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FSTL3 and its role in mediating fibrosis and hypertrophy in diet-induced obesity

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Thesis

FSTL3 AND ITS ROLE IN MEDIATING FIBROSIS AND HYPERTROPHY IN DIET-INDUCED OBESITY

by

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FSTL3 AND ITS ROLE IN MEDIATING FIBROSIS AND HYPERTROPHY IN DIET-INDUCED OBESITY

WILLIAM LONG

ABSTRACT

Metabolic syndrome (MetS) is a conglomeration of several risk factors for cardiovascular disease, with obesity currently being one of the common causes of disability and death in the United States. Underlying the obesity, however, there is metabolic imbalance that could be exacerbating the issue of metabolic syndrome. Approximately 34% of adults over 20 years old matched the criteria for metabolic syndrome. The risk factors for cardiovascular disease (CVD) associated with metabolic syndrome can, over time, lead to severe CVDs, such as heart failure (HF). Metabolic syndrome can also lead to developing metabolic heart disease over time. Understanding the development of cardiac hypertrophy and fibrosis in diet-induced metabolic heart disease allow development of an early treatment of metabolic heart disease (MHD) and HF.

This study looked at one potential mediator and its role in cardiac hypertrophy and fibrosis, follistatin-like 3 (FSTL3). FSTL3 is an extracellular antagonist of members of the TGF-β superfamily. The goal of our study was to determine the effect, if any, a knockout of FSTL3 would have on the development of cardiac hypertrophy and fibrosis after a high-fat, high-sucrose diet for five months. FSTL3 knockout mice were given a high-fat, high-sucrose (HFHS) diet for five months. These mice were then sacrificed and their hearts were analyzed.
for cardiac myocyte hypertrophy and interstitial fibrosis using histological methods. After five months on the HFHS diet, wild-type (WT) mice had cardiac hypertrophy. In FLRG KO mice the diet-induced cardiac hypertrophy was attenuated. WT HFHS-fed mice developed interstitial fibrosis, and FLRG KO HFHS developed more accentuated interstitial fibrosis than WT HFHS diet fed mice. This study is useful in suggesting that FTSL3 contributes to the pathogenesis of cardiac hypertrophy in MHD. FTSL3 may be a useful biomarker for cardiac hypertrophy in patients with suspected MHD, and may be a viable target for therapeutic interventions aimed at decreasing pathologic myocardial hypertrophy.
TABLE OF CONTENTS

TITLE ............................................................................................................................................. i
COPYRIGHT PAGE ....................................................................................................................... ii
READER APPROVAL PAGE ......................................................................................................... iii
ABSTRACT .................................................................................................................................... iv
TABLE OF CONTENTS ................................................................................................................... vi
LIST OF TABLES ............................................................................................................................ viii
LIST OF ABBREVIATIONS ............................................................................................................ x
INTRODUCTION ............................................................................................................................. 1

Metabolic Syndrome and Heart Failure ......................................................................................... 1
Cardiac Hypertrophy and Fibrosis ................................................................................................. 4
High fat, high sucrose diet as a model for metabolic syndrome ................................................. 6
Cardiokines ..................................................................................................................................... 7
Transforming Growth Factor β ..................................................................................................... 7
Protective members of the TGF-β family .................................................................................. 10
FSTL3 and its Role in Cardiac Hypertrophy and Fibrosis ......................................................... 10
Specific Aims and Objectives ....................................................................................................... 16
METHODS ..................................................................................................................................... 17

HFHS diet mouse model of MetS and MHD ............................................................................. 17
FSTL3 KO mouse model ............................................................................................................ 17
Plasma Collection ....................................................................................................................... 21
Histology Preparation ................................................................................................................ 22
Picrosirius Red Staining for Fibrosis ........................................................................................ 22
Hematoxylin and Eosin Staining for Hypertrophy ................................................................. 23
Statistical Analysis ..................................................................................................................... 24
RESULTS ...................................................................................................................................... 25

FLRG KO had no apparent impact on weight gain or heart development ............................... 25
FLRG KO attenuated cardiomyocyte hypertrophy ................................................................. 29
FLRG KO induced more fibrosis........................................................................................................33

DISCUSSION ..................................................................................................................................36

LIST OF JOURNAL ABBREVIATIONS ..........................................................................................40

REFERENCES .................................................................................................................................42

CURRICULUM VITAE ......................................................................................................................46
<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Adult values for the diagnosis of metabolic syndrome</td>
<td>1</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cumulative incidence of HF in normal vs. obese patients</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>Mortality associated with diastolic dysfunction</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Effects of TGF-β signaling</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>FSTL3’s effect on cardiac fibrosis and hypertrophy</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>Plasma levels of FSTL3 correlated with diastolic function and LVH</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>Genotyping FLRG KO mice</td>
<td>19</td>
</tr>
<tr>
<td>7</td>
<td>Breeding strategy for FSTL3 KO mice</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>Mice gained weight over time</td>
<td>25</td>
</tr>
<tr>
<td>9</td>
<td>Percent change in body weight after five months on diet</td>
<td>26</td>
</tr>
<tr>
<td>10</td>
<td>Heart weights</td>
<td>27</td>
</tr>
<tr>
<td>11</td>
<td>Individual heart weight/tibia length</td>
<td>28</td>
</tr>
<tr>
<td>12</td>
<td>Heart weight/tibia length for all groups</td>
<td>29</td>
</tr>
<tr>
<td>13</td>
<td>Microscopy of cardiomyocyte hypertrophy</td>
<td>30</td>
</tr>
<tr>
<td>14</td>
<td>Individual cardiac myocyte measurements</td>
<td>31</td>
</tr>
<tr>
<td>15</td>
<td>Mean diameter of cardiomyocytes</td>
<td>32</td>
</tr>
<tr>
<td>16</td>
<td>Microscopy of cardiac interstitial fibrosis</td>
<td>33</td>
</tr>
<tr>
<td>17</td>
<td>Individual fibrosis data</td>
<td>34</td>
</tr>
<tr>
<td>18</td>
<td>Mean percentage of interstitial fibrosis</td>
<td>35</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

