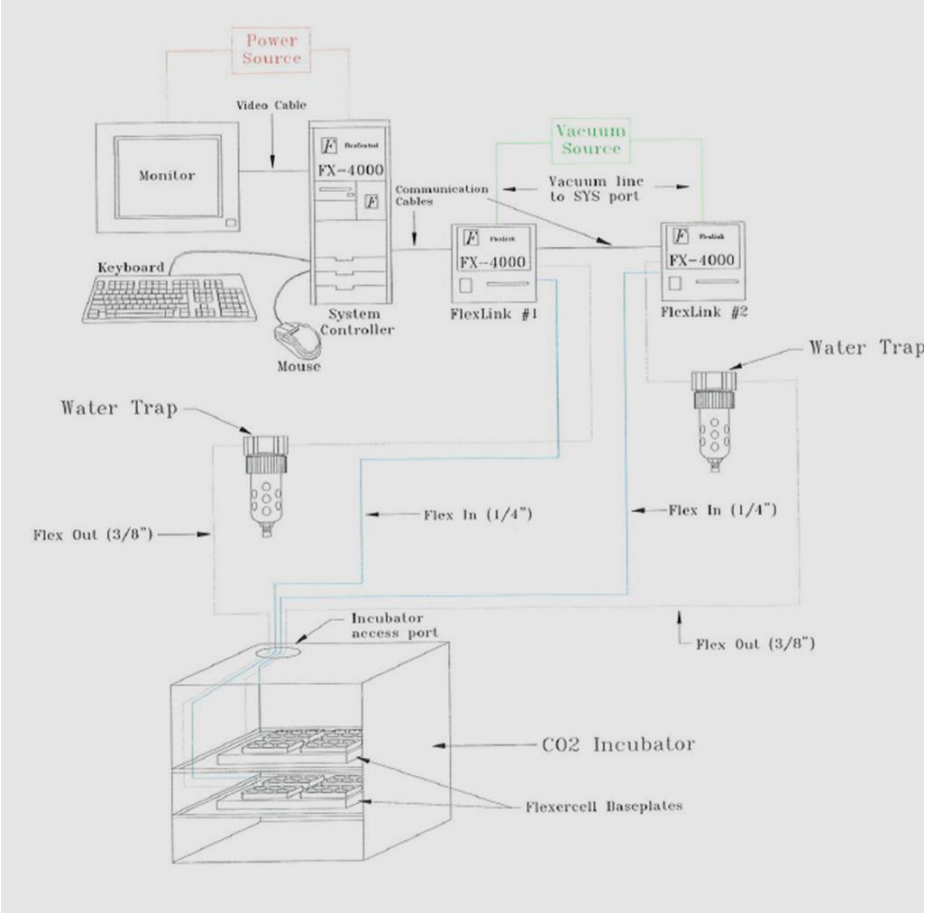

Figure 18: The Flexcell® FX-5000 Tension system set-up. The vacuum source is monitored by the system controller. The controller can be programmed for specific protocols using the provided software. The vacuum source pulls on the flexible bottom culture plates. The outline from the culture plates passes through a water trap that prevents humidity from the incubator from reaching the Flexlink control box. While this diagram depicts two separate baseplates and FlexLink controls, in our experiments, only one system was utilized.

2.3 Mechanical Strain and TGF-β1 treatment of AVICs

AVICs were seeded onto collagen-I coated BioFlex® culture plates at 8 x 10^5 cells per 35mm well for 24 h in complete media. Media was then removed and serum free medium was added to each well with either active recombinant human TGF-β1 (+ active TGF-β1; 0.5 ng/ml, R&D Systems, Minneapolis), latent TGF-β1 (+ latent TGF-β1; 1.7 ng/ml, R&D), or neither (NT). Serum free media was used to remove any growth factors present in the FBS. Treatments were chosen based on previous studies using active TGF-β1 [8], with latent TGF-β1 at the molar equivalent assuming that all latent TGF-β1 was active. AVICs were then exposed to cyclic strains (0.75 Hz) at either physiologic (10-15%) or pathologic (20-30%) levels for periods of 6 and 24 h. After treatment, conditioned medium was removed, snap-frozen, and stored at -80^{o}C. AVICs were either lysed using a mammalian cell lysis buffer (Promega, Madison, WI) for protein quantification or fixed in formalin for 19mmunoflourescence imaging.

2.4 TGF- β1 Quantification Assay

Active and total TGF-β1 were determined with a PAI-I promoter firefly luciferase assay as described previously [49]. Briefly, transfected mink lung epithelial cells **(TMLCs)** with the expression construct p800neoLUC containing a truncated PAI-1 promoter fused to the firefly luciferase reporter gene, kindly provided by Dr. Daniel Rifkin, were grown on 75-cm culture dishes in DMEM containing 10% FBS, 1% GlutaMAX-1 100x (Invitrogen), and 0.4% G418 Sulfate (MP Biomedicals, Solon, OH) to confluence. TMLCs were then transferred to a 24-well plate at 3x10^5 cells/well and incubated for 4 h to allow attachment. Wells were filled with conditioned medium from the strained cells to

quantify active TGF-β1. Conditioned medium was also heated (100ºC for 3 min) to activate latent TGF-β1 in order to quantify total TGF-β1. Reporter cells were incubated with conditioned media for 17 hours, after which cell lysates were assayed in triplicate and luciferase activity was quantified using a luminometer (SynergyHT, BioTek, Winooski, VT) **(Figure 7)**.

