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Molecular Basis of Sex Differences in Cardiac Hypertrophy and Heart Failure

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ABSTRACT

Transforming growth factor beta ligands and receptors are known to be pro-hypertrophic and pro-fibrotic factors in the heart, and are known to contribute to the development of cardiac hypertrophy and heart failure. It is well established that premenopausal females possess a lower incidence of these pathologies. We and others have observed a greater level of fibrosis in male hearts compared to female hearts in rodent models of cardiac hypertrophy. It is well established that estrogen is cardioprotective in that it can prevent the development of cardiac hypertrophy, as well as abrogate the development of heart failure following sustained cardiac hypertrophy. It is not fully understood how estrogen mediates these cardioprotective effects. In this study, we address the hypothesis that sex differences in the development of cardiac hypertrophy and heart failure is at least in part mediated by differential expression of TGFβ family members. To test this hypothesis, we utilized an angiotensin II pump infusion rodent model of cardiac hypertrophy in both male and female mice, followed by gene expression analysis and gene expression analysis of non-failing and failing human heart specimens. Interestingly, sex differences in cardiac hypertrophy were not observed in the angiotensin II pump mouse model, suggesting that angiotensin II may circumvent the cardioprotection afforded to females. Interestingly, TGFβ family members were more similarly expressed in this mouse model. Taken together these results provide evidence that sex specific differences in the development of cardiac hypertrophy are mediated in part by TGFβ signaling.

Human hearts reveal some sex differential gene regulation in both non-failing and failing hearts. Taken together, these results provide evidence that sex specific differences in the development of cardiac hypertrophy and heart failure are mediated in part by TGFβ signaling.

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Chapter I

INTRODUCTION & LITERATURE REVIEW

The morbidity and mortality of cardiovascular disease is on the rise in the western world (1). According to the CDC, over 67 million Americans have hypertension. Prolonged hypertension eventually leads to the development of cardiac hypertrophy, which is a significant risk factor for heart failure (HF). HF afflicts 6 million Americans and costs the United States $31 billion per year. Treatment options for patients with heart failure remain limited, and 50% of affected individuals will die within 5 years of being diagnosed (2). Therefore, new therapies and treatments are desperately needed. Of late, noticeable correlations have been established in the prognosis of heart failure such as the pathophysiologic differences between males and females with heart failure (1,3). For example, females with left ventricular hypertrophy are more likely to have preserved cardiac function and less likely to develop decompensated heart failure than men with similar levels of hypertrophy (4,5). Despite this, current guidelines recommend the same treatments (beta-blockers and drugs targeting the renin-angiotensin aldosterone system) for all patients despite these known hypertrophic sex differences. However, the molecular basis for sex-specific differences in heart failure is not yet understood.

To study the molecular and physiological components of cardiac hypertrophy, investigators primarily rely on animal models through specific gene transgenic models, a surgical transaortic constriction (TAC), or infusion of angiotensin II (ANG II) into individual animals in order to model hypertrophy. Our lab, as well as others, have observed sex-specific responses to TAC in the development of cardiac hypertrophy.

Specifically, heart weight to body weight ratios (HW:BW) significantly increased in TAC male mice as compared to females, which was accompanied by an increase in expression of hypertrophy gene expression markers. These include: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), alpha myosin heavy chain (α-MHC), and beta myosin heavy chain (β-MHC). Sex differential expression of transforming growth factor beta (TGFβ) superfamily members were also observed in our TAC model. TGFβ signaling is one of the major profibrotic signaling pathways studied in these models. Others have demonstrated that a greater upregulation of TGFβ profibrotic family members correlates with more severe left ventricular hypertrophy and fibrosis (6). In an effort to further investigate whether the TGFβ pathway is involved in sex specific differences in the development of cardiac hypertrophy and heart failure, we sought to establish the first survival surgeries at EKU using an ANG II infusion model. This model was established with the expertise of Dr. Lindsay Calderon. We were able to successfully model hypertrophy using this model. However, unlike that seen in the TAC model, sex-specific differences in the degree of hypertrophy were not observed. These animals were further characterized in terms of cardiac gene expression.

Because the ANG II infusion model of cardiac hypertrophy could not be used as a model to determine how sex specific gene expression, we sought another research model. Dr. Ken Campbell at the University of Kentucky provided us access to the human biospecimens bank, a source of nearly 100 human heart samples. Dr. Campbell also provided access to microarray data from 18 human hearts. Ten non-failing hearts (five females and five males) and eight failing hearts (1 female and 7 males) samples were analyzed with Affymetrix chips at the University of Kentucky Microarray Core. We

analyzed this expression data, specifically for genes of protein families known to affect fibrosis or contractility, identifying several gene candidates differentially expressed between failing hearts and non-failing hearts, as well as those differentially expressed in the non-failing hearts of males vs. females (Table 1).

Table 1. Differentially Expressed Heart Failure Candidate Genes in Midwall of the Heart.

Differentially Expressed Genes by Microarray Analysis				
TGFβ	**Collagen Deposition**	**Inflammation**	**Angiotensin**	**Other**
TGFβ	COL1α2	IL6R	ACE	ACER1
TGFβ1I1	COL12α1	NFκB2	AGTR2	ACER2
TGFβR3L	COL14α1	TNFαIP1		**βACE1**
	COL15α1	TNFαIP2		CTNN α 1
	COL21α1	TNFαIP8L1		CTNNβIP1
	COL27α1	TNFαIP8L2		CTNNβIP1
	COL4α5			DPAGT1
	COL6α1			HACE1
	MMP10			IMMP1L
	MMP11			IMMP2L
	MMP16			LACE1
	MMP2			MCOLN1
	MMP23B			MYOM2
	MMP27			PGLB1
	TIMP1			PTGFRN
	TIMP3			RTTN
				TGFα
				TNN

Notes: Microarray analysis was performed on 10 non-failing human hearts and 8 failing human hearts by Ken Campbell to assess transmural gene expression in failing vs. nonfailing hearts. Comparison of non-failing vs failing myocardium, regardless of sex, revealed numerous effects of heart failure when analyzed using multiple-comparison corrected t-tests (p-values < 0.05). These genes are organized by gene family or pathway. Of the 10 non-failing hearts, 5 were from female patients and 5 were from male patients, allowing for statistical comparison between sexes. Those shown by microarray to be sex regulated in non-failing hearts are shown in bold. Sex comparison in failing hearts was not possible because these data were initially generated for a separate project and only 1 of the 8 samples was from a female patient. MMP1 that demonstrated sex-specific expression in non-failing myocardium and differential expression in non-failing vs. failing are both bold and underlined.

Not enough female samples were present in the failing heart group to identify sex specific differences between non-failing and failing myocardia. However, six sex-specific genes were identified in non-failing myocardia, which are likely to play a role in heart disease. Due to both time and financial resources, the study was necessarily limited, but it did provide an impetus for further investigation of these genes and their sex specific expression in heart failure.

Cardiac Hypertrophy and Heart Failure Progression

Cardiac hypertrophy is the remodeling or thickening of the myocardium that leads to decreased chamber size (7). As the myocardium widens and the ventricular wall thins, the overall size of the heart increases as depicted in figure 1 (8).

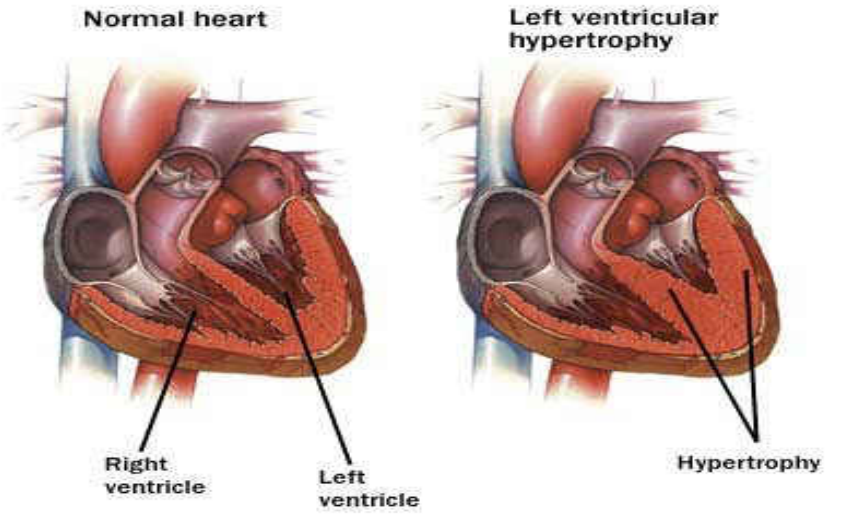

Figure 1: Visualization of Left Ventricular Hypertrophy. The myocardium surrounding the left ventricle is thickened in the heart depicted on the left in comparison to the left ventricle's myocardium of heart on the right. This thickening is due to the addition of sarcomeres in series rather than parallel and the fibrosis, or scarring of injured tissue (10).

Additionally, inflammation, fibrosis, and collagen deposition occur in ventricular remodeling (8). On a cellular level, cardiac hypertrophy is characterized by cardiomyocyte hypertrophy, not myocyte hyperplasia (7). In fact, as the diseased state progresses, cardiomyocytes are replaced by proliferative fibroblasts (8). These changes may result in a weakened and rigid heart that prevents systemic output and increases blood pressure within the heart (7). The presence of left ventricular hypertrophy is a strong independent risk factor for future cardiac events and mortality (9).

Cardiac remodeling is largely influenced by hemodynamic load, neurohumoral activation, and additional factors such as endothelin, cytokines, nitric oxide production and oxidative stress (11). The cardiac remodeling observed in cardiac hypertrophy can be physiological or pathological (11, 12, & 13).

The pathological or physiological remodeling that results from cardiac hypertrophy determines individual outcomes. Physiological remodeling in cardiac hypertrophy is a normalized change in the proportions and functions of the heart (11). Physiological remodeling may result in enhanced contractile function and elongated cardiac structure as observed in athletes and pregnancy (11 & 12). The Frank-Starling mechanism describes how heart muscle is able to increase contractile force when the ventricular wall is stretched, yet as cardiac remodeling progresses, this ability is greatly diminished (11). The increased volume and pressure loading in volume overload leads to the dilation of the left ventricle and an increase in left ventricular wall thickness; this is known as eccentric hypertrophy, in which sarcomeres are added in parallel and elongate the heart (11). The dual characteristics of eccentric hypertrophy allow for it to be

compensatory (11). Volume overload leads to the compensation and increase in cardiac output (11).

Pathological remodeling is due to an increased afterload, causing a need for increased intraventricular pressure to open the aortic valve, which in turn increases myocardial wall stress (11). Pressure overload is initially compensatory because increased pressure within the heart is offset by an increase in wall thickness; thus normalization occurs (14). It is sustained pressure overload that is deleterious. When sustained, pressure overload increases wall thickness without ventricle dilation because sarcomeres are added in series. This is in contrast to volume overload which adds in parallel and allows for lengthening (11). Pressure overload decreases cardiac output and is associated with ventricular remodeling, contractile dysfunction, interstitial and myocardial fibrosis, and re-expression of fetal cardiac genes (13). The production of collagen by myocardial fibrosis increases the stiffness of the myocardium, which interferes with heart filling (11). As fibroblasts replace cardiomyocytes within the ventricle, the contractile force and myocardial wall thickness both decrease, leading to ventricular dilation (11). Re-expression of the fetal cardiac genes, α-MHC and β-MHC, as well as ANP and BNP, results in characteristics associated with heart failure, (15). Decreased and increased expression of α-MHC and β-MHC, respectively, have been accepted as cardiac hypertrophy markers (16). Additionally, the natriuretic peptides have altered expression in cardiac hypertrophy; overexpression of BNP was strongly related to weakened left ventricular function, and overexpression of ANP has been correlated with hypertension and increased blood pressure, both precursors of heart failure (17). It

is when these potentially deleterious changes associated with cardiac hypertrophy are sustained that changes in myocardium lead to heart failure. (11).

Cardiac Hypertrophy Markers

Cardiac hypertrophy results from fetal gene reprogramming of alpha-MHC and beta-MHC, and increased levels of natriuretic peptides, ANP and BNP (15). The levels of these natriuretic peptides in plasma have been used to diagnose the severity of heart failure in patients, and thus serve as biomarkers for cardiac hypertrophy (18). Previous *in vivo* results in the lab indicated increased levels of cardiac hypertrophy markers ANP and BNP in TAC animals. To verify that hypertrophy was occurring in our angiotensin II mouse model of cardiac hypertrophy, we analyzed re-expression of these fetal cardiac genes. ANP and BNP are synthesized in the myocardium and brain as the precursors pro-ANP and pro-BNP that bind to receptors on target cells to mediate their biological effects (10 & 19).

The heart secretes natriuretic peptides as a homeostatic signal to maintain stable blood pressure and volume, and to prevent excess salt and water retention by inhibiting renin, vasopressin and aldosterone release (20 & 21). As depicted in figure 2, in inhibiting renin release, the natriuretic peptides maintain homeostasis by decreasing circulating levels of angiotensin II and aldosterone and preventing further natriuresis and diuresis, sodium excretion and fluid excretion (22).

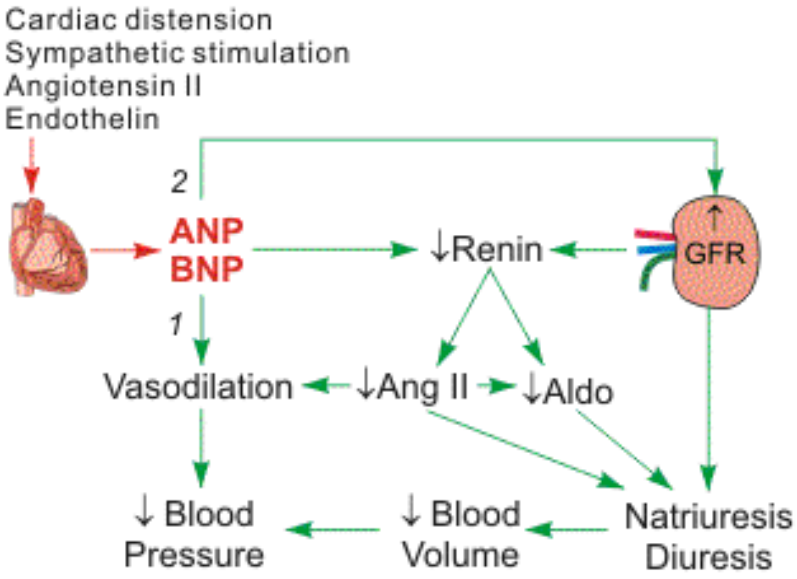

Figure 2: Mechanism of Atrial Natriuretic Peptide and Brain Natriuretic Peptide Expression. Both atrial and brain natriuretic peptides are secreted to maintain blood pressure and volume by contributing to vasodilation and inhibiting renin, vasopressin, and aldosterone release. Additionally, these peptides act on the kidneys directly to increase the glomerular filtration rate and fraction. Overexpression is over compensatory mechanism of the heart in attempt to prevent hypertrophy and subsequent heart failure (22).

Decreased angiotensin II also contributes to systemic vasodilation and decreased systemic vascular resistance (22). Natriuretic peptides maintain natriuresis and diuresis by increasing the glomerular filtration rate and filtration fraction (22). The heart releases these peptides in response to hypertrophy and subsequent heart failure, but rather than contribute to cardiac hypertrophy, these peptides stem its progression. ANP is released by atrial myocytes in response to atrial distension, angiotensin II stimulation, endothelin, and sympathetic stimulation (22).

Elevated levels of ANP are detected when blood volume is elevated and has been correlated with hypertension, both of which are precursors of heart failure (22). Overexpression of ANP is meant to be a preventative step in disease progression (22).

Brain-type natriuretic peptide is synthesized by the brain and ventricles (22). BNP is released in response to the same factors that release ANP (22). Increased expression of BNP is a means of abating cardiac hypertrophy; however, overexpression of BNP is strongly related to weakened left ventricular function (17 & 24). Increased expression causes the arteries to dilate, while reducing blood pressure and blocking adrenalin release (19), and also causes a decrease in myocardial fibrosis and remodeling (23).

The sarcomeres of cardiomyocytes are made up of the key contractile protein myosin (25). Myosin and actin work together to contract heart muscle (25). In the mammalian heart, myosin exhibits two myosin light chains and two myosin heavy chains (MHC). The heavy chains have two isoforms, alpha and beta (25 & 26). β-MHC is the predominant isoform in the fetal heart (25). Figure 3 depicts the myosin heavy chain switch and fetal gene reprogramming that occurs a s the heart develops. As the heart ages, α-MHC switches to become the predominant isoform (25).

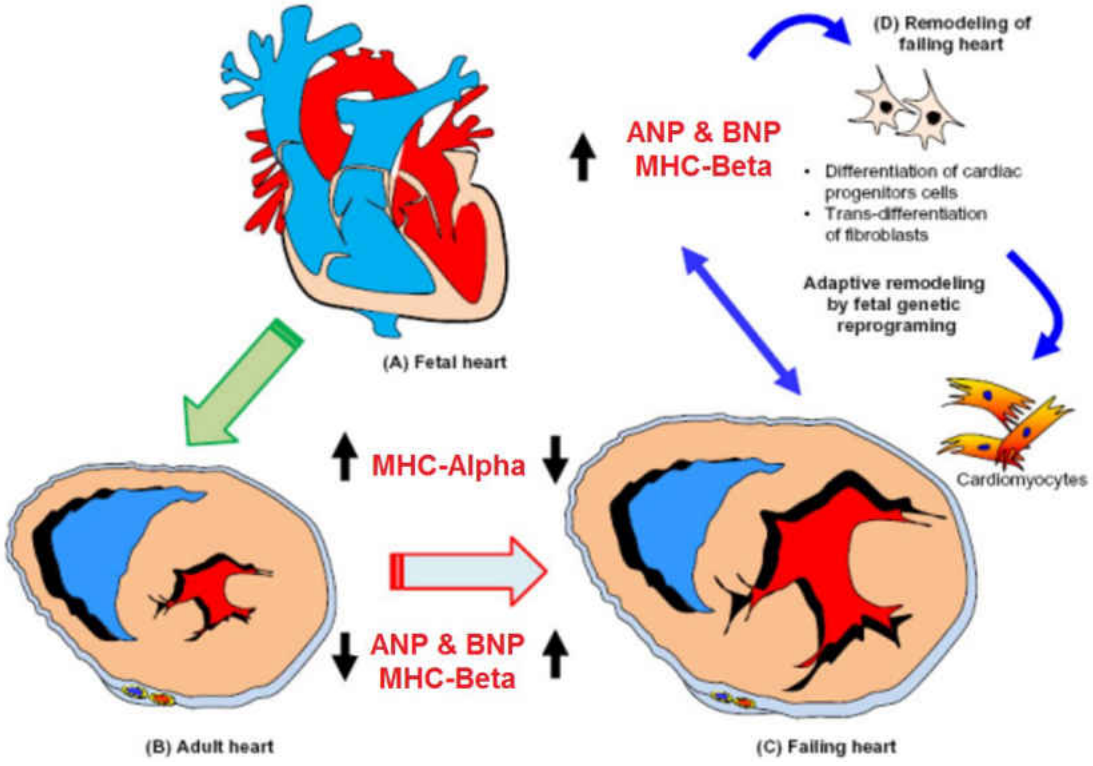

Figure 3: Cardiac Hypertrophy Marker Expression. In the fetal heart, α-MHC is the predominant isoform of the myosin heavy chain once upregulated after birth. (B) is the normal size structure of the adult heart where ANP and BNP are secreted to maintain cardiac homeostasis and β-MHC expression is inhibited. As (B) progresses to (C), a failure state, the ventricles are remodeled, the expression of ANP & BNP increases as a means of compensating cardiac dysfunction and disease, and β-MHC expression no longer suppressed but upregulated. These hypertrophy markers lead to proliferation and fibrosis of fibroblasts as the number of cardiomyocytes decreases (25).

Fetal reprogramming occurs when the adult heart re-expresses several fetal genes, such as β-myosin heavy chains (25). During cardiac hypertrophy and heart failure in mice and humans, the expression of the β-MHC genes increases as α-MHC expression decreases (16). Increased β-MHC expression decreases contractile work, and even slight changes in expression can significantly change cardiomyocyte power output (16 & 27). α-MHC is known to play a role in preserving heart function; therefore, decreases in α-MHC expression is an indication of decreased cardiac function (16 & 26). In mice models it has been suggested that the shift from alpha to beta is well tolerated and does not cause heart failure, whereas in human heart failure, it is suggested that the shift is not well tolerated and does contribute to disease development (27).

Fibrosis

Most cardiac diseases are associated with increased fibrosis, the thickening and scarring of connective tissue, in the heart (28). Thus, it has been suggested that increased fibrosis, not myocardial hypertrophy, may be the most significant cause of diastolic dysfunction in hypertrophic cardiomyopathy (28). Cardiac fibroblasts synthesize collagen and extracellular matrix proteins and are crucial in wound healing (28). Cardiac fibroblasts help maintain normal cardiac function by impacting cardiomyocyte function,

allowing for the electrical conduction required for contraction, and maintaining extracellular matrix (ECM) homeostasis (29).

As mentioned, fibrosis is a normal physiological response to wound healing (28). After injury, fibrosis of the myocardium occurs to replace necrotic cardiac tissue with extracellular matrix proteins to preserve the heart wall (28). Fibroblasts accomplish this by transforming into myofibroblasts, proliferating, and then migrating into the wound where they synthesize elevated levels of the extracellular matrix protein collagen (28). The remaining healthy heart tissue compensates for this loss of myocardium and ventricular function by increasing levels of fibrosis and inducing myocyte hypertrophy (28). As shown in figure 4, in cardiac hypertrophy cardiomyocytes are replaced by myofibroblasts (30).

