Molecular Mechanisms of Heart Valve and Skeletal Muscle Development and Disease

Margaret E. Benny Klimek
University of Miami, mbklimek678@gmail.com

Follow this and additional works at: https://scholarlyrepository.miami.edu/oa_dissertations

Recommended Citation
https://scholarlyrepository.miami.edu/oa_dissertations/1515
UNIVERSITY OF MIAMI

MOLECULAR MECHANISMS OF HEART VALVE AND SKELETAL MUSCLE DEVELOPMENT AND DISEASE

By

Margaret E. Benny Klimek

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

December 2013
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

MOLECULAR MECHANISMS OF HEART VALVE AND SKELETAL MUSCLE
DEVELOPMENT AND DISEASE

Margaret E. Benny Klimek

Approved:

Christian Faul, Ph.D.
Assistant Professor of Medicine

Alessia Fornoni, M.D, Ph.D.
Associate Professor of Medicine

Carlos Moraes, Ph.D.
Professor of Neurology

Nevis Fregien, Ph.D.
Associate Professor of Cell Biology

Steven Lipshultz, M.D.
Professor of Pediatrics

M. Brian Blake, Ph.D.
Dean of the Graduate School
Heart valves function to provide unidirectional blood flow during each cardiac cycle. The development of the heart valves from embryonic stages is a highly regulated process involving many signaling pathways in order to provide the proper extracellular matrix components in the trilaminar structure. When these processes are dysregulated, disease can persist in the valves. Here we examined additional levels of regulation in the heart valves at the level of miRNAs and phosphate homeostasis. There are many studies examining miRNA regulation in the heart, however, there is little knowledge about which miRNAs are expressed in the heart valves during development, maturation, homeostasis and disease. To address this gap and determine miRNA regulators of valve development and disease, RNA was extracted from mouse atrioventricular (AV) heart valves at mE11.5 (endocardial cushion), mE15.5 (remodeling), postnatal (maturing), and 4 months (4m) of age (maintained). The mechanism of miR-101 binding to the 3’UTR of Sox9, a SRY transcription factor required for proper valve development, was analyzed and the results suggest Sox9 may be regulated in the valves by miR-101 during development.

In addition to the miRNA valve studies, although elevated FGF23 and phosphate serum levels have been demonstrated to be associated with vascular calcification in patients with chronic kidney disease (CKD), the direct effect on the heart valves remains unknown. Here we show evidence for phosphate, but not FGF23 promoting calcification
in heart valve explants, valve interstitial cells and in mouse aortic smooth muscle cells. Sodium phosphate (NaPh) Co-transporters are required for this calcification and their expression is altered by phosphate and FGF23. 

Lastly, the data presented here also shows a mechanism by which skeletal muscle wasting or cachexia can be prevented in mouse models of cancer cachexia. These studies specifically look at inhibiting myostatin-family ligands in order to protect skeletal muscles from cancer induced wasting.

Taken together, these studies provide evidence as to examine both heart valve and skeletal muscle signaling pathways further in order to understand the developmental processes that have gone awry in diseases associated with these tissues. (Revised 7/2014 CREC)
Dedication

I want to dedicate this work to my family Bradley, Niko Parker and Alexandra Elizabeth. You have taught me the meaning of family and unconditional love.

Brad, thank you for constant support and love. You have given me the greatest gifts I could ever ask for with our children. So far, our entire married life I have been in graduate school and you have been my rock and supported me in every possible way. Although in the past year and half we have lived apart, been alone, or with Alex or Niko, it was nice to know that no matter the circumstance you were there for me and believed in me. I feel that if we can get through what we did in the last year and a half we can face any challenges together and be supportive of one another in every aspect of our lives. You are an amazing father and our children absolutely adore you. I love you with all my heart and am so happy that I have the rest of my life to learn even more about you.
Acknowledgements

There are so many people that helped me along the path toward my Ph.D. and I am grateful for everything that I have learned along the way.

My family – Mom and Dad, thank you for teaching me the importance of education. You have always been supportive of all my endeavors and for that I am forever grateful.

Karen and Joey, than you for always being there and supporting me along the way.

Canterbury teachers – with out this school and the staff it would not have been possible for me to have my children close tome during the day and resume my work after having Niko and Alex.

Committee members – Dr. Moraes, Dr. Fregien, Dr. Lipshultz and Dr. Fornoni, Thank you for all of our wonderful discussions for always being supportive and available to talk. It has been a pleasure working with all of you and I hope that in the future we will collaborate.

Molecular, Cell and Developmental Biology Department - Maria Penton, thank you for your help along the way, you are such an amazing woman and I wish all the best for you in all your endeavors. Our graduate student advisors, Dr. Salas and Dr. Fregien, thank you for always listening and helping me along the way. You both have taught me how to handle unique situations for students I advise in the future and have prepared me to do so in a professional manner.
Lab managers, Agata Levay & Jeff Vanwye, you have taught me so much over the years and for that I thank you. You both are more than “lab managers” and more like family members to me.

I have learned so much from previous students and mentors and just wanted to thank a few individuals that contributed to my education in graduate school.

Drs. Ge Tao, Jacque Peacock and Joy Lincoln for teaching me all there is to know about heart valves. Drs. Darci Moore, Noelia Kunzevitzky, Yuanli Duan and Jeff Goldberg for teaching me neuroscience along the way. Also, Drs. Teresa Zimmers and Tufan Aygodu and Paul Spalding for immersing me into the biology of cachexia and muscle wasting.

The Fornoni Lab Members – Thank you for always helping me when I needed cells or just someone to talk to. I especially would like to thank Sandra, Chris and Rodrigo for being so very helpful with planning experiments and Chris for always asking me questions and keeping me on my toes.

Dr. Alessia Fornoni you have been such an amazing support and role model for me. Thank you for being there always… even on a Sunday when I felt I was alone in the lab. At times I felt you were the only one that could understand and I am truly grateful for you to be a part of my scientific education and my life. I hope that our relationship continues to grow over the years even thought the distance may be great, our work can bring us together.
The Faul lab…

Alex, Britta, Mary, Christina and Karla. I only spent a small amount of time working with each of you, but am certainly glad I had the opportunity to do perfusions with Alex, share a bench with Britta and teach Carla how to work with RNA.

Saurav, you are a wonderful young man. Thank you for always listening and I wish you all the best in your career.

Ansel, one word… Salsa. Thank you for your support and advice along the way.

My dearest friends….

Alexis Sloan, it has been such a pleasure working with you. You are truly one of my best friends and one of the most amazing people I have met in my life. Thank you for always being there for everything. Not only has it been fun watching our scientific careers grow, but also our bellies during our pregnancies. Your family is absolutely beautiful and I hope that we always can stay close and one day… we will have that lab together just like we wished…

Linsey Lindley, if I didn’t have you I would not be here today. You have been such an amazing friend and confidant and I now understand what my professor was talking about in my undergrad when she said the friends you make in Graduate school will be the ones you have for life. You are one of the most brilliant young women I have ever met and I know you are going to be an amazing physician/scientist. Just remember Alexis and I will be getting that joint lab together in a few years and need a clinical liason to help.
Alexis and Linsey I truly hope the three of us can make a difference not only in science but for other young women that come through this career path.

Most of all I would like to thank my mentor, Dr. Christian Faul, for his overwhelming support. Without you I would not be finishing my Ph.D. You have taught me how to be a mentor that is well educated, close to the bench and excited about research. You made me realize the joy in science again and allowed me to be independent to learn on my own. Thank you for allowing me to be a part of your amazing group and I only wish the best for you and your family. I am looking forward to collaborating with you in the future and want you to know that learned from you the kind of mentor I hope to be one day.
# Table of Contents

## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>xi</td>
<td></td>
</tr>
</tbody>
</table>

## Chapter 1: Introduction

**Aging Heart Valves**
- History of Heart Valves .................................................. 1
- Valve Function ................................................................. 2
- Valve Structure: Gross Morphology ........................................ 3
- Valve Structure: Stratification of Layers .................................. 4
- Valve Development ............................................................... 5
- Valve Disease ................................................................. 6
- Calcific Valve Disease .................................................... 7
- Clinical Implications of Valve Disease .................................. 8

**Muscle**
- History of Muscle Biology ............................................... 8
- Skeletal Muscle Function .................................................. 9
- Skeletal Muscle Development: Structure ................................ 10
- Skeletal Muscle Development: Signaling Pathways .................... 10
- Skeletal Muscle Diseases ................................................ 11
- Cachexia ...................................................................... 12
- Clinical Implications of Cachexia ....................................... 12

**Concluding Thoughts** ......................................................... 13

## Chapter 2: miRNAs and Heart Valve Development

**Background**
- Heart Valves ................................................................. 15
- Heart Valve Disease .......................................................... 15
- Heart Valve Development ..................................................... 16
- Heart Valve Development: EMT ............................................. 16
- Heart Valve Development: Valve Remodeling ............................... 17
- Heart Valve Development: Sox9: A Key Player in Valve Organization .... 19
- Heart Valve Development: Valve Maturation ..................................... 20
- miRNAs: Discovery and Function ........................................... 20
- miRNAs and Heart Valves .................................................. 21

**Experimental Design**
- miR-101 Regulation of Sox9 mRNA ..................................... 22

**Methods**
- Collection of Murine and Avian Tissue ................................ 23
- RNA Extraction ............................................................... 23
- cDNA Generation and Quantitative Real Time PCR .................. 23
- miR-101 Overexpression Assays ......................................... 24
- Plasmid Cloning and Mutagenesis ........................................ 25
- Luciferase Assays ............................................................ 26
- Statistics .................................................................. 26
Results

miR-101 Functions to Repress Sox9 ......................................................... 27

Discussion

Conclusions ................................................................. 31
Future Directions .......................................................... 33

Chapter 3: The Role of Phosphate and FGF23 in Heart Valve Calcification ............ 35

Background

Chronic Kidney Disease and Vascular Calcification .................................. 35
Phosphate .......................................................... 35
Fibroblast Growth Factor 23 ......................................................... 37
Mouse Models of Valve Calcification with Altered FGF23 Signaling ............ 38

Experimental Design

Methods

Valve Explant Cultures ......................................................... 40
Cell Culture .......................................................... 40
RNA Isolation ........................................................ 40
qRT-PCR .......................................................... 41
Von Kossa Staining ........................................................ 41
Protein Analysis ........................................................ 42

Results

Phosphate Co-transporters are Expressed in MOVAS, Valves and pAVICs ........ 42
Phosphate Induces Calcification in Mouse Aortic Smooth Muscle Cells ........ 43
Phosphate Induces Calcification in Valve Explants and pAVICs ............... 45
Phosphate Co-transporters are Required for Phosphate-Induced Calcification . 48
FGF23 Does Not Induce Calcification ............................................ 51
Phosphate and FGF23 Alter Phosphate Co-Transporter Expression .......... 53
Phosphate Induces Apoptosis in MOVAS ........................................ 54
Phosphate and FGF23 Alter Bone Gene Signatures in pAVICs ............... 55

Discussion

Conclusions ................................................................. 56
Future Directions .......................................................... 58

Chapter 4: ACVR2b Soluble Receptor Therapy for Cancer Cachexia ............... 60

Background

Overview ................................................................. 60
Cancer Cachexia .......................................................... 60
Cancer Cachexia: Signaling pathways ............................................. 61
Myostatin: Discovery and Conservation ........................................ 62
Myostatin: Tonic Regulator of Muscle Development and Growth ............ 62
Myostatin: Biosynthesis and Signaling Mechanisms ................................ 63
Myostatin: Mouse Models with Altered Myostatin Signaling ............... 64
Cancer Cachexia: Animal models ............................................. 65

Experimental Design

Methods

Cell Lines ................................................................. 66
Mice.................................................................................................................66
Mouse Models of Cachexia..................................................................................67
RNA Extraction and Quantitative Real-Time (qRT-PCR)......................................67
Statistics.............................................................................................................67

Results
Myostatin Null Mice have Accelerated Wasting with Cancer Cachexia.............68
Trichostatin Fails to Inhibit Muscle Wasting in C26 Cancer Cachexia..............72
Soluble ACVR2b Increases Muscle Mass............................................................74
Soluble ACVR2b Prevents Cancer-induced Muscle Wasting in Mice.................76

Discussion
Conclusions..........................................................................................................78
Future Directions................................................................................................78

Chapter 5: Concluding Remarks........................................................................82

Citations.............................................................................................................83
List of Figures

Chapter 2
Figure 2.1 miR-101 Conservation and Predicted Consensus Sequence.........................27
Figure 2.2 miR-101 Expression in Heart Valves and Limbs........................................28
Figure 2.3 miR-101 Inhibits Endogenous Sox9 mRNA Expression..........................29
Figure 2.4 Sequence of Sox9-3’UTR-Luc (insert)..................................................29
Figure 2.5 Site-Directed Mutagenesis for generating the mSox9-3’UTR-Luc
Plasmid...........................................................................................................30
Figure 2.6 miR-101 Negatively Regulates Sox9.........................................................31

Chapter 3
Figure 3.1 Phosphate Co-Transporter Expression in Valves, pAVICs and MOVAS.....43
Figure 3.2 Phosphate induces Calcification in MOVAS............................................45
Figure 3.3 Phosphate induces Calcification in Valve Explants..............................46
Figure 3.4 Phosphate Induces Calcification in pAVICS.......................................47
Figure 3.5 Phosphate Phosphate Co-transporters are Required for
Phosphate-induced Calcification in MOVAS.................................................48
Figure 3.6 Phosphate Co-transporters are Required for Phosphate-induced
Calcification in Heart Valve Explants..........................................................49
Figure 3.7 Phosphate Co-transporters are Required for Phosphate-induced
Calcification in pAVICs................................................................................50
Figure 3.8 FGF23 does not Induce Calcification in MOVAS Cells.........................51
Figure 3.9 FGF23 does not Induce Calcification in Heart Valve Explants...............52
Figure 3.10 FGF23 does not Induce Calcification in pAVICS...............................53
Figure 3.11 Phosphate Co-transporter Expression is Down Regulated after Phosphate
and FGF23 Treatment in MOVAS................................................................54
Figure 3.12 Apoptosis is Induced after Phosphate Treatment in MOVAS.............55
Figure 3.13 mRNA Expression in pAVICs treated with Phosphate and FGF23........56

Chapter 4
Figure 4.1 Myostatin Null Mice Have Accelerated Wasting with Cancer Cachexia.....71
Figure 4.2 TSA does not Inhibit Muscle Wasting in C26 Cachexia.........................73
Figure 4.3 Systemic Administration of ACVR2B-Fc Promotes Weight Gain and Muscle
Growth............................................................................................................75
Figure 4.4 ACVR2B-Fc Inhibits Muscle Wasting in Cancer Cachexia.....................77
Chapter 1: Introduction

Aging

"Grow old along with me! The best is yet to be!”