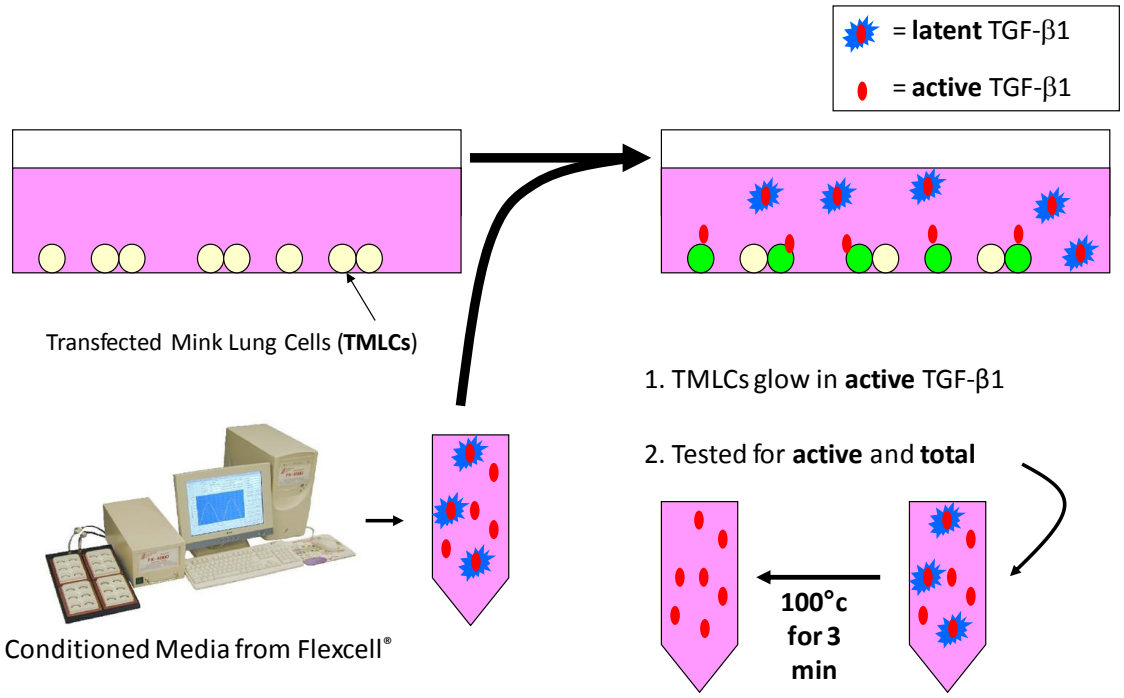

Figure 19: PAI-1 luciferase assay allows quantification of active and total TGF-β1.

2.5 Sandwich ELISA for αSMA

Samples were assayed for total protein using a standard Bradford Assay. Sandwich ELISA techniques were used to detect αSMA as described previously[50]. Polyclonal αSMA antibody (1:200, Abcam, Cambridge, MA) in carbonate/bicarbonate buffer (pH 9.6) was adsorbed onto the bottom of 96 well plates overnight at 4°C. Non-specific binding sites were blocked using 1% bovine serum albumin (BSA) in PBS. Cell

lysates were then diluted to a final protein concentration of 50 μg/ml in PBS and 100 μl of cell lysate solution was added to each well. Monoclonal αSMA antibody (Sigma, 1:200) and secondary antibody linked to a peroxidase enzyme (1:1000, Jackson, West Grove, PA) were added, followed by 100 μl of detection substrate. αSMA content was recorded in relative light units.

2.6 Indirect ELISA for Smad2/3

Smad2/3 and pSmad2/3 were quantified by indirect ELISA. Cell lysates were diluted to a final protein concentration of 20 μg/ml in a carbonate/bicarbonate buffer (pH 9.6). Antigen solutions were added to 96 well plates and incubated at 4^{o}C overnight to allow the proteins to adsorb to the plate surface. From this point onward, the indirect protocol follows the direct ELISA protocol detailed above. Smad2/3 and pSmad2/3 antibodies (Santa Cruz) were used at a 1:100 dilution. Luminescence was recorded in relative light units.

2.7 Immunofluorescence Microscopy

AVICs were fixed and permeabilized with Triton-X in a 3.7% formaldehyde, then blocked with 1% BSA in PBS. Monoclonal αSMA antibody (Sigma) and FITC-conjugated secondary antibody (Abcam) were added to the samples. Nuclei were stained with UV-excitable DNA stain, Hoechst 33342.

2.8 Indirect ELISA Soluble Protein Quantification

ED-A fibronectin (Santa Cruz), MMP-9 (Abcam), and Plasmin (Abcam) were each quantified from conditioned media of 6 h samples by indirect ELISA using specific primary antibodies at a 1:1000 dilution. HRP-conjugated antibodies were added (1:5000) and the previously described detection substrate was used.

2.9 Inhibition of Known TGF-β1 Activators

As above, AVICs were strained for 6 h at physiologic and pathologic levels after being treated with inhibitors for known molecular proteolytic activators of TGF-β1: 0.86 μg/ml Plasmin-inhibitor [51] (Recombinant Human Serpin E1/ PAI-1; R&D systems) and 5ng/ml MMP inhibitor (ONO 4817; Tocris). Media and cell lysates were assayed as described above.

2.10 Statistical Analysis

Due to animal variability, data is presented as fold change from the physiologic strain at 6 hours, which was designated as the control condition as it represented the most physiologic state at the earliest tested time point. Statistical significance was determined using a Holm-Sidak multiple comparison test followed by a post-hoc 2-tail t-test assuming unequal variances. Data is presented as mean ± SEM. Significant differences are represented by + for $p<0.05$ and ++ for $p<0.01$.

3.0 RESULTS

3.1 Pathologic Strain Increases TGF-β1 Activation and Synthesis

We hypothesized that pathologic strain applied to isolated AVICs would induce increased levels of TGF-β1 activation. To determine the effect of strain on AVICs alone or in the presence of exogenous latent or active TGF-β1, monolayer cultures of AVICs from 6 pigs (n=6) were exposed to either physiologic (10-15%) or pathologic (20%-30%) for 6 or 24 hours. Results for each cell line were normalized to the physiologic strain value at 6 h for each pig, with fold change reported for each treatment. At 6 h, there were significant increases (**Figure 8A**; $p<0.01$) up to 113%, in active TGF-β1 in groups subject to pathologic strain, whereas total TGF-β1 levels did not differ with strain or TGF-β1 treatment. At 24 h, similar trends were seen for active TGF-β1 levels but we also observed a strong correlation between pathologic strain and increases in total TGF-β1 (**Figure 8B**).