α-MHC................................................................... α-myosin heavy chain
CVD........................................................................... Cardiovascular disease
DD........................................................................... Diastolic dysfunction
ECM........................................................................... Extracellular matrix
FLRG......................................................................... Follistatin related gene
FSTL3......................................................................... Follistatin-like 3
HF........................................................................... Heart failure
HFHS........................................................................... High-fat, high-sucrose
KO........................................................................... Knockout
LV........................................................................... Left ventricle
LVH........................................................................... Left ventricular hypertrophy
MHD........................................................................... Metabolic heart disease
MMP........................................................................... Matrix metalloproteinase
MetS........................................................................... Metabolic syndrome
RAAS...................................................................... Renin-angiotensin-aldosterone system
ROS.......................................................................... Reactive oxygen species
TAC........................................................................... Transverse aortic constriction
TβRI......................................................................... TGF-β type I receptor
TβRII......................................................................... TGF-β type II receptor
TGF-β......................................................................... Transforming growth factor β
TIMP........................................................................... Tissue inhibitor of metalloproteinases
INTRODUCTION

Metabolic Syndrome and Heart Failure

Metabolic syndrome (MetS) is a conglomeration of several risk factors (Table 1) for cardiovascular disease including elevated glucose concentrations in the fasting state (≥100 mg/dL), high systolic (≥130 mmHg) or diastolic (≥85 mmHg) blood pressure, high triglycerides (≥150 mg/dL), low values of high-density lipoprotein (≤40 mg/dL in men and ≤50 mg/dL in women), and abdominal obesity (waist circumference of ≥40 inches in men and ≥35 inches in women).4,5

Table 1. Adult values for the diagnosis of metabolic syndrome. To be diagnosed with metabolic syndrome, a patient must display at least three of the symptoms listed in the table.6

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Defining Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abdominal obesity, given as waist circumference††</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>&gt;102 cm (&gt;40 in)</td>
</tr>
<tr>
<td>Women</td>
<td>&gt;88 cm (&gt;35 in)</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>≥150 mg/dL</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>&lt;40 mg/dL</td>
</tr>
<tr>
<td>Women</td>
<td>&lt;50 mg/dL</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>≥130/≥85 mm Hg</td>
</tr>
<tr>
<td>Fasting glucose</td>
<td>≥110 mg/dL††</td>
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</tbody>
</table>
Obesity, in particular, has reached an unfathomable prevalence in the United States. In fact, obesity is one of the common causes of disability and death in the U.S., and the prevalence of obesity in various subgroups of the population, such as youth and people of a lower socioeconomic status, has increased in recent years.\(^1\) Underlying the obesity, however, there is metabolic imbalance that could be exacerbating the issue of metabolic syndrome.\(^2\)

Approximately 34% of adults over 20 years old matched the criteria for metabolic syndrome.\(^3\) The risk factors for cardiovascular disease (CVD) associated with MetS can, over time, lead to severe CVDs, such as heart failure (HF).\(^4\) This is shown in individuals with MetS and their 2-fold higher chance of developing CVD than their healthy counterparts.\(^7\) In addition, over time, the risk of heart failure for obese individuals goes up dramatically (Figure 1).

Heart failure is capable of reducing cardiac output and limiting the delivery of oxygenated blood to the tissues, while also causing an accumulation of blood in the venous system.\(^8\) It is also strongly associated with high mortality rates. According to the Heart Disease and Stroke Statistics report from the American Heart Association, 1 in 9 death certificates in 2011 mention heart failure and of those, heart failure was the underlying cause of death in 20% of them.\(^1\) The high medical costs and increasing prevalence of heart failure demands that a new method of detection be put in place to ensure timely diagnosis and early treatment to avoid the mortality associated with HF.
Figure 1. Cumulative incidence of HF in normal vs. obese patients.

Over time, the incidence of HF in obese subjects goes up drastically. This exemplifies why a further knowledge of the biochemistry of the
development of heart failure as a result of obesity occurs. Figure from Kenchaiah et al. 2002.9

**Cardiac Hypertrophy and Fibrosis**

MHD is marked by LVH, myocardial fibrosis, diastolic dysfunction, and impaired cardiac energetics.10–12 Similarly, it has been shown in animal models that pressure overload through transverse aortic constriction (TAC) induces left ventricular hypertrophy (LVH) and cardiac remodeling and fibrosis.13 While the TAC model is seemingly a severe model of heart failure, metabolic syndrome develops much more slowly.

To understand the development of fibrosis, a background of collagen production is necessary. The structure of the myocardium consists of myocytes, fibroblasts, endothelial cells, and smooth muscle cells; the extracellular matrix (ECM) is composed of collagen fibers, elastin fibers, proteoglycans, among other important proteins.14 The primary types of collagen present in the myocardial ECM consists of type I and type III collagen fibers. During the formation of collagen fibers, a procollagen (a mature collagen precursor) is secreted into the ECM, and is then cleaved by endopeptidases. These new collagen fragments condense together to form collagen fibrils. Fibrosis occurs when there is a disruption in the metabolism and breakdown of collagen fibrils.15