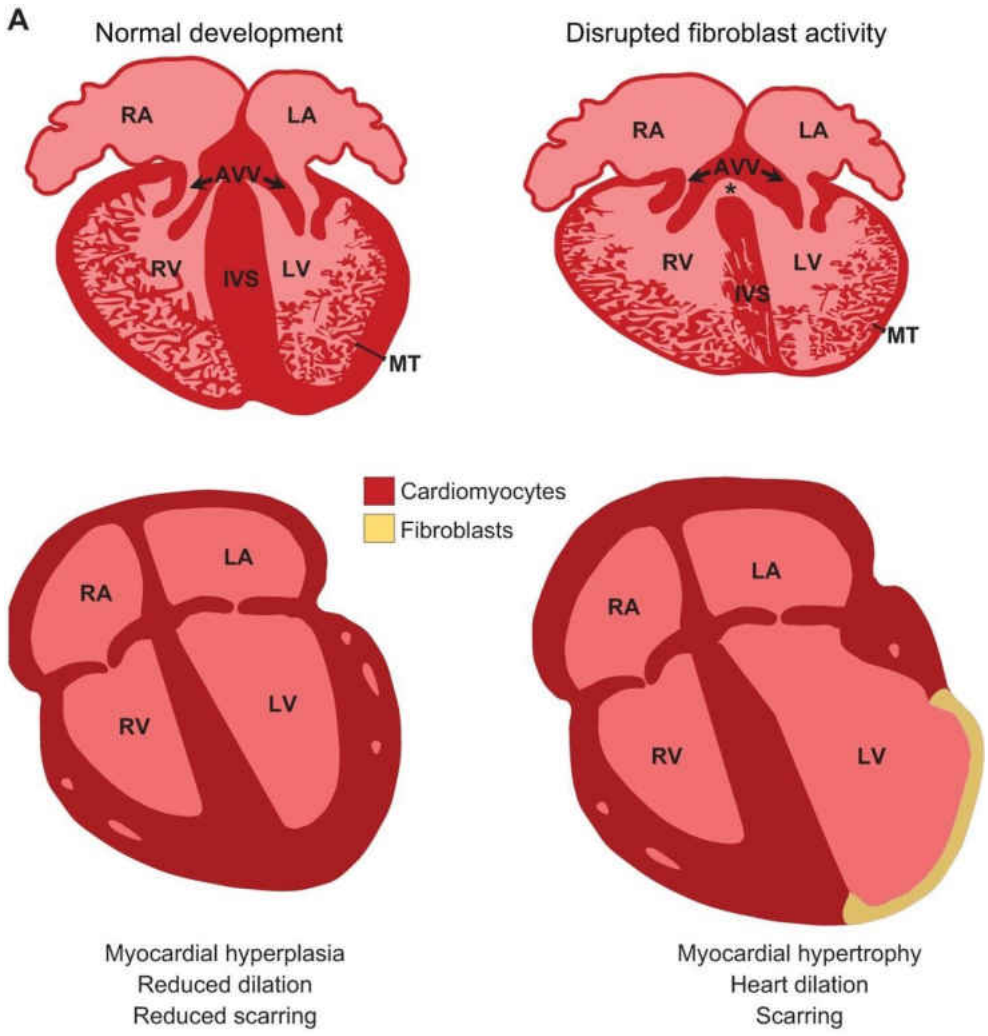

Figure 4: Cellular Makeup in Cardiac Development. Depiction of the changes in cellular makeup in normal cardiac development from a fetal to adult on the left in comparison to the hearts on the right in which the heart progresses from fetal to disrupted hypertrophy. In normal development fibroblasts are nearly nonexistent but as the heart progresses to a hypertrophic state, the fibroblasts replace the cardiomyocytes as they undergo necrosis leading to a major fibrotic lesion. This connective tissue cannot properly contract and disrupts cardiomyocyte electrical signaling and blood output leading to heart failure (31).

These myofibroblasts are characteristic of fibrotic lesions and produce a two to threefold rise in collagen synthesis (30). The fibrosis component of cardiac hypertrophy occurs when reparative fibrosis fails to terminate. The excessive production, deposition, and contraction of extracellular matrix and collagen results in increased mechanical

stiffness, disrupted electrical conduction and thus contractility. The inflammation and fibrosis may decrease the flow of oxygen and nutrients and increase the pathological remodeling observed in cardiac hypertrophy that results in heart failure (28). Recent research has identified the major fibrotic signaling pathway as the transforming growth factor beta superfamily. TGFβ signaling primarily occurs through SMADs, a family of proteins named for a portmanteau of the protein homolog in *Drosophila* known as mothers against decapentaplegic (MAD) and the protein homolog in *Caenorhabditis elegans* for small body size (SMA) (28,30,33).

Transforming Growth Factor Beta Family

TGFβ family members are reported as key upregulated components in the signaling pathway that results in LV remodeling in both pressure overload animal models and human patients with aortic valve stenosis (6). The TGFβ cytokine has cellular functions in regulating inflammation, extracellular matrix deposition, cell proliferation, differentiation, and growth (32). TGFβ regulates phenotype and function of cells involved in tissue injury, repair, and remodeling in cardiac tissue (32). Furthermore TGFβ is a component of cardiomyocyte and fibroblast phenotype and function, myofibroblast differentiation, and the fibrogenic mediator connective tissue growth factor (CTGF) (32). Results have demonstrated that *in vivo* TGFβ promotes myocardial hypertrophy when overexpressed in mouse models of hypertrophy (32). TGFβ was also found to be upregulated in human cases of hypertrophy and in *in vitro* cardiomyocytes (32). Yet, while overexpressed TGFβ leads to collagen deposition, increased myocardial stenosis, fibrosis, and diastolic dysfunction, a certain baseline level is required to

maintain cardiac structure and protect against cardiac dilation in cases of pressure overload (32). TGFβ signaling through SMADs, depicted in figure 5, in pressure overload models of hypertrophy are dependent on levels of active TGFβ (32).

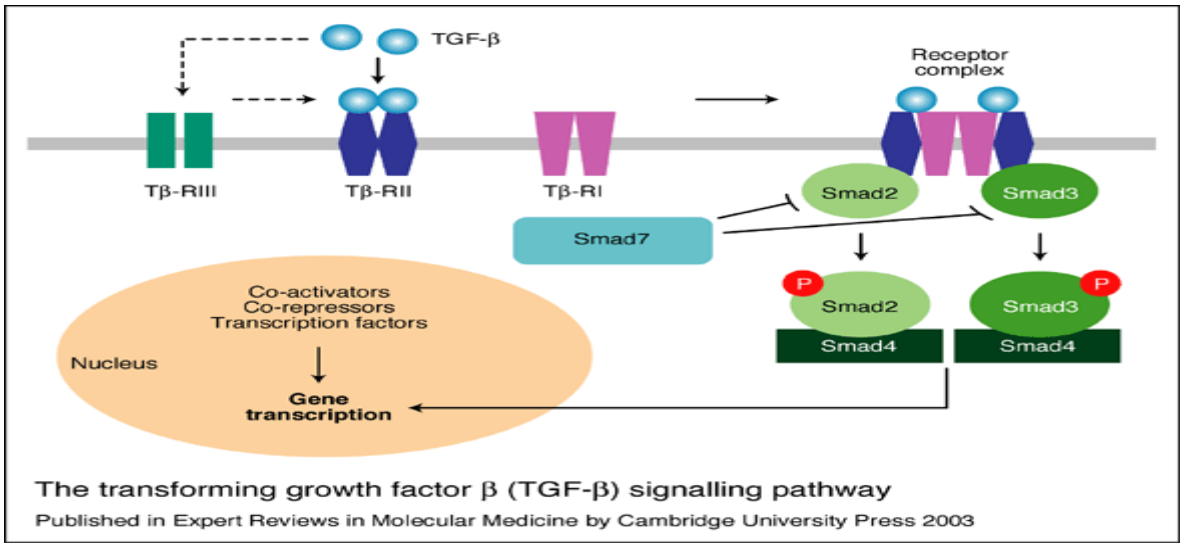

Figure 5: Mechanism of TGFβ Signaling. TGFβ binds to transforming growth factor beta receptor 2 (TGFβRII) forming a heteromeric receptor complex; this binding might be enhanced by the presence of transforming growth factor beta receptor 3 (TGFβRIII). Receptor I and Receptor II possess tyrosine kinase activity. Up-regulation of TGFβ and its receptors also contributes to increased activity of TGFβ signaling. After binding to TGFβ, TGFβ-RII recruits and phosphorylates transforming growth factor beta receptor 1 (TGFβR1), leading to activation of the Smad family of transcriptional activators. Smad2 and Smad3 are phosphorylated by TGFβR1 kinase, to bind as a heterodimer to Smad4 and translocate into the nucleus. This process is inhibited by Smad7. Together with co-activators, co-repressors and other transcription factors, the Smad complex regulates gene expression. In a recent experiment indicated that adult fibroblasts Smad3 is required for TGFβ induced gene expression (28, 30, & 33).

TGFB and the Renin Angiotensin Aldosterone System

Evidence of a link between the renin angiotensin aldosterone system (RAAS) and ANG II indicate that TGFβis downstream of ANG II in this pathway. The renin-angiotensin aldosterone system is an endocrine pathway that regulates blood pressure and fluid volume of the body (34). Research has verified the presence of RAAS in

independent local systems in the heart and blood vessels (35). The enzyme renin is secreted by the kidneys and cleaves angiotensin I, the precursor of ANG II (36). Renin production is a rate-limiting step in the RAAS and is stimulated by a decrease in arterial pressure, change in salt content, and/or increased sympathetic activity (36). ANG II, the byproduct of renin cleavage of angiotensinogen I, directly promotes cell growth, regulates gene expression of various bioactive substances, and activates multiple intracellular signaling in cardiovascular and renal cells (36). The role of ANG II in cardiac hypertrophy is widely accepted to be direct or combinatorial with other growth factors (35).

Angiotensin II is produced through two mechanisms (36). The first mechanism is production of angiotensinogen in the liver, cleavage by renin to form angiotensin I, and further cleavage of angiotensin I to angiotensin II. The second mechanism involves the production of angiotensin I or angiotensin II from angiotensinogen in non-renin specific pathways such as the conversion of angiotensin I to ANG II via the secretion of angiotensin converting enzyme (ACE) by the pulmonary circulation or RAAS tissues (34). Recent *in vitro* and *in vivo* evidence supports that the actions of ANG II include regulating blood pressure through vasoconstrictive effects, regulating the retention of sodium and water by the renal tubules, cell proliferation, fibrosis, inflammation, and aldosterone release in cardiovascular and renal diseases, depicted in Figure 6 (34).

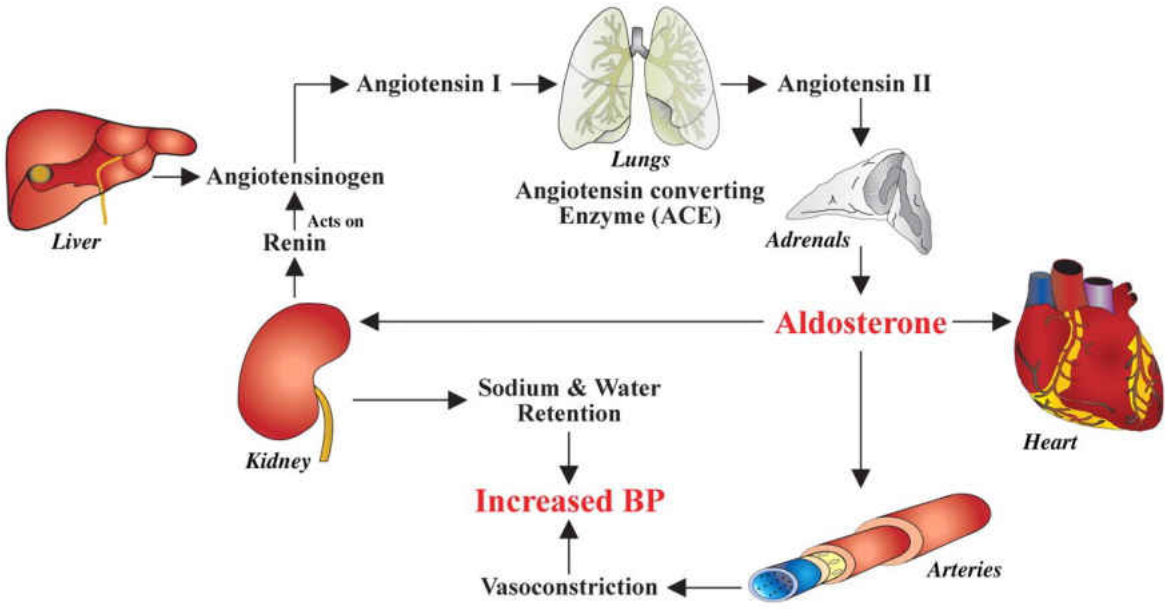

Figure 6: The Renin Angiotensin Aldosterone System. Angiotensinogen is produced by the liver and cleaved into angiotensin I by renin, an enzyme produced by the kidneys. angiotensin I is converted into angiotensin II by the angiotensin converting enzyme to then act on the adrenal glands to produce aldosterone. Aldosterone acts on the heart, arteries, and kidney to produce vascular effects such as vasoconstriction, renin release, natriuresis, and diuresis (39).

These actions are almost exclusively mediated by binding to the angiotensin type one receptor (AT_1 receptor) (34). AT_1 receptors are located in the kidney, heart, brain, smooth muscle, adrenal glands, and other tissue and cell types (34). On a molecular level, ANG II binding to AT_1 impairs nitric oxide synthesis, a molecule required for vasodilation (36). Additionally, ANG II may exert effects through binding to intracellular AT_1 receptor–like proteins (37). AT_1's counterpart, the angiotensin type two receptor (AT_2 receptor), is expressed in developing fetal tissues, although expression rapidly decreases after birth, and is limited mainly to the uterus, ovary, certain brain nuclei, heart, and adrenal medulla (38). Furthermore it is antihypertrophic in that it mediates vasodilation and inhibits fibrosis in addition to playing a role in blood pressure regulation

(38). AT_1 receptors are responsible for growth and remodeling ANG II–mediated effects in the heart, while AT_2 receptors counteract the effects of AT_1 receptors (37).

Signaling by angiotensin II is a key factor in the development of cardiac hypertrophy (40). ANG II signaling leads to the induction of prohypertrophic genes (40). Additionally, hypertrophy is characterized by interstitial fibrosis caused by increased expression of collagen genes (40). Both angiotensin II and TGFβ induce proliferation of fibroblasts to myofibroblasts and thus collagen synthesis in the infarctic heart (41). Figure 7 illustrates how ANG II activation induces TGFβ transcription by cardiomyocytes and fibroblasts (32).

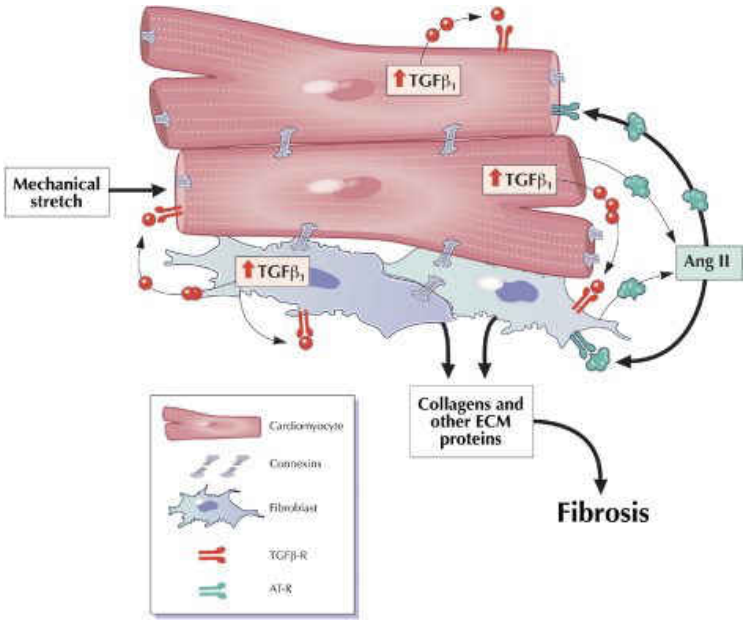

Figure 7: ANG II and TGFβ Signaling. Both cardiomyocytes and fibroblasts exhibit TGFβ receptors and angiotensin receptors. Cardiomyocytes undergoing mechanical stress release growth-promoting factors, such as transforming growth factor beta. Upregulation of TGFβ in cardiomyocytes leads to hypertrophic growth. Additionally, a number of *in vitro* and *in vivo* studies have indicated that TGFβis up-regulated by angiotensin II in myofibroblasts and cardiac fibroblasts as well. Binding of ANG II to the AT_1 receptor upregulates TGFβ expression in fibroblasts, proliferative growth, and leads to increased expression of collagen and ECM proteins characteristic markers of fibrosis. Kupfahl et al. noted that angiotensin II did not directly stimulate collagen expression, but rather caused TGFβup-regulation, which then altered collagen production (44 & 45).

In vitro, stored ANG II released from the secretory granules of cardiomyocytes in response to mechanical stretch, a means of stretching cardiomyocytes, induces cardiac hypertrophy (42). Inducing hemodynamic overload, a form of mechanical stress, is considered to induce a growth response in the overloaded myocardium (43). Furthermore, mechanical stress induces the release of growth-promoting factors, such as angiotensin II, endothelin-1, and TGFβ, which provide a second line of growth induction (43). Mechanical stretching of cardiomyocytes activates the phosphorylation cascade of protein kinases, the re-expression of immediate early and fetal-type genes, and increases the protein synthesis rate (42). Sadoshima and Izumo in 1993 reported increases in protein synthesis but not DNA synthesis in cardiomyocyte culture experiments (35). Similar to cardiomyocytes, fibroblasts *in vitro* had increased protein expression in response to mechanical stretching, but also underwent hyperplasia and DNA synthesis (35). The re-expression of immediate early genes and fetal-type genes was also observed in fibroblast cultures (35). These phenotypic changes observed by Sadoshima and Izumo are reported to closely resemble those of load-induced hypertrophy *in vivo* and suggest that this may occur through a positive feedback regulation of angiotensinogen and TGFβ.

Reports indicate that the use of ACE inhibitors or AT_1 receptor blockers, decreased TGFβ expression in hypertrophied hearts (32). Therefore TGFβ has been proposed as a therapeutic target for hypertrophy because increased levels indicate cardiac remodeling and are associated with the activation of TGFβ signaling pathway (32). Future clinical use of TGFβ as a therapeutic agent faces difficulties because of its broad range of effects and potentially interfering role in immune regulation (32). Therefore,

with the known cardioprotective role of estrogen in cardiovascular disease, it is of interest to determine how sex-differences affect TGFβ signaling in hypertrophy.

Additional Genes in Fibrosis and Collagen Deposition

Tumor necrosis factor-alpha (TNF-α), a proinflammatory cytokine, is involved in wound healing and antifibrotic through the inhibition of matrix genes (30). TNF-α and its receptor also trigger intracellular signaling cascades through phosphorylation of protein kinase B (AKT), activation of nuclear factor-kappa B (NFκB), and the phosphorylation of c-JUN N-terminal kinase (JNK) (46 &47). These intracellular signaling cascades allow TNF- α binding to modulate host defense against injury, facilitate growth and survival, and promote apoptosis and matrix metalloproteinase (MMP) expression (46 & 47). TNF-α is induced in the myocardium under volume or pressure overload (48). Prolonged overexpression is implicated in pathogenesis of myocarditis, ischemic heart disease, cardiac hypertrophy, and left ventricular dysfunction (49). Patients with HF exhibit increased levels of TNF-α in the myocardium (47). Transgenic mice that overexpress TNF-α in the myocardium develop LVH, dilated cardiomyopathy, and premature death (48).

There is evidence that suggests significant cross-talk between neurohormonal and inflammatory cytokine signaling in cardiac hypertrophy and failure (46). For example, ANG II activates NF-κB to initiate the transcriptional activation of increased expression of proinflammatory cytokines, nitric oxide, chemokines and cell adhesion molecules (46). TNF-α provokes the activation of the RAAS in the heart through increased ACE activity (46). Figure 8 illustrates the crosstalk between ANG II activation of NF-κB and the activation of the RAAS through ACE TNF-α. Recent experiments indicate that

pathophysiological concentrations of angiotensin II are sufficient to provoke TNF-α mRNA and protein synthesis in the adult cardiomyocytes through a NF-κB dependent pathway (46).

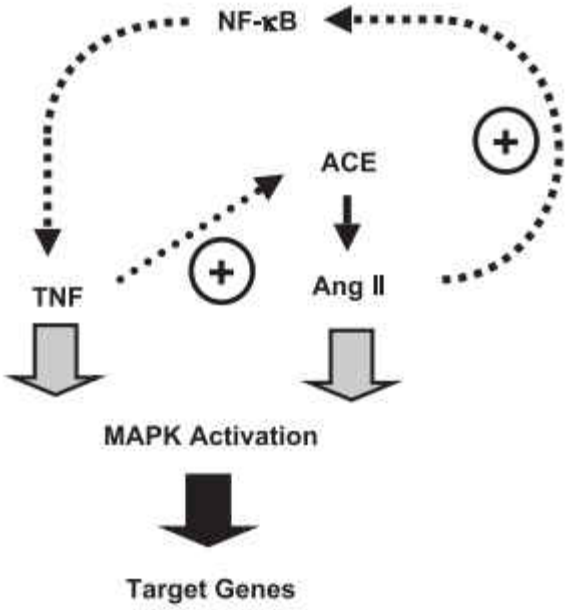

Figure 8: Cross-regulation of Tumor Necrosis Factor-α and ANG II Pathways. ACE cleaves ANG II to provoke an inflammatory response through NFκB pathway. This pathway then acts on TNF to provoke increase ACE activity. Both pathways converge in the MAPK signaling pathway (46).

Evidence also suggests that TNF-α stimulation has been shown to increase the density of AT_1 receptors on cardiac fibroblasts and increase their profibrotic sensitivity to ANG II (46). Furthermore, the RAAS and inflammatory mediators converge on the mitogen activated protein kinase (MAPK) pathway (46). This, however, leads to the hypothesis that these two pathways on MAPK signaling may serve to amplify or propagate stress signals within the heart (46). One such pathway in the heart involves Jun kinases (JNKs), which are activated in TNF-α signaling. (46). Recent studies suggest that angiotensin II induced TNF-α signaling activates JNKs in cardiomyocytes (46).

As indicated in Table 1, microarray analysis identified TNFαIP1, part of the TNF-α pathway, as exhibiting sex-regulated expression. As previously mentioned, TNF-α has a cross-regulatory relationship with the renin angiotensin aldosterone system, is antifibrotic, and proinflammatory. Signaling cascades of TNF-α lead to activation of NFκB and the phosphorylation of JNK that modulates host defense against injury, facilitates growth and survival, and promotes apoptosis and MMP expression (46 & 47). Lastly, TNF-α is induced in the myocardium in cardiac hypertrophy and left ventricular dysfunction (49). Specific aim two of my project is the identification of the role of TNFαIP1 in collagen deposition and if it is indeed involved in the proposed interplay of genes that result in heart failure.

Proteolytic enzymes such as matrix metalloproteinases (MMPs) function in the extracellular environment of cells and degrade both matrix and non-matrix proteins (50). They play central roles in morphogenesis, wound healing, tissue repair and remodeling in response to injury, further biological effects are listed in table 2 (50). Their main function is the degradation and removal of ECM molecules from tissue. MMPs are implicated in the progression of myocardial infarction, atheroma, arthritis, cancer, chronic tissue ulcers, idiopathic dilated cardiomyopathy, tachycardia induced heart failure, and pressure-overload hypertrophy (50 & 51). Collagen deposition, as depicted in figure 9, is caused by imbalanced concentrations of MMPs or tissue inhibitors of metalloproteinases (TIMPs) (51). TIMPs regulate the activation of MMPs by binding to and preventing the degradation of the collagen matrix (50 & 51). Thus, the balance between the two proteins is crucial for ECM remodeling as depicted in figure 10 (50).