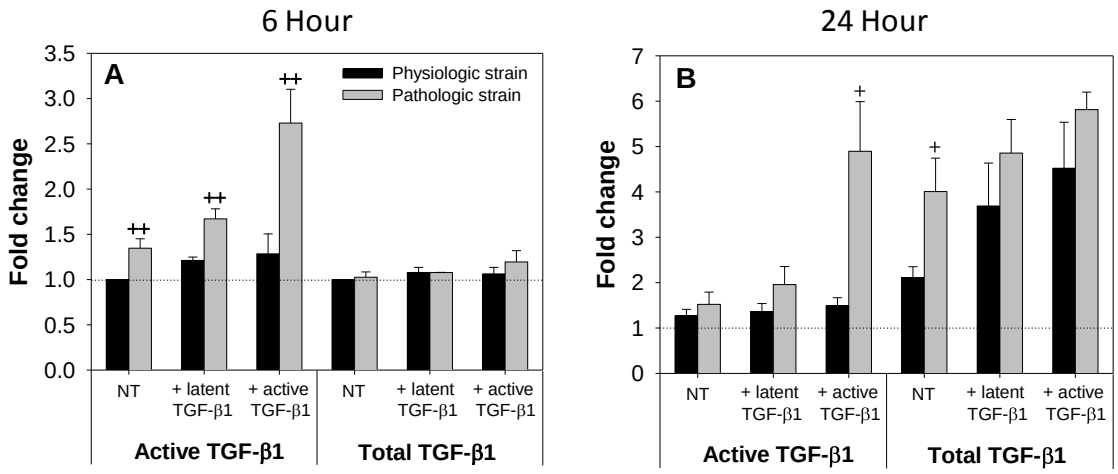

Figure 20: Pathologic strain increases active and total TGF-β1. (A) At 6 h, TGF-β1 is activated under pathologic strain for all treatments, but total TGF-β1 was not increased. (B) At 24 h, there is further increase in active TGF-β1 with an increase total TGF-β1, suggesting feed-forward TGF-β1 synthesis. NT=no TGF-β1 addition; +latent TGF-β1=1.7ng/ml latent TGF-β1; +active TGF-β1=0.5ng/ml active TGF-β1; +=$p<0.05$; ++=$p<0.01$. Note scale difference between **A** and **B**.

3.2 AVIC Phenotypic Switch is Induced by Pathologic Strain

AVICs exposed to pathologic strain exhibited a two-fold increase in αSMA expression at 6 h (**Figure 9A**; $p<0.05$). AVICs treated with latent and active TGF-β1 also had increases in αSMA expression (not significant) when exposed to pathologic strain. At 24 h, there are significant strain-dependent αSMA increases in the AVICs that received no treatment and those that received active TGF-β1 (**Figure 9B**; $p<0.01$). Under either strain level, samples given active TGF-β1 exhibited large increases in αSMA levels when compared to untreated samples, further corroborating active TGF-β1's ability to induce the myofibroblast state. Regardless of treatment or strain level, αSMA content increased from 6 to 24 h for each respective treatment. To qualitatively verify ELISA results, immunofluorescence microscopy was performed. At 6 h, AVICs exposed to pathological strain (**Figure 10C**) and pathologic strain + active TGF-β1 (**Figure 10D**) exhibited

greater amounts of αSMA than the corresponding treatments exposed to physiological strain (**Figure 10A and B**).

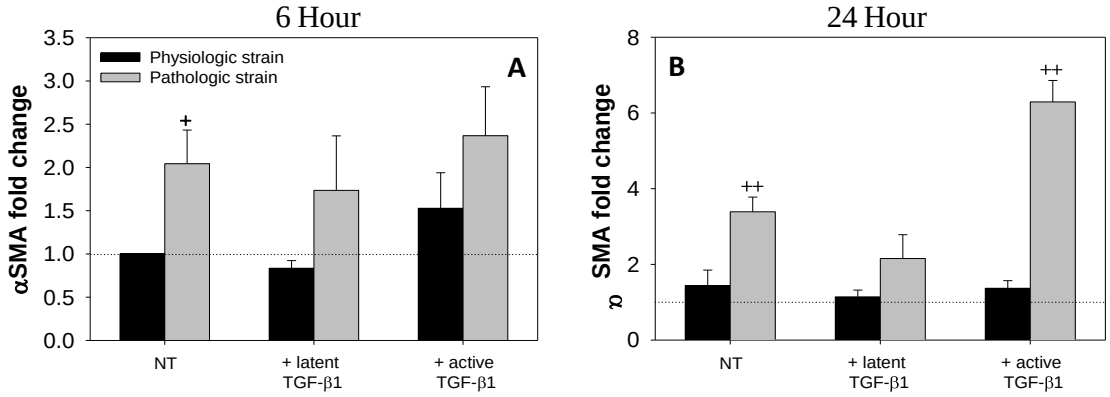

Figure 21: αSMA increases with pathologic strain. **(A)** αSMA content is significantly increased ($p < 0.05$) at 6 h due to pathologic strain in the NT group. Groups receiving TGF-β1 treatment also had αSMA that trend up with pathologic strain. **(B)** Significant increases in αSMA content were seen at 24 hours in NT and +active TGF-β1 ($p < 0.01$).