There are two separate, but related, methods of fibrotic remodeling: reparative and reactive.16 Generally in instances of cardiac injury, the heart
needs to repair itself using scar tissue in instances of cell death, such as ischemia. On the other hand, reactive fibrosis is remodeling of the interstitial space in response to increased demand on the heart, such as is present with hypertension and cases without extensive cell damage, as in MHD.\textsuperscript{17} The accumulation of unneeded collagen fibers in the interstitial matrix to a pathologic level has been attributed to contributing to the onset of diastolic dysfunction.\textsuperscript{18} Diastolic dysfunction has no cure, and contributes to morbidity and mortality at an astonishing rate. Only five years after the diagnosis of moderate to severe diastolic dysfunction (DD), there is more than a 20\% mortality in patients (Figure 2).\textsuperscript{19}
Figure 2. Mortality associated with diastolic dysfunction. Even five years after the diagnosis of diastolic dysfunction, moderate to severe DD has more than a 20% mortality rate compared to that of a patient with normal diastolic function. From Redfield MM et al. 2003.\textsuperscript{19}

High fat, high sucrose diet as a model for metabolic syndrome

A HFHS diet used in animal models has been shown to duplicate the symptoms of metabolic syndrome in humans.\textsuperscript{10,12,20–22} Using the three-symptom requirement for diagnosis in humans, metabolic syndrome can also be crudely diagnosed in mice. Mice on a HFHS diet develop obesity,\textsuperscript{10} insulin resistance leading to high fasting plasma glucose levels,\textsuperscript{21} and high blood pressure.\textsuperscript{20,21} In addition, a HFHS diet led to tissue-specific changes in the heart such as LVH,\textsuperscript{10,20} chronic inflammation,\textsuperscript{21} diastolic dysfunction,\textsuperscript{10,12,20} and cardiac interstitial fibrosis.\textsuperscript{12,20}

Another mediator of cardiac hypertrophy and fibrosis as a result of a HFHS diet is reactive oxygen species (ROS). After only four months on a HFHS diet, mitochondrial function declined, LVH developed, and ROS production increased.\textsuperscript{10} The effect of ROS on the cardiac structure and function has been studied previously. If the effect of ROS is negated with either a polyphenol with antioxidant effects or an enzyme to reduce the amount of ROS present, the cardiac structural changes are prevented.\textsuperscript{10,20}
Cardiokines

Much like other tissues, the heart is capable of releasing extracellular proteins in the interstitial space and the bloodstream. These proteins have the potential to communicate in normal cellular growth as well as a reaction to pathological activities occurring in the body. In the heart, these secreted proteins are known as cardiokines. With the increasing prevalence of obesity and heart disease, there is an increasing desire to identify new biomarkers for early diagnoses as well as potential targets for therapeutics. In addition, cardiokines are capable of playing a role in pathologic cardiac remodeling through processes like fibroblast stimulation and apoptosis or even have a contributing role in muscle wasting processes.

Currently, levels of B-type natriuretic peptide (BNP) present in circulation are the gold standard to help in the diagnosis of HF; however, in patients with diastolic dysfunction or heart failure with preserved ejection fraction (HFpEF), the sensitivity of BNP tests have been brought into question. As such, new biomarkers in models of MetS or diastolic dysfunction are needed for early diagnosis.

Transforming Growth Factor β

The transforming growth factor beta family (TGF-β) consists of pleiotropic cytokines which is involved in many aspects of cellular functions, spanning from cell growth, proliferation, and differentiation to inflammation and ECM protein
Three isoforms of TGF-β are encoded in mammals, which include TGF-β1, TGF-β2, and TGF-β3. These glycoproteins are secreted into the ECM as an inactive complex with latency associated peptide (LAP). This inactive secretion prevents the immediate binding and activation of TGF-β receptors. Enzymes such as plasmin and cathepsin, or substances such as reactive oxygen species can activate TGF-β. Once this complex is cleaved, the protein is active.

There are many possible pathways towards cardiac remodeling, such as TGF-β, the renin-angiotensin-aldosterone system (RAAS), and activin A. One method of fibrosis and hypertrophy that has been heavily studied is the transforming growth factor beta (TGF-β) pathway. A TGF-β superfamily member will bind to the extracellular region of the type II receptor (TβRII), which then combines with and phosphorylates the type I receptor (TβRI). This complex initiates signalling through Smad-dependent and Smad-independent pathways as shown in Figure 3. The activated receptor complex recruits the receptor-activated (R-) SMADS 2/3 or SMAD 1/5. This complex then binds with Smad 4 which allows the complex to translocate to the nucleus and interact with either co-activators or repressors.

TGF-β1 is known to have effects on the function of every cell in tissue injury, repair and remodeling; this includes cardiomyocytes and fibroblasts. Through TGF-β1 signaling, there is upregulation of ECM protein expression, such as collagen types I and III; there is inhibition of tissue inhibitors of
metalloproteinases (TIMPs), which inhibit the breakdown of collagen fibrils leading to more interstitial fibrosis; and there is hypertrophy of the cardiomyocytes.\textsuperscript{25} To test TGF-\(\beta\)\textsubscript{1}’s impact in the heart further, Lijnen PJ et al. found that providing neutralizing TGF-\(\beta\)1 antibodies \textit{in vivo} prevented the increase of ECM protein expression and reduces the amount of myocardial fibrosis.\textsuperscript{28}

\textbf{Figure 3. Effects of TGF-\(\beta\) signaling.} TGF-\(\beta\) signaling influences several key events in the production of cardiac fibrosis. The downregulation of MMPs and upregulation of TIMPs inhibits the degradation of excess collagen fibrils, it encourages fibroblast proliferation, and increases ECM protein deposition. From Biernacka A et al. 2011.\textsuperscript{29}
Protective members of the TGF-β family

Two members of the TGF-β family are myostatin and activin A. Both of these ligands send signals through SMAD 2 and SMAD 3 intracellular signaling and are negative regulators of cellular growth. Working through SMADs 2/3, myostatin inhibits the differentiation and hypertrophy of skeletal myotubes. The inhibition of skeletal muscle growth can lead to cachexia, or muscle wasting disease, which increases the risk of HF.