COL6α1

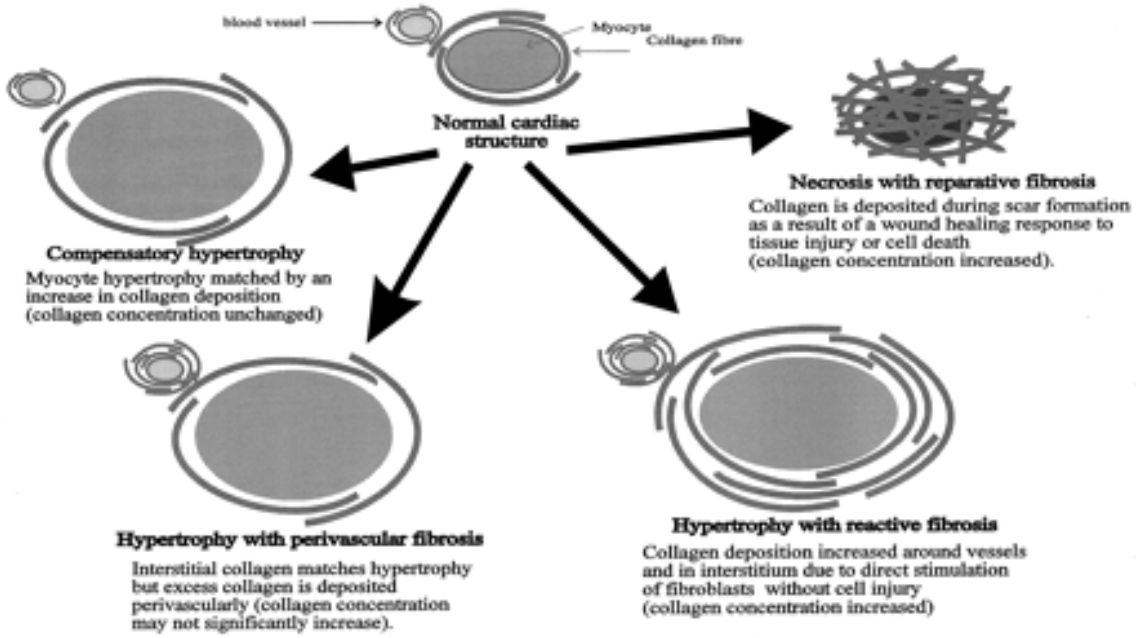

Figure 9: Collagen Deposition. Collagen deposition in the ECM by fibroblasts is known to promote scarring of the heart and decrease contractibility of surrounding cardiomyocytes leading to heart failure (28). Fibrosis rather than hypertrophy is now thought to be the most significant cause of cardiac dysfunction in hypertrophy (see page 20 for more information on fibrosis) (28). In the depicted image, normal cardiac structure can be observed to be disrupted in various form of hypertrophy with fibrosis. When the collagen concentration is increased is when fibrosis becomes pathophysiological. Here I wish to tie the COL6α1 gene, which was found to be sex-specifically regulated by microarray analysis in Table 1, into our project. Specific aim two of my project seeks to identify the role of COL6α1 in collagen deposition and if it is indeed in the proposed interplay of genes involved with heart failure through. (53)

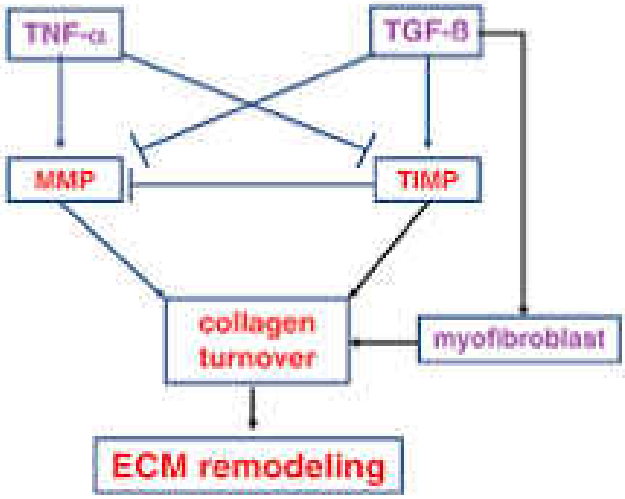

Figure 10: Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases Activation. TNF-α activates MMPs to induce collagen turnover and ECM remodeling It is known that an imbalance between TIMPs and MMPs leads to cardiac dysfunction. TNF-α inhibits TIMPs unlike the profibrotic cytokine that inhibits MMPs and activates TIMPs to cause ECM remodeling. (52)

Recently, it has been suggested that MMPs become activated within the failing myocardium (50). In mouse models of LVH, MMP expression disrupted the ECM surrounding myocytes and was shown to increase in a time-dependent manner as the LVH progressed (28). In patients with hypertrophic cardiomyopathy, findings suggest that changes in collagen metabolism by the interactions between MMPs and TIMPs may be associated with LV remodeling and the progression of LV systolic dysfunction (51).

As indicated in Table 1, microarray analysis identified MMP2 as exhibiting sex-specific expression in non-failing myocardium and differential expression in non-failing vs. failing hearts. MMP2 has been implicated in cell migration, increased bioavailability of TGFβ, as a vasodilator or vasoconstrictor, and anti-inflammatory among other properties (Table 2).

Table 2: Biological Effects of MMP2.

Biological Effects of MMP2
Cell migration
Neurite outgrowth
Mesenchymal cell differentiation with inflammatory phenotype
Enhanced collagen affinity
Increased bioavailability of IGF1 and cell proliferation
Anti-inflammatory
Epithelial cell migration
Increased bioavailability of TGFβ
Generation of vasoconstrictor
Conversion of vasodilator to vasoconstrictor
Neuronal apoptosis leading to neurodegeneration

Source: Nagase, Hideaki, Robert Visse, and Gillian Murphy. "Structure and Function of Matrix Metalloproteinases and TIMPs." *Cardiovascular Research* 69, no. 3 (February 15, 2006): 562–73. doi:10.1016/j.cardiores.2005.12.002.

Experiments using MMP gene knockout mice have shown that MMP2 plays a key role in cardiac rupture after myocardial infarction. MMP2 cleaves and degrades some types of collagen. In cardiomyocytes, intracellular MMP2 degrades structures and enzymes that contribute to cardiac dysfunction (50). Specific aim two of my project is the identification of the role of MMP2 in collagen deposition and if it is indeed involved in the proposed interplay of genes that result with heart failure. I specifically seek to determine its relationship with TGFβ.

The roles of TGFβ and MMPs in cardiac remodeling may be intertwined. In fact, both proteins may be part of one complex pathway for fibrosis that we propose in figure 11.

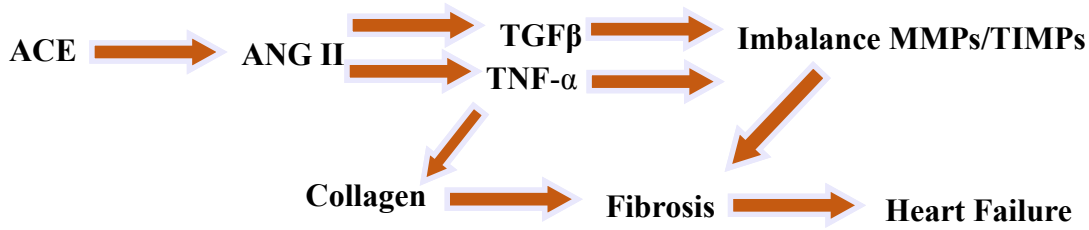

Figure 11: Proposed Interplay of Genes in Fibrosis and Heart Failure. We propose an interplay of genes and signaling pathways that lead to the fibrosis observed in heart failure. Evidence for this interplay exists in our literature. Upregulation of ACE leads to increased ANG II synthesis by the renin angiotensin aldosterone system which in turn activates some of the same pathways TNF-α activates as seen in Figure 8. As mentioned in Figure 7, ANG II receptors and TGFβ receptors exist in cardiomyocytes and fibroblasts and TGFβ is upregulated by ANG II binding AT_1. TNF-α and TGFβ appear to work independently of each other to alter the balance of MMPs and TIMPS as seen in Figure 9. TGFβ expression is known to be profibrotic and to affect collagen deposition. Ultimately we propose that each of these individual components work together to cause fibrosis and its subsequent result, heart failure.

Increasing evidence suggests that TGFβ increases MMP activity within the myocardium (28). The breakdown of the ECM stimulates the release of growth factors that are bound to the ECM, such as TGFβ (50). It has been noted that levels of TGFβ and MMP2 expression are higher in terminally failing hearts (28). TGFβ appears to up-regulate MMP2 expression in fibroblasts to facilitate migration and motility allowing for increased fibrosis (28).

Estrogen is Cardioprotective

Premenopausal women are at a lower risk of developing heart failure, yet the risk of heart failure in women significantly increases after the age of 55 (1 & 54). Furthermore, risk factors of heart failure including diabetes, anemia, high blood pressure, and cardiac hypertrophy occur at different rates between men and women (1). Women in particular are protected against cardiac hypertrophy more than men as seen in cases of early diagnosis (1). With cardiac hypertrophy being one of the strongest predictors of

mortality in women, premenopausal females with cardiac hypertrophy are more likely to have preserved cardiac function and less likely to develop decompensated heart failure than men (1, 9, 10,11). This sex-related incidence has been correlated to the cardioprotective effects of estrogen in premenopausal women (1, 9). In comparison, hormone replacement therapy (HRT) for postmenopausal women was initially thought to have numerous beneficial effects (13). Findings, however, differ on the efficacy of HRT. While some studies report the cardioprotective benefits of HRT when treatment is begun at an early age, other studies have reported HRT to be ineffective overall or even harmful if begun at a later age (14). Though this finding seems to negate estrogen's cardioprotective effects, in actuality it provides the need to maximize the potential use of estrogen therapies by understanding the molecular mechanism of its beneficial effects without activating pathways leading to negative side effects (54). Furthermore, these findings attest to the mechanism through which estrogen is cardioprotective; however, other potential sex differences still remain elusive and not well understood. Most cardiac hypertrophy studies involve primarily male subjects. The study defined here will provide insight into biochemical differences between males and females that could lead to better treatment for women with heart disease.

Estrogen is a steroid hormone that binds to the estrogen receptors alpha or beta (ERα and ERβ) to mediate transcriptional regulation as well as non-nuclear effects. Figure 12 illustrates estrogen mediating transcriptional and non-nuclear effects.

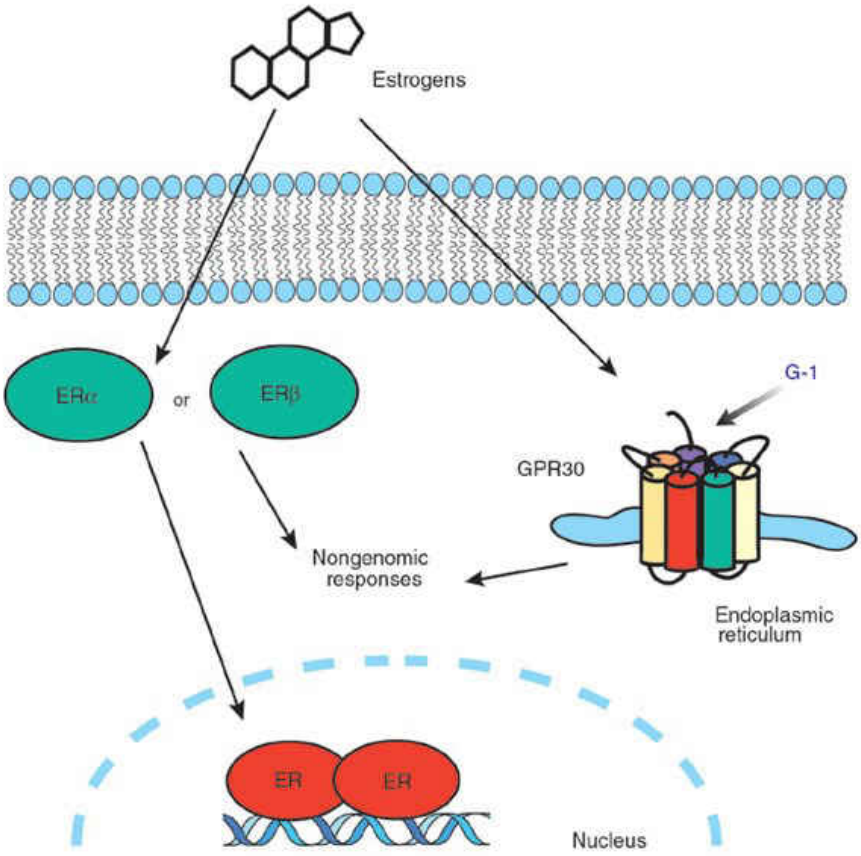

Figure 12: Estrogen Signaling and Cardioprotection. Estrogens can bind to either ERα and ERβ or GPR30 to regulate transcription after translocating to the nucleus. Binding to the latter two generates nongenomic or non-nuclear responses. As depicted, G-1 binds to GPR30 (56).

Transcriptional estrogenic effects, when the estrogen-nuclear receptor complex binds to estrogen response elements of the target gene's promoter region, are the result of the recruitment of coactivators and displacement of corepressors at DNA binding sites (54). Estrogen can bind independently of estrogen response elements by binding to transcription factors and regulatory elements, such as cyclic AMP response element sites (54). Estrogen has been implicated in mediating vascular tone in response to vasoactive agents with effects ranging from vasodilation and reverse acetylcholine-induced vasoconstriction through the synthesis and release of nitric oxide (55). Additionally, vascular prostacyclin synthesis, inhibition of aortic smooth muscle cell proliferation, and

decreases in hemostatic factors are thought to be mechanisms through which estrogen can affect the heart (55). Estrogen has proved to be cardioprotective, both *in vivo* and *in vitro*, in modulating vascular tone, arterial resistance, vasodilation, and blood flow, in addition to regulating blood pressure and protection against cardiovascular injury (40). The effects of estrogen on the cardiovascular system include increased expression of the genes for nitric oxide synthase and prostacyclin, rapid vascular endothelialization after injury, inhibiting the proliferation of vascular smooth muscle cells, and preventing apoptosis (54).

Numerous mechanisms for the cardioprotection effected by estrogen have been proposed (54). This cardioprotection occurs in cardiovascular diseases including coronary artery disease, ischemia, reperfusion injury on the myocardium, and cardiac hypertrophy (54). Its effects are thought to be mediated either by its receptors or its non-transcriptional effects (55). ER-α and ER-β are both implicated in increasing intracellular concentrations of calcium and in membrane ER binding effects through receptor tyrosine kinase and protein kinases such as P13K, Akt, mitogen-activated protein kinase (MAPK), Src, and protein kinase A and C (54). Endothelialization is mediated by ER-α activation of antiapoptotic and proapoptotic MAPKs. (54). ER-β has been shown to mediate antihypertrophic effects of estrogen through inhibiting angiotensin II induced cardiac hypertrophy in a mouse model (40). Estrogen receptors are thought to attenuate cardiac hypertrophy through the use of three possible mechanisms: degrading calcinuerin, changing the expression of fetal-type genes, and suppressing TGFβ family members. Current drugs that use the signal transduction pathways of estrogen include selective estrogen receptor modulators (SERMs) as seen in treatments of breast cancers and

osteoporosis, a promise for the development of future therapies (54). Furthermore, with cardiovascular disease mortality and morbidity on the rise, treatments for its prevention and lessening its severity are essential (1).

Effect of Sex on Cardiac Hypertrophy through Profibrotic Genes

Numerous scientific studies have reported that sex has an impact on the cardiac modeling response to pressure overload (6). More specifically, more favorable cardiac remodeling to protect systolic pumping efficiency in premenopausal women is observed in cases of hypertrophy (6). Postmenopausal women, lacking the protection of estrogen, experienced less favorable remodeling, similar to cases of male hypertrophy (6). These observations have been mimicked in a TAC model of myocardial remodeling in mice (6). Two proposed mechanisms of mediation by estrogen are: (1) estrogen is a transcriptional regulator of genes implicated in hypertrophy; or (2) that estrogen may regulate angiotensin mRNA levels and ACE activity in the RAAS system (57). Therefore, although the mechanism is not well understood, the potential use of estrogen in studies of cardiac hypertrophy are necessary (6). Sex differences in TGFβ signaling have previously been reported. In a two-week TAC mouse model entitled "Androgens Contribute to Sex Differences in Myocardial Remodeling under Pressure Overload by Mechanism Involving TGF- β", TGFβ levels were elevated only in males and the use of anti-TGFβ antibodies in a TAC group of male mice greatly reduced fibrosis (6). Furthermore, when a group of male mice underwent an orchiectomy, surgical removal of the testicles, they experienced more favorable remodeling because sex-related differences were eliminated both in the physical and molecular assessments of hypertrophy (6). The unfavorable remodeling in cardiac hypertrophy can be attributed to sex differences in a downstream

mechanism involving TGFβ (6). However, this report attributed this to the detrimental effects of androgens in males rather than the protective effects of estrogen in females (6). Additionally, estrogen has been identified to affect individual gene expression in our proposed pathway. Estrogen has been shown to downregulate ACE and thus ANG II production (58). Estrogen is anti-inflammatory and may therefore inhibit the pro-inflammatory TNF-α (30 &54). As mentioned previously, estrogen suppresses TGFβ expression (6). Finally, estrogen improves the balance of MMPs and TIMPs, thus inhibiting collagen deposition (59). Therefore, estrogen may mediate its cardioprotective effects by downregulating or inhibiting the expression of ACE, MMP2, TNF-α, TGFβ, and COL6α1. This effect would result in reduced risk of heart failure by lessening the degree of hypertrophy, according to our proposed hypertrophic pathway.

Research Models for Studying Heart Failure

With 67 million Americans suffering from hypertension, a significant risk factor for cardiac hypertrophy and HF, research has focused on identifying these signaling pathways of disease progression (8). *In vivo*, studies have reported hemodynamic overload induces cardiac hypertrophy in mice through increases in RAS tissue in the heart, mRNA and protein levels of renin, ACE, angiotensinogen, and ANG II receptors (42). Cardiac hypertrophy induced by angiotensin acts in cardiomyocytes *in vivo* and *in vitro* and fibroblast cultures in an AT_1 receptor dependent manner (35 & 42).

With the understanding that ANG II is essential for the development of cardiac hypertrophy, previous research in our lab has utilized TAC, phenylephrine, and estrogen to model cardiac hypertrophy *in vivo* and *in vitro*. TAC is a well-established pressure overload system of cardiac hypertrophy (60). TAC is a surgical procedure in which a

suture is incompletely tightened around the transverse aorta, thereby increasing the resistance to blood flow from the left ventricle as depicted in figure 13 (60).

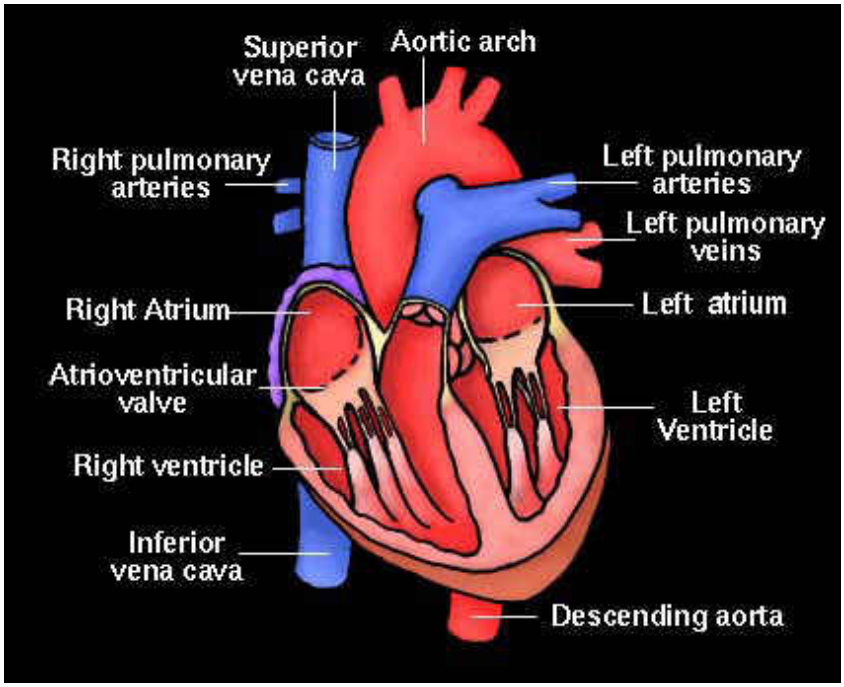

Figure 13: Schematic of Transaortic Constriction. Using the above image as a mental guide, transaortic constriction is done by tying a 6-0 suture around aortic arch between right and left carotids.

While initially compensatory, the chronic pressure overload that TAC induces leads to cardiac hypertrophy by increasing hemodynamic load on the heart (60). The suture placed on the heart leads to a pressure overload system of cardiac hypertrophy. TAC contrasts from the angiotensin II infusion model of cardiac hypertrophy in that angiotensin II is reportedly not required for pressure overload-induced hypertrophy as observed in experimental AT_1-α receptor knockout mice (14 & 42). Phenylephrine is another means of directly inducing hypertrophy directly in culture. One of the central neurohormonal abnormalities of heart failure is the chronic elevation of epinephrine and norepinephrine (NE) (61). The mechanism of phenylephrine, a synthetic drug that stimulates α_1 –receptors on blood vessels and cardiac myocytes, is similar to that of

epinephrine, and results in increased contraction, heart rate, blood pressure, and vasoconstriction (61 & 62). Activation of the α1-receptor with phenylephrine induces hypertrophy and upregulation of ANP and β-MHC (61).

Brief Recap

My research sought to elucidate the molecular basis of the cardioprotective effects of estrogen in models of cardiac hypertrophy and heart failure by identifying genes affected by its hormonal effects. In the mouse model, the TGFβ pathway was the primary research focus. Estrogen is known to suppress the profibrotic TGFβ gene and gene expression analysis was used to characterize its expression in an angiotensin infusion model of hypertrophy. In the human study, genes identified by microarray analysis that demonstrated sex-specific regulation or differential expression in non-failing and failing hearts were analyzed for sex-specific regulation. Our question remains: how does estrogen mediate its cardioprotective effects?

Overall Aim and Overarching Hypothesis

The overall goal of my thesis research seeks to expand our understanding of the molecular basis of sex on hypertrophy and heart failure, using an angiotensin II infusion mouse model of cardiac hypertrophy and clinical study of non-failing and failing human hearts. Our hypothesis was that sex influences cardiac hypertrophy and heart failure via sex-specific expression of profibrotic genes. To test this hypothesis, we proposed two specific research aims. The first specific aim established a new (to EKU) mouse model of cardiac hypertrophy to analyze further sex-specific gene expression. To do this, we utilized the ANG II pump model of cardiac hypertrophy in male and female C57/BL6

mice in order to assess sex-specific gene expression of cardiac hypertrophy markers and the prohypertrophic TGFβ pathway. The second specific aim sought to analyze the interplay between estrogen and genes in a clinical study of heart failure**.** To do this, we utilized failing and non-failing hearts male and female hearts to assess sex-specific gene expression of profibrotic COL6α1, ACE, TNFαIP1, and TGFβ all of which were genes that were identified as sex-regulated by microarray analysis as shown in Table 1.