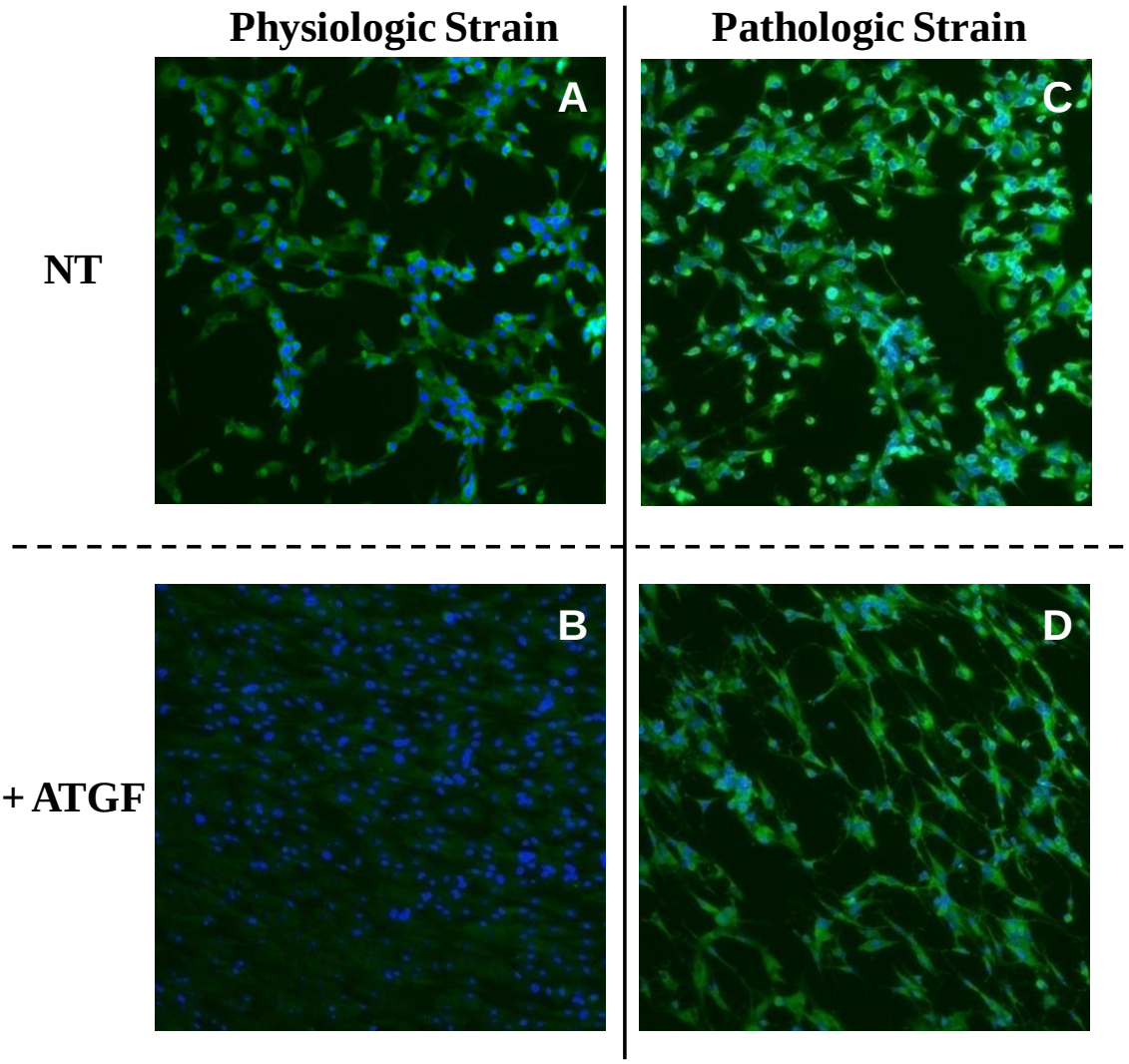

Figure 22: (A and B) AVICs under physiological strain for 6 h receiving NT or active TGF-β1, respectively. **(C and D)** AVICs under pathologic strain for 6 h receiving NT or active TGF-β1, respectively. Qualitatively, pathologically strained samples express higher levels of αSMA (shown in green). Cell nuclei stained blue.

3.3 Intracellular TGF-β1 Signaling Under Pathological Strain

Intracellular TGF-β1 signaling along the canonical pathway was quantified using indirect ELISAs. Smad proteins are phosphorylated at the intracellular portion of an activated TGF-β1 receptor complex. In static cultures of cardiac fibroblasts, levels of Smad remain relatively constant; however, levels of pSmad increase with increasing

levels of active TGF-β1 [52]. Thus, we present our data as the ratio of pSmad to Smad in order to represent intracellular TGF-β1 signaling along the canonical pathway. At both 6 and 24 h, there were increasing trends in this ratio (up to 38%) with increased strain and added TGF-β1; however, none of these groups were significantly different (**Figure 11A-B**). When quantifying pSmad data alone, we did not observe any significant differences between any groups. To verify our ELISA results, AVICs were seeded on tissue culture plastic and treated with increasing dosages of active TGF-β1. From these, we observed a corresponding increase in pSmad/Smad ratio as expected (**Figure 12**).

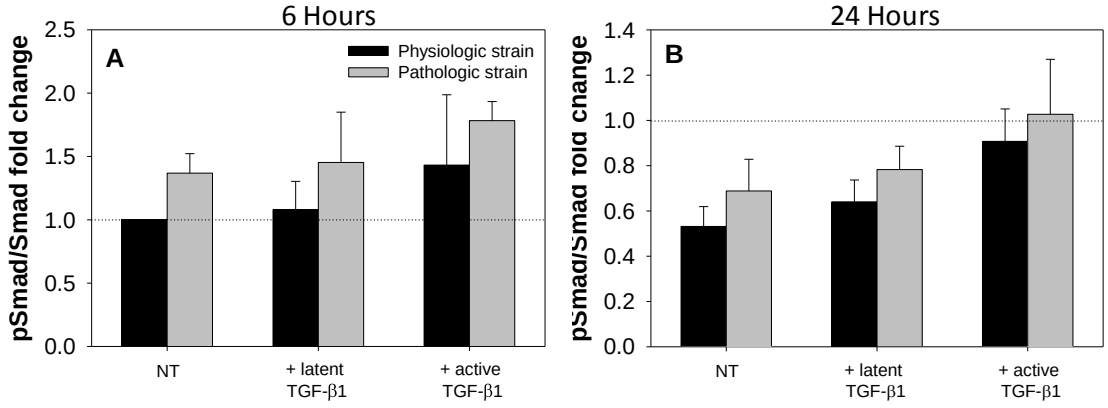

Figure 23: Changes in canonical Smad signaling due to pathologic strain is insignificant at both 6 and 24h. Matched pair ratios of pSmad to Smad fold changes from ELISA data show increases, but not significant, in phosphorylation at 6 h **(A)** and 24 h **(B).**