This quote by Robert Browning is what most would like to think about aging. Growing older is accompanied by gaining knowledge and maturity. Life’s lessons are learned through experiences in success and failure. Despite what is learned, the biological process of aging is inevitable and from a medical standpoint can be defined as:

Gradual change in an organism that leads to increased risk of weakness, disease, and death. It takes place in a cell, an organ, or the total organism over the entire adult life span of any living thing. There is a decline in biological functions and in ability to adapt to metabolic stress. Changes in organs include the replacement of functional cardiovascular cells with fibrous tissue. Overall effects of aging include reduced immunity, loss of muscle strength, decline in memory and other aspects of cognition, and loss of colour in the hair and elasticity in the skin. [154]

This definition is from Webster’s Dictionary and is very fitting for the scope of this thesis project. There are many diseases that are prevalent in the aging population including diabetes, cancer and even neurodegenerative disorders. Despite major advancements in science and medical breakthroughs, there are still many diseases that exist without therapies. Most often these diseases have a negative impact on quality of life for patients and are associated with significant costs for healthcare. Heart valve and muscle wasting diseases are two examples that are very prevalent in the population but remain without treatment. Heart valves and skeletal muscles have critical developmental pathways that
are involved with the regulation of proper development and maintenance of these structures and to prevent disease. It has been hypothesized that many diseases are the result of developmental pathways that have gone awry. This highlights why understanding basic regulatory mechanisms during development is important for scientists today. Without this knowledge, the disease etiologies remain elusive and prevent involvement by the medical community. This project focuses on two such diseases that may arise from important developmental pathways: heart valve disease and muscle wasting. These two diseases will be outlined here and this study will provide mechanisms by which developmental pathways might be targeted to prevent the onset or progression of these currently untreatable diseases.

**Heart valves**

“The heart is the household divinity which, discharging its function, cherishes, nourishes, quickens the whole body, and is indeed the foundation of life, the source of all action.”
— William Harvey

**History of Heart Valves**

During the lifespan of an individual the heart beats 100,000 times per day, pumping blood in a unidirectional manner [1]. Throughout the course of this constant extensive muscular activity the heart valves maintain the amount of bloodflow that travels through the heart with every cardiac cycle. This is of course common knowledge today, however, it was not the accepted idea of circulation in the 1600’s. Until then it was believed the blood had no particular directional flow. It wasn’t until the scientist William Harvey published findings indicating that blood flow was circulating and unidirectional, mainly because of valves. Harvey and his teacher, Fabricius, first observed bloodflow was directed by valves and when an artery was blocked the flow stopped. As Harvey
described, the heart valves function to maintain unidirectional blood flow in the heart throughout the cardiac cycle. “So it is proved that a continual movement of the blood in a circle is caused by the beat of the heart.” [2]. He was against the Galenic view at the time and proposed the heart was the pump by which the blood circulated throughout the body. Harvey is now considered the father of systems biology and made significant contributions in anatomy and physiology throughout his life.

**Valve Function**

Harvey established the idea of unidirectional circulation and additionally noted the importance of the valves in the heart after examining and calculating the amount of blood flowing in the body:

> Then we may suppose in man that a single heart beat would force out either a half ounce, three drams, or even one dram of blood, which because the valvular block could not flow back that way into the heart. The heart makes more than a thousand beats in half an hour, in some two, three, or even four thousand. Multiplying by the drams, there will be in half an hour either 3,000 drams, 2,000 drams, five hundred ounces, or some other such proportionate amount of blood forced into the arteries by the heart, but always a greater quantity than is present in the whole body” (Reviewed in [3])

It is now known that the heart valves are critically important to the function of the heart. They provide the exact right volume of blood flow to enter the chambers of the heart between cycles in a unidirectional manner, and when they are not functioning properly have detrimental effects throughout the heart due to increased or decreased pressure and volume. Harvey described they provide the blockade for maintaining the unidirectional blood flow to the body and lungs and was the first to stand behind this observation.
Valve Structure: Gross Morphology

Interpretations of the function of the heart valves based on the structure were made back to Hippocrates time. One of the oldest accounts was published in, “The Heart” by an unknown author describing the anatomy and structure of the heart valves in detail:

membranes and other muscles resembling spiderwebs spread out, gird everywhere the outlets and send fibers into the solid parts of the heart.... There is a pair of [veins] at the entrance to which there have been constructed three membranes for each, rounded at the extremity at least, to the extent of a half-circle, and when they come together it is marvellous that they close the outlets, the end of the veins.... If someone ... removes the heart of a dead man and takes up one of these membranes and bends another up against it, water will not go through into the heart, nor even the breath when forced in." The Heart X (Hurlbutt, 1939) Reviewed in [4].

This demonstrates the importance of the valves and their structure and was recognized even back to antiquity. Using available tools doctors and scientists were able to determine where the valves were in the heart and examine diseased valves. Valve structure has always been a topic of interest to clinicians interested in studying the heart, since the heart is such a vital organ. There are four valves in the heart: aortic and pulmonic or semilunar (SL) valves and mitral and tricuspid or atrioventricular (AV) valves. The pulmonic and tricuspid valves are responsible for maintaining the flow of blood from the body to be oxygenated in the lungs and the mitral and aortic valves are provide the regulation of oxygenated blood to flow to the rest of the body [1]. The AV valves are composed of leaflets that are connected to the papillary muscle by the chordae tendineae and separate the atria from the ventricle. This differs from the SL valves, which are anchored in a root deep in the aorta and pulmonary artery.
Valve Structure: Stratification of Layers

It was not until molecular techniques were developed that the actual components of the valves became evident. The first comprehensive review describing the extracellular matrices in the heart valves was published by Kugel and Gross in 1931 where they describe in great detail each distinct layer of the valves [5]. Although they note others have observed the valves and components, this study was comprehensive including all valves of the heart. With the tools available today, the stratified layers of the valves have become very well characterized through manipulation both in vitro and in animal models. The valves have a trilaminar structure consisting of the fibrosa, spongiosa and ventricularis (or atrialis). The fibrosa is situated at the side of the valve opposite from the bloodflow and is composed mainly of type I and III collagens or fibrillar collagens [1]. The spongiosa is mainly composed of proteoglycans and provides the compressibility, and ventricularis or atrialis is made up of elastic fibers.

Valve Development

It is important to know the structure and function of the heart valves, but understanding the molecular mechanisms underlying the proper establishment of the various extracellular matrices and cells in the valves is just as critical. Significant work has already been done to determine how the heart valves are established during embryonic development and the processes that they undergo until becoming mature valves. These processes are outlined in detail in Chapter 2. Briefly, the initial step in valve development involves endothelial to mesenchymal transition (EMT) where the endothelial cells lining the endocardial cushion begin to transform and migrate into the
cardiac jelly [6]. During valve remodeling, the trilaminar structure of the valve is established from ECM secreted from valve interstitial cells (VICs) [7]. Lastly, the valves mature and the VICs become relatively quiescent throughout the lifetime of the organism [1].

**Valve Disease**

Valve disease is one of the most common problems with the cardiovascular system and can be congenital or arise from an acquired disease. Up to 25% of the aging population has valve disease [8]. There are several ways the function of the valve can be compromised and these include regurgitation and stenosis. Regurgitation typically arises when the valve is “floppy” or myxomatous and cannot close properly allowing backflow of blood into the atria. This usually presents clinically in the form of mitral valve prolapse, which is commonly seen in patients with connective tissue disorders [9].

Stenotic valves are usually calcified and do not open completely for the blood traveling across the valve. This results in increased velocity of blood flow across the valve when measured by echocardiography. Both types of disease cause the heart to work harder and will eventually result in heart failure. The most common valve affected from disease is the aortic valve, although the other valves can also be susceptible [10]. Understanding the molecular mechanisms during development has identified regulatory pathways that are deregulated in valve disease. This is mainly through models of genetic manipulation *in vivo* (Reviewed in [1]). Many of the pathways involved include ECM components like collagens, proteoglycans or elastic fibers or alter the cell number during valve development resulting in hypoplastic or hyperplastic valves [1].
Calcific Valve Disease

Calcified valves or stenotic valves prevent the valve leaflets from opening all the way and create a smaller orifice for the blood to flow through during the cardiac cycle. The gross morphology of calcified valves demonstrates nodules of calcification in the valve leaflets. Stenotic valves result in a higher velocity of blood flow over the valve, which over time results in enlargement of the ventricles. Ultimately, this increased pressure and larger ventricular mass leads to heart failure in these patients. Molecularly, calcified valves have been shown to be composed of a “bone-like” matrix, since it has been discovered that these valves exhibit gene expression patterns similar to those found in bone [11]. Originally this form of ectopic calcification was thought to be passive process, however, it is now accepted that the calcification of the valves and other soft tissues (vasculature) is a highly regulated process similar to bone development.

Normally, VICs in the mature valves are quiescent having little proliferation and gene expression. However, upon activation they take on a myofibroblast-like phenotype and express matrix metalloproteases (MMPs) and smooth muscle α-actin (SMA). Some of the factors that are upregulated in calcified valves are those that are expressed during osteochondrogenesis and include but are not limited to Runx2, alkaline phosphatase (ALP) and osteopontin (also called Spp1) [1]. Atherosclerosis also plays a large role in inducing calcification in heart valves and it has been demonstrated that VICs become activated in response to signaling cascades perturbed by inflammatory cytokines.
Clinical Implications of Valve Disease

Despite significant progress unraveling many of the molecular mechanisms involved with valve disease, there are no treatments available. The only option that patients have for clinical intervention is valve replacement surgery. This is surprising, since the components of the valves have been described for almost a century. The first valve replacement was conducted in 1952, and along with technology the prosthetic valves that can withstand the demands of the heart have evolved over the last half century [12]. Currently, there are approximately 300,000 valve replacement surgeries performed worldwide every year. Today valve replacements can even be performed via catheterization decreasing risks involved with open-heart surgery. This can also provide an alternative for patients that may not be healthy enough to withstand the stress of surgery to have the catheterization for repair. It is estimated that annually 1 billion US dollars are spent for valve disease. With a growing aging population in the world, the number of heart valve replacement surgeries is predicted to triple by 2050 [12]. This indicates a need for both treatments and improving the surgical interventions, highlighting the importance of understanding underlying mechanisms responsible for valve disease pathologies.

Muscle

History of Muscle Biology

For centuries anatomists and artists have been fascinated with the human form. As interest in heart valves dates back to antiquity, ancient scholars studied and were
captivated by structure and function of muscles. Some of the earliest accounts describing muscle function are from the early 11\textsuperscript{th} century from Avicenna:

The power of locomotion is that which contracts and relaxes the muscles whereby the members and joints are moved, extended or flexed. This power reaches the limbs by way of the nerves and there are as many forms of power as there are of movement. Each muscle has its own peculiar purpose and it obeys the decree of the composite sense [13].

During the Renaissance period (fifteenth and sixteenth centuries) artists like Leonardo da Vinci demonstrated this by drawings depicting amazing detail in the muscles. The father of systems biology, William Harvey, used these ancient ideas to provide an understanding of muscles in \textit{Lectures on the Whole of Anatomy} (1653) where he stated:

"[Muscle] fibers [are] for the sake of motion, sinews, threads. Straight: [1] long longitudinally opening by contraction [2] transverse compressing by contraction. The oblique are unable to detain by tonic motion, because [according to] Fallopius the straight are opened and the transverse are not compressed, by contraction [14]."

\textit{Skeletal Muscle Function}

As the ancient scholars described, muscles function to provide locomotion and movement. The neuromuscular junction provides an interface by which the nerves can innervate the muscle to provide proper signaling events to set the muscle in motion allowing conductivity in the muscle. Muscles can contract (become tense) and then relax by returning to a resting state. Additionally, forces outside the muscle can cause the distention and elasticity allows the muscle to stretch uninjured as long as it is the physiological limits of the muscle [15]. When extended outside these limits the muscles become irritated and can tear. Although the shapes and sizes of muscles can be quite different, together all of the muscles give form to the body that marveled artists and scientists alike for centuries.
**Skeletal Muscle Development: Structure**

In order to further understand and classify muscles based on their structure, they can be in one of two main groups: smooth and striated (skeletal and cardiac). Striated muscle is composed of fibers, which are aligned in a parallel fashion. Skeletal muscle has many levels of compartmentalization. Fibers are composed of myotubes, which are fused myoblasts. Muscle fibers are surrounded by the perimysium. Groups of muscle fibers are surrounded by the endomysium to form the fasciculus. Lastly, groups of the fascicles are surrounded by the epimysium to form the skeletal muscles.