Chapter II

SPECIFIC AIM ONE

To characterize the gene expression of prohypertrophic TGFβ family members during cardiac hypertrophy, we utilized the well-characterized ANG II mouse model for the development of cardiac hypertrophy and heart failure. Our laboratory previously used a surgical model of cardiac hypertrophy and HF, but this invasive survival surgery is not currently possible at EKU, thus necessitating the less invasive ANG II model.

The working hypothesis of this aim is that ANG II administered over a period of 4 weeks induces hypertension, as well as direct effects on cardiomyocytes, leading to cardiac **hypertrophy and subsequent heart failure**

a. Our first objective was to establish a model of cardiac hypertrophy by way of ANG II infusion in BL-6 mice and compare ANG II induced cardiac hypertrophy in male and female mice. A higher heart-to-body ratio and heart-to-tibia ratio would indicate a greater degree of cardiac hypertrophy. Taqman gene expression assays of the standard markers of cardiac hypertrophy, ANP, BNP, α-MHC, and β-MHC, were used to verify signature gene expression patterns in hypertrophy.
b. The second objective of the experiment was to use RT-PCR to determine sex specific expression levels of TGFβ target genes. It would be expected that estrogen mediates its antihypertrophic and cardioprotective effects by inducing transcription of miRNAs, which silence the expression of prohypertrophic members of the TGFβ family. We hypothesized that estrogen

suppresses expression of TGFβ either by increasing miRNAs which target TGFβ family members.

Specific Aim One Materials and Methods

Rodent Use and Surgery

Six female and six male C57BL6 mice were purchased from Jackson Laboratories. In addition to marking their ears with identifiable tags, mice of both sexes were numbered one through six upon arrival. Pump implantation took place in collaboration with Dr. Lindsay Calderon. The desired infusion dosage of angiotensin was 500ng/kg/min. The recorded weight of each mouse was used to calculate the concentration of ANG II or saline for each corresponding subcutaneous osmotic pump. Calculations using the largest mouse's initial starting weight and estimated weight gain provided the dose per hour per animal. The concentration of the ANG II had to be individualized per pump/mouse in order to ensure the proper dosage. The pump releases fluid at a constant rate and therefore the concentration of the released fluid determines the dosage. The Alzet osmotic pumps pumped at a 0.25uL/hr rate. Pumps were loaded according to this spreadsheet, weighed, and primed in an incubator overnight in individual tubes of ANG II or saline solution. A carprofen injection of 50cc with saline was prepared according to the spreadsheet then the surgery room was set up with the appropriate use of the isoflurane and oxygen tank. A four-week infusion period occurred before the mice were euthanized using isoflurane and cervical dislocation. A mouse was placed in the isoflurane chamber until it was anesthetized and transferred onto a nose cone that continuously fed isoflurane to maintain a proper plane of anesthesia during surgery. The hair on the back of the mouse was shaved and Nair and alcohol pads were used to remove any excess hair. The injection of carprofen was applied by pulling the skin away from the

hip region. Using forceps and scissors, an incision was made into the mouse's shaved back. A path was made along the side of the body and the spine into which the pump was inserted before suturing and glue was lightly applied on top of the sutures. The mouse was then taken off the nose cone and placed back into the cage after movement was exhibited. This procedure was repeated for each mouse. Mice were monitored for an hour after surgery and daily for signs of distress or removing of their sutures.

Heart and Tibia Collection

After euthanasia and recording the body weight and pump weight, the heart tissue was removed from the body cavity, washed in sterile PBS and dried before being weighed. The base of the heart was stored in formalin overnight before being transferred to a tube of ethanol for histology lab use. The apex was stored in RNALater at 4°C. The tibia was extracted, length measured, recorded, and discarded.

RNA Isolation

RNA isolation from the heart utilized the Trizol reagent, lysing D matrix tubes, and FastPrep. The apex of the heart was place in the lysing tube with 1mL of Trizol for homogenization in the FastPrep at 6.0m/s for 45 seconds three or four times with rest periods on ice for 1 minute in between each run. RNA isolation then followed Ambion by Life Technologies Trizol Reagent Protocol. RNA was extracted from the heart using Trizol (Invitrogen). The media was removed from the cells and washed with 3mL of PBS, which was then removed. Next, 1mL of Trizol was added to each flask and the cells were scraped off the flask using a cell scraper, then removed from the flask and placed in a 1.5mL tube. The tubes were incubated and shaken at room temperature for 5 minutes

before being centrifuged 2,000xg for 10 minutes at 4°C and supernatant transferred to a new tube. To the new tube, 200mL of molecular biology grade chloroform (MP Biomedicals) was added, shaken vigorously for 15 seconds by hand and incubated at room temperature for 3 minutes. Centrifugation was repeated at 12,000xg for 15 minutes at 4°C. The colorless upper aqueous phase was mixed with 0.5mL molecular biology grade isopropanol (Fischer Scientific) in a new 1.5mL tube. The samples were incubated for a minimum of 30 minutes.

After the incubation period the samples were centrifuged at 12,000xg for 10 minutes at 4°C. The supernatant was removed and the pellet was washed with 1mL molecular biology grade 75% ethanol (Fischer Scientific). The samples were centrifuged at 7,500xg for 10 minutes at 4°C and supernatant removed. The pellets were allowed to air dry for 5 minutes and resuspended in 100ul of nuclease free water (Fischer Scientific) if an RNA pellet was visible, or 50ul of nuclease free water if an RNA pellet was not present.

RNA quality was determined using a Nanodrop (ThermoScientific), in which the RNA concentration and 260/280 values were recorded. A 260/280 value of approximately 2.0 was used to denote pure RNA. The RNA was stored at -80°C.

RT Reaction

Total RNA was converted to cDNA through reverse transcription using the high capacity cDNA reverse transcription kit and protocol by Applied Biosystems. RNA was converted to cDNA using a thermal cycler set for the following program: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds, and hold at 4°C. The cDNA products were stored at -20°C for a maximum of thirty days.

Taqman PCR

Taqman RT-PCR was performed using the following commercially available primers for the following genes: glyceraldehyde 3-phosphade dehydrogenase (GAPDH), BNP, ANP, α-MHC, β-MHC, TGFβ, TGFβR1, TGFβR2, and TGFβR3 purchased from Applied Biosystems. Taqman assays were performed according to the Fast Mastermix protocol, using 20ul total volume and 2ul of cDNA. The plate was covered with MicroAmp 48 well optical adhesive film (Applied Biosystems) and centrifuged at 1000xg for 1 minute. The step one real-time PCR system by Applied Biosystems was set to the following program: Step One: 50°C for 2 minutes, 95°C for 10 minutes; Step Two: 95°C for 15 seconds, 60°C for 1 minutes, repeat 40 times. Relative expression will be determined using the delta, delta CT calculation (2^-(delta CT experimental- average delta CT control) to determine fold change relative to GAPDH expression. Results are expressed as mean ± SEM. ANOVA and Welch's T-test statistical analysis was run to compare expression of each primer per treatment and sex. The expression data was interpreted to correspond to degree of hypertrophy or prohypertrophic signaling of TGFβ and its receptors.

Specific Aim One Results

To determine the impact of sex on cardiac hypertrophy induced by an ANG II infusion model, we implanted ANG II infusion pumps in six male and six female C57/BL6 mice. Three mice of each sex were infused with the saline while the other three were infused with ANG II at a 0.25uL/hr rate. During the surgery one female mouse was euthanized early. Therefore, the final numbers were three males with saline, three males with ANG II, two females with saline, and three females with ANG II (Table 3). After a four-week infusion period, heart weight to body weight ratios and heart weight to tibia length ratios in Table 4 & Table 5 and hearts were collected to determine hypertrophy by gene expression with primers detailed in Table 6.

Table 3: Mouse Sample ID Numbers and Respective Surgery.

Sample ID	Sex	Surgery	Sample ID	Sex	Surgery
1001	Male	ANG II	2001	Female	ANG II
1002	Male	ANG II	2002	Female	ANG II
1003	Male	ANG II	2003	Female	ANG II
1004	Male	Saline	2004	Female	Saline
1005	Male	Saline	2005	Female	Saline
1006	Male	Saline	2006	Female	Saline

Table 4: Mouse Sample ID Numbers with Results after Surgery.

Sex	Number	Body Weight (g)	Tibia Length (mm)	Heart Weight (g)	Pump Weight (g)
Male	1001	27.6	16.45	0.1409	1.4
Male	1002	26.5	16.48	0.163	1.4
Male	1003	25.5	16.46	0.1376	1.4
Male	1004	26.7	17.8	0.12	1.3
Male	1005	28	17.43	0.1367	1.3
Male	1006	26.1	16.67	0.1343	1.4
Female	2001	21.9	17.34	0.1294	1.3
Female	2002	20.5	17.64	0.1074	1.3
Female	2003	23.2	18.851	0.1301	1.4
Female	2004	22.4	16.72	0.104	1.3
Female	2006	23.4	17.19	0.1055	1.4

Table 5: Mouse sample ID Numbers with Results after Surgery.

Sex	Number	Heart Weight/Tibia Length (g/mm)	Heart Weight/(Body Weight-Pump Weight) (g/g)
Male	1001	0.0086	0.00538
Male	1002	0.0099	0.0065
Male	1003	0.0084	0.0057
Male	1004	0.0067	0.0047
Male	1005	0.0078	0.0051
Male	1006	0.0081	0.0054
Female	2001	0.0075	0.0063
Female	2002	0.0061	0.0056
Female	2003	0.0069	0.0060
Female	2004	0.0062	0.0049
Female	2006	0.0061	0.0048

Table 6: Real Time PCR Primers used in Specific Aim One.

Name	Gene Name	Assay ID
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Mm99999915_g1
NPPB	Natriuretic peptide B (BNP)	Mm01255770_g1
NPPA	Natriuretic peptide A (ANP)	Mm01255747_g1
Myh6	Myosin Heavy Chain Alpha (α-MHC)	Mm00440359_m1
Myh7	Myosin Heavy Chain Beta (β-MHC)	Mm00600555_m1
TGFβ	Transforming Growth Factor Beta Ligand	Mm01227699_m1
TGFβR1	Transforming Growth Factor Beta Receptor 1	Mm01353997_m1
TGFβR2	Transforming Growth Factor Beta Receptor 2	Mm03024091_m1
TGFβR3	Transforming Growth Factor Beta Receptor 3	Mm00803538_m1

Heart weight to body weight ratios were analyzed in Figures 14 and 15 and statistical analysis of these results are detailed in tables 7 and 8. Heart weight to tibia length ratios were analyzed in Figures 16 and 17. Hypertrophy marker expression were analyzed in Figures 18 through 25 and statistical analysis of these results are detailed below each subsequent figure in tables 9 through 24. TGFβ family member expression were analyzed in Figures 26 through 33. Statistical analysis of TGFβ family member expression is listed in tables 25 through 36 under each corresponding experiment's results.

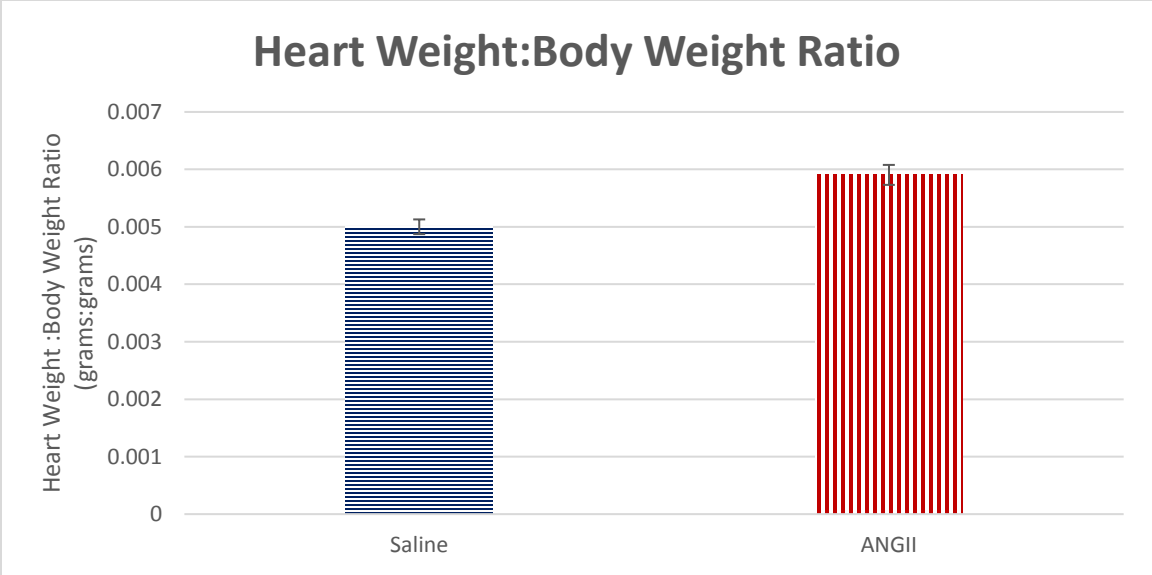

Figure 14: Average Heart Weight:Body Weight Ratios by Treatment. Average saline HW:BW ratio was 0.0050. Average ANG II HW:BW ratio was 0.0059. ANGII treatment induced a 118% increase in HW:BW. This result was statistically significant according to a Welch's T-test.

Table 7: T Test of Average Heart Weight:Body Weight Ratios.

Comparison Group	P-value
Saline vs. ANG II	0.0026**

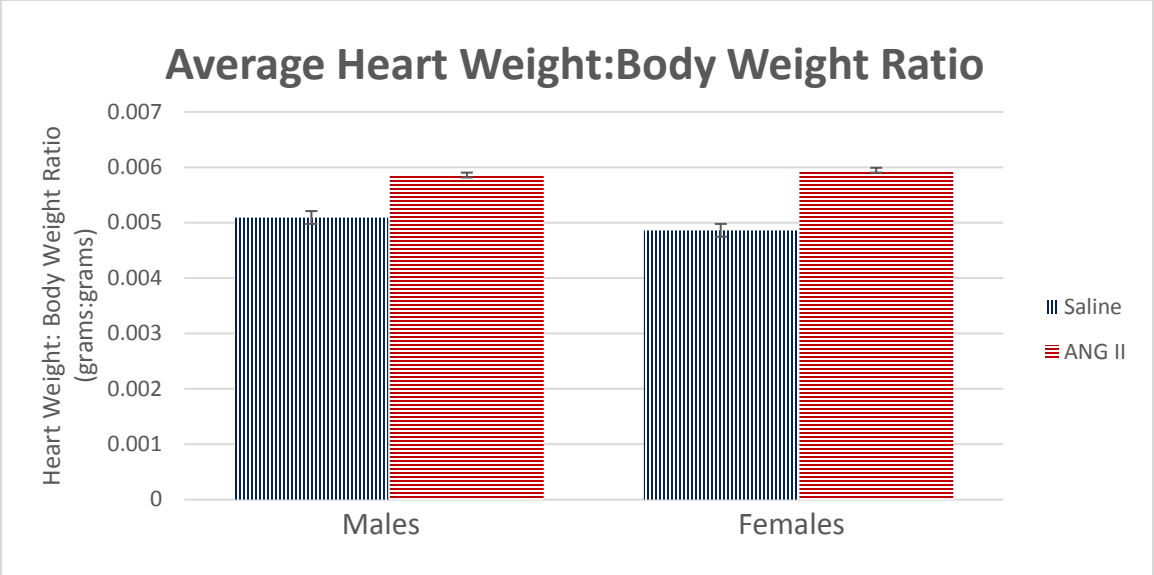

Figure 15: Average Heart Weight:Body Weight Ratios of each Sex-Specific Treatment Group. Male saline HW:BW ratio was 0.0051. Male ANG II HW:BW ratio was 0.0059. Female Saline HW:BW ratio was 0.0049. Female ANG II was 0.0059. The value increase between male saline and male ANG II was 116% although this was not significant by ANOVA or Welch's T-test p-values. The valued increase between female saline and female ANG II was 120%. This was found to be significant by Welch's T-Test analysis indicated statistical significance (p-value 0.0234) between female saline and ANG II treatments.

Table 8: ANOVA of Average Heart Weight:Body Weight Ratios.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.2847
Female Saline vs. Female ANG II	0.1262
Male Saline vs. Female Saline	0.9920
Male ANG II vs. Female ANG II	>0.9999

Table 9: T Test of Average Heart Weight:Body Weight Ratios.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.1343
Female Saline vs. Female ANG II	0.0234*
Male Saline vs. Female Saline	0.3811
Male ANG II vs. Female ANG II	0.8346

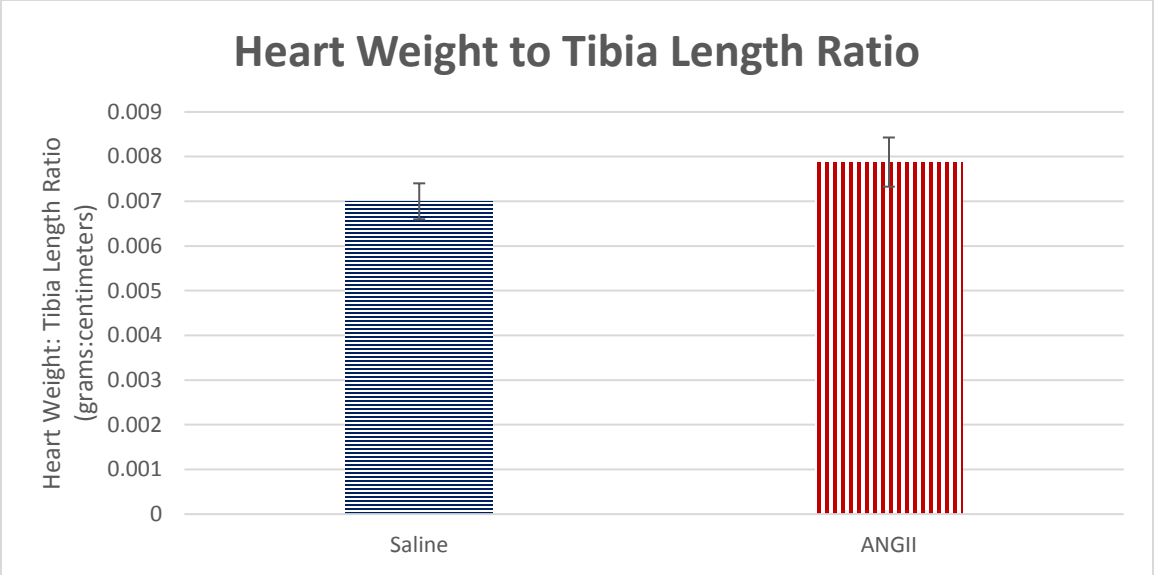

Figure 16: Average Heart Weight:Tibia Length Ratios by Treatment. Average saline HW:TL ratio was 0.0070. Average ANG II HW:TL ratio was 0.0079. ANGII treatment induced a 113% increase in HW:TL ratio. This result was not statistically significant according to a Welch's T-test.

Table 10: T Test of Average Heart Weight:Tibia Length Ratios.

Comparison Group	P-value
Saline vs. ANG II	0.2310

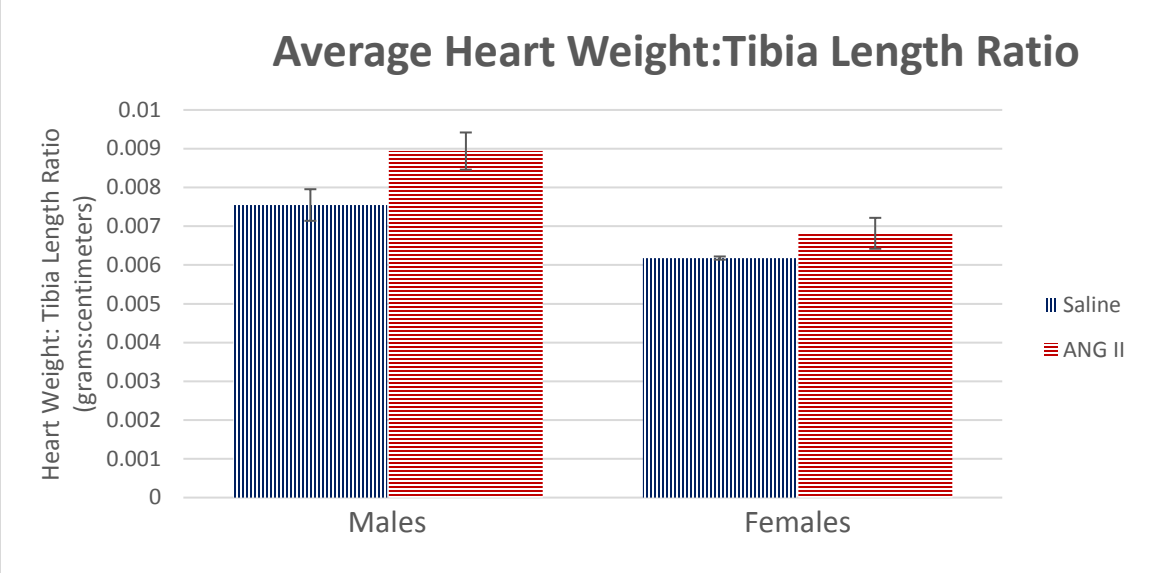

Figure 17: Average Heart Weight:Tibia Length Ratios of each Sex-Specific Treatment Group. Male saline HW:TL ratio was 0.0075. Male ANG II HW:TL ratio was 0.0089. Female Saline HW:TL ratio was 0.0062. Female ANG II HW:TL ratio was 0.0068. The value increase between male saline and male ANG II was 118% although this was not significant by ANOVA or Welch's T-test p-values. The valued increase between female saline and female ANG II was 109%. This too was not significant by ANOVA or Welch's T-test p-values.