Both myostatin and activin A, on the other hand, are capable of attenuating cardiac hypertrophy in vitro, and activin A transcript levels rise in cases of congestive heart failure. Activin A is capable of protecting cardiomyocytes from hypoxia/reoxygenation-induced apoptosis in vitro and also protected cardiac tissue from ischemia/reperfusion injury. In addition, it has been shown that activin A limits the hypertrophic response of cardiomyocytes in vitro, but that suppression is reversed with overexpression of follistatin-like 3 (FSTL3). It is believed that overexpression of FSTL3 results in excess binding of activin A, leading to decreased activation of the cardioprotective SMAD2/3.

FSTL3 and its Role in Cardiac Hypertrophy and Fibrosis

Follistatin-like 3 (FSTL3) is a member of the follistatin family, a group of extracellular glycoproteins which antagonize the effects of the members of the TGF-β family, such as the cardioprotective activin A and myostatin. Once a member of the TGF-β family binds to its receptor, the Smad signaling cascade
transcriptionally regulates the expression of FSTL3 to provide a negative feedback loop for TGF-β.\textsuperscript{34} Follistatin (FST) and FSTL3 are similar in both structure and function. However, these two extracellular regulators differ in their expression distribution. While FST expression is highest in the ovary, testes, and kidney, FSTL3 expression is preferentially expressed in the heart.\textsuperscript{13,35}

Genetic ablation of follistatin-related gene (FLRG), the gene that encodes for the FSTL3 glycoprotein, has already been studied in a full-body over-expression and knockout models.\textsuperscript{35,36} In the full-body over-expression model, following a high-fat diet over 12 weeks, there was a decrease in visceral fat imaged by MRI in addition to improved glucose tolerance as compared to the control group on high-fat diet.\textsuperscript{36} A genetic knockout of FSTL3 also showed several interesting results, including similar results to the over-expression model. The pancreatic islet sizes in the knockout mice were disproportionately large, leading to increased insulin concentrations. First believed that this was a sign of insulin resistance, a glucose tolerance test showed enhanced glucose tolerance tests.\textsuperscript{35}

FSTL3’s actions in the heart have also been explored, although not as extensively as whole-body targets. Through these experiments, FSTL3 was shown to promote LVH and cardiac fibrosis in models of pressure overload-induced hypertrophy, ischemic injury, and β2 adrenergic agonist-induced hypertrophy.\textsuperscript{34} Both FSTL3 and activin A have been found to be regulated by
levels of stress in the heart, and were markedly upregulated following TAC after 1 week. The cardioprotective role of activin A is highlighted by Oshima et al. who injected a recombinant human activin A protein and witnessed a large reduction in infarct size following ischemia/reperfusion (I/R) injury. The opposing actions of FSTL3 on activin A were demonstrated by Shimano et al., who generated FSTL3 knockout (KO) mice. These KO mice showed reduced cardiac hypertrophy, LV wall thickness and dilatation following TAC than control mice.

The role of FSTL3 on cardiac fibrosis has also been investigated. Panse et al. discovered that FSTL3 is necessary and sufficient to begin the process of cardiac fibrosis, although fully developed cardiac fibrosis requires additional factors. The interaction of the cardiokine FSTL3 and the components of the structure of the heart are shown in Figure 4.
Figure 4. FSTL3’s effect on cardiac fibrosis and hypertrophy. FSTL3 is secreted from cardiac myocytes and is thought to act in a paracrine manner to develop hypertrophy. In addition, FSTL3 acts upon fibroblasts to promote proliferation and increased expression of collagens. From Panse et al. 2012.34

Interestingly, FSTL3 has been found to be upregulated in end-stage HF and correlated with disease severity.32,34 Initial findings from Tu show that FSTL3 is correlated to parameters of HF including LV wall thickness and declining
diastolic function in instances of MetS.\textsuperscript{17} Using plasma from 63 patients diagnosed with MetS, and 14 control patients, the plasma levels of FSTL3 was significantly increased in patients with low mitral annular myocardial diastolic velocity ($E_m$) and left ventricular hypertrophy (Figure 5).\textsuperscript{17} While this certainly doesn't speak to the role of FSTL3 in cardiac remodeling, it does suggest it could be included in the pathway.

Through these experiments, it appears that FSTL3 is involved in the regulation of cardiac fibrosis and hypertrophy following pressure overload. While pressure overload and MHD have several features in common, it is important to determine the role of FLRG in MHD.
Figure 5. Plasma levels of FSTL3 correlated with diastolic function and LVH. These scatter plots show the correlation between plasma levels of FSTL3 and EM and LVH in certain human subjects. A) Higher levels of
FSTL3 are correlated ($r^2=0.13$, $p=0.002$) with lower values of $E_m$ indicating diastolic dysfunction in patients with metabolic syndrome. B) Higher levels of FSTL3 are also seen with higher values of LVH, indicating the level of FSTL3 is indicative of structural change in the heart. Figures from Tu 2015.17

**Specific Aims and Objectives**

The purpose of this study is evaluate the impact of the genetic ablation of a potential mediator of cardiac remodeling to determine its effect in a HFHS diet-induced obesity model of metabolic syndrome. The hypothesis for this project is that the genetic ablation of FLRG will prevent the hypertrophy and fibrosis that is associated with a HFHS diet and metabolic syndrome.