During development the muscle arises from the mesoderm and the somite compartment in the embryo. Mesodermal cells or “muscle precursor cells” give rise to myoblasts, which are spindle-shaped and contain many ribosomes, microtubules and filaments. Myoblasts fuse to form myotubes, which then arrange to form fibers as described above. These fibers are complete with sarcomeres composed of actin and myosin outlined with Z disks. It is important to note that there are different types of fiber types and these include slow (type I) and fast (type II) twitch fibers [16]. Slow twitch muscle fibers contract slowly allowing for extended activity and are highly aerobic. On the other hand, fast twitch muscle fibers contract quickly producing small amounts of fast movements and are mainly anaerobic. Overall, the organization and specificity of skeletal muscle provides proper structure and function for movement in an organism.

**Skeletal Muscle Development: Signaling Pathways**

Due to the structural complexity of skeletal muscle, several critical processes have been outlined during development; determination, differentiation and maturation [17].
The onset of these processes is mainly due to myogenic regulatory factors (MRF) which are basic helix loop-helix (bHLH) transcription factors that contain an E box (sequence CANNTG) DNA binding motif [18]. The MyoD Family including Myf5, Myogenein, MyoD and MRF4 have been found to be necessary at different stages of muscle development [19]. For example, Myf5 is expressed in early somites followed by myogenin expression in myoblasts. Expression of MyoD is established after myogenin and MRF4 is expressed throughout fetal and the adult life. In addition to these genes, there are transcription factors, myocyte enhancer factors (MEFs), that bind to promoters of MRFs to induce gene expression [17]. During development it is important that both types and sizes of muscle lineages are established. Growth factors have been shown to modulate muscle growth and one example of a negative regulator of muscle size during development is myostatin (previously described as growth differentiation factor 8). The studies outlined in this thesis describe myostatin in detail in Chapter 4 and add an additional level of complexity in the regulation of skeletal muscle development.

**Skeletal Muscle Diseases**

The complexity of the structure and development of skeletal muscle provides many factors that can be impaired in disease. This can arise from a variety of causes including genetic, acquired and secondary to other syndromes. Genetic diseases of the skeletal muscle include the muscular dystrophies and mitochondrial myopathies. Inflammatory myopathies and sarcopenia (atrophy with aging) are examples of acquired forms of muscle disease. In some cases, muscle atrophy will present secondary to a
primary neurotrophic disease or cancer. Although there are many interesting skeletal muscle diseases to examine, the focus for this thesis will be on cachexia.

*Cachexia*

Cachexia is Greek and translates to “bad condition”. This translation suggests cachexia has tremendous deleterious effects on the individual including a positive correlation with mortality. One often hears of muscle loss as a result of muscular dystrophies, however, cachexia presents secondarily to HIV/AIDS, cancer, burns, chronic kidney disease (CKD), sepsis, chronic heart failure, chronic obstructive pulmonary disease, diabetes, chronic kidney disease (CKD), and cystic fibrosis. Cachexia can be present in patients with these disease states at percentages as high as 67% [20]. Cachexia leads to muscle weakness and decreases the quality of life for the individual preventing them from performing normal activities. Although the term has been around for a very long time, the definition of cachexia is constantly evolving. Currently, cachexia has been defined as “a multifactorial syndrome involving ongoing loss of skeletal muscle mass that cannot be reversed by conventional nutritional support and progresses into functional impairment” [21]. As the definition of cachexia changes the clinical diagnostic criteria also evolves. Currently, cachexia is diagnosed as a weight loss greater than 5% over 12 months.
Clinical Implications of Cachexia

The complexities that arise from the multifactorial nature of cachexia have challenged researchers and clinicians with developing treatments. Although treating cachexia would help to make patients better candidates for therapies and/or surgeries that would target their primary diseases, currently there are limited options. This taken together the complexity of the disease highlights why it is important to determine which regulatory pathways in muscle biology to target in order to treat and manage cachexia.

Concluding Thoughts

Heart valve and skeletal muscle development are highly regulated biological processes and have associated diseases when these pathways are disrupted. Despite both the heart valves and skeletal muscle being studied by ancient scholars, there is still a need today to further study molecular mechanisms in order to develop therapies. Subsequent chapters provide scientific data and evidence for further understanding these developmental and disease processes.

Although, there have been many advances in understanding the development and with providing surgical interventions for heart valve disease, there are several mechanisms of heart valve development and disease that were still not well studied at the beginning of this project. One avenue of research that has been studied in many other systems, but not in heart valves is examining expression patterns of microRNAs (miRNAs) in the developing valves. This would lead to an understanding of which miRNAs were important at critical time points during valve development and potentially point to individual miRNAs to be further studied in disease models. The following
chapter, Chapter 2, outlines the background and reasoning behind studying miRNA regulation in the heart valves and provides data generated for a specific mechanism of miR-101 targeting Sox9.

Another area of research that is lacking is examining valve calcification in chronic kidney disease (CKD) and the effects of phosphate on heart valves. Knowing that patients with CKD often have various forms of cardiac calcification, this pathway pertaining to valve calcification is not very well studied. Chapter 3 provides background and experimental evidence as to why this is necessary to understand these mechanisms in the heart valves.

Lastly, Chapter 4 presents a novel mechanism by which muscle wasting induced by cancer can be ameliorated by targeting myostatin-family ligands. Understanding these mechanisms could create new avenues by which treatments could be developed in order to ease the burden of valve replacement and muscle wasting in medicine and provide better quality of life for patients suffering from either disease.
Chapter 2: miRNAs and Heart Valve Development

Background

Heart Valves

Mature heart valves are highly complex structures that coordinately function to maintain unidirectional blood flow during the cardiac cycle. This is largely achieved by stratification of elastic fibers, proteoglycan and collagen matrices that collectively provide the valve with all the necessary biomechanical properties to withstand constant changes in hemodynamic forces.

Heart Valve Disease

Pathological changes in the distribution and/or composition of the valve connective tissue affect the ability of the valve leaflets to fully open during diastole, collapse during systole, and are associated with stenosis or regurgitation, respectively. Myxomatous or “floppy valves” typically arise when there is an accumulation of glycosaminoglycans and proteoglycans in the valve preventing it from closing properly [22]. Conversely, calcific valves are the result of the valve leaflets not opening enough and restricting the blood flow in the heart. Many of the molecular mechanisms that control valve development are disrupted in disease models, thus stressing the importance of understanding these signaling pathways and their contribution to both development and subsequently disease.
Heart Valve Development

In order to provide proper function throughout life and to prevent valve disease, it is essential to maintain homeostasis of the highly organized connective tissue within the heart valves. Additionally it is also important that this intricate compartmentalization of matrix and VICs is correctly established during embryonic development.

Heart Valve Development: EMT

Valvulogenesis begins at the “looping heart stage” where bulges called endocardial cushions arise in the outflow tract and AV region. This occurs in the mouse embryo around embryonic day 9-10. The endocardial cushions arise following EMT in a subset of cells in the AV canal and outflow tract regions in response to growth factor signaling, including bone morphogenetic protein-2 (BMP2) [23] and vascular endothelial growth factor (VEGF) [24], emanating from the myocardium. The endothelial cells lining the endocardial cushion transform and migrate away from the endothelium into the hyaluronan-rich cardiac jelly of the developing endocardial cushions [7]. These mesenchymal cells are highly proliferative and are necessary to form the pool of precursor cells that give rise to the mature valve structures [25, 26]. Through lineage tracing studies it has been determined that many of the cells that make up the valve arise from these endothelial origins [27, 28]. Many of the key signaling pathways required for the establishment of the endocardial cushions have been identified from studies using mouse models and in vitro culture systems. Transforming growth factor-β (TGFβ) and Wnt/β-Catenin signaling promote EMT in the endocardial cushion and simultaneously Notch1 expression during EMT inhibits endothelial phenotypes [23, 24, 29, 30].
Decreases in expression of the transcription factor Snail1 prevent migration and transformation of cells during EMT, thus demonstrating a role in the initiation of valvulogenesis [31, 32]. The mesenchymal cells in the cushions express Twist1, Msx and Tbx20, all of which promote expression of mesenchymal genes. Defects in these signaling pathways in vivo lead to premature lethality due to improper endocardial cushion formation including the proteoglycans hyaluronan and versican [33-35]. The complexity of this initial step of valvulogenesis, EMT, is evident through all of the regulatory networks involved. Therefore, it is essential that components of these signaling pathways be tightly regulated in order to generate the proper number of mesenchymal cells that will secrete the necessary amounts of ECM during the development of the valve primordia.

**Heart Valve Development: Valve Remodeling**

Following EMT, endocardial cushions elongate to form valve primordia at the mitral, tricuspid, aortic and pulmonic positions and undergo extensive remodeling. During this time, valve progenitor cell proliferation is reduced and cells begin to lose their mesenchymal phenotype. These valve interstitial cells become differentiated and secrete diversified extracellular matrices including collagens and proteoglycans to develop trilaminar structure of the mature valve. Nuclear factor of activated T-cells (NFATc1) activity is required for activating expression of the cathepsin K (CtsK) protease in the valve endothelial cells to promote ECM remodeling [36-38]. This process is similar to bone development and interestingly the valves at the remodeling stage activate signaling pathways that are also present in other types of connective tissue.
BMP2 has been shown to activate expression of “cartilage” genes Sox9 and aggregan in the developing valves and FGF4 is responsible for activating tenascin and scleraxis expression giving the valve and associated structures “tendon” like qualities [39, 40]. The valve stratification involves many distinct signaling pathways in order to establish the trilaminar structure of the valves. The fibrosa layer situated furthest away from the blood flow, is comprised of a dense network of type I and III collagen fibers and is established in part by Wnt signaling. This layer provides strength required for the valve to function consistently throughout lifetime of the organism. The spongiosa layer is mainly composed of proteoglycans like versican and aggregan, which form elastic fibers, that provide compressibility necessary for the valve to withstand the pressure of the blood flowing during the cardiac cycle. The third layer is the atrialis in AV and the ventricularis layers in SL valves. Mainly composed of filamentous elastic fibers, this layer provides the flexibility required for the valve to open and close properly when exposed to hemodynamic forces. Additional structures facilitate proper function of the valve, and these include the annulus, which is where the valve leaflets attach to the myocardium. In the AV valves, there is also a supporting network of tissues made up of chordae tendineae and the papillary muscle, where the SL valves are anchored in the root of the arteries. Together, these diversified connective tissues throughout the valve and supporting structures provide the valve with all the necessary biomechanical properties to function throughout life.
Heart Valve Development: Sox9: A Key Player in Valve Organization

The molecular events that regulate valve remodeling have been studied, however there are still many gaps in deciphering the pathways involved with this highly regulated process. Previous reports show that signaling pathways common to cartilage and tendon development and maturation [41-45] are required for valve precursor cell differentiation and ECM stratification which indicates the importance of these diverse structures for the proper function of the valves. Sox9 is an SRY-transcription factor that has been shown to be a master regulator of cartilage development [46]. Mutations in Sox9 cause campomelic dysplasia in humans and mice [46-49]. Since the valves express factors that are also found in cartilage, studies have shown that targeted loss of Sox9 in the heart valves leads to hypoplastic endocardial cushion by E10.5 [44]. Additionally, reduced function around E15.5 attenuates expression of cartilaginous matrices within the fibrosa and spongiosa layers. It has also been shown that conditional heterozygous loss of Sox9 promotes valve calcification phenotypes in mice when under the control of a collagen II (Col2a1) promoter [43, 44]. Homozygous animals have tracheal defects and do not survive past birth, thus only heterozygotes could be examined [46, 50]. These studies highlight Sox9 as a factor required for proper heart valve development and disease prevention. In addition to Sox9, other transcription factors, growth factors and signal mediators are emerging as key players of valve remodeling and alterations in their function can lead to attenuated valve function and disease. Therefore, understanding the regulation of these factors on a molecular level might have important implications in defining mechanisms of valve pathology associated with alterations in connective tissue organization and dysfunction.
**Heart Valve Development: Valve Maturation**

The mechanisms involved with development and establishment of the valves are highly regulated. Once the valve has matured and the ECM is established, the valve interstitial cells become quiescent and only provide the necessary matrix for maintaining the adult valve. Valve interstitial cells are activated in disease and results in an alteration of the molecular signaling, which contributes to differences in the valve ECM. Many of the heart valve diseases have emerged as mutations in components that make up the valves or are responsible for the development and maturation of the valves. For example, mutations in the Notch1 gene have been found in patients with bicuspid aortic [51]. The mature valve must maintain proper homeostasis of the number of cells and amount of ECM necessary to provide strength, durability and plasticity to endure cardiac cycles over a lifetime.