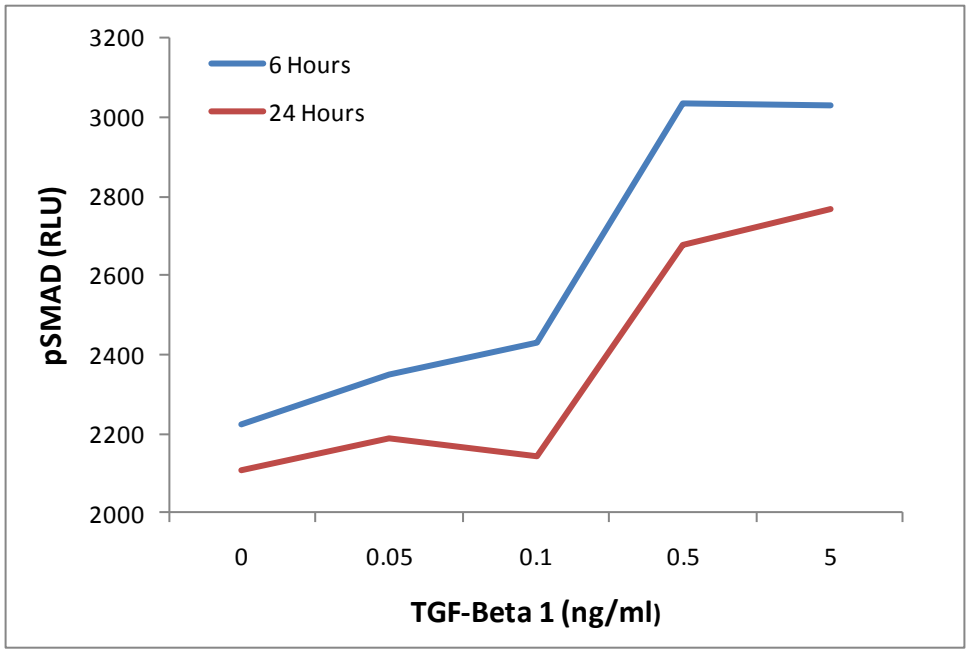

Figure 24: AVICs cultured on tissue culture plastic were given increasing doses of TGF-β1. After 6 or 24 h cells were lysed and pSmad2/3 concentrations were quantified using ELISA. As expected, increasing TGF-β1 dosages yielded larger pSmad quantities. Results are given in relative light units.

3.4 Pathologic Strain of AVICs Increases Extracellular Protein Concentration

Select secreted proteins in the conditioned medium were quantified from physiologic and pathologic strained, NT samples at 6h to elucidate the mechanism underlying strain-dependent TGF-β1 activation. Specifically, known biochemical activators of latent TGF-β1, MMP-9 and plasmin, and the ECM component ED-A FN, which is known to be increased in the presence of active TGF-β1, were quantified [53]. MMP-9 is known to activate TGF-β1 as well as be over expressed in stenotic aortic heart valves [37, 38, 40, 54, 55]. Plasmin proteolytically cleaves the LAP and releases the active molecule to bind to the cell. We observed a significant increase in MMP-9 and ED-A FN

expression due to pathologic strain (**Figure 13**; $p<0.05$). The effect of pathologic strain on known activators of TGF-β1 was also quantified. Plasmin expression was not significantly increased with pathologic strain, however still increased by 20%.

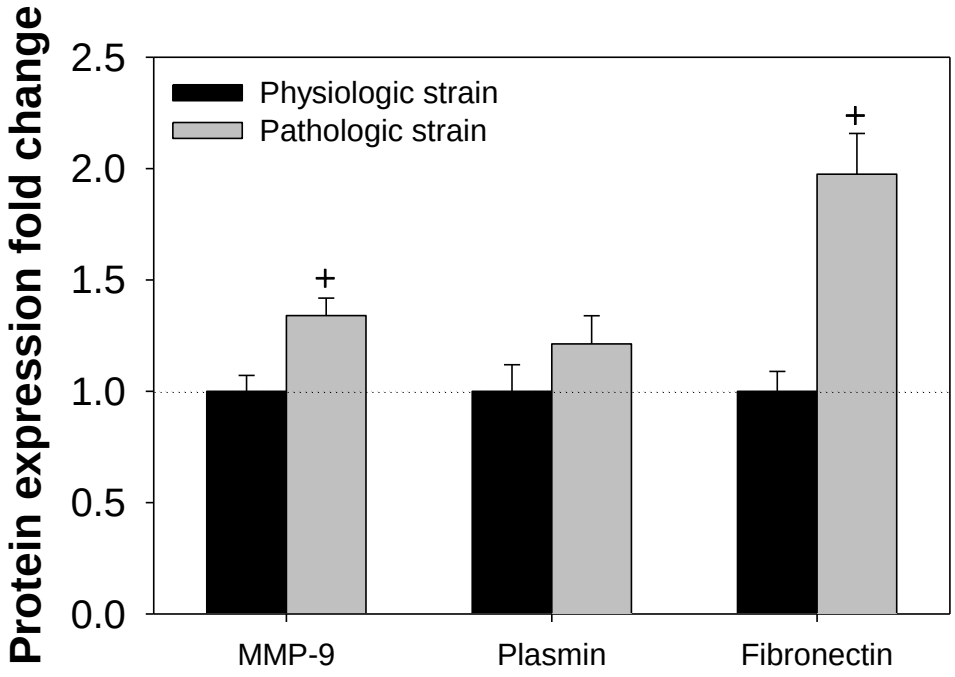

Figure 13: At 6h pathologic strain of the AVICs results in significant increases in bboth MMP-9 and ED-A FN ($p<0.05$).

3.5 Inhibition of Known TGF-β1 Activators Eliminates Mechano-dependent TGF-β1 Activation, but does not Eliminate Strain-induced Phenotypic Change in AVICs

To determine the potential contributions of MMP-9 and plasmin to the activation of latent TGF-β1 under pathologic strain, each was inhibited individually and active TGF-β1 and αSMA were quantified. We found that all mechano-dependent activation of TGF-β1 at pathologic strain was abolished in the presence of either MMP-9 or plasmin inhibitors (**Figure 14A;** $p<0.01$) when compared to the no treatment, pathologic strained samples. Interestingly, αSMA was minimally reduced in the presence of the inhibitors, but still demonstrated a strain-dependent response, suggesting that a portion of the AVIC phenotypic change may be caused by pathologic strain but independent of TGF-β1 (**Figure 14B**).