Understanding the development of cardiac hypertrophy and fibrosis in diet-induced MHD is an important milestone to get closer to early treatment of MHD and HF. By identifying the cardiokines that are the most influential on cardiac remodeling, there is potential for therapeutics to delay, or prevent, the development of cardiac remodeling that eventually leads to diastolic dysfunction, heart failure, and mortality. Through these experiments, we hope to:

- Test whether HFHS diet-induced cardiac hypertrophy is prevented by a knockout of FSTL3 in cardiac myocytes.
- Test whether HFHS diet-induced myocardial fibrosis is prevented by cardiac myocyte-specific FSTL3 KO.
METHODS

**HFHS diet mouse model of MetS and MHD**

To accurately demonstrate the effect of diet on MHD, a HFHS diet model was used. A HFHS diet has been found to show to the same clinical features of MetS, including obesity, high fasting plasma glucose, and hypertension.\(^{20,21}\) In addition, a HFHS model of MetS has been shown to lead to a cardiac phenotype of MHD, including left ventricular hypertrophy, diastolic dysfunction, and interstitial fibrosis, all of which are critical to the purpose of this project.\(^{37,38}\) Twenty-four CL57BL/6 mice were fed a HFHS diet for 5 months beginning at two months of age, which is standard for the HFHS diet model of MHD.

**FSTL3 KO mouse model**

To investigate the role of the FSTL3 KO in particular, a Cre-Lox method was used to delete exons 3-5 in a cardiomyocyte-specific method.\(^{13}\) To obtain the desired cardiomyocyte-specific FSTL KO, female homozygous \(Fstl3^{flox/flox}\) were crossed with male mice heterozygous for overexpressed Cre-recombinase promoted by the \(\alpha\)-myosin heavy chain promoter (\(\alpha\)-MHC-Cre). These mice had the C57BL/6 background and were obtained from The Jackson Laboratory. To ascertain the genotype of the progeny, tail snips from the mice were digested using the REDExtract-N-Amp™ Tissue PCR Kit (Sigma) to extract the genomic DNA. The DNA products were then multiplied using the polymerase chain
reaction (PCR). This PCR protocol consisted of an annealing temperature of 56°C and ran for 40 cycles. To detect the knockout of FSTL3, the following loxP site-specific primer pair was used:

**SJL 954** 5’-TCTGAGAAGAGGGATTCAAG-3’

**SJL 955** 5’-ATTTACACCTAGCCACATCTCTG-3’

The wild type allele produces a 270-bp fragment while the loxP site generates a 310-bp fragment. To test for cardiomyocyte-specific knockout of FSTL3, the heart specific α-MHC-Cre transgene was detected with the following primers:

**9543 Fwd** 5’-ATGACAGACAGATCCCTCCTATCTCC-3’

**9544 Rev** 5’-CTCATCACTCGTTGCATCATCGAC-3’

In addition, an internal control was used to detect the presence of DNA. These are the primers to bind to the T-cell receptor delta locus (Tcrd):

**olMR8744 iFwd** 5’-CAAATGTTGCTTGTCTGGTG-3’

**olMR8745 iRev** 5’-GTCAGTCGAGTGCACAGTTT-3’

The PCR products were then run on a 2% agarose gel and 0.01% GelRed™ nucleic acid stain (Biotium) using electrophoresis. The resulting fragment bands can be seen in Figure 6.
Figure 6. Genotyping FLRG KO mice. Using PCR, the genotype of each mouse was determined to ensure the correct genetic manipulation was used for the experiment. A) Shows bands for FLRG KO vs. WT allele to determine the status of the floxed allele. B) Bands showing expression of α-MHC Cre. The internal control is a combination of the forward and reverse primers for Tcrd, T-cell receptor delta, which is endogeneous in all mice.

The breeding strategy shown in Figure 7 was used to generate $Fstl3^{\text{flox/flox}}$, $\text{Cre}^{\text{+/+}}$. 
After three generations, the desired genotype of $Fstl3^{flox/flox}$, Cre$^{+/}$ was available (See Figure 7). These mice were then put on a high-fat, high-sucrose diet or a control diet for five months. Mice were then sacrificed and whole heart tissue was collected, placed on cold phosphate buffered saline (PBS, Bio-Rad) on ice, cut into three to four pieces, and snap frozen using liquid nitrogen. Prior to freezing the tissue, a small piece of the heart tissue was placed in a 10% formalin solution (Sigma) for 24 hours and, using an EDTA-coated pipette tip, a blood sample was taken from the chest cavity and placed into a separate EDTA-coated BD Microtainer®.
**Figure 7. Breeding strategy for FSTL3 KO mice.** Mice were bred so that they progeny would be homozygous for FSTL3 KO, and hemizygous for α-MHC-Cre or α-MHC-Cre<sup>−/−</sup>. Figure from Tu V. Dissertation, BU. 2015.

**Plasma Collection**

Upon collection of a blood sample from the chest cavity of the mice, the EDTA-coated tubes were placed on ice until all samples were collected. These blood samples were then centrifuged at 4,615 rpm at room temperature for five
minutes to form a loose pellet of cellular debris. The supernatant was then extracted and placed in a new 1.5 mL Eppendorf microcentrifuge tube. This supernatant was centrifuged again at 20,000 rpm for 15 minutes at room temperature. The supernatant (plasma) was collected, placed in a new, labeled 1.5 mL Eppendorf microcentrifuge tube, dipped into liquid nitrogen to snap freeze the sample, then stored at -80°C.

**Histology Preparation**

The small samples of heart tissues placed in formalin during the heart tissue collection were left to sit for 24 hours at room temperature. Then, the formalin was removed, and replaced with 1X PBS (Bio-Rad). The samples were then embedded in paraffin and sectioned with a microtome to produce 4 µm thick sections that were then placed in a warm water bath. The sections were positioned on labeled, positively charged glass slides and left to dry overnight.