**miRNAs: Discovery and Function**

Although many of the mechanisms governing heart valve development have emerged, one type of regulation that has not been described thoroughly in valve development is at the level of miRNAs. MiRNAs are an abundant class of non-coding RNAs that play essential roles in ‘fine-tuning’ gene expression by controlling development and tissue homeostasis in many systems [52]. MiRNAs are transcribed as a primary miRNA (pri-miRNA), by RNA polymerase II. Pri-miRNAs are then cleaved into a precursor miRNA (pre-miRNA) by Drosha (RNase III enzyme) and subsequently exported by exportin-5 from the nucleus into the cytoplasm. The pre-miRNA is then further processed by Dicer, which removes the stem-loop structure and results in a duplex
of miRNA and its antisense strand. The duplex is unwound, and the mature miRNA strand is assembled into an RNA-induced silencing complex (RISC). This complex of RISC and miRNA most commonly binds to the target mRNA at the 3’-untranslated regions (3’UTR). This results in the miRNA inhibiting translation of the target mRNA and inducing mRNA degradation, thus imposing a repressive effect [53]. miRNAs can each have hundreds of targets and one mRNA can have consensus sequences for several adding another the level of complexity to the function of miRNAs [54].

In addition to regulating developmental processes, miRNAs are dysregulated in diseases such as cancer and heart disease. For example, studies have been performed examining global miRNA expression patterns in cancer cells compared to control cells and also in hearts that have undergone fibrosis after myocardial infarction [55, 56]. Studies in cardiomyocytes demonstrate miRNA regulation of collagens during fibrosis, and these studies may be applicable when examining collagen distribution in the valves during development and disease (reviewed in [57]). Given the high level of regulation in heart valve development, it would not be surprising if miRNAs were also involved.

**miRNAs and Heart Valves**

Although miRNAs have been well studied in the developing heart and diseased myocardium to date, there are still few studies that have focused on identifying miRNAs in the valves. Of these studies, a few key miRNAs have been identified to be involved in valve development. One study in humans examined differences in miRNA expression between stenotic (usually calcific) and insufficient valves and identified reduced levels of several miRNAs including miR-26a, miR-195 and miR-30b [58]. Human aortic valve
interstitial cells over expressing miR-26a and miR-30b also show decreases in expression of “calcification” promoting genes. Similarly, another recent study identified miR-30b as playing a role in aortic stenosis and that miR-30b directly targets Runx2, Smad1, and caspase-3 [59]. Furthermore, miR-23 has been identified in Zebrafish to be required for proper endocardial cushion formation mainly through inhibiting hyaluronan synthase 2 (Has2) [60]. It has also been demonstrated that miRNAs are differentially expressed in response to blood flow since miRNA expression patterns from endothelial cells isolated from both sides of the heart valve differ [61]. miRNAs add an additional level of regulation of the heart valves, and could potentially lead to therapeutic interventions in the future.

**Experimental Design**

*miR-101 Regulation of Sox9 mRNA*

For the purposes of these studies one miRNA, miR-101, was examined in detail on a mechanistic level. Initially, miR-101 was predicted to bind to the 3’UTR of the Sox9 mRNA using software and therefore, we examined expression patterns during valve and limb development. Additionally, the miR-101 consensus binding sequence in the 3’UTR of Sox9 mRNA was tested in order to determine whether this interaction was functionally relevant. Overall, these studies provide new insights into the possible roles of miRNAs regulating important signaling pathways essential for normal heart valve formation and prevention of disease.
Methods

Collection of Murine and Avian Tissue

Whole hearts or mitral valve tissue was collected 4-month-old mice and postnatal day 1 (PN) pups (C57BL/6, Jackson Labs, Bar Harbor, Maine), in addition to remodeling mitral valves and endocardial cushions from mouse E15.5 and E11.5, respectively. Furthermore, tissue for avian valve development from comparable time points was collected from White Leghorn Chicken eggs (Charles River Laboratories, Portage, MI) at E4, E7, E10 and E14. Valves and endocardial cushions from at least three mice or chicks at each time point were pooled for each biological sample and subject to RNA isolation (see below). All animals were maintained and experiments were performed according to the animal ethics for the care and use of laboratory animals of the Institutional Animal Care and Use Committees at the University of Miami Miller School of Medicine.

RNA Extraction

Total RNA from pooled samples (see above) was isolated using the miRVana Kit (Ambion, Grand Island, New York) according to manufacturer’s instructions or by homogenizing in Trizol, followed by chloroform treatment and subsequent isopropanol precipitation. Pellets were washed in 70% ACS grade ethanol and dried before resuspending in DEPC water.

cDNA Generation and Quantitative Real Time PCR

cDNA for miRNA expression analysis was prepared from 20 ng of total RNA using Taqman miRNA reverse transcriptase PCR (RT-PCR) kit and specific miRNA RT
primers for miRNA-101 (both from Applied Biosystems, Grand Island, New York). For Sox9 and 18S expression, cDNA was generated from 200 ng RNA using the fast cDNA master mix (Quanta Biosciences, Gaithersburg, Maryland) following the manufacturer’s instructions.

For miRNA analysis, 1 µL of cDNA was subject to quantitative real time PCR (qRT-PCR) analysis using Taqman assays specific for miRNA-101 and the Taqman Universal PCR Master Mix (No AmpErase UNG, Applied Biosystems). qRT-PCR for Sox9 and 18s mRNAs were performed using Taqman probes and the Taqman fast qRT-PCR master mix (Quanta Biosciences). Cycle counts (Ct) of miRNAs and Sox9 were normalized to 18sCt and respective fold changes and statistical significance were determined based on delta Ct values [43]. Student’s t-test was used to determine statistical significance between experimental and control samples using the delta Ct values (p<0.05).

**miR-101 Overexpression Assays**

For miR-101 overexpression studies, miR-101-GFP and Scrambled (Scr)-GFP clones were purchased from GeneCopoeia (catalog numbers: HmiR0009-MR01 and CmiR0001-MR01, respectively). The miR-101-GFP plasmid contains the human (hsa) miR-101-1 precursor sequence (hsa-miR-101-1 miRBase# MI0000103 - UGCCUGCUCAGUUAUCACAGUGUGUGUGUCUAUUCUAAAGGUACA GUACUGUGAUACUGAGGAGGCA) in the pEZX-MR01 vector under the control of the histone H1 promoter. Two mature miRNAs can arise from the human (hsa) hsa-miR-101-1 precursor sequence, hsa-miR-101-3p (miRBase #MIMAT000099 UACAGUACUGUGAUACUGAA) and hsa-miR-101-5p (miRBase #MIMAT0004513
CAGUUAUCACAGUGCUAGCU). Of these miRNAs, hsa-miR-101-3p shares 95% homology with the mature form of the mouse (mmu) mmu-miR-101b (precursor - mmu-mir-101b miRBase #MI0000649)

AUCUGAGACUGAACCGCCUUUUCGGUUAUCAGGUACUGCUUGA
CUCUGAAAGGUACUGCUUGAUCAGAAUGGCUGCGCGCAUC), and mmu-miR-101b-3p (mature – mmu-miR-101b-3p miRBase #MIMAT0000616

UACAGUACUGUAAGCUGAA). Both mouse and human contain the same consensus binding sequence 5’ ACAGUAC 3’ for targeting the Sox9-3’UTR. The mouse mesenchymal stem cell line, C3H10T1/2, was maintained in BME media supplemented with 10% FBS, 1% Pen/Strep, L-glutamine and sodium bicarbonate. Cells were seeded in 6-well plates 24 hours prior to transfection at a density of 0.5 x 10^5 cells per well and transfected with 1 µg miR-101-GFP or SCR-GFP using 5 µL lipofectamine/well for 5 hours in serum-free medium (Optimem, Gibco). Transfection efficiencies were determined by examining GFP expression from miR-101-GFP or SCR-GFP plasmids and then lysed 48 hours post-transfection using the miRVana lysis buffer and RNA was isolated as above.

**Plasmid Cloning and Mutagenesis**

For luciferase assays, the 3’UTR of Sox9 containing the miR-101 consensus sequence at -435-441 was PCR amplified using primers 5’-

ACGctgagGCTGGGATTCCAGGAGAGA

AAcgccggCGGACGAACGACCGACGT 3’ and cloned into psiCheck2 (Sox9-3’UTR-Luc) using Not1 and Xho1 restriction sites. A similar construct containing mutations
(GTACTGT → GgAgGg) in the miR-101 seed sequence (mSox9-3’UTR-Luc) was generated using the Quickchange Lightning Site-Directed Mutagenesis Kit (Agilent) as per manufacturers directions using primers designed to generate three mismatches

(Forward - 5’-
GGAATATTCTCTATTTAAATACTTTTAGTATGGACGGGTATGACTCATTAC
CATTTTGAGGGGAT-3’ and Reverse - 5’-
ATCCCTCAAAATGGTAATGAGTCATACCCCGTCCATACGTAAAAAGTATTTAAA
ATAGAGAATATTTCC-3’).

**Luciferase Assays**

HEK293 cells were seeded in 6-well plates at a density of 250,000 cells per well 24 hours prior to transfection. At 70% confluency cells were co-transfected with 1 µg of miR-101-GFP or SCR-GFP, and 1 µg of Sox9-3’UTR-Luc or mSox9-3’UTR-Luc using 6µL of Fugene reagent per well. Luciferase assays were performed using active lysis of the samples followed by dual luciferase assays (Promega, Madison, WI). Data is represented as average percent of renilla luciferase activity of the SCR-GFP co-transfected with psiCheck2 empty (set at 1) and normalized to Firefly luciferase (n = 3).

**Statistics**

Student’s t-test was used to determine statistical significance when comparing data and significance was determined by a p-value less than or equal to 0.05.
Results

miR-101 Functions to Repress Sox9

In order to focus on a regulatory mechanism, we examined which miRNAs were predicted to target Sox9, a transcription factor critical to the development of the heart valves. Using TargetScan software, we identified a highly conserved seed sequence for miR-101 in the murine 3′UTR (435-441) of Sox9 mRNA (Figure 2.1 A-C).

Next, we examined expression levels in mouse and chicken heart valves and limbs during development. Using qRT-PCR analysis we observed opposing expression patterns of miR-101 and Sox9 in developing mouse and chick heart valves (Figure 2.2). As valve development progresses, expression levels of miR-101 moderately increase, while Sox9 decrease. We also observed similar changes during limb development (Figure 2.2),

Figure 2.1 miR-101 Conservation and Predicted Consensus Sequence
A) TargetScan images representing conservation between species of the miR-101 consensus binding site in the Sox9 3′UTR (mouse (mmu), human (hsa)) B) location of the site in the murine Sox9 mRNA, (C) and the nucleotide sequences of the consensus sequence in the Sox9 3′UTR and miR-101.
which appears to be a relevant finding since the regulatory mechanisms controlling limb development overlap in valve development.

![Figure 2.2 miR-101 Expression in Heart Valves and Limbs](image)

**Figure 2.2 miR-101 Expression in Heart Valves and Limbs**

A) *miR-101* mRNA expression levels during development in mouse valves (from miRNA array) and C) limbs (via qRT-PCR). Data is represented as fold change compared to mE11.5. B & D) *miR-101* expression levels in chicken valves and limbs as determined by qRT-PCR. Data is represented as fold change compared to mE11.5 or cE4 with an experimental N=3, where *p*≤0.05 determined by Student’s T-test.

In order to determine whether *miR-101* could directly regulate *Sox9* at the consensus sequence a number of assays were performed. Initially, overexpression studies were performed in C3H10T1/2 cells. In these cells, *miR-101* overexpression leads to a significant decrease in endogenous *Sox9* mRNA levels (Figure 2.3). These results are consistent with a repressive effect of *miR-101 on Sox9* expression.
Figure 2. miR-101 Inhibits Endogenous Sox9 mRNA Expression

Fold change in miR-101 and endogenous Sox9 mRNA expression compared to untransfected cells after transient transfection with miR-101-GFP and SCR-GFP in C3H10T1/2 cells. N=3 independent experiments, *p≤0.05 Student’s T-test.

To examine functionality of miR-101 at region 435-441, psiCheck2 vectors were used to clone the 3’UTR of the murine Sox9 sequence containing only the miR-101 consensus site (GTACTGT) and none of the other miRNA binding sites previously examined in Sox9 (Figure 2.4).

**Figure 2.4 Sequence of Sox9-3’UTR-Luc (insert)**
The 3’ end of human Renilla luciferase (hRLuc) sequence is outlined in blue
The XhoI and NotI restriction sites that were added during cloning are outlined in yellow and purple, respectively. The miR-101 consensus binding site is outlined in green.
After cloning, site-directed mutagenesis was performed on the Sox9-3’UTR-Luc plasmid to introduce nucleotide substitutions from GTACTGT to GGACGGG (mSox9-3’UTR-Luc) in order to determine whether the miR-101 consensus sequence was responsible for the regulation of Sox9 (Figure 2.5).

**Figure 2.5 Site-Directed Mutagenesis for Generating the Murine Sox9-3’UTR-Luc Plasmid**

A) Sequence alterations as a result of site-directed mutagenesis using the primers outlined above.

B) Final sequencing of the cloned, mutated plasmid indicates the only altered sites were those in the miR-101 consensus binding site outlined in blue.

After the generation of these plasmids, luciferase assays were implemented in HEK293 cells to determine whether miR-101 was binding to the consensus sequence in the Sox9 3’UTR. Co-transfections were performed with either Sox9-3’UTR-Luc or
mSox9-3'UTR-Luc and a non-sense scrambled control vector (SCR) or *miR-101*. As shown in Figure 2.6, compared to controls overexpression of miR-101 significantly decreased luciferase activity of the Sox9-3’UTR, and site directed mutagenesis of the seed sequence abolished this repressive effect. Taken together, these data suggest that miR-101 negatively regulates Sox9 expression and that this repressive effect is mediated by nucleotides 435–441 in the Sox9-3’UTR.