Table 11: ANOVA of Average Heart Weight:Tibia Length Ratios.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.2312
Female Saline vs. Female ANG II	0.9208
Male Saline vs. Female Saline	0.3381
Male ANG II vs. Female ANG II	0.0415*

Table 12: T Test of Average Heart Weight:Tibia Length Ratios.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.0993
Female Saline vs. Female ANG II	0.2496
Male Saline vs. Female Saline	0.0769
Male ANG II vs. Female ANG II	0.0287*

I. Cardiac Hypertrophy Marker PCR Results

a. ANP Expression

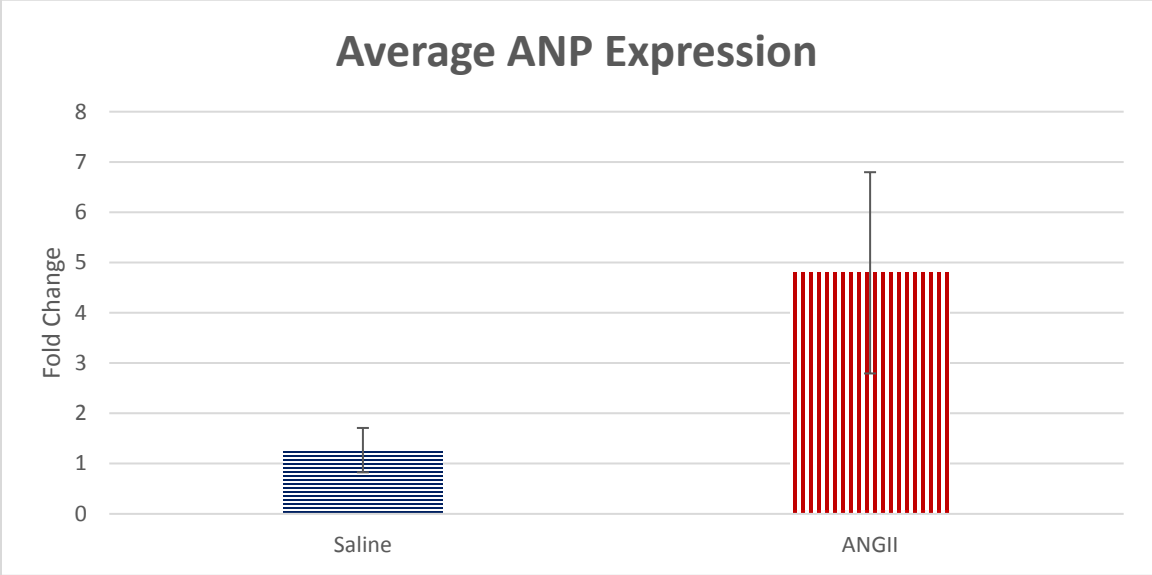

Figure 18: Average ANP Expression by Treatment. Average saline ANP expression was 1.2680. Average ANG II ANP expression was 4.7950. ANGII treatment induced a 378% increase in average ANP expression. This result was not statistically significant according to a Welch's T-test.

Table 13: T Test of Average ANP Expression.

Comparison Group	P-value
Saline vs. ANG II	0.1407

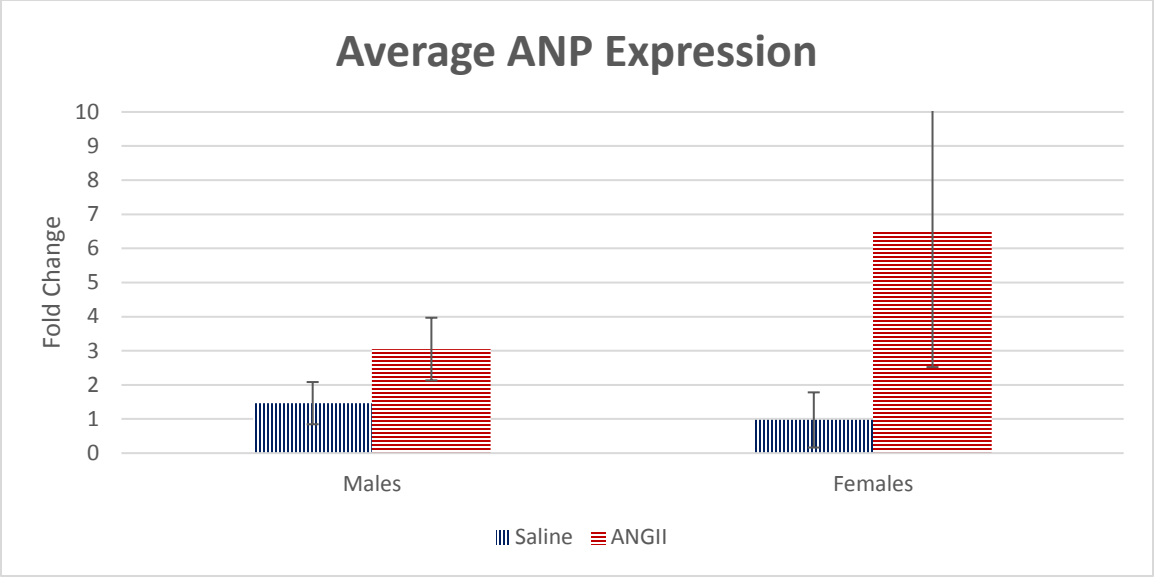

Figure 19: Average ANP Expression by Sex-Specific Treatment. Average ANP expression for male saline was 1.4650 and for male 3.0503. Female average ANP expression is 0.9724 and 6.5397. The value increase between the male treatment groups was 208%. This result was not statistically significant according to ANOVA or Welch's T-test. The value increase between the female treatment groups was 673%. This result was not statistically significant according to ANOVA or Welch's T-test.

Table 14: ANOVA of Average ANP Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.9975
Female Saline vs. Female ANG II	0.6493
Male Saline vs. Female Saline	>0.9999
Male ANG II vs. Female ANG II	0.8897

Table 15: T Test of Average ANP Expression

Comparison Group	P-value
Male Saline vs. Male ANG II	0.2349
Female Saline vs. Female ANG II	0.2989
Male Saline vs. Female Saline	0.6733
Male ANG II vs. Female ANG II	0.4790

b. BNP Expression

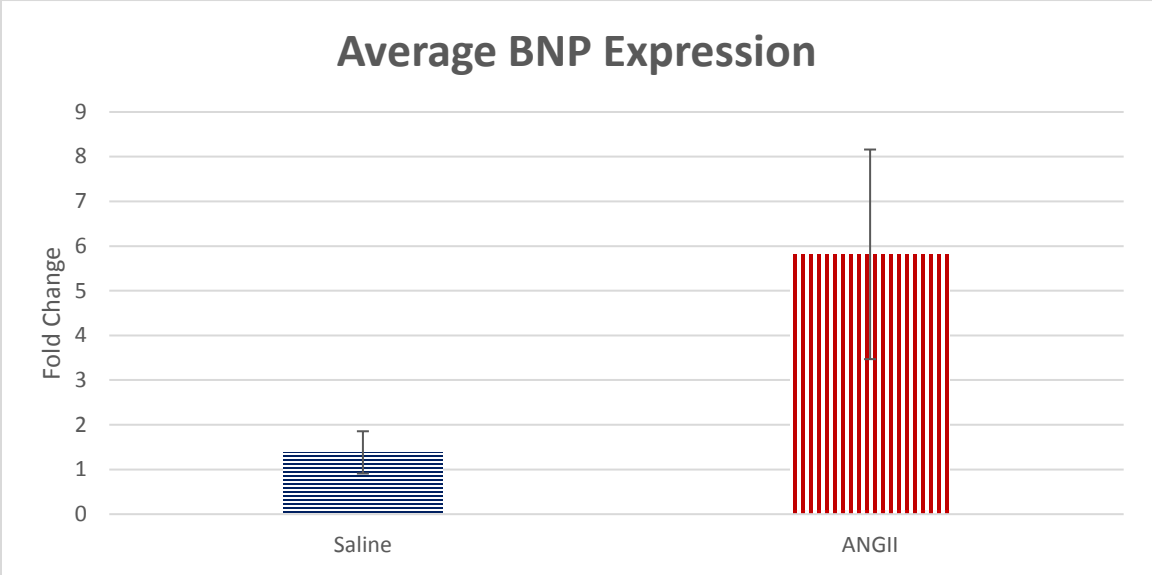

Figure 20: Average BNP Expression by Treatment. Average saline BNP expression was 1.3824. Average ANG II BNP expression was 5.8140. ANGII treatment induced a 421% increase in average BNP expression. This result was not statistically significant according to a Welch's T-test.

Table 16: T Test of Average BNP Expression.

Comparison Group	P-value
Saline vs. ANG II	0.1188

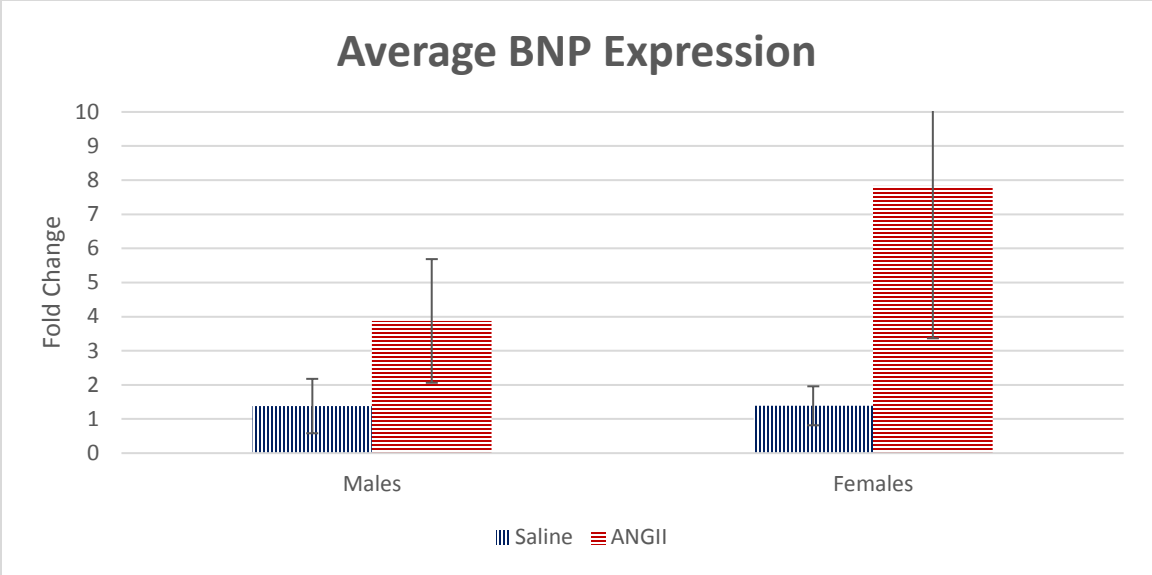

Figure 21: Average BNP Expression by Sex-Specific Treatment. Average BNP expression was 1.3794 for saline males and 3.8745 for ANG II males. Average BNP expression was 1.3868 for saline females and 7.8275 for ANG II females. The value increase between the male treatment groups was 281%. This result was not statistically significant according to ANOVA or Welch's T-test. The value increase between the female treatment groups was 673%. This result was not statistically significant according to ANOVA or Welch's T-test.

Table 17: ANOVA Average BNP expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.9900
Female Saline vs. Female ANG II	0.6617
Male Saline vs. Female Saline	>0.9999
Male ANG II vs. Female ANG II	0.8964

Table 18: T Test of Average BNP Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.3301
Female Saline vs. Female ANG II	0.2849
Male Saline vs. Female Saline	0.9944
Male ANG II vs. Female ANG II	0.4730

c. α-MHC Expression

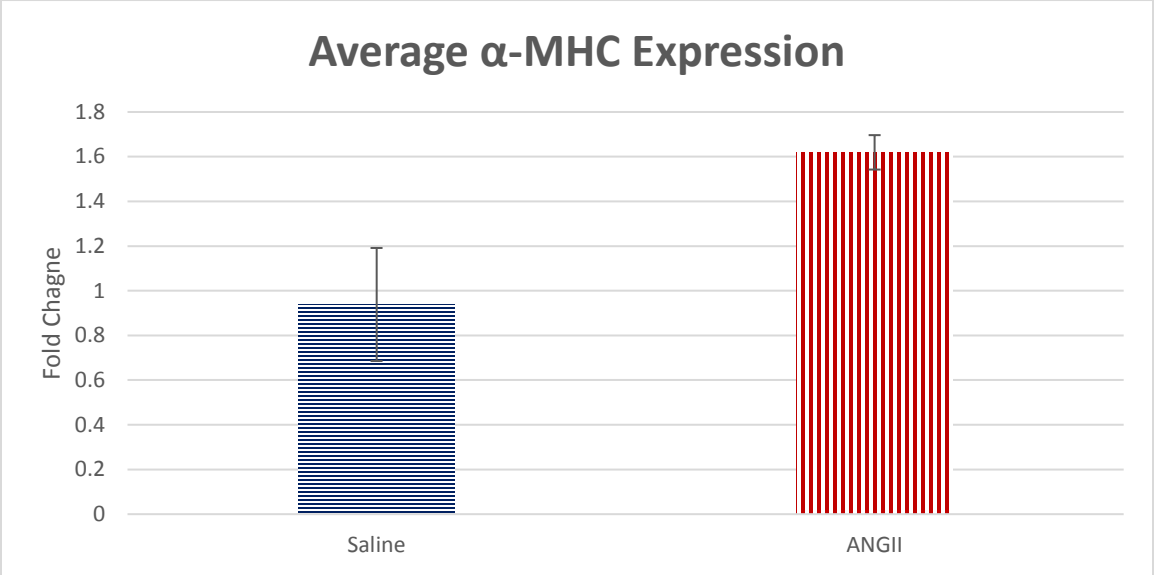

Figure 22: Average α-MHC Expression by Treatment. Average saline α-MHC expression was 0.9384. Average ANG II α-MHC expression was 1.6194. ANGII treatment induced a 173% in average α-MHC expression. This result was not statistically significant according to a Welch's T-test.

Table 19: T Test of Average α -MHC Expression.

Comparison Group	P-value
Saline vs. ANG II	0.0523

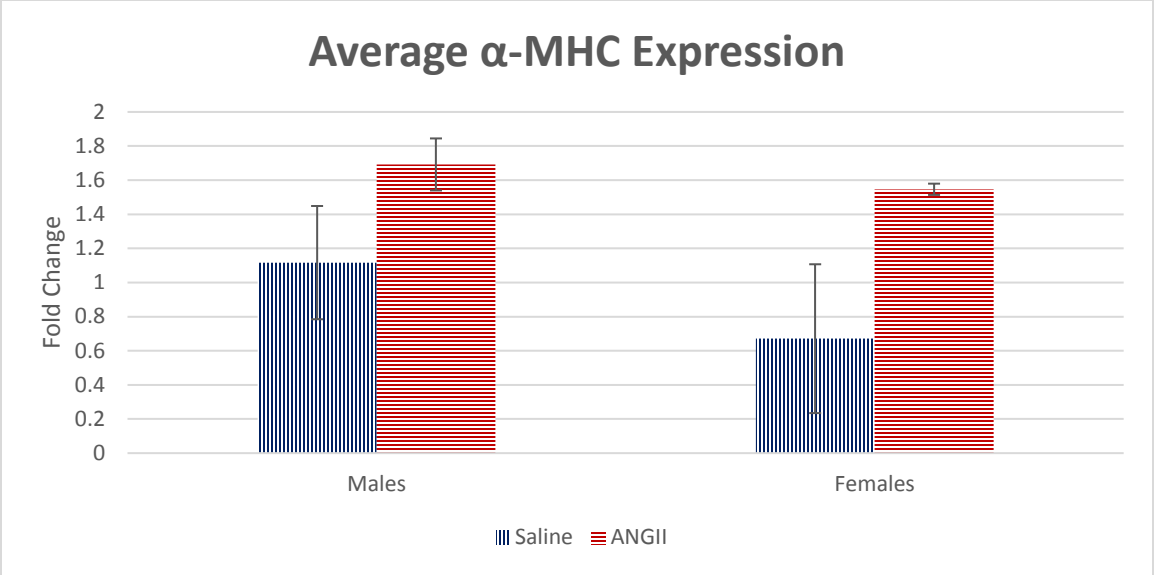

Figure 23: Average α-MHC Expression by Sex-Specific Treatment. Average α-MHC expression was 1.1166 and 1.6922 for saline and ANG II males respectively. Average α-MHC expression was 0.6709 and 1.5466 for saline and ANG II female respectively. The value increase between the male treatment groups was 152%. This result was not statistically significant according to ANOVA or Welch's T-test. The value increase between the female treatment groups was 231%. This result was not statistically significant according to ANOVA or Welch's T-test.

Table 20: ANOVA of Average α-MHC Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.5687
Female Saline vs. Female ANG II	0.2774
Male Saline vs. Female Saline	0.8542
Male ANG II vs. Female ANG II	0.9989

Table 21: T Test of Average α -MHC Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.2193
Female Saline vs. Female ANG II	0.2928
Male Saline vs. Female Saline	0.4968
Male ANG II vs. Female ANG II	0.4419

d. β-MHC Expression

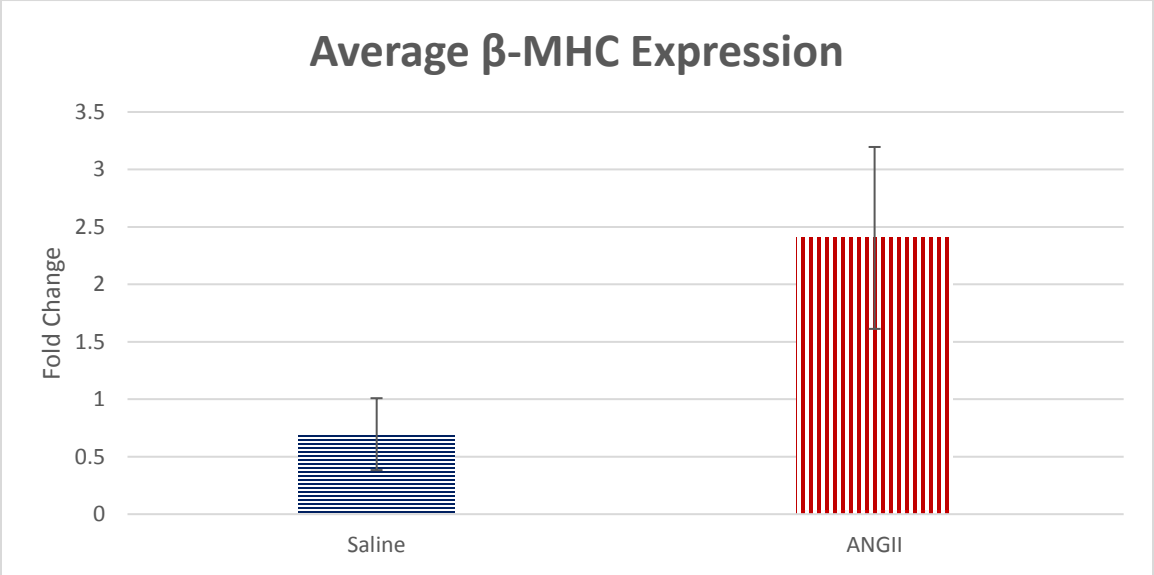

Figure 24: Average β-MHC Expression by Treatment. Average saline β-MHC expression was 0.6962. Average ANG II β-MHC expression was 2.4039. ANGII induced a 345% increase in average β-MHC expression. This result was not statistically significant according to a Welch's T-test.

Table 22: T Test of Average β-MHC Expression.

Comparison Group	P-value
Saline vs. ANG II	0.0523

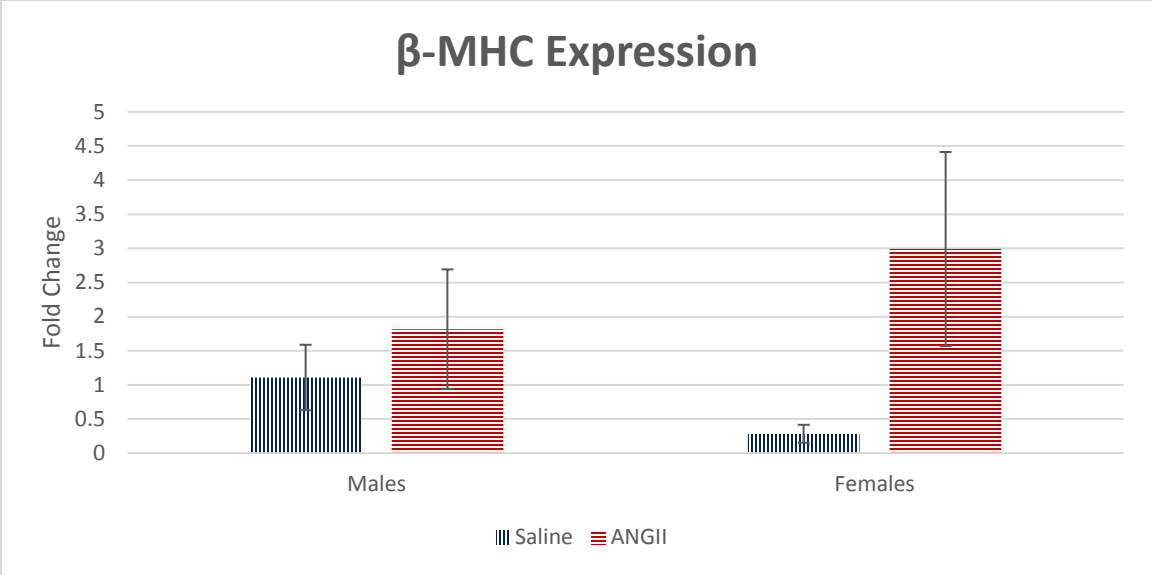

Figure 25: Average β-MHC Expression by Sex-Specific Treatment. Average β-MHC expression was 1.1090 and 1.8173 for saline and ANG II males respectively. Average β-MHC expression was 0.2835 and 2.9905 for saline and ANG II female respectively. The value increase between the male treatment groups was 164%. This result was not statistically significant according to ANOVA or Welch's T-test. The value increase between the female treatment groups was 1055%. This result was not statistically significant according to ANOVA or Welch's T-test.

Table 23: ANOVA of Average β-MHC Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.9656
Female Saline vs. Female ANG II	0.3781
Male Saline vs. Female Saline	0.9592
Male ANG II vs. Female ANG II	0.8305

Table 24: T Test of Average β-MHC Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.5312
Female Saline vs. Female ANG II	0.1964
Male Saline vs. Female Saline	0.3205
Male ANG II vs. Female ANG II	0.5284

II. TGFβ Family Member PCR Results

a. TGFβ expression

Figure 26: Average TGFβ Expression by Treatment. Average saline TGFβ expression was 2.1376. Average ANG II TGFβ expression was 2.3223. ANGII induced 109% increase in average TGFβ expression. This result was not statistically significant according to a Welch's T-test.

Table 25: T Test of Average TGFβ Expression.

Comparison Group	P-value
Saline vs. ANG II	0.8600

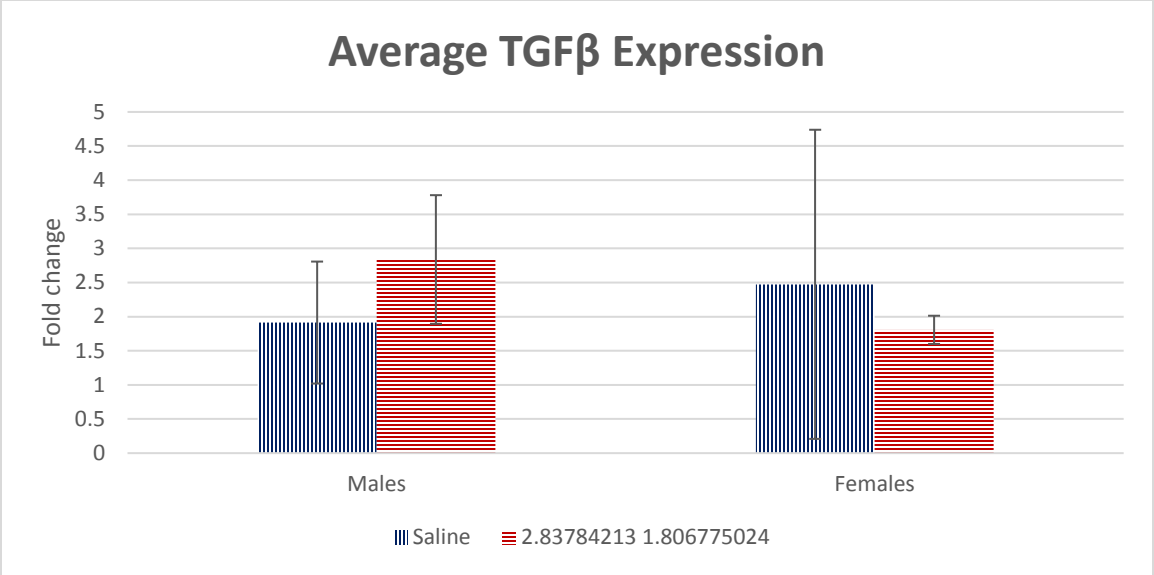

Figure 27: Average TGFβ Expression by Sex-Specific Treatment. Male saline TGFβ expression was 1.9135. Male ANG II TGFβ expression was 2.8378. Female Saline TGFβ expression was 2.4538. Female ANG II was 1.8068. The value increase between the male treatment groups was 148%. This result was not statistically significant according to ANOVA or Welch's T-test. The value increase between the female treatment groups was 74%. This result was not statistically significant according to ANOVA or Welch's T-test.