**Picrosirius Red Staining for Fibrosis**

The dried slides of tissue sections were rehydrated with xylene and ethanol baths in decreasing concentrations of 100%, 95% for 10 minutes each, and 80% and 70% for five minutes each. The slides were placed in Bouin’s Fixitave (Polysciences) that had been prewarmed to 55°C and allowed to sit in the 55°C water bath for 1 hour. The samples were then removed and washed with tap water for approximately 10 minutes, or until the visible yellow stain from
the Bouin's washed away. The slides were then placed in Wiegert's Iron Hematoxylin (Sigma) for 7 minutes, then removed and washed again with tap water for another 10 minutes. The sections were then placed in the Picosirius Red A solution (Polysciences) at room temperature for 2 minutes, rinsed with distilled water, then stained with Picosirius Red B solution (Polysciences) for 1 hour at room temperature. Before the dehydration process, the excess Picosirius Red solution was wiped away from the slides. The sections were then dehydrated using ascending concentrations of ethanol of 95%, and 100% for 5 seconds in each, then were cleared with xylene. The dehydrated sections were then mounted using Eukitt quick-hardening mounting medium (Fluka Analytical) and left to dry overnight. The images were captured using an Olympus BX 40 microscope on 20x magnification. Analysis was done using histology sections. About 6-8 sections were obtained from each animal’s heart. Then, about 2-3 fields of view from each section were imaged to use for analysis. The limited amount of fields of view from some of the samples prevented extensive analysis. Therefore, a maximum of 13 fields of view were quantified. The amount of fibrosis identified with the Picosirius Red stain was quantified using ImageJ (NIH, Bethesda, MD).

**Hematoxylin and Eosin Staining for Hypertrophy**

The tissue sections were rehydrated with 15 minutes of a xylene bath, then 10 minutes each of descending ethanol concentrations of 100% and 95%,
then 5 minutes each of 80% and 70%. The slides were then rinsed with distilled water for 2 minutes. The slides were then stained with hematoxylin (Vector) for 5 minutes, washed with tap water for 5 minutes, placed in 95% ethanol for 30 seconds, and then stained with eosin (Sigma) for 3 minutes. The slides were then dehydrated with ascending concentrations of ethanol of 95% and 100%, and were then placed in 3 baths of xylene for 5 minutes each. Tissue sections were mounted using Eukitt quick-hardening mounting medium (Fluka Analytical) and left to dry overnight. The images were captured using an Olympus BX 40 microscope on 40x magnification. Analysis was done using histology sections. Approximately 8-10 sections were obtained from each animal's heart. Then, about 10-15 different fields of view from each section were imaged, and the cells with clear cell membranes and centered nuclei were used for measurements. Each heart provided anywhere from 30-70 myocytes for measurements. The diameter of cardiomyocytes was measured using ImageJ (NIH, Bethesda, MD).

**Statistical Analysis**

Results are displayed as mean ± SEM. Differences among groups were tested using unpaired, non-parametric Mann-Whitney t-tests. Due to the low sample sizes in the groups, non-Gaussian distribution was assumed in all of the calculations. Statistical analyses were executed using GraphPad Prism version 5 (GraphPad Software, Inc.). Statistical significance was identified with a p-value < 0.05.
RESULTS

*FLRG KO had no apparent impact on weight gain or heart development*

Over the course of the experiment, all animals were weighed monthly to track progress. A total of 23 mice were followed in this experiment, with four mice in the WT CD group, five mice in the WT HFHS diet group, five mice in the FLRG KO CD group, and nine mice in the FLRG KO HFHS diet group. Each heart generated approximately 8-10 sections for histology that were 4 µm thick. Diet-induced weight gain was not affected by FLRG KO (Figure 8). The HFHS diet-fed groups gained the expected amount of weight after five months on diet (Figure 9).
Figure 8. Mice gained weight over time. This chart shows body weight (in grams) gained by the animals over time. All animals gained similar amounts of weight; however, the FLRG KO groups gained less weight in both diet groups. There was statistical significance between every group (p<0.05).

Figure 9. Percent change in body weight after five months on diet. HFHS diet groups gained weight (vs. CD fed) and the weight gain was not affect by FLRG KO. The asterisk indicated statistical significance compared to WT CD, with a p-value < 0.05.
Upon completion of the diet portion of this study, the hearts were weighed to get the raw heart weight for each animal (Figure 10), and then compared separately with each animal’s respective tibia length to normalize the body weight. There were four hearts in the WT CD group, five hearts in the WT HFHS, five hearts in the FLRG KO CD group, and nine hearts in the FLRG KO HFHS group. HW/tibia length seemed to be lower in FLRG KO HFHS mice than the WT HFHS group; however, this result was not statistically significant (p=0.10) (Figures 11 and 12).

**Figure 10. Heart weights.** This figure displays the average heart weight for each animals group. The FLRG KO HFHS was statistically different from the WT HFHS, suggesting that a knockout of FLRG would help to
protect from the extent of hypertrophy seen in the WT HFHS diet fed group.

**Figure 11. Individual heart weight/tibia length.** This figure shows the individual data points for each heart weight/tibia length from each mouse in the study.
Figure 12. Heart weight/tibia length for all groups. This chart shows no difference between all of the groups. However, there is a slight trend for the FLRG KO HFHS heart weight/tibia length to be slightly lower than the WT HFHS group (p=0.10).

**FLRG KO attenuated cardiomyocyte hypertrophy**

Following five months on a HFHS diet, WT cardiac myocytes in the HFHS-fed group were larger (131%) than in WT CD-fed mice. The ablation of FLRG attenuated the extent of hypertrophy seen in HFHS-fed wild-type mice (Figures 13, 14, and 15). Although there was still hypertrophy of the cardiomyocytes, the extent of the hypertrophy seen in the FLRG KO HFHS group (mean
cardiomyocyte diameter = 20.29 ± 0.23) was significantly different than that seen in the WT HFHS group (mean cardiomyocyte diameter = 21.76 ± 0.34).