**Figure 2.6 miR-101 Negatively Regulates Sox9**

**A)** Fluorescence images demonstrating similar transfection efficiencies by examining GFP expression of HEK 293 cells transiently transfected with miR-101-GFP or SCR-GFP. **B)** Luciferase data showing no differences between empty vector, mSox9-3’UTR when transfected with either miR-101 or scrambled control (SCR) expression vectors. Only when miR-101 is transfected with Sox9-3’UTR-Luc a 25% reduction in luciferase activity can be detected. **∗∗p≤0.01, Student’s t-test. N=3 independent experiments.**

**Discussion**

**Conclusions**

The formation of highly organized valvular structures from endocardial cushions requires tight control of many regulatory hierarchies consisting of growth factors, transcriptional factors and downstream structural genes [35]. This study provides evidence for a regulatory mechanism of miR-101 targeting Sox9 in the consensus
sequence in the 3’UTR. The data show increased expression levels of miR-101 in adult stages and indicate that this miRNA functions as a repressor of Sox9; a transcription factor previously shown to be important for normal valve development and prevention of valve disease [40,43]. Limitations of this study prevented further analysis for direct effects of miR-101 in heart valves; however, data here provide new insights regarding potential regulatory events that control stages of heart valve development and maturation.

Here we demonstrate Sox9 is highly expressed during early stages of valve (and limb) development and expression is progressively reduced to low levels by 4 months of age (Figure 2.2). High levels of Sox9 at early timepoints in embryonic development have previously been shown to be required for mesenchymal cell proliferation during endocardial cushion formation and lower expression levels of Sox9 coincide with its role in regulating expression of cartilaginous matrix genes [44]. In adult valves, Sox9 is expressed, however at very low levels. These previous studies suggest that Sox9 expression levels must be tightly regulated for differential functions during valve development and maintenance.

Sox9 has been shown to transcriptionally regulate the expression of miR-574-3p and miR-140 and is inhibited by miR-145 in chondrocytes [62][63-65]. Here we support recent findings in chondrocytes [66] and human hepatocellular carcinoma cells [67] and show that Sox9 is a direct target gene of miR-101 (Figure 2.6). Their opposing expression patterns (Figure 2.2) suggest that miR-101 plays a role in fine tuning Sox9 expression levels and therefore function in embryonic and adult valve structures. In mouse models, dysregulation of molecular pathways important for valve development lead to premature lethality or phenotypes of human disease associated with histopathological changes in the
composition of valve connective tissue. This can include calcification, defined by ectopic formation of calcific nodules similar to bone, myxomatus changes characterized by an abnormal excess of proteoglycans, and fibrosis as a result of increased collagen deposition.

**Future Directions**

There were several limitations of our studies and there are additional experiments that could be performed to demonstrate direct effects of miR-101 on Sox9 in heart valves during development and in disease. Because of similarities with signaling networks these effects could also be examined in cartilage and cartilage disorders. Here we show evidence of miR-101 targeting Sox9 mRNA, thus the next step would be to investigate this effect directly in heart valves or valve interstitial cells. This could be mediated by viral infection of valve explant cultures from mouse and chicken followed by downstream microarrays to determine the global change in genes associated with a decrease in Sox9 mRNA expression. Additionally, the adenoviruses for miR-101 and scrambled control could be used in cultured cells to overcome the challenges with transfecting the pig aortic valve interstitial cells and to make gene transfer in these cells possible. Once the infection protocol is established, several downstream Sox9 target genes could be investigated including those inhibited and activated, osteopontin (Spp1) and Col2a1, respectfully, by Sox9.

In parallel with these studies, it would be important to study the spatial location of miR-101 in valves and cartilage. Using new in situ hybridization probes that are double labeled (5’ and 3’ ends) to detect miR-101 expression in heart valves (whole mount,
bisected hearts). It is possible that the expression is too low to detect; however, this could provide a tool to examine differences in disease and normal heart valves.

Since other miRNAs have been identified to regulate Sox9 (miR-30 and miR-145), combinatorial treatments could be performed using several miRNAs to target Sox9 and to determine whether there is an additive effect of miRNA regulation of Sox9. For these experiments, the entire Sox9-3’UTR would need to be cloned containing all miRNA binding sites, since the clone for our studies only included the consensus sequence for miR-101 (Figure 2.4).

To translate these results and to potentially utilize this pathway for treating disease phenotypes, it would be important to determine whether miR-101 plays a role in the pathology or progression of calcific valve disease. There are several models of calcific valve disease (Sox9^{0/+:Col2aCre, klotho^-}, Notch1) and these could be utilized to determine whether miR-101 is upregulated in these mouse models of calcific valve disease. If present and elevated, one could use antagonirs to block the increased miR-101 expression followed by phenotypic analysis to determine if valve calcification is reduced. To further identify the underlying mechanism, potential upstream stimuli of miR-101 could be examined in these mice in order to study potential transcriptional regulators of this miRNA.
Chapter 3: The Role of Phosphate and FGF23 in Heart Valve Calcification

Background

Chronic Kidney Disease and Vascular Calcification

Patients with CKD have an increase in the incidence of vascular calcification (VC) [68-70]. VC is more prevalent in later stage CKD patients and has emerged as a risk factor for predicting mortality related to cardiovascular events in these patients [71-73]. Other factors that have been identified as potential “biomarkers” in serum from patients with CKD include: phosphate [74], fibroblast growth factor 23 (FGF23), osteopontin (OPN), osteoprotegerin (OPG), matrix Gla protein, fetuin-A, magnesium and pyrophosphate (PPi) (reviewed in [75]). Similar to what is found in valve calcification, VC has emerged as being an active process involving the differentiation of SMCs to become more osteochondrogenic in nature [76]. Despite evidence from clinical studies showing such strong correlations, the molecular events leading to calcific pathologies of VC in CKD are not clearly understood. Elucidating these mechanisms would provide avenues by which clinicians could potentially prevent and/or treat VC safely in patients with CKD, as well as in the aging population. Currently, treatments for cardiovascular calcification are not available although it has been associated with increased mortality in CKD patients.

Phosphate

One of the first “biomarkers” that was examined in CKD patients was serum phosphate. Increased serum levels of phosphate have been associated with VC in both CKD patients and the general population [68, 77-80]. Serum levels higher than 2
mm/liter are considered as hyperphosphatemia, compared to the normal range that is approximately 1-1.5mm/liter [81]. From a biological point of view, inorganic phosphate is necessary for life. It forms the backbone of DNA and RNA, and it is the main component of bones, which serve as the main storage compartment for phosphate in the body. The intestines function to absorb most of the phosphate that is taken in through the diet. Additionally, the homeostasis of phosphate is very highly controlled through hormones and other metabolic factors that allow for proper renal excretion [82].

Phosphate homeostasis at the cellular level is controlled via sodium phosphate (NaPh) co-transporters, which are present in many tissues. The first NaPh co-transporter was identified in 1991 and since, many elegant studies have emerged identifying the mechanisms, functions and locations of these co-transporters [83, 84]. There are three families (NaPh-I-III) each of which has been identified as having tissue specific roles in maintaining intra- and extra-cellular phosphate levels. The NaPh-I family mainly functions in the liver, kidney and brain, whereas the NaPh-II (SLC34) family is primarily involved with phosphate flux in the kidney and intestine. Lastly, the most recently identified family, NaPh-III, includes two transporters, Pit-1 and Pit-2 (SLC20a1 and SLC20a2, respectively). They are expressed in many tissues and are believed to serve as housekeeping NaPh co-transporters. Pharmaceutical phosphate binders are a common form of treatment in the clinical setting for balancing phosphate levels in patients with CKD [85-89]. One inhibitor of NaPh co-transporters that has been used previously in clinical studies is phosphonoformic acid (PFA), a pyrophosphate derivative [90]. Studies have shown that VC is highly regulated in vascular smooth muscle cells (VSMC) and that these cells transdifferentiate to a bone-forming phenotype where they increase expression
of the NaPh co-transporter Pit-1 and BMP-2. On the other hand, some VSMCs cannot adapt to the environment and the mineral imbalance and undergo apoptosis. Both events result in the formation of calcified SMCs. It has been shown that elevated phosphate can promote extracellular matrix degradation in vessel walls by activating MMPs. Again, this process is similar to matrix reorganization during valve disease. Phosphate promotes osteochondrogenic differentiation through binding to elastin–laminin and subsequent degradation of elastin. All of these factors contribute to mineral deposition and both vascular and valve calcification arise from similar molecular mechanisms. In VSMCs, phosphate increases calcification \textit{in vitro} mainly via Pit-1; however this mechanism of calcification is not well examined in the heart valves [91-93]. Interestingly, knockout mice for Pit-1 also demonstrate vascular defects early during embryonic development [93]. Many studies have been performed examining effects of phosphate on human VSMCs; however to date no experimental studies have addresses potential direct effects of phosphate on heart valves. Some evidence points towards the interstitial cells in the valve suggesting that they behave in a similar manner. It is also known that inflammation plays a role in the activation of VICs and the onset of the initiation of osteogenic phenotypes in these cells.

\textit{Fibroblast Growth Factor 23}

Interestingly, FGF23, which has been shown to maintain phosphate homeostasis in the body, has emerged as another potent biomarker for CKD. High levels of serum FGF23 have been correlated with increased mortality in patients with CKD [94-96]. FGF23 is released by the bone via an unknown mechanism in response to elevated serum phosphate levels in order to reduce serum phosphate. FGF23 regulates phosphate
homeostasis by down regulating NaPh co-transporter expression in the kidney [97],
resulting in increased phosphate excretion in the urine. FGF23 also down regulates 1-
alpha hydroxylase (the enzyme which converts vitamin D into its active form), which
results in a reduction in serum levels of active vitamin D [98] and decreased absorption
of dietary phosphate by the gut [99].

FGF23 is similar to FGF21 and FGF19, the two other endocrine members of the
FGF family [100], and mediates signaling through FGF receptors (FGFRs). It was
thought that FGF23 can only induce cell signaling when a co-receptor, called klotho, was
present, and klotho was thought to provide tissue specificity for FGF23 signaling [101].
However, recent studies by our group have demonstrated a direct role of FGF23 in
inducing cardiac hypertrophy independent of klotho [102]. This study provides the first
evidence that FGF23 can mediate signaling in the absence of klotho and it indicates that
FGF23 may act on other tissues that do not express klotho.

**Mouse Models of Valve Calcification with Altered FGF23 Signaling**

To accompany these studies of klotho-independent FGF23 signaling there is a mouse
model where klotho was genetically deleted and the mouse exhibits signs of early aging
including cardiac defects [103]. In addition to exhibiting an aging phenotype including
graying hair and kyphosis, these mice have elevated phosphate and calcium.
[103, 104] serum levels and develop valve calcification [105]. These mice also show
elevated expression of “bone” inducing genes like Runx2 and osteopontin (Spp1) in the
heart valves with calcification.
Therefore it seems that klotho is not required for valve calcification, since this study demonstrated that mice lacking klotho still develop valve calcification. This is likely to be due to the dysregulation of phosphate homeostasis in these mice. Additionally, this study showed limited inflammation in the valves indicating that the mechanism of calcification is not induced by inflammation as it is in atherosclerosis. The mechanism of calcification still remains elusive, and this study did not indicate whether phosphate or FGF23 played a direct role in the calcification process. Lastly, another study has demonstrated in humans that a klotho variant (KL-VS) that has been associated with cardiovascular disease does not correlate with mitral or aortic valvular calcification [106]. Although these studies do not indicate if elevated phosphate and/or klotho-independent FGF23 signaling is responsible for calcification, they provide evidence that klotho is not required for the manifestation of valve calcification.

**Experimental Design**

We wanted to determine whether phosphate and/or FGF23 were directly responsible for the induction of calcification in heart valves. This hypothesis was tested by using *ex vivo* and *in vitro* methods including mouse aortic smooth muscle cells (MOVAS), pig aortic valve interstitial cells (pAVICS) and murine aortic and mitral valve explants. In each system, cells or valves were treated for several time points with varying concentrations of phosphate-supplemented media or recombinant FGF23. To determine if phosphate-mediated calcification in heart valves requires phosphate transporters, treatments were performed with phosphate in the presence of the phosphate co-transporter inhibitor PFA. Additionally, experiments were performed to examine
expression levels of phosphate co-transporter after treatments and to study whether phosphate can induce apoptosis in these systems.

**Methods**

*Valve Explant Cultures*

Aortic and mitral valves were dissected from postnatal mice and pooled for at least an N=3 valves per condition and valve explants were cultured as described previously [43]. Briefly, valves were placed on filter papers (Millipore 0.1um VCWP) and incubated in media (DMEM, 4 mM L-glutamine, 1% P/S and 2% FBS) overnight. The following day valves were treated with either phosphate (3 mM Sodium dihydrogen phosphate), recombinant FGF23 (25 ng/mL in PBS; R&D Systems) or in combination with PFA (0.1 M). Explants were incubated for two to five days.

*Cell Culture*

pAVICs were maintained in M-199 media (supplemented with 10% FBS, 1% P/S, gentamicin and amphotericin), and treatments were performed using 1.0-1.5 mM phosphate, 0.1 M PFA or 25-100 ng/mL FGF23 for four days. MOVAS cells (ATCC® CRL-2797) were maintained in DMEM media (supplemented with 10% FBS and 1% P/S), and treatments were performed using anywhere from 3-9 mM Phosphate, 0.1 M PFA and 25-100 ng/mL FGF23 (as previously described [102] for 3-21 days.