Table 26: ANOVA of Average TGFβ Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.9893
Female Saline vs. Female ANG II	0.9990
Male Saline vs. Female Saline	0.9996
Male ANG II vs. Female ANG II	0.9816

Table 27: T Test of Average TGFβ Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.5161
Female Saline vs. Female ANG II	0.8178
Male Saline vs. Female Saline	0.8485
Male ANG II vs. Female ANG II	0.3886

b. TGFβR1

Figure 28: Average TGFβ-Receptor 1 Expression by Treatment. Average saline TGFβR1 expression was 0.7852. Average ANG II TGFβR1 expression was 0.8810. ANGII induced 112% increase in average TGFβR1 expression. This result was not statistically significant according to a Welch's T-test.

Table 28: T Test of Average TGFβ-Receptor 1 Expression.

Comparison Group	P-value
Saline vs. ANG II	0.8897

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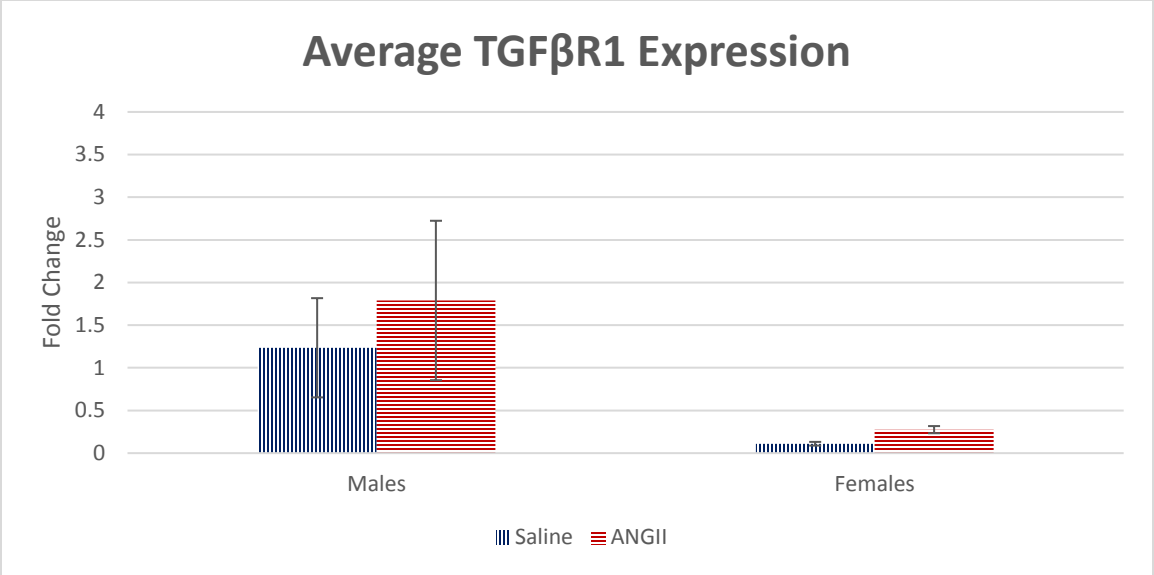

Figure 29: Average TGFβ-Receptor 1 Expression by Sex-Specific Treatment. Male saline TGFβR1 expression was 1.2352 Male ANG II TGFβR1 expression was 1.7895. Female Saline TGFβR1 expression was 0.1102. Female ANG II was 0.2753. The value increase between the male treatment groups was 145%. This result was not statistically significant according to ANOVA or Welch's T-test. The value increase between the female treatment groups was 250%. This result was statistically significant (0.0436) according to Welch's T-test.

Table 29: ANOVA of Average TGFβ-Receptor 1 Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.9872
Female Saline vs. Female ANG II	>0.9999
Male Saline vs. Female Saline	0.7598
Male ANG II vs. Female ANG II	0.4993

Table 30: T Test of Average TGFβ-Receptor 1 Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.7189
Female Saline vs. Female ANG II	0.0436*
Male Saline vs. Female Saline	0.1926
Male ANG II vs. Female ANG II	0.4118

c. TGFβR2

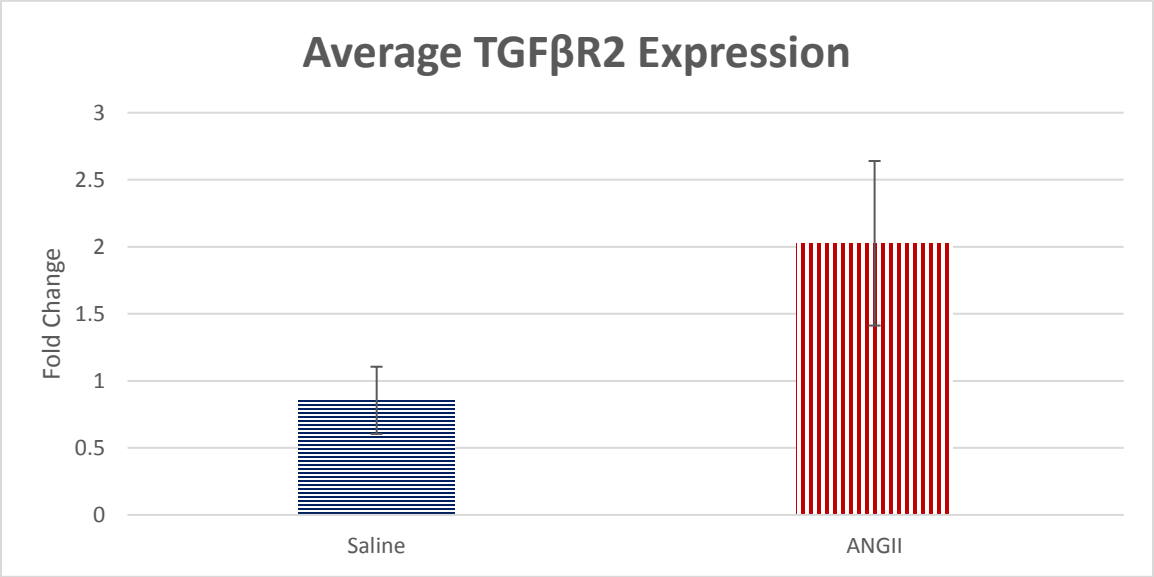

Figure 30: Average TGFβ-Receptor 2 Expression by Treatment. Average saline TGFβR2 expression was 0.8548. Average ANG II TGFβR2 expression was 2.0262. ANGII induced 237% increase in average TGFβR2 expression. This result was not statistically significant according to a Welch's T-test.

Table 31: T Test of Average TGFβ-Receptor 2 Expression.

Comparison Group	P-value
Saline vs. ANG II	0.1233

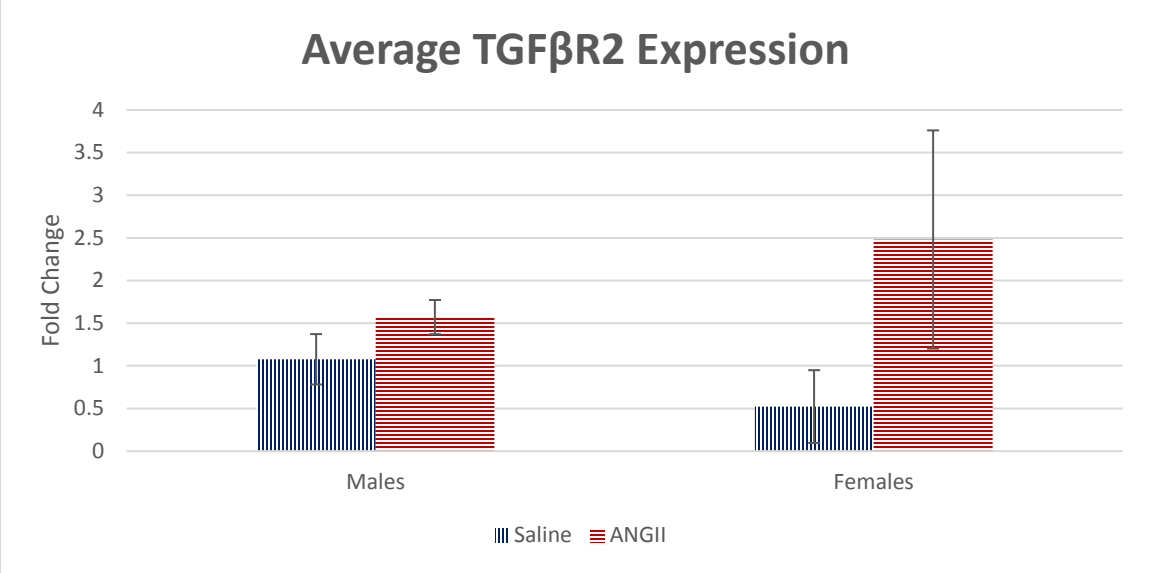

Figure 31: Average TGFβ-Receptor 2 Expression by Sex-Specific Treatment. Male saline TGFβR2 expression was 1.0766. Male ANG II TGFβR2 expression was 1.5712. Female Saline TGFβR2 expression was 0.5222. Female ANG II was 2.4812. The value increase between the male treatment groups was 146%. This result was not statistically significant according to ANOVA or Welch's T-test. The value increase between the female treatment groups was 475%. This result was not statistically significant according to ANOVA or Welch's T-test.

Table 32: ANOVA of Average TGFβ-Receptor 2 expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.9979
Female Saline vs. Female ANG II	0.5659
Male Saline vs. Female Saline	0.9979
Male ANG II vs. Female ANG II	0.9545

Table 33: T Test of Average TGFβ-Receptor 2 Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.2471
Female Saline vs. Female ANG II	0.2629
Male Saline vs. Female Saline	0.3991
Male ANG II vs. Female ANG II	0.5520

d. TGFβR3

Figure 32: Average TGFβ-Receptor 3 Expression by Treatment. Average saline TGFβR3 expression was 0.9574. Average ANG II TGFβR3 expression was 1.9338. ANGII induced 202% increase in average TGFβR3 expression. This result was not statistically significant according to a Welch's T-test.

Table 34: T Test of Average TGFβ-Receptor 3 Expression.

Comparison Group	P-value
Saline vs. ANG II	0.3106

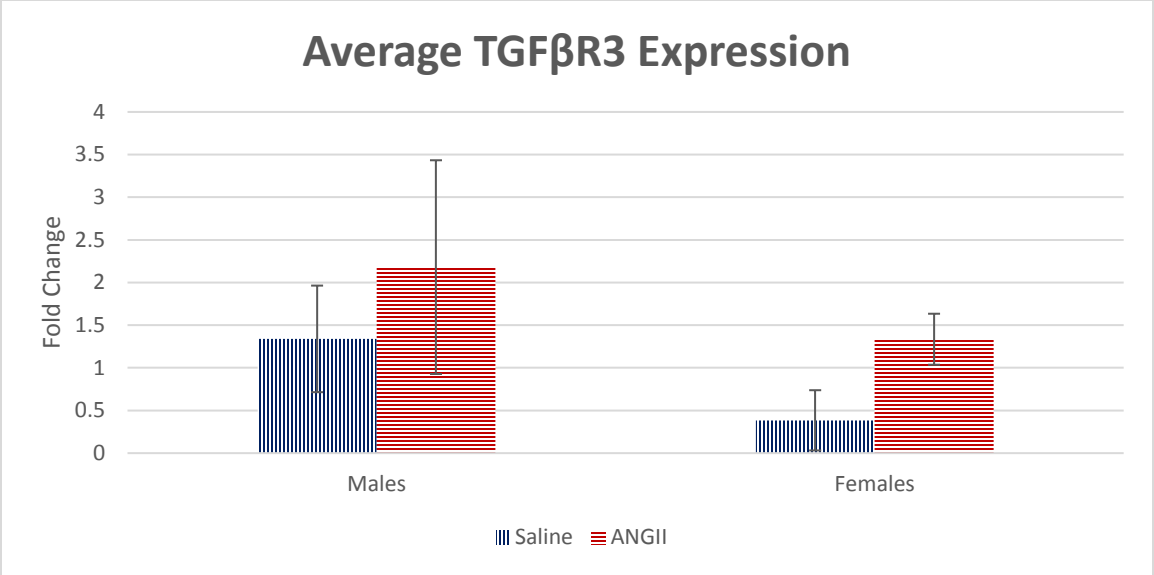

Figure 33: Average TGFβ-Receptor 3 Expression by Sex-Specific Treatment. Male saline TGFβR3 expression was 1.3392. Male ANG II TGFβR3 expression was 2.1805. Female Saline TGFβR3 expression was 0.3846. Female ANG II was 1.3353. The value increase between the male treatment groups was 163%. This result was not statistically significant according to ANOVA or Welch's T-test. The value increase between the female treatment groups was 347%. This result was not statistically significant according to ANOVA or Welch's T-test.

Table 35: ANOVA of Average TGFβ-Receptor 3 Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.9770
Female Saline vs. Female ANG II	0.9758
Male Saline vs. Female Saline	0.9753
Male ANG II vs. Female ANG II	0.9765

Table 36: T Test of Average TGFβ-Receptor 3 Expression.

Comparison Group	P-value
Male Saline vs. Male ANG II	0.5911
Female Saline vs. Female ANG II	0.1571
Male Saline vs. Female Saline	0.2786
Male ANG II vs. Female ANG II	0.5730

Specific Aim One Discussion

Comparison between the ANG II group versus the saline group indicated that our model was successful in inducing hypertrophy. Male-female differences were not observed in our model, though significance was observed between the female saline and female ANG II group. These observations differed from our previous TAC model which demonstrated a clear male-female difference in degree of hypertrophy as little as 2 weeks post TAC. This was a preliminary study intended to be expanded once the hypertrophy phenotype was established, and therefore only a small number of mice were used. Both HW:BW and HW:TL ratios indicated hypertrophy between the treatment of each sex; however, the ratios either did not vary or did not vary greatly across sex and treatment. HW:BW ratios increased in each sex between saline and ANG II treated mice, indicating that ANG II did induce hypertrophy in each sex. However, the difference did not translate across sex and treatment. The male and female HW:BW ratio of ANG II treated mice was 0.0059 for both sexes. HW:TL ratio increased between male saline and male ANG II groups by 0.0014 but not as drastically in female saline and ANG II groups. Between treatments and sex, the difference was 0.0013 between male saline and female saline and 0.0021 between male ANG II and female ANG II. However, statistical analyses indicated no significant difference for the majority of our comparison groups based on sex and treatment. HW:BW ratios were expected to be decreased in females when compared to males, due to the cardioprotection afforded to their hearts. The HW was expected to increase in both sexes, but to a greater degree in males, an indication of greater hypertrophy. This was not observed. The HW:BW ratio in ANG II groups of both sexes did not differ, indicating that hypertrophy occurred almost to the same extent. Sex-

difference did not seem to affect HW:BW ratios. TL was another means measuring the growth of the mice over the infusion period. HW:TL ratios were expected to be decreased in females when compared to males, due to the cardioprotection afforded to their hearts. Again HW was expected to increase in both sexes, but to a greater degree in males, an indication of greater hypertrophy. This was not observed. The HW:TL ratios indicated hypertrophy did occur in each sex, though the ratios were not as large as the HW:BW ratios per sex. The HW:TL ratios did not greatly differ between the ANG II receiving groups, supporting the evidence that hypertrophy may have occurred to the same degree. Sex-difference did not seem to affect HW:TL ratios. A future mouse model with a greater or longer infusion period may result in greater degrees of hypertrophy. Additionally, a larger sample size may allow for significant statistical analysis results.

Re-expression of fetal genes is known to be a biomarker of cardiac hypertrophy. ANP and BNP expression increase in hypertrophy, whereas α-MHC decreases as β-MHC increases (15,16, &17). In our model, ANP and BNP expression increased in our ANG II treatment groups in comparison with our saline treatment groups in both sexes. ANG II males exhibited a 3-fold increase in ANP expression and BNP expression in comparison with their saline male counterparts. Females exhibited a 6.5-fold and >7-fold increase in ANP and BNP expression respectively in comparison to saline female expression. ANG II-treated females exhibited greater expression of both natriuretic peptides. Though we expected hypertrophy, and therefore the natriuretic peptides that increase with its progression to be expressed greater in our male mice because of a greater degree of hypertrophy, we know that increased expression of these peptides is meant to be cardioprotective. Therefore, the increased peptide expression in females would indicate

increased hypertrophy than their male counterparts and an attempt to afford cardioprotection. Males may have expressed lesser amounts of these peptides because less of their cardioprotection was afforded. Sex-difference in the females may have further upregulated the expression of the cardioprotective fetal genes in our model. Further investigation is necessary to conclude this with confidence. A future mouse model with a larger sample size may allow for better statistical analysis results.

Expression of the myosin heavy chains alpha and beta did not occur as hypothesized. Myosin alpha concentration is known to decrease in hypertrophy and myosin beta concentration is known to increase. We expected decreased alpha expression in our males undergoing hypertrophy in comparison with our females undergoing hypertrophy due to the cardioprotection afforded them. Our results indicated decreased alpha and increased beta expression between sex and treatment groups. Average α-MHC expression increased based on treatment with ANG II in each sex. More importantly, alpha expression between males and females receiving ANG II was decreased, 1.6922 vs. 1.5466, in females in comparison to males. This supports our surgical and natriuretic peptide PCR results that hypertrophy occurred at a lesser degree in our males in comparison to our females receiving ANG II, because alpha expression decreases in hypertrophy. Beta expression increased based on treatment in both sexes, indicating that ANG II upregulates beta expression. Females saw marked increase in beta expression when receiving ANG II treatment, 2.9905 in comparison with their male counterparts at 1.8173. These results support that females underwent greater levels of hypertrophy versus their male counterparts. Statistical analysis did not indicate significant differences between sexes and treatments. The myosin heavy chain expression trend of alpha and

beta observed in our model indicates that females underwent greater degrees of hypertrophy than their male ANG II counterparts. We hypothesized greater expression of α-MHC and decreased expression of β-MHC in our female group afforded by the cardioprotection of their sex difference. This was not the case. Further analysis is necessary to determine if the sex-difference affords cardioprotection in this model, because our results indicate otherwise. Again, a larger sample size may allow for significant statistical analysis results.

TGFβ family members are reported as key upregulated components in the signaling that results in LV remodeling in animal models of pressure overload and human patients with aortic valve stenosis (6). Evidence of a link between the RAAS and ANG II indicate that TGFβis downstream of ANG II in this pathway. ANG II activation induces TGFβ transcription by cardiomyocytes and fibroblasts (32). Both angiotensin II and TGFβinduce proliferation of fibroblast to myofibroblast and thus collagen synthesis in the infarctic heart (41). In our model of ANG II induced hypertrophy, we hypothesized that profibrotic and prohypertrophic TGFβ family signaling would be decreased in our female mice because of the cardioprotection afforded to females. Ligand expression in our model increased with treatment in our male treatment groups. In our females, expression decreased between our saline and ANG II groups by 0.6670. Although signaling was decreased in our ANG II females in comparison with our ANG II males (by 1.0310), which is possibly indicative of less hypertrophy in our females, female saline expression of TGFβ signaling was increased. Statistical analysis did not indicate significance.

Treatment with ANG II increases TGFβ receptor expression. However, expression of TGFβ receptors appeared to be decreased in our female ANG II treatment groups in comparison to their male counterparts as hypothesized. TGFβR1 increased in both sexes in the ANG II groups in comparison with the saline groups. Expression in the females was greatly diminished, 0.1102 and 0.2753 in the saline and ANG II groups respectively. TGFβR2 expression increased based on sex and treatment. The females expressed TGFβR2 >2-fold more than their saline counterparts. Females exhibited greater TGFβR2 expression, 0.9100, than their male counterparts. TGFβR3 expression increased in both sexes with ANG II treatment. Females exhibited lesser TGFβR3 expression, 0.8452 less, when treated with ANG II than their male ANG II counterparts. Analyses of receptor PCR results did not indicate statistical differences expression based on treatment or sex. Our PCR results indicated that females exhibited lower levels of TGFβ receptor expression than their male counterparts. Previous studies have shown an increase in receptor expression in models of hypertrophy, though not in a sex-specific manner. Therefore, our PCR results agreed with our hypothesis that TGFβ receptor expression would be decreased in our female mice because they experienced cardioprotection. Receptor expression in females did not reflect that females underwent a greater degree of hypertrophy than males. Estrogen may be downregulating receptor expression in our females treated with ANG II. However, if the receptor expression is downregulated, we would expect to see lesser hypertrophy in our female mice, which we did not observe. Therefore, further analysis is necessary to determine if this model correctly measured TGFFβ expression in relation to ANG II treatment and sex differential expression.

Chapter III

SPECIFIC AIM TWO

To determine sex differential expression in male and female heart failure patients, hearts from heart failure patients and from deceased donors who were never diagnosed with heart failure or heart disease were analyzed. The hypothesis of this experiment is that sex specific gene expression profiles can be established for failing and non-failing hearts. It is also expected that genes that propagate fibrosis will be more highly expressed in failing male hearts with a greater degree of fibrosis.

Hypothesis

a. Our objective was to analyze sex-specific regulation of genes known to regulate collagen deposition. We predicted that our study of heart failure would detect sex-specific patterns of collagen deposition. The working hypothesis was that female myocardium would exhibit lower levels of gene products that promote fibrosis collagen, matrix metalloproteinase, angiotensin converting enzyme, tumor necrosis factor alpha, and TGFβ, than comparable tissue from male hearts. In addition, it was anticipated that gene expression would positively correlate to observed collagen deposition and contractility phenotypes. We anticipated that male myocardium would contain more collagen with particularly elevated levels in the midmyocardium. Conversely, we anticipated that female myocardium would demonstrate a collagen deposition pattern with less fibrosis in the mid-wall.