Figure 13. Microscopy of cardiac myocyte hypertrophy. These four images show sections of heart tissue sections. The size differences in diameter between all four experimental groups are shown in the slides. Although the differences are most easily seen between the control diet and HFHS groups, the hypertrophy in the FLRG KO HFHS is significantly different than that in the WT HFHS group. All images show heart tissue stained with hematoxylin and eosin. Images captured at 40X.
magnification. A) WT CD B) WT HFHS C) FLRG KO CD D) FLRG KO HFHS

Figure 14. *Individual cardiac myocyte measurements*. This figure shows the individual cardiac myocyte diameter measurements for all mouse hearts in the experimental study.
Figure 15. Mean diameter of cardiomyocytes. The mean diameter of the cardiomyocytes between the WT HFHS group and FLRG KO HFHS group are both significantly different from the WT CD group with p-values of <0.0001 in each group. In addition, the FLRG KO protected the cardiomyocytes from the full extent of the hypertrophy seen in the WT HFHS (p=0.0009). The asterisk indicates a statistically significant difference between the indicated group and the WT CD group. The pound sign indicates a statistically significant difference between the WT HFHS and FLRG KO HFHS groups.
FLRG KO induced more fibrosis

To further explore the cardiac remodeling in a FLRG KO during a HFHS diet, cardiac interstitial fibrosis was imaged and quantitated (Figure 16). In WT CD-fed mice there was approximately 1.5% fibrosis. Following five months on a HFHS diet, this was increased to around 2%. With CD feeding, fibrosis was increased in FLRG mice relative to WT mice. Likewise with HFHS feeding fibrosis was increased in FLRG vs. WT mice.
Figure 16. *Microscopy of cardiac interstitial fibrosis*. These four images show the difference in fibrosis between all four experimental groups. Collagen shows as red with this particular stain. Images were captured at 20x and analyzed with ImageJ. All images show heart tissue stained with picrosirius red. **A) WT CD B) WT HFHS C) FLRG KO CD D) FLRG KO HFHS**

![Fibrosis %](image)

**Figure 17. Individual fibrosis data.** This figure shows the thirteen individual cardiac interstitial fibrosis measurements for all mouse hearts in the experimental study.
Figure 18. **Mean percentage of interstitial fibrosis.** Both the FLRG KO control diet group and the FLRG KO HFHS diet group showed increased interstitial fibrosis following five months on diet as compared to the WT CD group. The p-value between the control diet groups is 0.024, and the p-value between the HFHS groups is <0.0001. The asterisk shows statistical significance between the group identified and the WT CD group. The pound sign indicates statistical significance between the WT HFHS and FLRG KO HFHS group.
DISCUSSION

The interactions between FSTL3 and members of the TGF-β family, such as TGF-β1 and activin A, have been investigated to discover more about their roles in cardiac remodeling. Previous studies have shown that whole body knockouts and overexpressions of FSTL3 lead to dramatic improvements in endocrine function as related to insulin resistance and visceral fat deposition.\textsuperscript{35,36} In studies of I/R injury and pressure overload, cardiac-specific knockout of FSTL3 has shown to be cardioprotective in that activin A can induce its effects via the SMAD pathway to prevent hypertrophy after TAC, and also prevent apoptosis in cultured cardiac myocytes.\textsuperscript{13,32}

Myocardial hypertrophy. The attenuated hypertrophy seen with the FLRG KO group matches the notion from past research that a cardiac-specific ablation of FLRG is beneficial. All of the past studies involved with cardiomyocyte specific ablation of FLRG are related to the study of TAC, or pressure overload.\textsuperscript{13} Since we expected the mice in our study to develop metabolic syndrome, including hypertension, after five months on a HFHS diet, the attenuated hypertrophy is not surprising. As it relates to our hypothesis, however, we believed the FLRG KO would prevent the hypertrophy. In fact, the FLRG KO did attenuate the extent of hypertrophy seen in FLRG KO HFHS mice.

Fibrosis. We observed the expected increase in fibrosis with HFHS in the wild-type group. Surprisingly, there was an increase in fibrosis in the FLRG KO CD and HFHS diet groups compared to their respective WT counterparts.
In both CD and HFHS-fed mice the FLRG KO groups had a striking increase in fibrosis compared to their WT counterparts. This is in contrast to the hypothesized decrease in fibrosis with FLRG KO, and furthermore, is in contrast to the decrease in myocyte hypertrophy observed with FLRG KO. While the reasoning is unknown we can speculate on certain possible causes.

A potential pathway that could explain these results come from two known mediators in cardiac remodeling: FSTL3 and members of the TGF-β family. It has been shown that FSTL3 is an antagonist of members of the TGF-β superfamily, including TGF-β1 and activin A. With a reduction in the amount of antagonizing effects of FSTL3 on these two components, each can influence cardiac remodeling in their respective ways. Several studies have shown TGF-β1’s role in heart failure and cardiac fibrogenesis. Without the negative regulator of TGF-β, it leaves TGF-β1 to promote collagen deposition.

The potential pathway for the attenuated hypertrophy consists of activin A’s cardioprotective action to prevent hypertrophy and apoptosis in stressed the stressed heart. Without FSTL3, the negative regulator of activin A, the cardioprotective SMAD 2 signalling pathway would be increasingly active, which would lead to attenuated hypertrophy.

**Limitations.** The HFHS diet induced increase in HW/tibia length was not significant, although the trend between the WT HFHS and FLRG KO HFHS was strong (p=0.1). This may reflect low numbers or could have been a result of experimenter error while trimming the atria off of the heart after excision. Any
additional, unneeded tissue that was removed from the heart could have impacted the heart weight, and led to inaccurate results. However, myocyte size measured with histology showed the expected increase with HFHS diet, suggesting that there was cardiac hypertrophy.

A second limitation of this study was that the HFHS diet mice did not develop as much fibrosis as expected. This could be due to several factors, however. For example, there were small sample sizes in this project, with the WT CD group only having four viable animals, and five animals each in the WT HFHS and FLRG KO CD group. In doing our statistical analysis, we assumed non-Gaussian distribution to help correct for this, although the impact to the analysis is still present. In addition, there could have been errors in the quantification of the fibrosis using ImageJ. There are still many subjective decisions to be made regarding the closeness of the quantitated image as compared to the original image captured from the microscope.