*RNA Isolation*

Heart valves were isolated from neonatal mice and immediately placed into Trizol on ice for RNA extraction. Treated valve explants were removed from filter paper and
placed directly into 200 uL Trizol on ice. For cells, 200 uL of Trizol was added to each well of a 6-well plate after washing once with water. Total RNA was isolated using chloroform and subsequent isopropanol precipitation and was resuspended in water. Quantification of the concentration of RNA was performed using Nanodrop analysis (Thermo Scientific, Wilmington, DE).

**qRT-PCR**

200 ng of total RNA was used to generate cDNA using cDNA master mix (Quanta Biosciences). QRT-PCR was performed using taqman probes for Runx2 or Spp1 and Sybergreen probes for BMP2. Sybergreen primers were designed and validated for all phosphate transporters, and primers for FGFRs were used as previously described [102]. Fast master mixes were used for taqman and sybergreen reactions (Quanta Biosciences).

**Von Kossa Staining**

Valves were placed on superfrost plus glass slides and cells in 6-well plates were fixed in 4% PFA for 20 minutes followed by two rinses in water. Silver nitrate was added and samples were placed under direct light for 10-60 minutes. Once completed, based on evidence of black staining, the silver nitrate was removed and cells were washed in water, followed by being washed in hypo solution and then washed in water again. Valves were also counterstained with alcian blue for 15 minutes and washed in water before mounting.
Protein Analysis

MOVAS cells were lysed in CHAPS extraction buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.5% CHAPS, protease inhibitor cocktail [Roche], protein phosphatase inhibitors [Sigma-Aldrich]) as described [102]. Protein extracts were subsequently boiled in sample buffer and analyzed by 12% SDS-PAGE gels and immunoblotted, as in [102]. Antibodies used for immunoblotting include the primary antibodies for caspase 3, cleaved caspase 3 (Cell Signaling; both 1:1,000), and GAPDH (Abcam; 1:10,000), and the secondary antibodies, horseradish peroxidase–conjugated goat anti-mouse and goat anti-rabbit (Promega; 1:20,000). The cleaved caspase 3 fluorometric kit to determine caspase 3 activity was used per manufacturers instructions (Biovision, Malpitas, California).

Results

Phosphate Transporters are Expressed in MOVAS, Murine Aortic and Mitral Valves and pAVICs

Initial studies were performed in order to determine which NaPh co-transporters were expressed in our three systems examined here. RNA was isolated from cells or tissues (neonatal aortic and mitral valves) and qRT-PCR was performed with primers specific for each co-transporter for either mouse or pig. Not surprisingly, the transporters with the highest expression levels were Pit-1 (SLC20a1) and Pit-2 (SLC20a2) in MOVAS, pAVICs (Figure 3.1). These are also the transporters that have previously been described to play a role in vascular calcification in human SMCs, specifically Pit-1[91].
Figure 3.1 Phosphate Co-Transporter Expression in Murine Aortic and Mitral Valves, pAVICs and MOVAS

qRT-PCR analysis showing approximate transcript numbers of phosphate co-transporter expression in mitral and aortic valves from postnatal mice (A), pAVICs (B) and MOVAS (C). In all three systems the most highly expressed phosphate co-transporters are the SLC20 family members, which are the housekeeping transporters that are ubiquitously expressed. Data represents N=3 independent biological replicates for valves, pAVICs and MOVAS.

Phosphate Induces Calcification in Mouse Aortic Smooth Muscle Cells

Since phosphate co-transporters were expressed in these systems and previous studies identify phosphate as a key mediator of calcification in human VSMCs we utilized MOVAS cells to establish a positive control for our system. Previous studies have been performed in rat and human aortic SMCs [92, 107] showing that phosphate is in fact capable of inducing calcification; however, mouse cells were not tested. Since SMCs can be isolated and purified from the aorta of transgenic mice and thereby enable researchers to generate primary cell lines with genetic alterations, working with mouse
cells should provide advances over the human and rat systems. In order to further understand the mechanism of phosphate-induced calcification, we treated MOVAS with increasing concentrations of inorganic phosphate in order to establish a dose response at which the calcification is initiated. Phosphate-induced calcification became evident as early as three days after treatment with various concentrations of phosphate (Figure 3.2). In CKD patients serum phosphate levels from 5.5 to 6 mg/dL [108] correlate with increased mortality, and even patients with levels in the high-normal range 3.5-4.5 mg/dL show signs of vascular calcification [78]. A treatment concentration of 3 mM that we chose for in vitro studies represents a serum level similar to those in patients. MOVAS cells survived even high levels of phosphate and exhibited a dose response of calcification as measured by von Kossa reactivity. This finding was verified by examining gene expression of several bone promoting genes, including Runx2 (a mater regulator of bone), Spp1 (or osteopontin), and BMP2 ten days after treatment.
Phosphate Induces Calcification in MOVAS

A) Von Kossa staining to detect calcification is dose- and time-dependent in mouse aortic smooth muscle cells (MOVAS) treated with phosphate at concentrations and time points indicated.

B) Bright field images at 10X demonstrating von Kossa staining at 3 and 10 days post phosphate treatment. C) Quantification of von Kossa staining from images in B. At least six images were used per well and an n=3 wells for each treatment. Statistical analysis reveals a significant increase in the percentage of von Kossa reactivity in the phosphate treated cells compared to control. (** p<0.01 comparing to control, # p<0.05 comparing to 3 mM phosphate treatment.

D) qRT-PCR shows Runx2 and BMP2 expression is increased in a dose-dependent manner and Spp1 expression is also significantly increased after 10 days of phosphate treatment. (* p<0.05, ** p<0.01, *** p<0.001 comparing to control). N=3 for 9mM and N=2 for other treatment conditions.

Phosphate Induces Calcification in Valve Explants and pAVICs

To test if phosphate could directly induce calcification in aortic and mitral valve explants, valves explanted from postnatal mice were treated with 3 mM phosphate for five days. Valves treated with phosphate took on an opaque color in comparison to translucent controls, which was evident before fixing the tissue for staining (Figure 3.3).
After fixation and von Kossa staining the phosphate-treated valves were approximately 80% positive for von Kossa reactivity, whereas controls were only at 5% (most likely due to melanocytes in the valve which are dark brown cells).

Figure 3.3 Phosphate Induces Calcification in Valve Explants
(A) Bright field images at showing gross morphology of the valve explants treated with 3 mM Ph for 5 days in culture demonstrate the valves become opaque instead of translucent after phosphate treatment. Von Kossa reactivity and alcian blue staining are in the right panel. (B) Quantification of the percentile of von Kossa reactivity normalized to the area of the valve explant show phosphate treatment increases reactivity to almost 80% (*** p<0.001). Experimental N=3
Similar results were obtained when pAVICs were treated with 1-1.5 mM phosphate. The cells treated with 1 mM phosphate for just four days formed calcific nodules, whereas controls did not (Figure 3.4). Interestingly, the pAVICs did not tolerate nearly as high levels as the MOVAS cells and died already when exposed to phosphate levels as low as 1.5 mM. MOVAS cells were able to tolerate up to 9 mM phosphate. This finding indicates that cells in the heart valves may be more susceptible to calcification when exposed to increased levels of phosphate.

Figure 3.4 Phosphate Induces Calcification in pAVICS
(A) Bright field images at 10X showing von Kossa reactivity of pAVICs treated with 1-1.5 mM phosphate for 4 days in culture.
(B) Quantification of the percentage of von Kossa reactivity normalized to the area evaluated demonstrate a significant increase in von Kossa reactivity after treatment with Phosphate. At least six images were utilized from three independent wells for each experiment. N=2.
**Phosphate Co-Transporters are Required for Phosphate-Induced Calcification**

Next, we wanted to test whether NaPh co-transporters were responsible for the observed calcific phenotypes. To do so, we utilized PFA, which acts as a pyrophosphate analog and has been demonstrated in previous studies to prevent phosphate uptake [90]. Initially, MOVAS cells were used in order to determine the effective concentration of PFA. Cells survived in the presence of 0.1M PFA, but died when PFA was used at higher concentrations. At 0.1M PFA no calcific lesions could be observed after co-treating cells with 6 mM Ph for seven days (Figure 3.5).

![Figure 3.5 Phosphate Co-transporters are Required for Phosphate-Induced Calcification in MOVAS](image)

Von Kossa reactivity demonstrates PFA can protect MOVAS from phosphate-induced calcification when treated with 6 mM phosphate for 7 days. A) Images at 10X showing von Kossa staining and B) quantification of the stain when using 6 mM Ph with and without PFA for 7 days. N=3 *P<0.05, **P<0.01, ***P<0.001.
To test whether NaPh co-transporters were required for phosphate-induced calcification in valve explants, the cultures were treated with PFA for 15 minutes prior to adding 3 mM phosphate for five days. After fixation and von Kossa staining the valve explants pre-treated with PFA revealed reactivity that was similar to the levels of control valve explants which was significantly reduced compared to the phosphate treated valves (Figure 3.6).

Figure 3.6 Phosphate Co-transporters are Required for Phosphate-induced Calcification in Heart Valve Explants.
A) Bright field images at 10X showing von Kossa and alcyan blue staining of heart valve explants after 5 days of treatment. The presence of PFA prevents the increase in von Kossa reactivity.
B) Quantification in both mitral and aortic valve explants reveals that the levels of calcification in explants treated with PFA is similar to the levels in controls. N=3 *P<0.05, **P<0.01, ***P<0.001.
Additionally, pAVICs grown in the presence of PFA were protected from phosphate-induced death and even when treated with 1.5 mM (which normally would be toxic for the cells and induce apoptosis) phosphate did not induce any von Kossa reactivity (Figure 3.7).

**Figure 3.7 Phosphate Co-transporters are Required for Phosphate-Induced Calcification in pAVICs**

A) Bright field images at 10X showing reduced von Kossa reactivity in pAVICS co-treated with 0.1 M PFA and 1.5 mM phosphate for 4 days.  B) Quantification demonstrates that PFA can protect pAVICs from phosphate-induced calcification. N=2.
**FGF23 Does Not Induce Calcification**

Since several groups have analyzed phosphate-induced calcification in SMCs, we wanted to examine potential effects of FGF23, a major regulator of serum phosphate. Elevated serum FGF23 levels correlate with increased mortality in CKD patients [94-96], and we wanted to determine whether this factor by itself had the ability to induce calcification in MOVAS, valve explants or pAVICs. First, dose responses were performed to examine whether FGF23 could induce calcification alone in MOVAS when treated for up to 14 days. No von Kossa staining was observed in any of treatment groups. Although MOVAS cells express FGFRs 1, 3 and 4, treatment with increasing concentrations of FGF23 for up to 14 days did not cause positive von Kossa reactivity (Figure 3.8).

**Figure 3.8 FGF23 Does Not Induce Calcification in MOVAS Cells**

A) MOVAS cells express FGFRs 1, 3 and 4. FGFR2 expression was not detected. qRT-PCR analysis demonstrates approximate transcript numbers in these cells.

B) Bright field images at 10X represent 3 and 7 days post FGF23 treatment demonstrate the absence of von Kossa reactivity when MOVAS cells were treated with increasing concentrations of FGF23. N=3.

To explore further whether FGF23 had effects on valve explants, we incubated explants with FGF23 for two to five days and examined whether this treatment induced calcification. We did not detect any von Kossa reactivity in explants treated with FGF23 at 25 or 100 ng/mL for up to five days even though the valves express FGFR1 and FGFR3 (Figure 3.9).
Figure 3.9 FGF23 Does Not Induce Calcification in Heart Valve Explants

A & B) Heart valves express FGFR1 and FGFR3 during development; however, klotho was not detected in the heart valves. Von Kossa staining revealed minimal staining and no significant differences between the groups treated with FGF23 compared to control for aortic or mitral valves at 2 days (C) or 5 days post treatment (D). This analysis was performed in triplicate for a biological replicate of 2.

Lastly, we tested whether FGF23 would have any effects on pAVICs and found that these cells also did not undergo calcification after treatment with FGF23 (Figure 3.10).
Overall these studies in the different model systems indicate that phosphate, but not FGF23 is capable of inducing valve calcification.

**Phosphate and FGF23 Alter Phosphate Co-Transporter Expression**

Although FGF23 did not induce calcification in our systems, we wanted to determine whether phosphate and/or FGF23 had effects on the expression levels of the NaPh co-transporter and may mediate signaling by changing the availability of these transporters and thereby acting as a switch for gene expression.
Figure 3.11 Phosphate Co-Transporter Expression is Down-Regulated after Phosphate and FGF23 Treatment in MOVAS

Fold Change in mRNA expression levels when compared to untreated control for SLC20a1 (A) SLC20a2 (B) shows down regulation after treatment with phosphate or co-treatment with phosphate and FGF23 (C) SLC34a1 mRNA expression is down regulated when FGF23 is introduced. N=3 independent experiments.