Specific Aim Two Materials and Methods

Heart Collection

The University of Kentucky's Cardiac Biobank directed by Ken Campbell, provided the non-failing and failing female and male heart specimens. Every sample was procured directly from the operating room and flash-frozen within a few minutes of being removed from the patient. A member of Dr. Ken Campbell's lab group was paged to the operating room every time a consented patient was to receive a heart transplant or a ventricular assist device. When a sample that would otherwise be discarded was cut free, the surgeon would hand it directly to a scientist who immediately placed the tissue in ice-cold saline slush. The samples were then dissected, flash-frozen in liquid nitrogen, and subsequently placed in the vapor phase of liquid nitrogen for long-term storage. The samples are stable under these conditions for at least a decade. Identical procedures are used to obtain samples from donors who do not have a history of ventricular disease. These specimens (currently ~350 from 13 people) were obtained with the assistance of KODA, the regional organ procurement agency, from 3 hospitals in Lexington, KY when the heart could not be transplanted for technical reasons including inappropriate size and/or blood group. Clinical data from each patient's medical records (or the federally organized UNOS database in the case of organ donors) were de-identified and stored in a computer database along with a randomly generated hash code linking them to the banked samples.

Experimental Design

Experiments were performed using samples from sub-epicardial, mid-myocardial, and sub-endocardial regions of 3 non-failing male individuals, 2 non-failing female individuals providing three LV locations, 6 failing male individuals with three of those individuals providing three LV locations and the other three providing LV2 locations, and 8 female individuals with one of those individuals providing three LV locations. All banked hearts were linked to clinical data (including information from echocardiography and magnetic resonance imaging studies) collected from the medical records. The transmural pattern of gene expression in male vs. female and failing vs. non-failing myocardium were determined using Taqman gene expression analysis for candidate genes presented in Table 1 using the same samples analyzed in objectives 1 and 2. Human myocardium samples were homogenized via Fast Prep using fast prep lysing matrix D tubes (MP Biomedicals) and Trizol (Invitrogen). Total RNA was extracted and converted to cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems). Taqman gene expression analysis was performed to determine relative levels of target gene expression. Relative expression was determined using the delta, delta CT calculation. Results from quantitative studies are expressed as mean ± SEM. Comparisons between groups were performed by ANOVA and Welch's T-tests.

Table 37: Real Time PCR Primers used in Specific Aim Two

Name	Gene Name	Assay ID
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	***Hs04420697_g1***
MMP2	Matrix Metallopeptidase 2	***Hs01548730_m1***
ACE	Angiotensin Converting Enzyme	***Hs01104606_m1***
COL6α1	Collagen Type VI Alpha 1	***Hs01095580_m1***
TNFαIP1	Tumor Necrosis Factor Alpha Induced Protein 1	***Hs00221705_m1***
TGFβ	Transforming Growth Factor Beta Ligand	***Hs00998129_m1***

Specific Aim Two Results

To directly to determine sex differential expression in male and female heart failure patients, I analyzed hearts from heart failure patients and from deceased donors who were never diagnosed with heart failure or heart disease. Dr. Campbell provided access to microarray data from 18 human hearts. Ten non-failing hearts (five females and five males) and eight failing hearts (1 female and 7 males) samples were analyzed with Affymetrix chips at the University of Kentucky Microarray Core. This data was used to establish the hypothesis of the human study: sex specific gene expression profiles can be established for failing and non-failing hearts. It is also expected that genes that propagate fibrosis will be more highly expressed in failing male hearts with a greater degree of fibrosis. Experiments were performed using samples from sub-epicardial, mid-myocardial, and sub-endocardial regions of 3 non-failing male individuals, 2 non-failing female individuals providing three LV locations, 6 failing male individuals with three of those individuals providing three LV locations and the other three providing LV2 locations, and 8 female individuals with one of those individuals providing three LV locations. Primers detailed in Table 37 were used to analyze gene expression. ACE expression was analyzed in Figures 24-26. COL6α1 expression was analyzed in Figures 27-29. TNFαIP1 expression was analyzed in Figures 30-32. TGFβ expression was analyzed in Figures 33-35.

a. ACE Expression

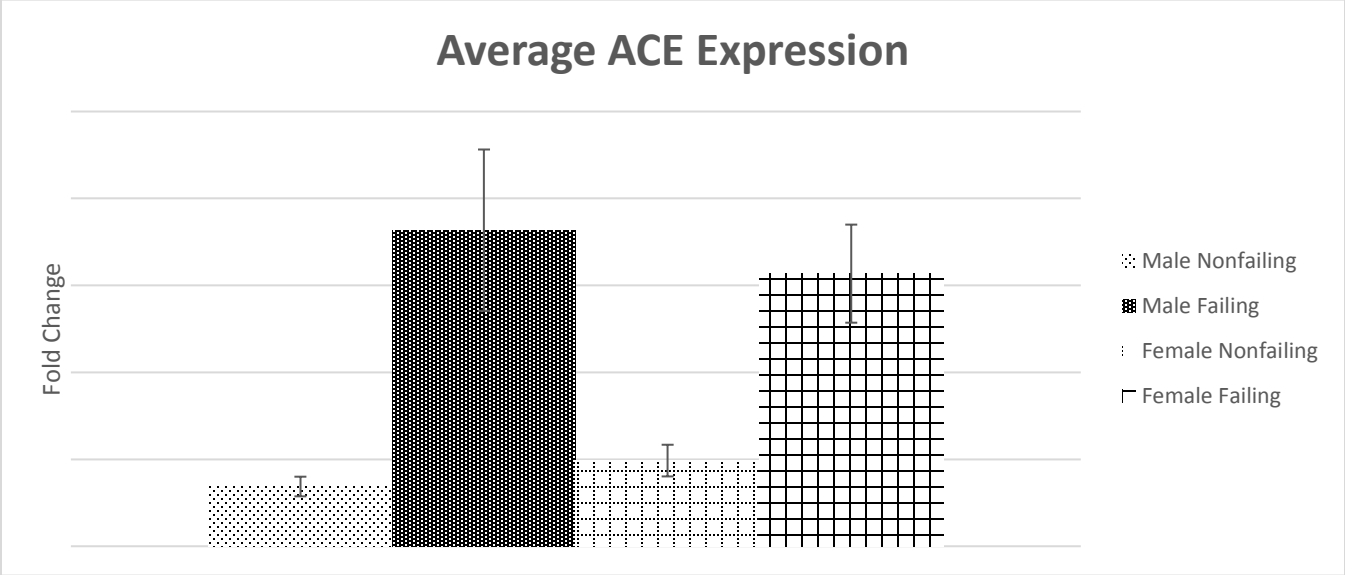

Figure 34: Average ACE Expression in Male and Female Nonfailing and Failing Hearts. Male nonfailing expression was 0.3436. Male failing expression is 1.8177. Female nonfailing expression was 0.4927. Female failing expression 1.5677. The value increase between male nonfailing and male failing was 529% although this was not significant by ANOVA p-values. The valued increase between female nonfailing and female failing was 318%. ANOVA statistical analysis did not indicate statistical significance.

Table 38: ANOVA of Average ACE Expression.

Comparison Group	P-Value
Male Nonfailing vs. Male Failing	.3205
Female Nonfailing vs. Female Failing	.4902
Male Nonfailing vs. Female Nonfailing	>.9999
Male Failing vs. Female Failing	.9971

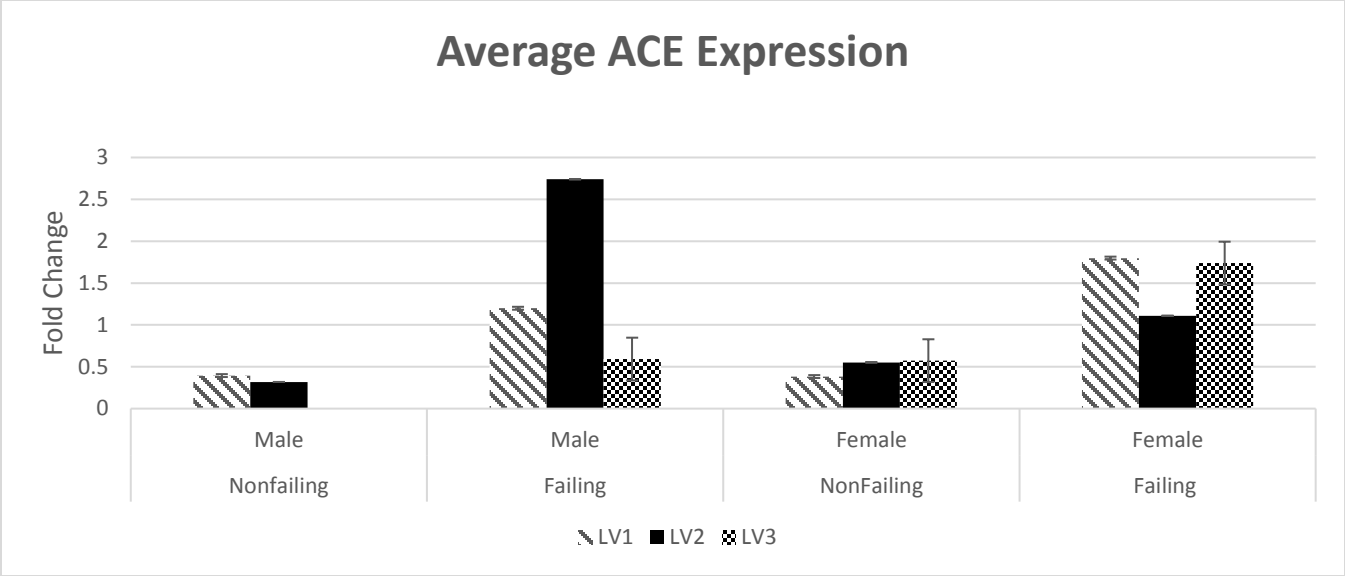

Figure 35: Average ACE Expression. Male nonfailing LV1 expression was 0.3946. Male nonfailing LV2 expression was 0.3181. Male failing LV1 expression was 1.1991. Male failing LV2 expression was 2.7399. Male failing LV3 expression was 0.5920. The valued increases between the male nonfailing and failing LV1 and nonfailing and failing LV2 locations were 304% and 861% respectively. Female nonfailing LV1 expression was 0.3827. Female nonfailing LV2 expression was 0.5521. Female nonfailing LV3 expression was 0.5730. Female failing LV1 expression was 1.7984. Female failing LV2 expression was 1.1089. Female failing LV3 expression was 1.7388. The valued increases between the female nonfailing and failing LV1s, LV2s, and LV3s locations were 470%, 201%, and 303%.

Figure 36: Average ACE Expression by Sample. The graphed average ACE expression of each sample by sex and location.

b. COL6α1 Expression

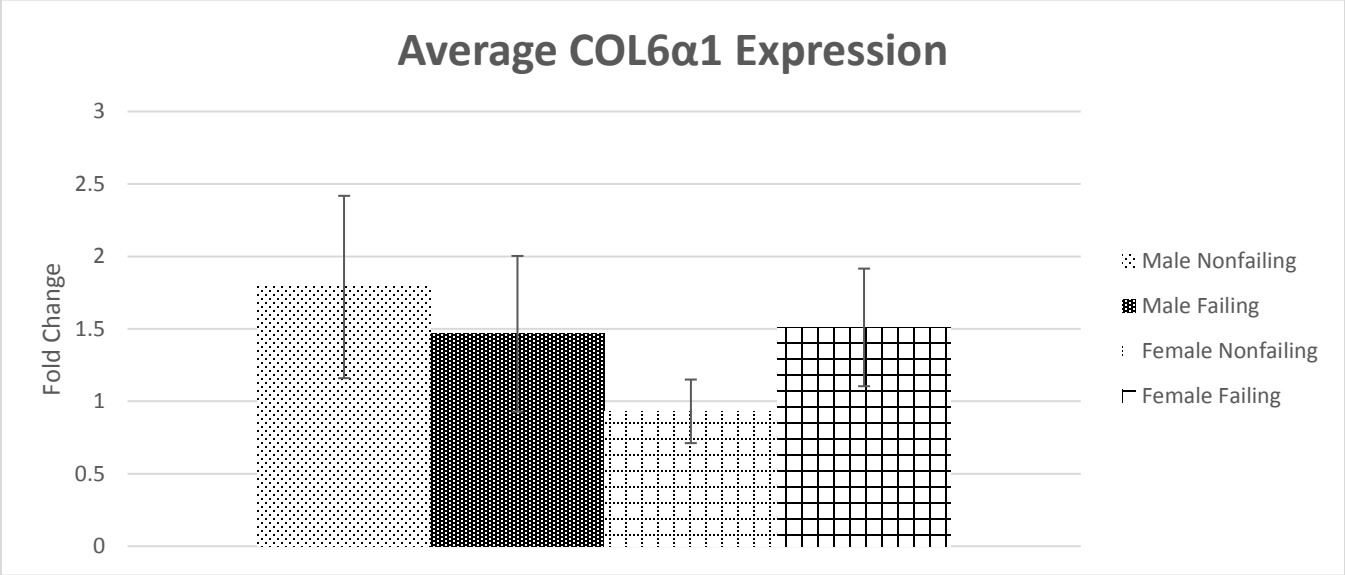

Figure 37: Average COL6α1 Expression in Male and Female Nonfailing and Failing Hearts. Male nonfailing expression was 1.7891. Male failing expression is 1.4661. Female nonfailing expression was 0.9311. Female failing expression 1.5103. The value increase between male nonfailing and male failing was 819% although this was not significant by ANOVA p-values. The valued increase between female nonfailing and female failing was 162%. ANOVA statistical analysis did not indicate statistical significance.

Table 39: ANOVA of Average COL6α1 Expression.

Comparison Group	P-Value
Male Nonfailing vs. Male Failing	.9996
Female Nonfailing vs. Female Failing	.9693
Male Nonfailing vs. Female Nonfailing	.9553
Male Failing vs. Female Failing	>.9999

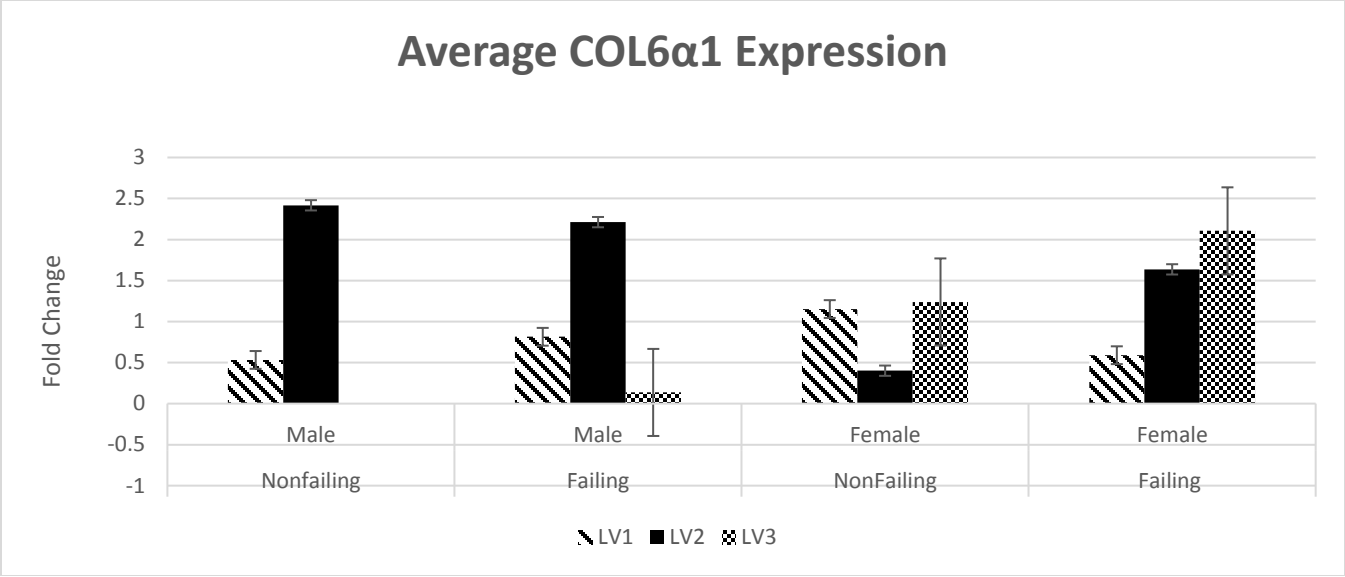

Figure 38: Average COL6α1 Expression. Male nonfailing LV1 expression was 0.5325. Male nonfailing LV2 expression was 2.4174. Male failing LV1 expression was 0.8149. Male failing LV2 expression was 2.2122. Male failing LV3 expression was 0.1364. The valued increases of male nonfailing and failing LV1 and nonfailing and failing LV2 were 153% and 91.5% Female nonfailing LV1 expression was 1.1527. Female nonfailing LV2 expression was 0.4014. Female nonfailing LV3 expression was 1.2392. Female failing LV1 expression was 0.5901. Female failing LV2 expression was 1.6365. Female failing LV3 expression was 2.1058. The valued increases between the female nonfailing and failing LV1s, LV2s, and LV3s were 51.2%, 408%, and 170%.

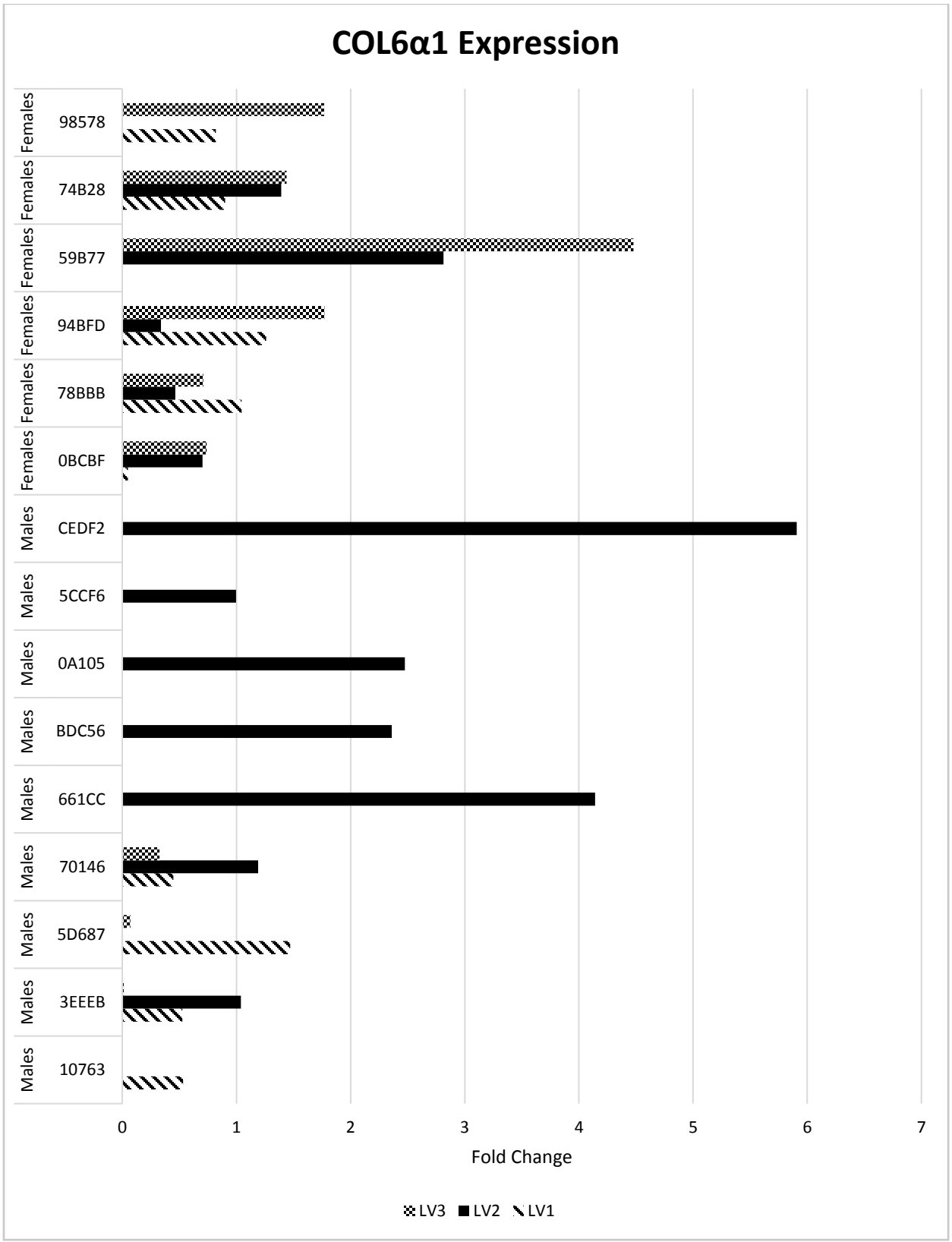

Figure 39: Average COL6α1 Expression by Sample. The graphed average COL6α1 expression of each sample by sex and location.

c. TNFαIP1 Expression

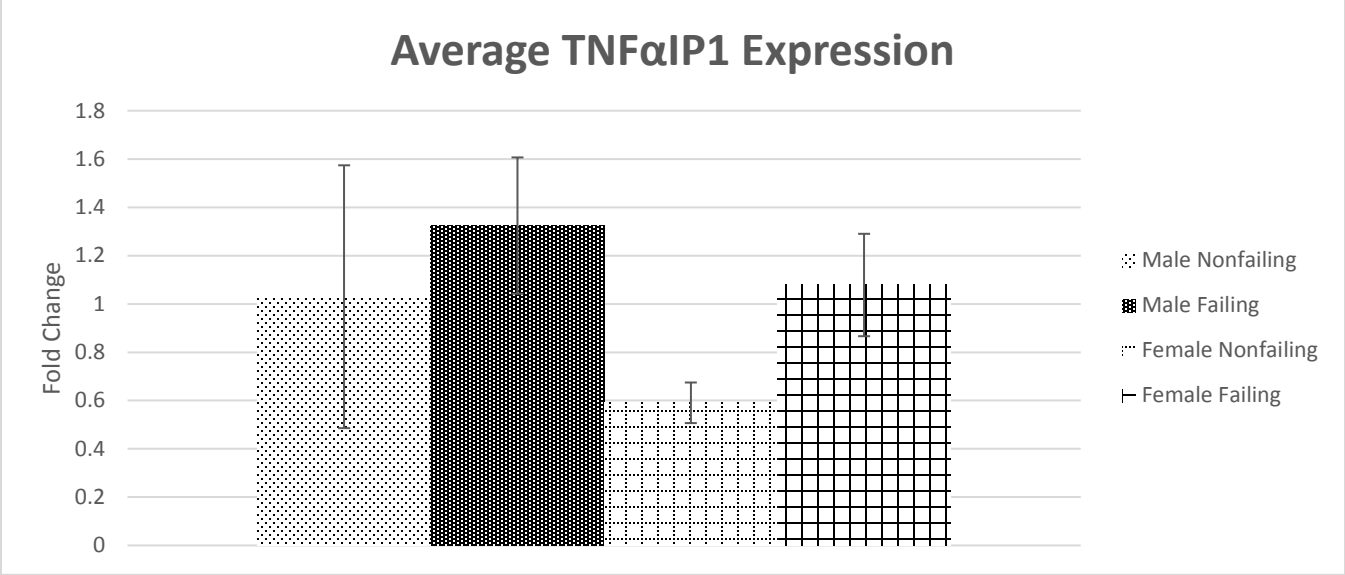

Figure 40: Average TNFαIP1 Expression in Male and Female Nonfailing and Failing Hearts. Male nonfailing expression was 1.0299. Male failing expression is 1.3245. Female nonfailing expression was 0.5907. Female failing expression 1.0784. The value increase between male nonfailing and male failing was 129% although this was not significant by ANOVA p-values. The valued increase between female nonfailing and female failing was 183%. ANOVA statistical analysis did not indicate statistical significance.