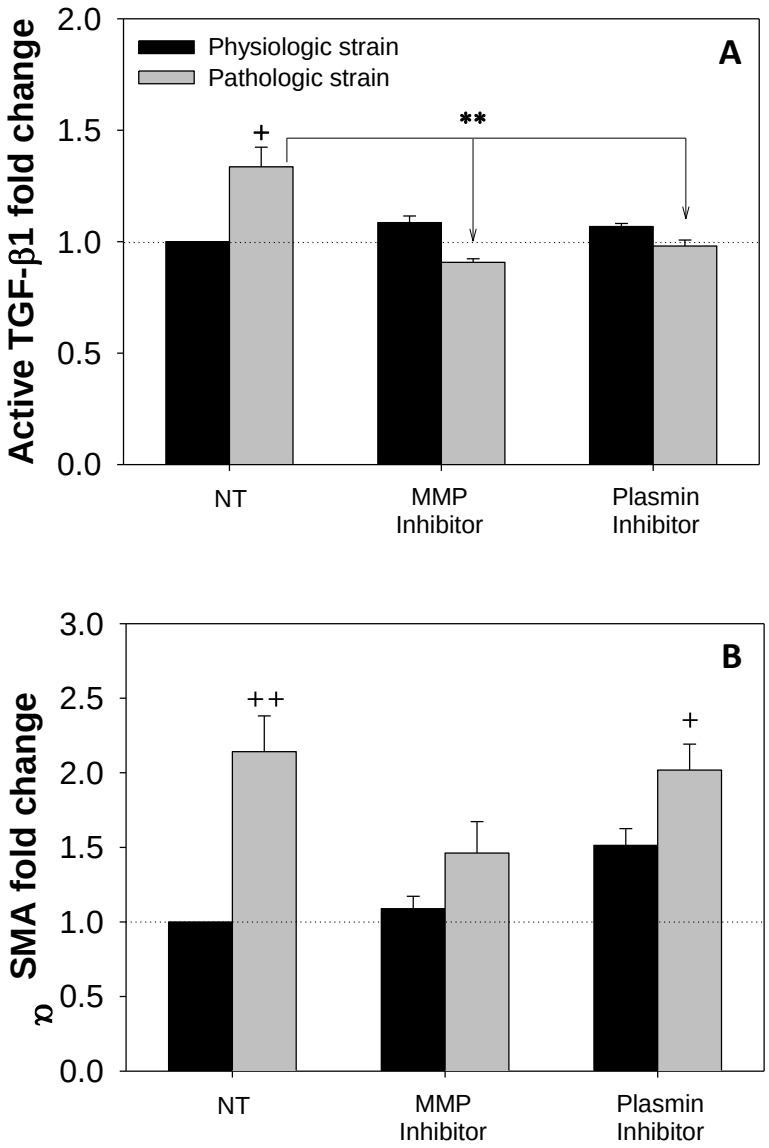

Figure 14: (A) Inhibiting MMP-9 and plasmin caused a significant drop in active TGF-β1 in pathologic levels compared to NT. (B) However, inhibition of MMP-9 and plasmin did not eliminate the mechano-dependent increase in αSMA expression at 6 h. **=$p<0.01$, compared to NT under pathological strain.

4.0 DISCUSSION

AVICs are known to play an integral part in the maintenance of the AV leaflets, with a phenotype shift to myofibroblast cells when repair of the tissue is needed [17]. In healthy tissue, these myofibroblasts are believed to become apoptotic or revert back to the quiescent phenotype yet, in AV disease, the myofibroblast phenotype persists [21]. The active form of TGF-β1 has been shown to cause and perpetuate this phenotypic change in monolayer cultures, eventually leading to calcific nodule formation characteristic of end – stage AV disease. Previous studies have suggested that infiltration by inflammatory macrophages into the leaflet tissue may be the initial step in AV disease. While these macrophages may play a substantial role in the release of inflammatory cytokines, such as TGF-β1, our study focused on how increased strain may initiate AV disease by shifting the phenotype of AVICs, possibly via mechano-dependent activation or synthesis of TGF-β1. Our previous studies have indicated that a link exists between cyclic strain and TGF-β1 expression [8]; however, this link has not been examined to date. Therefore, we sought to determine how cyclic mechanical strain affects TGF-β1 activation by the AVIC. Our hypothesis was that pathologic levels of cyclic mechanical strain, through an undetermined mechanism, activates latent TGF-β1 in the ECM, leading to an increase in TGF-β1 signaling via phosphorylation of Smad proteins, and culminating with a phenotypic switch of AVICs to the myofibroblast state, characterized by increased αSMA

expression. We believe that the supporting evidence of this strain-dependent mechanism could be an important initiator in the etiology of DAVD.

4.1 TGF-β1 Activation is Strain Dependent

Our results support our hypothesis that pathological strain significantly increases TGF-β1 activation. At 6 h there was a significant increase in active TGF-β1 in the pathologically strained AVICs. However, there was no evident change in total TGF-β1, indicating a mechano-sensitive activation process (**Figure 8A**). At 24 h, strain-dependent activation continues; however it is at a smaller, less than significant degree. This lowered strain dependent response may be explained by the transient nature of cytokine signaling, or possibly the TGF-β1 response to pathologic strain has already been exhausted in samples that did not receive exogenous active TGF-β1. At 24 h there was also an increase in total TGF-β1 in all groups (**Figure 8B**). The levels of total TGF-β1 increase in a strain dependent manner as well as increasing over time in both physiologic and pathologic strained samples. This increase in synthesis could be due to the feed-forward properties of the TGF-β1 signaling pathway that causes AVICs to synthesize more TGF-β1 at 24 h after the signaling process has become active near the 6 h time point. These mechano-sensitive increases in active TGF-β1 are further corroborated by αSMA results (**Figure 9**). Interestingly, Smad signaling results showed no significant differences at this time point. This may indicate that TGF-β1 is signaling through a non-canonical pathway. On the other hand, it may indicate a need to observe the Smad pathway at a different time point or a need to optimize our assay using both positive and negative controls for verification. To our knowledge this is the first time it has been shown that the level of