Phosphate Induces Apoptosis in MOVAS

Other studies in human VSMCs have shown that calcification involved the induction of apoptosis [109]; however this effect was only studied in the presence of inflammation. We wanted to determine whether phosphate and/or FGF23 could induce apoptosis in MOVAS. Cells were treated with phosphate and PFA for up to seven days, and we then determined caspase 3 activity by studying the level of caspase 3 cleavage via Western blotting and by using a cleavage assay for fluorochrome-labeled caspase 3 substrate. Phosphate, but not FGF23, increased caspase 3 activity in both, Western blotting and the fluorometric assays. When PFA was added this phosphate-induced apoptotic effect could not be detected indicating that phosphate mediates apoptosis through NaPh co-transporters (Figure 3.12).
Apoptosis is Induced after Phosphate Treatment in MOVAS

In MOVAS cells cleavage of caspase 3 is increased in the presence of 6 mM phosphate by itself and in combination with 100 ng/mL FGF23. An increase in the caspase 3 cleavage product is not detected when the cells are treated with 0.1 M PFA and 6 mM Ph or FGF23 alone at 50 or 100 ng/mL. Similar results were obtained from the fluorometric assay for caspase 3 activity; a dose dependent increase in caspase 3 activity was observed when cells were treated with phosphate, which was prevented by co-treatment with 0.1 M PFA. N=2

Phosphate and FGF23 Alter Bone Gene Signatures in pAVICs

We also examined whether phosphate and/or FGF23 treatment on pAVICs altered gene expression of “bone” promoting genes. We treated cells with various concentrations of phosphate and FGF23 and examined expression levels of Spp1 and BMP2. Based on our preliminary results FGF23 seems to play a role in regulating BMP2 expression, since BMP2 levels were similar to those observed in cells that were treated with PFA.
Figure 3.13 mRNA Expression in pAVICs Treated with Phosphate and FGF23.
qRT-PCR analysis for Spp1 and BMP2 demonstrates changes compared to control. Phosphate induces expression of both genes, and this is reduced to normal levels by PFA treatment. FGF23 may have repressive effects on phosphate-induced BMP2 expression. N=2.

Discussion

Conclusions

Here we show further evidence for phosphate induced calcification in MOVAS cells, another SMC model. In addition to demonstrating that these cells are sensitive to phosphate, we show that NaPh co-transporters mediate phosphate effects, since their pharmacological inhibition completely blocks calcification. Additionally, FGF23 by itself does not induce calcification of MOVAS cells, but may act on a different regulatory level in this process. FGF23 might control gene expression and alter NaPh co-transporter levels as well as their availability at the cell surface. Based on the function of FGF23 in other systems like the kidney where FGF23 regulates the expression of NaPh co-transporters and thereby phosphate uptake, this would be a likely scenario.
In addition to solidifying studies in SMCs, our data provides the first evidence indicating that phosphate can directly act on heart valves. Phosphate but not FGF23 can induce calcification in the valve explants, and this occurs likely through Pit-1 and/or Pit-2, since these are the most highly expressed NaPh co-transporters in the valves. This finding provides a potential mechanism for the etiology of valve calcification due to elevated serum phosphate levels. Although serum FGF23 is often elevated and associated with increased mortality in CKD, we could not detect a direct and causative involvement of FGF23 in the calcification process. However, FGF23 could play a role in modulating the expression levels of NaPh co-transporters in order to alter signaling cascades in these systems, which might be dysregulated in disease states. These studies also support other findings indicating that klotho is not required for FGF23 responsiveness. Combinational in vitro studies using phosphate and FGF23 in addition to klotho in human SMCs did not detect an induction of calcification when klotho was present [110]. Similarly, this study showed in a cohort of patients with CKD that phosphate, but not FGF23, correlated with VC. Recently another experimental study has shown combinatorial effects of FGF23 and phosphate on calcification in uremic rat models and vascular SMCs overexpressing Klotho [111]. The effects of FGF23 further driving calcification were only detected in cells overexpressing klotho, but not in cells lacking klotho. Since FGF23 is not inducing calcification it might play a role in the regulation of NaPh transporters in heart valves (as it does in other systems) and the onset of the osteochondrogenic gene programs that arise during valve calcification.
**Future Directions**

The data presented here sets the stage for further experiments in order to dissect the molecular pathways involved in phosphate-induced valve calcification.Outlined here are a few studies that could be performed to elucidate mechanisms of ectopic calcification.

**Apoptosis:**

Since we describe a link between levels of cleaved caspase 3 and phosphate-induced calcification, further studies could be performed in pAVICs and valve explants in order to examine the effects of phosphate-induced calcification in further detail. To determine the requirement of apoptosis for this mechanism, treatments could be performed using the pan-caspase inhibitor zVAD (with zFA used as negative control). This experiment would demonstrate whether apoptosis is required for phosphate-induced calcification. Other studies have examined whether apoptosis plays a role in calcification of SMC. However, whether phosphate could induce calcification in this context was not analyzed and future studies could utilize valves and pAVICs to determine the role of apoptosis in these systems.

**Pathway analysis:**

To further elucidate the signaling pathways that are altered after phosphate treatment, pAVICs, MOVAS, and valve explants could be treated with phosphate in the presence of established inhibitors of several signaling cascades including pan-FGFR, PD173074; ERK1/2, U0126; ERK1, PD98059; PI3K, wortmannin; Akt1/2, A6730; PLC and A2, U73122; calcineurin, cyclosporine A at concentrations previously described in
[102]. This would provide evidence to whether the calcification phenotype is altered when specific pathways are inhibited. Inhibition of these pathways could be examined by Western blot analysis.

Co-transporter requirement:

Since we show evidence that blocking NaPh co-transporters ameliorates phosphate-induced calcification, it is important to identify inhibitors that can target each transporter individually. A gene knockdown study of phosphate co-transporters in valves would determine which specific transporter is required for phosphate-induced calcification. This could be done lentiviral knockdown in the valve explant cultures, which are then treated with phosphate.

In vivo studies:

Subsequent mouse studies could also be performed to examine the effects of a high phosphate diet on the heart valves and vascular calcification.
**Chapter 4: ACVR2b Soluble Receptor Therapy for Cancer Cachexia**

**Background**

**Overview**

Cachexia, muscle wasting, has been defined as muscle loss despite adequate nutritional intake. This occurs in individuals with AIDS, diabetes, CKD, heart disease and certain cancers, and patients become poor candidates for receiving surgical intervention or therapies [112, 113]. Chapter 1 provides additional background on the history and composition of muscle and implications of cachexia. Chapter 4 focuses on cancer cachexia and the molecular pathways that could be targeted in order to provide therapies for cancer-induced muscle wasting.

**Cancer Cachexia**

Muscle wasting can be the result of several different diseases as outlined in Chapter 1, but is specifically prominent in advanced cancer patients [114]. Often, unexplained significant weight loss brings individuals to be examined by their doctor and later it is discovered they have some form of cancer. Patients with cancer cachexia become poor candidates for surgical intervention and do not respond well to treatments like chemotherapy. Up to 30% of cancer-related deaths are caused from the effects of cachexia, not the cancer itself [115, 116]. This is in part because there are no treatments for cancer cachexia and a major contributing factor to this has been due to a lack of a clinical definition for diagnosing patients. Recently panels have established these diagnostic criteria:
weight loss >5% over the past 6 months (in absence of starvation); or BMI <20 and any degree of weight loss >2%; or appendicular skeletal muscle index consistent with sarcopenia and any degree of weight loss >2%. [21, 117]

This highlights a need for therapeutic intervention in order to treat muscle wasting to increase not only quality of life for cancer patients, but to make them strong enough to withstand the necessary therapies or surgical interventions.

**Cancer Cachexia Signaling Pathways**

Cancer cachexia has been defined as a…

‘multifactorial syndrome involving ongoing loss of skeletal muscle mass (with or without fat mass) that cannot be reversed by conventional nutritional support and leads to progressive functional impairment [21].

Cytokines such as tumor necrosis factor-α, interleukin-6 (IL-6) [118, 119] and interleukin-1β [117] have all been identified as molecular mediators of signaling cascades that induce cachectic phenotypes. Additionally, it has been shown that the tumors themselves secrete these cytokines (tumorkines) thus contributing to muscle wasting. The liver responds by increasing the release of acute phase proteins, which can then act on both skeletal muscle and fat to deplete these stores. Skeletal muscle wasting results from an anabolic and catabolic protein imbalance partly due to activation of E3 ubiquitin ligases. For example, atrogin-1 and Murf-1 are activated in models of cachexia thus targeting proteins for ubiquitination and degradation by the proteasome. Additionally, Foxo transcription factors have been shown to induce expression of these E3 ligases in skeletal muscle [120].
**Myostatin: Discovery and Conservation**

Diseases are often a result of aberrant signaling in critical developmental pathways, therefore it is important to understand molecules controlling muscle development to potentially target these pathways for treating diseases with muscle wasting. One of these proteins is myostatin (previously called Growth Differentiation Factor 8 or GDF-8) and was first discovered by the Lee laboratory at Johns Hopkins University in 1997 [121]. Lee and colleagues performed screens in an attempt to identify novel TGF-β family members and predictions based on the amino acid sequence demonstrated that myostatin exhibited the hallmarks of a TGF-β family member and had homology when compared to other family members especially GDF-11. In addition to homology, the myostatin sequence is conserved across many species and thus provides further evidence for the importance of myostatin signaling during development.

**Myostatin: Tonic Regulator of Muscle Development and Growth**

Myostatin is expressed in the developing myotome of mouse embryos [121], and in adults it is primarily found in skeletal muscle. This led the group to believe that myostatin played a role in the formation of skeletal muscle during development and was proven to be true when a portion of the myostatin gene, encoding the C-terminal domain required for downstream myostatin signaling, was genetically deleted in mice. *Myostatin* null (MSTN \(^{-/-}\)) mice exhibited a double-muscling phenotype from both muscle hyperplasia (increase in number) and hypertrophy (increase in fiber size). Additionally, myostatin heterozygous mice (MSTN \(^{+/}\)) had increased muscle size and number compared to controls, but were not as large as the MSTN\(^{-/-}\), indicating that myostatin
signaling functions in a dose-dependent manner [121]. Mice are not the only animals affected by mutations in myostatin, as this has been shown in Belgian blue cattle, whippets and one human [113, 122]. Since, myostatin has been demonstrated to be a tonic inhibitor of muscle growth during development [113, 121] this raised the question of whether inhibition of myostatin signaling would have clinical implications in muscle diseases.

**Myostatin: Biosynthesis and Signaling Mechanisms**

In order to establish potential therapies that could target the myostatin pathway, it is important to understand the mechanisms of myostatin activation and signaling. It has been shown that myostatin is synthesized as a precursor protein and requires several proteolytic events in order to produce the active form of the protein responsible for downstream signaling. Like other TGF-β family proteins, myostatin undergoes two proteolytic cleavage reactions generating a C-terminal fragment which is responsible for binding to activin type II receptors (ActRIIB and with lesser affinity to ActRIIA) and activin type I receptors (ALK4 and ALK-5) [122]. The kinase activity from these receptors results in Smad2/3 phosphorylation and by mechanisms that are not fully understood muscle hypertrophy and differentiation is inhibited.

The active form of myostatin is capable of binding to the activin type II receptors, ActRIIA and ActRIIB, and this interaction has been demonstrated *in vivo* using a mouse model which expresses a skeletal muscle specific (myosin light chain promoter, MLC) dominant-negative (lacking the kinase domain) form of ActRIIB and as a result develops a doubling muscle phenotype [122]. In classical TGF-β signaling the ligand first binds to
the type II receptors, which then recruits and forms dimers with the type I receptors. These interactions provide a spatial interaction in order for the type II receptor to phosphorylate the type I receptor, thus activating the kinase domain providing a docking site for further signaling to occur via Smad phosphorylation and its subsequent translocation to the nucleus to mediate gene expression [113]. The C-terminal dimer of myostatin is inhibited by several molecules including the myostatin propeptide, follistatin, and a soluble form of the type-two receptor (ACVR2b-Fc). These inhibitors can act as ligand “traps” for myostatin thus allowing muscle growth [122, 123]. As mentioned above, several pathways have been shown to play a role in muscle wasting but myostatin provides a pathway responsible for regulating muscle development that when manipulated by inhibition caused muscle growth.

**Myostatin: Mouse Models Altering Myostatin Signaling**

Increases in muscle size have been shown in mouse models where myostatin signaling has been inhibited [113, 122]. For example, mice with a genetic deletion of the myostatin C-terminus (forms active ligand, MSTN -/-) show an increase in skeletal muscle mass [121]. Conversely, mice that are systematically administered myostatin develop cachexia [123].

It has also been shown that mice with alterations in the myostatin signaling cascade develop double muscling phenotypes similar to those seen in the myostatin null mice. These include: mice with skeletal muscle specific expression under the MLC promoter of a dominant negative version of the type two receptor for myostatin (MLC-dnACVR2B) and mice over expressing the myostatin inhibitor follistatin (MLC-
Follistatin) [124]. The MLC-dnACVR2B mice express a truncated form of the receptor lacking the kinase domain and although the muscles are larger, they seem to be normal and display no evidence of fibrosis. In addition to these genetically manipulated mouse models, systemic administration of a soluble form of the ACVR2b (ACVR2B-Fc) also increased muscle mass in nude mice.

In addition to the genetic mouse models, studies in animals have been performed using pharmaceutical approaches to target muscle wasting, but none have been specific to myostatin. These include examining Trichostatin (TSA), a histone deacetylase (HDAC) inhibitor that has been shown to prevent dystrophic muscles [125, 126]. Another study demonstrated that the soluble form of ACVR2B could increase muscle size in nude and wild type mice. Neither approach has been utilized in the context of cancer cachexia.

**Cancer Cachexia: Animal Models**

Cancer is one of the predominant causes for cachexia and there are several widely used mouse models to study the molecular mechanisms involved with muscle wasting. Several of these models involve injecting mice with cancer cell lines such as the colon-26 carcinoma, Lewis lung carcinoma (LLC) or melanoma. These models induce muscle wasting in mice and the tumors secrete similar inflammatory cytokines that are found in the circulation of humans with cancer.