Conclusion. This study was the first to test the role of FLRG in MHD using a cardiac-myocyte-specific FLRG KO mouse. The major new finding is that FLRG appears to mediate cardiac myocyte hypertrophy in MHD. Conclusions regarding the role of FLRG in fibrosis were not possible due to the lack of a change in the WT HFHS-fed group. Knowledge of the mechanism of cardiac fibrogenesis and hypertrophy in a model of metabolic syndrome could prove extremely beneficial to determine the early signs of heart failure in obese men
and women with metabolic syndrome and lead to early treatment and, perhaps eventually, prevention of heart failure due to metabolic heart disease.
LIST OF JOURNAL ABBREVIATIONS

Am J Cardiol. .................................................. American Journal of Cardiology
Am J Physiol - Cell Physiol. .... American Journal of Physiology – Cell Physiology
Arterioscler Thromb Vasc Biol. ...................... Arteriosclerosis, Thrombosis, and Vascular Biology
Cardiol Res Pract. ......................................... Cardiology Research and Practice
Cell Mol Life Sci. ........................................... Cellular and Molecular Life Sciences
Circ Cardiovasc Imaging ............................... Circulation Cardiovascular Imaging
Circ Res. ................................................................. Circulation Research
Cytokine Growth Factor Rev. ....................... Cytokine & Growth Factor Reviews
Eur Heart J. ......................................................... European Heart Journal
FASEB J. .............................................................. The FASEB Journal
Int J Cardiol ....................................................... International Journal of Cardiology
J Am Coll Cardiol. ......................................... Journal of the American College of Cardiology
J Am Heart Assoc .............................. Journal of the American Heart Association
JAMA ................................................................. Journal of the American Medical Association
J Biol Chem. ....................................................... Journal of Biological Chemistry
J Card Fail ............................................................. Journal of Cardiac Failure
J Cardiovasc Transl Res. .......... Journal of Cardiovascular Translational Research
J Mol Cell Cardiol. ........................... Journal of Molecular and Cellular Cardiology
Mol Genet Metab. ........................................ Molecular Genetics and Metabolism
Pharmacol Ther ........................................... Pharmacology and Therapeutics

Physiol Rev .................................................. Physiological Reviews

Proc Natl Acad Sci. ...................... Proceeding of the National Academy of Sciences

Transl Res J Lab Clin Med. ............... Translational Research, The Journal of Laboratory and Clinical Medicine
REFERENCES


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EDUCATION

M.S. in Medical Science Anticipated 2016
Boston University School of Medicine, Boston, MA

B.S. in Biology, cum laude May 2012
Regis University, Denver, CO

Research Experience

Whitaker Cardiovascular Center, Boston University School of Medicine Research Assistant – Master of Science Thesis, July 2015 – May 2016
• Planned and implemented an independent project detailing the role of FSTL3 in cardiac hypertrophy and fibrosis in metabolic syndrome.
• Updated the lab manager regularly using presentations at weekly lab meetings.
• Collected all samples for analysis using carefully organized plans, and detailed notes.
• Conducted histological preparation and staining procedures using picrosirius red, hematoxylin, and eosin.
• Analyzed all data for statistical significance using Friedman, Wilcoxon, Kruskal-Wallis, and Mann-Whitney tests.

National Renewable Energy Laboratory, Golden, CO
Internship in Microalgal Biofuels, August 2011 – May 2013
• Performed detailed chemical analysis on algae samples using a variety of solvents and laboratory instruments
• Recorded detailed results of fatty acid methylated ester (FAME) analysis, optical density, and soxhlet extractions
• Established a firm foundation in basic biological science research while strengthening work ethic by working in a professional environment and government lab

**Regis University**, Denver, CO
Lab Research Assistant – Ichthyology, June 2010 – August 2010
• Recorded anatomical characteristics of fish skeletal systems resulting in a new, updated phylogenetic tree
• Interpreted results using the computer program MacClade

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**Clinical Experience**

**Penrose Hospital**, Colorado Springs, CO
Patient Transport Aide, June 2013 – June 2014
• Served patients by transporting them to schedule appointments in various departments throughout the hospital
• Oversaw the logistics of oxygen transportation throughout the hospital
• Ensured sanitization of transportation vehicles

**Penrose Hospital**, Colorado Springs, CO
• Observed the general and administrative responsibilities of an internist involved with patient care
• Interacted with other health care professionals (e.g. physicians, nurses, respiratory therapists, case managers, etc.) and observed their roles in patient care

**University of Colorado Cancer Center**, Aurora, CO
Clinical Research Assistant, May 2011 – August 2011
• Conducted follow-up interviews with patients who had participated in clinical trials for cancer treatment regarding their preset condition and updated their file accordingly
• Attained experience with the role of planning, budgeting, and follow-up care associated with a formal research study

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**Other Work Experience**

**Regis University**, Denver, CO
Writing Consultant, January 2009 – May 2012
• Provided students critical feedback regarding written works, specifically on topics of grammar, organization, formatting, and research practices
Coached students on professional accepting and using constructive criticism and feedback, as needed

**Regis University, Office of Residence Life, Denver, CO**
Resident Assistant, August 2009 – May 2011
- Designed programs that contributed to a healthy floor community and learning opportunities for students
- Counseled residents in dealing with personal and academic issues
- Served as a first responder to emergency situations on-campus

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**Significant Coursework Experience**

**Denver Health Medical Center, Denver, CO**
Clinical Trial Enrollment Assistant, September 2010 – December 2010
- As part of the Biomedical Clinical Research course at Denver Health Medical Center, learned steps involved in planning, implementing, and statistically analyzing clinical research data.
- Gained teamwork experience in a medical setting while enrolling patients into ongoing clinical studies at the hospital.