strain directly affects the activation of TGF-β1. This finding is clinically significant as it provides a potential target for preventative therapies that seek to delay the onset of DAVD. These data suggest that the activation of TGF-β1 due to pathologic strain may be a key step in the formation of constitutive myofibroblasts and in the beginning stages of DAVD. We believe that pathologic strain will continue to activate TGF-β1 being synthesized by the myofibroblast AVICs, and that this cycle will be further perpetuated, leading to tissue-wide myofibroblast AVICs, indicative of a diseased state.

4.2 Pathologic Strain Activates TGF-β1 via Secondary Molecular Pathways

While our data indicate that pathologic strain directly leads to the activation of TGF-β1, we sought to determine if activation occurred via direct physical forces or through secondary, molecular processes. In AVIC samples receiving no TGF-β1 treatment, we observed that levels of ED-A FN in the conditioned medium doubled under pathologic strain (**Figure 13**). This finding has numerous implications. TGF-β1 signaling is known to up-regulate FN expression [56], which further corroborates the strain dependent TGF-β1 activation data above. It also indicates an attempt by the AVICs to remodel the ECM in response to pathologic strain. ED-A FN deposition has been shown to precede αSMA expression and allow TGF-β1 to induce phenotypic change in fibroblast cells [53]. Furthermore, ED-A FN binds to numerous surface receptors at focal adhesion points, providing a mechano-transduction link between extracellular strain and intracellular signaling.

Similar to ED-A FN, known TGF-β1 activating proteases, MMP-9 and plasmin, were increased when AVICs were exposed to pathologic strain (**Figure 13**). Since these

are known activators of TGF-β1, we speculate that these molecules may be utilized by pathologically strained AVICs to activate TGF-β1. To examine this, we inhibited each activator independently and found that inhibiting either MMP-9 or plasmin eliminated strain dependent activation of TGF-β1 (**Figure 14**). This suggests that both MMP-9 and plasmin are necessary, but not sufficient, in the activation of TGF-β1 by AVICs under pathologic strain (**Figure 15**). Additionally, the ability of these inhibitors to eliminate strain mediated activation presents the possibility that increased strain may change the conformation of the ECM or the ECM-bound TGF-β1 latent complex via physical force to allow these activators to work more effectively. This has potential implications for the observation that TGF-β1 activation is increased with Marfan's syndrome [57, 58], where the mutated ECM fibrillin-1 gene results in defective connective tissue with elongated collagen architecture. We speculate that this elongated architecture makes latent TGF-β1 more 'bioavailable' to proteases and known activators. Similarly, our *in vitro* system likely mimics this architectural bioavailability by exposing the necessary proteolytic sites on the latent TGF-β1 complex, allowing MMP-9 and plasmin to liberate the active cytokine (**Figure 15**).

Despite the complete elimination of TGF-β1 activation using molecular inhibitors for MMP-9 or plasmin, the mechano-dependent αSMA increase was reduced but not eliminated (**Figure 14**). While we still observe a mechano-sensitive increase in αSMA content when the inhibitors are added, it is only 20% of the increase observed in the untreated samples. Although the phenotypic switch is not completely eliminated with the inhibition of TGF-β1 as expected, the result simultaneously indicates a potential pathway for mechano-sensitive phenotypic switch outside of the TGF-β1 pathway, and

demonstrates the contribution of the TGF-β1 pathway in significantly inducing the myofibroblast phenotype.

In summary, we show that pathologic strain leads to a phenotypic change to the myofibroblast phenotype in AVICs via up-regulation of known TGF-β1 activators with subsequent activation of TGF-β1 (**Figure 15**). This finding has potential therapeutic clinical implications. For instance, reducing strain on AV leaflets or preventing the synthesis of these molecular activators may be possible as preventative or therapeutic options for AV disease.