Table 40: ANOVA of Average TNFαIP1 Expression.

Comparison Group	P-Value
Male Nonfailing vs. Male Failing	.9932
Female Nonfailing vs. Female Failing	.8045
Male Nonfailing vs. Female Nonfailing	.9673
Male Failing vs. Female Failing	.9776

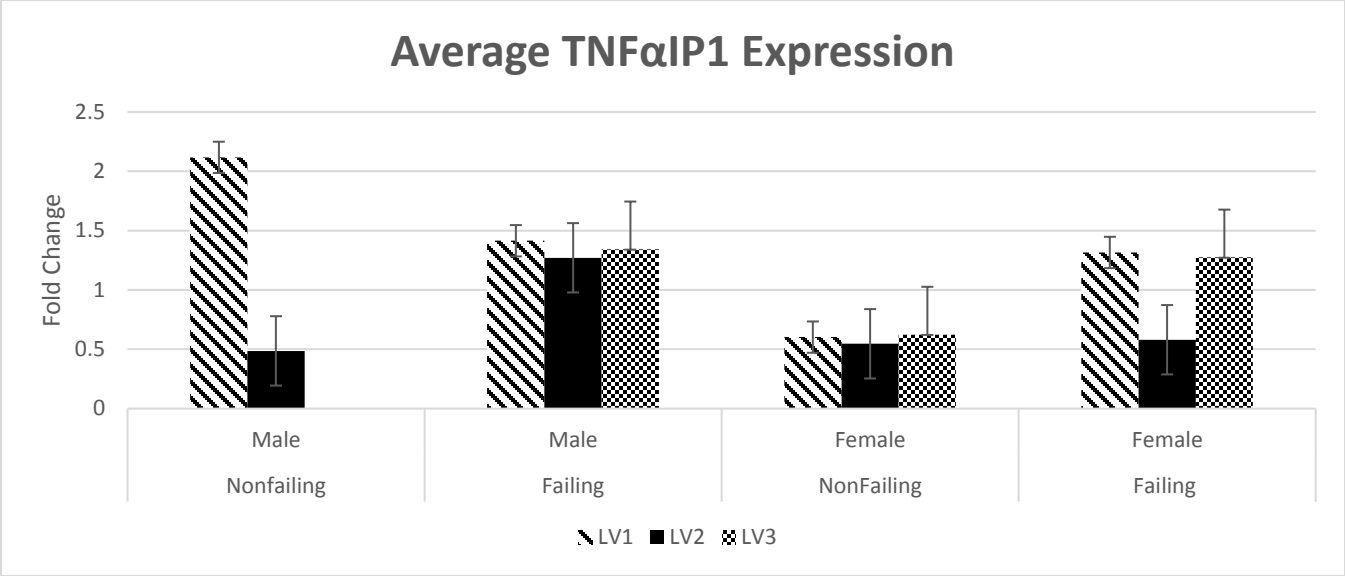

Figure 41: Average TNFαIP1 Expression. Male nonfailing LV1 expression was 2.1172. Male nonfailing LV2 expression was 0.4863. Male failing LV1 expression was 1.4144. Male failing LV2 expression was 1.2708. Male failing LV3 expression was 1.3420. The valued increases between the male nonfailing and failing LV1 and nonfailing and failing LV2 locations were 66.8% and 261% respectively. Female nonfailing LV1 expression was 0.6024. Female nonfailing LV2 expression was 0.5461. Female nonfailing LV3 expression was 0.6236. Female failing LV1 expression was 1.3158. Female failing LV2 expression was 0.5802. Female failing LV3 expression was 1.2739. The valued increases between the female nonfailing and failing LV1s, LV2s, and LV3s locations were 218%, 106%, and 204%.

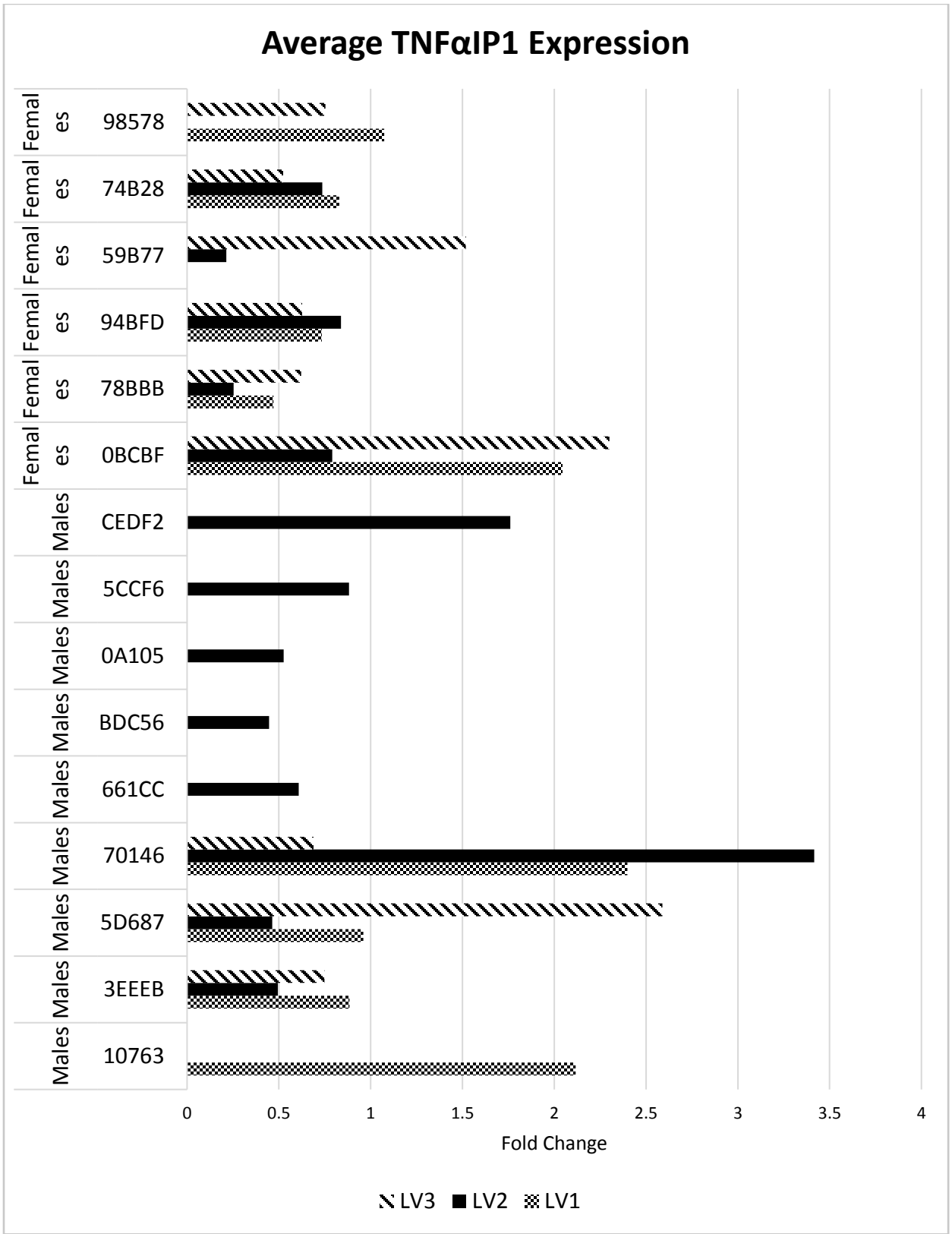

Figure 42: Average TNFαIP1 Expression by Sample. The graphed average TNFαIP1 expression of each sample by sex and location.

d. TGFβ Expression

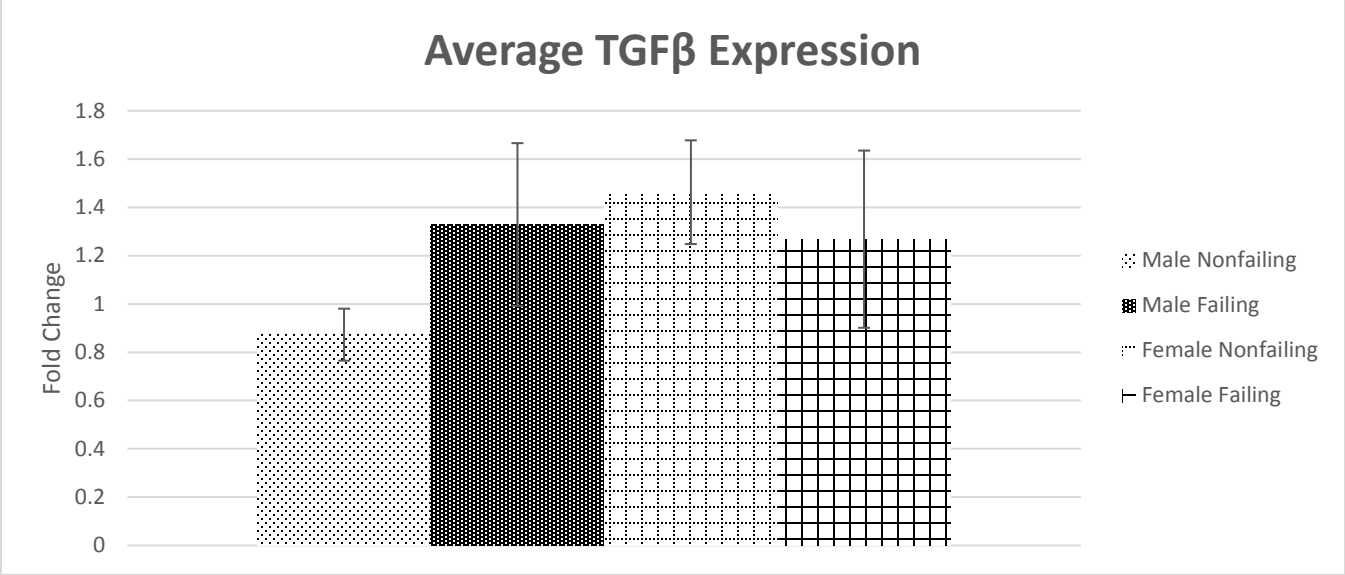

Figure 43: Average TGFβ Expression in Male and Female Nonfailing and Failing Hearts. Male nonfailing expression was 0.8727. Male failing expression is 1.3289. Female nonfailing expression was 1.4626. Female failing expression 1.2682. The value increase between male nonfailing and male failing was 152% although this was not significant by ANOVA p-values. The valued increase between female nonfailing and female failing was 87%. ANOVA statistical analysis did not indicate statistical significance.

Table 41: ANOVA of Average TGFβ Expression.

Comparison Group	P-Value
Male Nonfailing vs. Male Failing	.9839
Female Nonfailing vs. Female Failing	.9995
Male Nonfailing vs. Female Nonfailing	.9636
Male Failing vs. Female Failing	>.9999

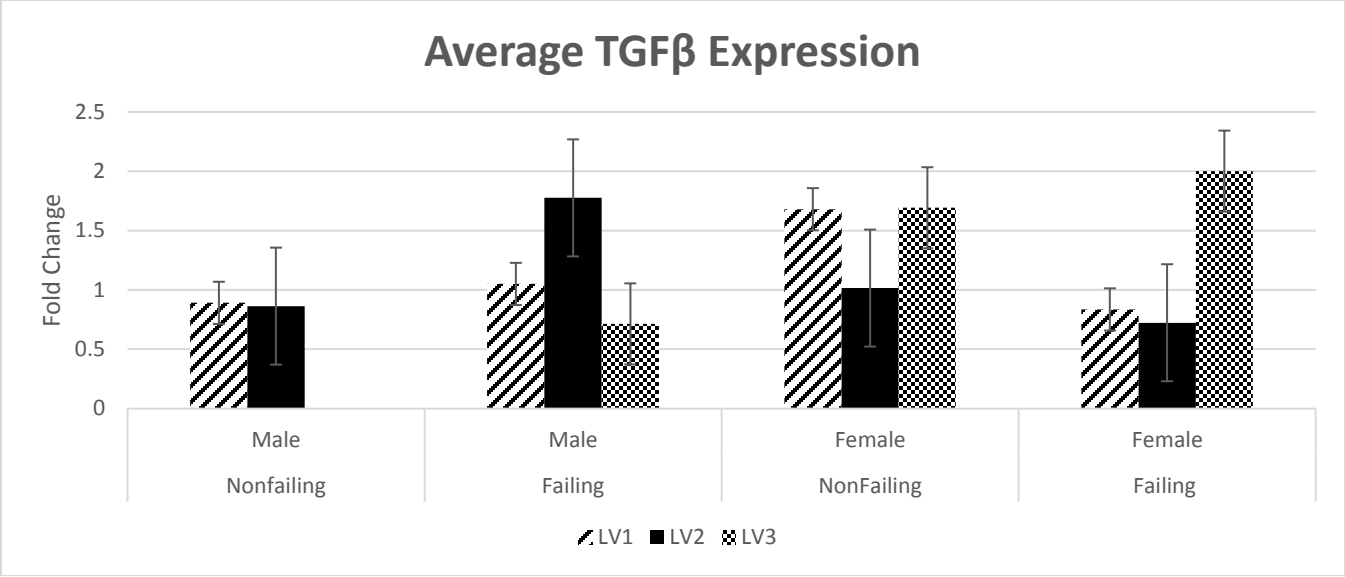

Figure 44: Average TGFβ Expression. Male nonfailing LV1 expression was 0.8911. Male nonfailing LV2 expression was 0.8636. Male failing LV1 expression was 1.0505. Male failing LV2 expression was 1.7757. Male failing LV3 expression was 0.7138. The valued increases between the male nonfailing and failing LV1 and nonfailing and failing LV2 locations were 186% and 206% respectively. Female nonfailing LV1 expression was 1.6801. Female nonfailing LV2 expression was 1.0155. Female nonfailing LV3 expression was 1.6923. Female failing LV1 expression was 0.8356. Female failing LV2 expression was 0.7235. Female failing LV3 expression was 2.0011. The valued increases between the female nonfailing and failing LV1s, LV2s, and LV3s locations were 49.7%, 67.7%, and 118%.

Figure 45: Average TGFβ Expression by Sample. The graphed average TGFβ expression of each sample by sex and location.

Specific Aim Two Discussion

As depicted in Figure 11, we proposed an interplay of genes and signaling pathways that lead to the fibrosis observed in heart failure. Upregulation of ACE leads to increased ANG II synthesis by the renin angiotensin aldosterone system, which in turn activates some of the same pathways as TNF-α. ANG II receptors and TGFβ receptors exist in cardiomyocytes and fibroblasts and TGFβ is upregulated by ANG II binding AT_1 receptor. TNF-α and TGFβ appear to work independently of each other to alter the balance of MMPs and TIMPS as seen in Figure 9. TGFβ expression is known to be profibrotic and to affect collagen deposition. Using the genes identified in the microarray analysis of nonfailing and failing hearts as being sex-regulated or exhibiting differential expression, we analyzed these genes to further investigate the sex-specific patterns of expression of collagen deposition in failing and nonfailing human hearts.

ACE expression was sex-differentially expressed in human nonfailing and failing human hearts according to microarray analysis. We hypothesized that ACE expression would be lessened in failing female hearts due to the cardioprotection afforded by estrogen. It has been proposed that estrogen may mediate its cardioprotection through regulating angiotensin mRNA levels and ACE activity in the RAAS system (57). The decreased expression observed in the failing female hearts in comparison to their male counterparts may support this proposed mechanism of estrogen cardioprotection in our model. Furthermore, by LV location, it was observed that overall ACE expression was greater in failing hearts of both sexes in comparison with nonfailing hearts. Additionally, failing male LV2 and female LV1 exhibited the greatest expression levels of ACE. Our hypothesis remained that females would exhibit lesser expression of ACE due to

cardioprotection; in addition, we expected less fibrosis in the midwall of the females. This expectation was met in that the failing LV2 location of the females expressed 1.631 less of ACE than their male failing LV2 counterparts.

COL6α1 expression was previously identified by microarray analysis to be sex-regulated. Interestingly, COL6α1 expression in our failing male hearts was slightly less than that of our nonfailing male hearts (1.7891 vs 1.4661). Also of note was that expression in our nonfailing female hearts was nearly 2-fold less than our nonfailing male hearts (0.9311). Finally, failing female expression was less than nonfailing expression of the males but greater than the failing expression of the males (1.5103). By LV location, it was observed that overall COL6α1 expression was greater in failing hearts of both sexes in comparison to nonfailing hearts, with the exceptions of the LV1 location of female hearts and the LV2 location of the males. Additionally, male nonfailing LV2 and female failing LV3 exhibited the greatest expression levels of COL6α1. Our hypothesis was that females would exhibit lessened expression of COL6α1 due to cardioprotection and thus less collagen deposition in the midwall. Failing females did exhibit lesser expression of COL6α1 than their male counterparts. However, nonfailing locations exhibited greater COL6α1 expression at the nonfailing male LV2 and female LV1 locations than their failing counterparts. The failing LV2 location of the females expressed 0.5757 less of COL6α1 than their male failing LV2 counterparts. ANOVA statistical analysis did not indicate statistical significance in our findings. We hypothesized that collagen deposition in fibrosis would lead to greater expression of COL6α1 in our male failing heart samples than our female failing heart samples. Our findings do not support that COL6α1 is sex-regulated in our model. Further investigation is necessary to determine if collagen is sex-

regulated as we previously concluded through microarray analysis, especially since estrogen is suspected to impact the pathway of genes leading to collagen deposition in fibrosis and subsequent heart failure.

TNFαIP1 expression was determined by microarray analysis to be sex-regulated. Additionally, the role of the TNF-α cytokine family in fibrosis has been identified. Our results indicated that expression of TNFαIP1 was decreased in female hearts in comparison with male hearts of both nonfailing and failing conditions. Furthermore, TNFαIP1 was upregulated in the failing hearts of both sexes in comparison with the nonfailing hearts. Statistical analysis did not indicate that these differences were significant. We hypothesized that TNFαIP1 expression would be less in failing female hearts due to the cardioprotection afforded by estrogen. Estrogen is known to be anti-inflammatory and may suppress the TNF-α pathway. It is also known that estrogen regulates components of the RAAS pathway, which has some crossover points with the TNF-α pathway (46 & 57). The decreased expression observed in the failing female hearts in comparison to their male counterparts may support this proposed mechanism of estrogen's cardioprotection in our model. By LV location, it was observed that overall TNFαIP1 expression was greater in failing hearts of both sexes in comparison with nonfailing hearts except LV1 males. Additionally, failing LV1s of both sexes exhibited the greatest expression levels of TNFαIP1. We hypothesized that females would exhibit lessened expression of TNFαIP1 due to cardioprotection and lesser fibrosis in the midwall of the females. This hypothesis was observed in that the failing LV2 location of the females expressed .6906 less of TNFαIP1 than their male failing LV2 counterparts. Interestingly, TNFαIP1 expression was lessened in the female LV2 location of the heart

contradicting our hypothesis. Additionally, other locations of female hearts exhibited more pronounced differences in expression compared to the LV2 location. Further investigation is necessary to determine if TNFαIP1 is sex-regulated as we previously concluded through microarray analysis.

Central to this thesis is that TGFβ family members are reported as key upregulated components in the signaling that results in LV remodeling in animal models of pressure overload and human patients with aortic valve stenosis (6). Overexpression of TGFβ leads to collagen deposition, increased myocardial stenosis, fibrosis, and diastolic dysfunction; however, a certain baseline level is required to maintain cardiac structure and protect against cardiac dilation in cases of pressure overload (32). In our human study of heart failure, we hypothesized that profibrotic and prohypertrophic TGFβ family signaling would be decreased in our failing female hearts because of the cardioprotection afforded to females. Female failing heart expression in our study was less than that of male expression (1.2682 vs. 1.3289). We expected this difference to be more pronounced because of the cardioprotection females are afforded. Of interest, nonfailing females exhibited the greatest expression in both sexes in failing and nonfailing groups. (1.4626). The LV2 location of the heart was hypothesized to undergo the most profibrotic expression in our human study. Results of our TGFβ PCR indicated that male LV2 and female LV3 exhibited the greatest expression of TGFβ. The female LV2 location expression was 0.7235 while the male LV2 location expression was 0.8636. Female LV2 did have less TGFβ expression as hypothesized and therefore this location may have undergone less fibrosis than the male LV2 location, potentially due to estrogen cardioprotection. Statistical analysis did not indicate significance in these findings.

Chapter IV

SUMMARY AND FUTURE DIRECTIONS

Our aims and working hypotheses sought to investigate the molecular basis of sex specific effects on hypertrophy and heart failure. While much work has been done regarding sex-specific differences in cardiac remodeling, a complete understanding of the mechanism underlying these differences in relation will require further study. We sought to test a mechanistic hypothesis that sex differences in the development of cardiac hypertrophy and heart failure are regulated by differential expression of genes important in regulation by sex specific factors, specifically TGFβ. Furthermore, the human study sought to establish whether expression of genes regulating collagen deposition exhibit sex-biased expression. Our mouse model, although overall a successful model of cardiac hypertrophy, did not demonstrate sex differences. Cardiac hypertrophy markers were shown to be regulated by treatment with ANG II, and TGFB family members were regulated by both sex and treatment. In our human study, expression of genes known to promote fibrosis deposition was higher in failing vs non-failing hearts. Additionally, the greatest expression of profibrotic genes were detected in the midwall of failing hearts; the expression patterns hint at sex differential expression of profibrotic genes, with greater expression of profibrotic genes in male patients. Estrogen, a sex-specific factor, may be playing a role in the sex-specific expression of profibrotic genes. Therefore, establishing the role of estrogen as a sex-specific factor will better determine whether and why hormone replacement therapy is beneficial. This research is significant because the negative and questionable effects of HRT in postmenopausal women make it imperative

to understand the molecular mechanism of estrogen's beneficial effects in order to develop new therapies without activating pathways that could lead to negative side effects (5). Future directions for this research include expanding on the human study of cardiac hypertrophy with a larger cohort of non-failing and failing heart samples, increasing our studies' parameters to include more patient health information, investigating estrogenic regulation of miRNAs implicated in cardiac hypertrophy and heart failure, and increasing the number of genes in our RT-PCR analysis to include a wider panel of agents that cause collagen deposition in order to more fully define sex differences in heart failure, specifically fibrosis. Our research has direct implications in the study of human cardiovascular disease and is of particular interest in our community which exhibits an extremely high incidence of cardiovascular disease.

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