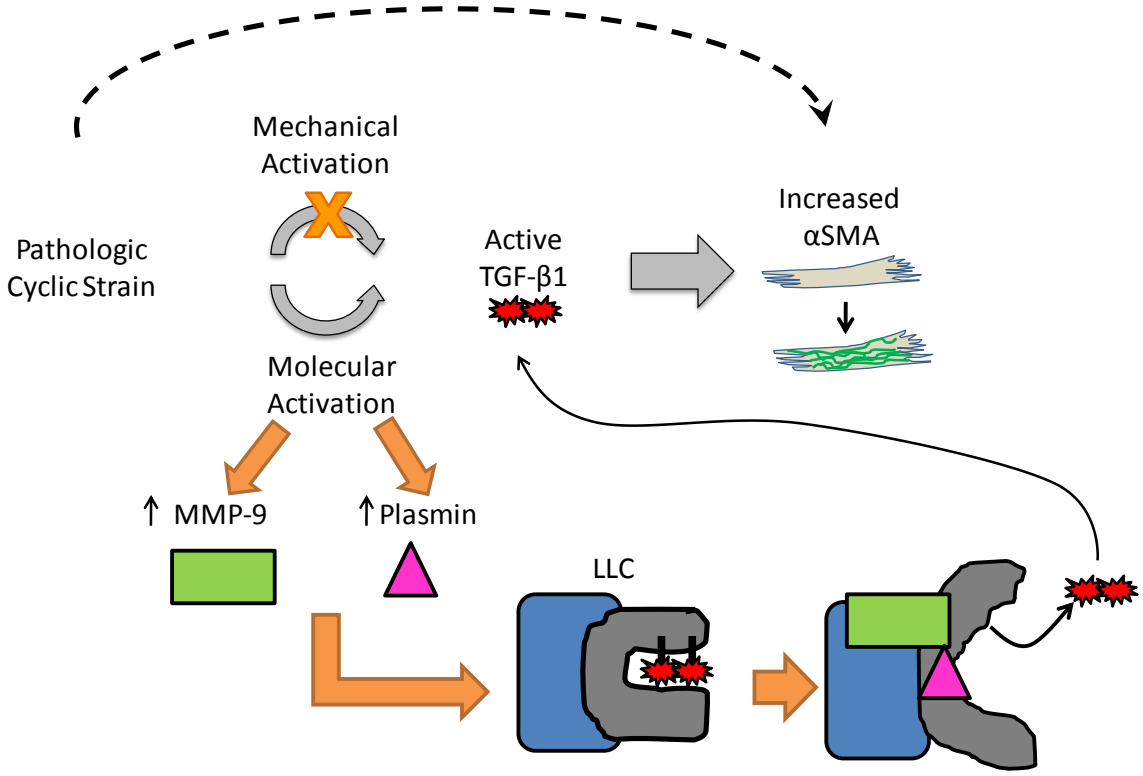

Figure 15: Pathologic strain activates TGF-β1 and leads to a phenotypic change in the AVIC. We show that this activation occurs via mechano-sensitive up regulation of known TGF-β1 activators: MMP-9 and Plasmin. While inhibiting TGF-β1 activation, we still observed a smaller scale mechano-sensitive phenotypic change in the AVIC, opening the door for an uninvestigated, non TGF-β1, mechano-dependent pathology.

5.0 RECOMMENDATIONS

This project resulted in many interesting and significant results concerning the connection between pathologic strain, TGF-β1, and DAVD. While there was a lack of significance within some of our data, these findings indicate the need to conduct further studies that will clarify this link. What follows are suggestions related to potential explanations of the lack of significance within our data as well as possible additional experiments that will lead to a better understanding of DAVD pathology.

While we showed encouraging trends in several of our experiments, we often failed to show significant differences. The most obvious solution to solve this problem will be to repeat these experiments to increase the number with a more consistent protocol. Since we were the first group to perform many of these FlexCell protocols, it took a large amount of time to develop optimal methods. Increasing the number of experiments should enhance the differences by a significant degree.

One factor that may explain our lack of significance, especially after the six hour time point is cell viability. While cells were seeded at the same density for all experiments, no analyses of cell viability were ever taken. IF images presented in the results suggest that viability may not be consistent with different treatments. AVICs exposed to pathologic strain may not survive as well as the physiologic strained cells. The extreme strain placed on the cells could cause programmed cell death in numerous ways; however, this study never quantified this. Cell viability tests may explain some of

the results we have presented as well as present interesting results in their own right. If cell viability was significantly lower in the pathologic strain groups, as the images suggest, then some normalization must be done to account for this. This adjustment may illuminate significant, mechano-dependent differences in TGF-β1, Smad signaling, αSMA expression and ECM proteins.

Correcting for cell viability may help explain some of our results; however, it will not eliminate the need to report results as fold-changes from a base condition. We decided to use the physiologically strained samples that received no TGF-β1 as our base condition. This was our choice because, of all our experimental groups, we believe that this group most closely represented normal *in vivo* conditions at the earliest time point. AVICs from each separate pig valve had levels of TGF-β1 and αSMA that were orders of magnitude different yet expressed similar % changes in response to pathologic strain; thus, data is reported in this manner. Since we only used two time points (6 and 24h), further studies should test levels at several other time points and may find that there is a more suitable base condition.

To link the mechano-sensitive activation of TGF-β1 to the mechano-sensitive αSMA expression, we quantified Smad2/3 and pSmad2/3 whose relationship represents the intracellular signaling of TGF-β1 along the canonical pathway. Previous groups have used Western Blot techniques to quantify these signals. Our group unsuccessfully attempted to replicate several protocols to also use Western Blots. Despite attempts to vary cell density, cell lyses techniques, and analysis techniques, no results were ever attained. Therefore, we switched our technique to ELISAs. While we attained results with trends that support our claims that the activation of TGF-β1 is connected to phenotypic

change of AVICs via the canonical signaling pathway, none of these trends were significant. We also attempted to image Smad and pSmad content using IF, with no success. Without a way to quantify these molecules, such as the development of a standard curve, it is difficult to ascertain what these results could signify. Further studies will either need to develop a method to quantify Smad proteins via ELISA, or form a way to have Western Blot techniques work for this system.

I believe this study opens up many avenues for further studies. I think that further investigation into the connection between pathologic strain and DAVD, both within the TGF-β1 signaling pathway and outside the influence of TGF-β1, is very much warranted. The inhibition and quantification of other ECM proteins linked to TGF-β1 may help elucidate how strain affects TGF-β1 activation. By knocking out specific aspects of the TGF-β1 signaling pathway using siRNA, studies may be able to determine how TGF-β1 affects the AVIC in this dynamic environment. I also believe that investigating the role of integrins, and how their expression and function are affected by strain will be extremely useful to expand on the information that this study presents.

6.0 CONCLUSIONS

This thesis project was meant to test the hypothesis that pathologic strain leads to the activation TGF-β1, which may be important knowledge in the onset of DAVD. Our initial results demonstrate this phenomenon after six hours of increased strain. Further, we showed that this strain also resulted in an increase in myofibroblast AVICs inferred by the increase in αSMA. Thus we showed that pathologic strain caused TGF-β1 activation and αSMA synthesis. Since we also knew that TGF-β1 has been shown to cause production of αSMA, the logical progression was that strain causes the activation of TGF-β1 which causes the phenotypic switch in AVIC's in turn increasing αSMA production. We then set out to determine how strain activated TGF-β1, by inhibiting known activators whose expression increased due to strain. We found that inhibiting these activators eliminated the mechano-sensitive activation of TGF-β1. Interestingly, it did not eliminate, only reduced, the increase in αSMA expression due to pathologic strain. This forced us to modify our "logical progression" to include the ability of AVICs to increase αSMA in a mechano-sensitive manner outside of the canonical TGF-β1 signaling pathway. The knowledge of the effects of increased strain on AVICs is important to further the understanding the pathology of DAVD as well as opening up potential targets for prevention and therapy.

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