**Experimental Design**

Since myostatin inhibition has been show to increase muscle size using several *in vivo* studies, we wanted to test whether myostatin inhibition could prevent muscle
wasting in cancer cachexia. Here, we utilized transgenic mouse models \( MSTN^{-/-} \) and MLC-dnACVR2b), C57BL/6 and Athymic nude mice and injected them with several different cancer cell lines, and/or Chinese hamster ovary (CHO) cells that secreted a soluble form of ACVR2b (CHO-ACVR2b-Fc) or control (CHO-Control) cells. Then, we examined whether we could induce wasting in mice using LLC, colon adenocarcinoma (C26), and melanoma (B16F10) cells and prevent cachexia by acutely inhibiting myostatin-family ligands. This work is part of a published journal article in BBRC [127].

Methods

Cell lines

C26 cells (from Donna McCarthy) were maintained in RPMI 1640 with 10% FBS, 1% Pen/Strep. LLC (LLC1, ATCC #CRL-1642) and B16F10 melanoma cells (B16F10, ATCC #CRL-6475) were maintained in DMEM with 10% FBS, 1% P/S. CHO-Control, CHO-ACVR2B-Fc and CHO-MSTN (from Se-Jin Lee at Johns Hopkins University) cells were maintained as described [128].

Mice

Experiments were approved by the Institutional Animal Care and Use Committee at the University of Miami Miller School of Medicine. Mice were housed with a 12 hour light-dark cycle with free access to chow and water. Female CD2F1 and athymic nu/nu mice were from Harlan (Tampa, FL). C57BL/6J mice were from Jackson Laboratories (Bar Harbor, Maine). C57BL/6J and C57:\textit{Mstn}^{-/-} mice (backcrossed >10 generations to C57BL/6J) and MLC-dnACVR2b (both a gift of Se-Jin Lee) were bred in our colony.
**Mouse Models of Cachexia**

Mice (>12 weeks) were injected with $10^6$ cells in 100 µL sterile PBS. *Mstn-/-* mice and wild-type sex matched littermates were injected with LLC or B16F10 melanoma cells. Controls were PBS injected wildtype and *Mstn-/-* mice. CD2F1 mice with C26 cells were treated daily with TSA (s.c., 0.6 mg/kg body weight in DMSO) from days 9-16 after tumor injection. Control C26 mice received DMSO only. Normal mice received either TSA or DMSO on the same schedule. Athymic nude mice were injected with either the LLC or C26 cells and with either CHO cells expressing a soluble form of the ACVR2B (CHO-ACVR2B-Fc) or with a similarly selected cell line, CHO-control, not expressing recombinant proteins.

**RNA Extraction and Quantitative Real-Time (qRT-PCR)**

Total RNA was extracted from flash frozen quadriceps using Trizol. Reverse transcription was with random primers using a High-Capacity cDNA Archive Kit (Applied Biosystems) and PCR reactions with iQ™ SybrGreen Supermix (BioRad Laboratories). Amplification was 1 cycle 95°C 10 minutes, then 40 cycles of 95°C for 15 seconds and 65°C for 1 minute. Standard PCR was also performed and products analyzed on ethidium bromide-agarose gels to verify specificity, and melting and standard curves were obtained for each primer pair. Six replicates for each sample were normalized to the GAPDH transcript content. Relative changes were quantified using the $2^{-\Delta\Delta Ct}$ method [129]. Primers were:

- Myostatin, 5’-CTGTAACCTTCCCAGGACCA-3’, 5’-GCAGTCAAGCCCAAGAGTCTC-3’;
- Follistatin, 5’-AGAGGTCGCTGCTCTCTG-3’,
and 5’-AGCTTCCTTCATGGCACACT-3’
GAPDH 5’-TGCACCACCAACTGCTTAG-3’, 5’-GGATGCAGGGATGATGTTC-3’.

Statistics

Results shown are representative of experiments performed at least twice.
Results are mean ±SD. Data were analyzed with Student’s unpaired two-tailed t-test, 1-way ANOVA with Tukey-Kramer post test, or 2-way ANOVA for time and treatment effects.

Results

Myostatin Null Mice have Accelerated Wasting with Cancer Cachexia

Mstn−/− mice have a 2-3-fold increase in skeletal muscle mass, with concomitant reduction in fat [121]. Therefore, we decided to challenge them with various cancer models that have been shown to induce cachexia in mice to determine whether they were protected from muscle wasting [113]. Surprisingly, the myostatin null mice actually lost a greater percentage of muscle mass relative to their body weight when compared to wild type mice after being challenged with two different cancer cell lines. Mstn−/− mice and wild type controls were challenged with two well-established models of cancer cachexia, LLC [130] or B16F10 melanoma [131] (Figure 4.1). Percentage weight loss over time was indistinguishable between genotypes (Figure 4.1A). By 14 days, overall tumor free body weight was reduced 18.1% ± 5.9% in wild-type mice and 19.9% ± 4.8% in knockout mice (N.S.) (Figure 4.1B). All muscles are affected and because Mstn−/− null mice are larger at baseline, a greater quantity of actual mass was lost in knockouts. Wild-
type mice lost 3.3±1.28 g of total body weight, while *Mstn*−/− mice lost 5.3±0.84 g (*P*< 0.01). *Mstn*−/− mice lost proportionately more muscle mass and also more absolute mass, as shown for gastrocnemius (-13.9% wild-type vs. -25.6% knockout, *P* > 0.05) and quadriceps (-11.9% wild-type vs. -15.0% knockout) (Figure 4.1D). Tumor mass was not different between the wild type and *Mstn*−/− mice (Figure 4.1C). Thus more absolute and greater fractional muscle mass were lost in *Mstn*−/− mice, even though they had a lower percentage tumor burden (compared to their body weight) relative to wild-type mice. *Mstn*−/− mice also showed enhanced wasting in a second tumor model, B16F10 melanoma (Figure 4.1E-H). Although often used to model cachexia, the cell line we obtained from ATCC grew well in wild-type mice without inducing wasting and these mice actually gained total body mass (+18.9 ± 7.9%). Unlike the wild-type controls, *Mstn*−/− mice still lost weight (-7.8 ±10.5%, *P* < 0.001) (Figure 4.1E). As with the LLC model, B16F10 tumor sizes in wild-type and *Mstn*−/− mice were similar (Figure 4.1F). Body weight changes were at least partly due to coordinate changes in muscle mass. Gastrocnemius mass was increased in wild-type mice but reduced in *Mstn*−/− (+9.0 ± 8.38% wild-type vs. -14.0 ± 11.0% knockout, *P* >0.001) (Figure 4.1G). Similarly, quadriceps mass was increased in wild-type mice but reduced in *Mstn*−/− (+9.7 ± 9.9% wild-type vs. -12.9 ± 9.8% knockout, *P* > 0.001) (Figure 4.1G). The growth in skeletal muscle in the wild-type mice may have been due to the weight load imparted by tumor growth. Both wild-type and *Mstn*−/− mice lost adipose mass, and percentage losses were not statistically different (Figure 4.1G). This might be indicative that myostatin signaling is actually required for the establishment and maintenance of skeletal muscle and when the animal does not have this functioning signaling pathway, then they are more susceptible to wasting. Thus
Mstn−/− mice on average lost far more percent and absolute body weight and skeletal muscle mass in response to a lower fractional tumor burden. By plotting individual changes in quadriceps or epididymal fat pads versus tumor weight demonstrates that Mstn−/− mice were more sensitive to cachexia (Figure 4.1H). Taken together with the LLC model, these results indicate clearly that Mstn−/− mice were not protected from cancer cachexia. It is important to note that the Colon-26 model could not be assayed because it will not grow in C57BL/6 mice.

**Figure 4.1 Myostatin Null Mice Have Accelerated Wasting with Cancer Cachexia**

(A–D) Lewis lung carcinoma (LLC), N = 7–9 per group. (A) Changes in body weight, (B) tumor-free body weight, (C) LLC tumor size, and (D) gastrocnemius (gast), quadriceps (quad) and abdominal fat mass in wild-type (WT) and myostatin null (null) mice. (E–H) B16F10 melanoma, N = 8–15 per group. (E) Tumor-free body weight, (F) B16F10 tumor mass, (G) muscle and fat mass, and (H) muscle or fat loss versus tumor size in wild-type and myostatin null mice killed on day 17 after injection. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure on Following Page
**Trichostatin Fails to Inhibit Muscle Wasting in C26 Cancer Cachexia**

We then took a pharmacologic approach using TSA, a HDAC inhibitor, to determine whether it was protective in mice with cancer cachexia. This was done since *Mstn<sup>−/−</sup>* mice lack myostatin from development and because such mutations are exceeding rare among humans. TSA has been shown previously to increase muscle mass and strength in wild-type and dystrophic mice [125, 126] mainly by increasing expression of follistatin, a myostatin inhibitor. We injected CD2F1 mice with colon-26 (C26) adenocarcinoma cells; a well-established, robust model of cachexia [132] and TSA was given daily for seven days once tumors were visible (Figure 4.2A). Total body weight loss over time was indistinguishable in TSA versus carrier-treated C26 mice (Figure 4.2A). Non tumor-bearing mice showed increased total body weight with TSA treatment versus carrier controls, however the difference was not statistically significant (Figure 4.2B). As expected, C26-injected mice lost total body mass; TSA did not protect mice from C26-induced weight loss (Figure 4.2B). TSA increased muscle mass in normal mice, as expected. Gastrocnemius and quadriceps mass were both increased (+14.7 and +13.5, respectively). However, TSA did not prevent muscle wasting in C26 cachexia; both tumor-bearing groups showed equivalently reduced gastrocnemius and quadriceps mass (Figure 4.2C). TSA treatment not only increased skeletal muscle mass in normal mice, but also increased adipose tissue mass. Differences in fat in carrier versus TSA-treated C26 mice were not significant. TSA did not affect tumor mass (Figure 4.7D). TSA treatment did increase quadriceps follistatin RNA ~2-fold in normal mice and ~1.6-fold in C26 mice. Moreover, TSA treatment resulted in a ~50% reduction in myostatin RNA expression in normal mice but not C26 mice (Figure 4.2E). Thus, unlike a prior
report [133], our treatment regimen succeeded in increasing the mRNA expression of follistatin, both in the normal mice and tumor-bearing mice although the magnitude was less in the latter. Despite the changes in expression, these data indicate that TSA is not protective in cancer-induced wasting.

Figure 4.2 TSA does not Inhibit Muscle Wasting in C26 Cachexia
A) Changes in body weight, B) tumor-free body weight, C) gastrocnemius, quadriceps and abdominal fat mass, D) tumor mass and E) quadriceps follistatin and myostatin RNA levels (relative to GADPH) in mice with no tumors treated with carrier (DMSO) or with TSA, or with tumor and carrier (C26-DMSO) or tumor and TSA (C26-TSA). N=8 per group. *P<0.05, **P<0.01, ***P<0.001.
**Soluble ACVR2b Increases Muscle Mass**

Next, we decided to examine the effects of acutely blocking myostatin-family proteins in mice. Since TSA did not prevent cancer-induced wasting, we utilized an approach that was more specific to blocking myostatin-family ligands. Here we used ACVR2B-Fc, the extracellular domain of Activin receptor type IIB fused to murine IgG-Fc, which has been shown to inhibit myostatin receptor binding and biological activities of myostatin and other TGF-β family members. Additionally, this molecule has been shown to induce muscle growth in normal mice when administered systemically [124]. To deliver sustained high levels of ACVR2B-Fc, we injected nude mice with CHO-ACVR2B-Fc cells or with CHO-control cells. We have used this approach successfully to study various secreted proteins [123, 124, 128, 134-138]. Over 15 days, CHO-ACVR2B-Fc injected mice gained 12% total body weight (Figure 4.3A) and 12.9% tumor-free body weight (Figure 4.8B). CHO tumor size was not different between groups (Figure 4.3C). Muscle mass was increased by CHO-ACVR2B-Fc, 14.5% in gastrocnemius and 15.9% in quadriceps, while abdominal fat pad mass was increased 248% (Figure 4.3D). These results indicate that administration of the soluble receptor ACVR2B-Fc by CHO cells results in muscle growth in nude mice, as purified protein does in C57BL/6 mice.
**Soluble ACVR2b Prevents Cancer-induced Muscle Wasting in Mice**

Next, we wanted to determine whether soluble ACVR2b was protective in mouse models of cancer cachexia. We injected athymic nude mice with CHO–Control or CHO-ACVR2B cells and performed co-injections with either C26 or LLC cells. Remarkably, over 12 days mice with LLC and CHOACVR2B-Fc cells (LLC/ACVR2B-Fc mice) showed a 4.1% increase in body weight in this model of cachexia (Figure 4.4A). In contrast, LLC/control mice lost 4.0% total body weight (p<0.0001). Tumor masses were similar between groups, indicating that both the CHO cell lines and C26 cells grew fine (Figure 4.4B). Skeletal muscles were significantly larger in LLC/ACVR2B-Fc mice; with
the pectoralis increased 28% and triceps increased 25% (Figure 4.4C). Leg muscles were not measured due to altered gait from tumors. LLC/ACVR2B-Fc mice were also protected against fat loss while LLC/Control mice had virtually no abdominal fat at necropsy (Figure 4.4C). Furthermore, LLC/ACVR2B-Fc mice remained pink, active and grossly normal despite their tumors, while LLC/Control mice were blue, inactive and near moribund at euthanasia on day 12.

Finally, athymic nude mice were injected with C26 cells (intrascapular fat pad) and with either CHO-ACVR2B-Fc or CHO-control cells (upper leg). C26/ACVR2B-Fc mice showed increased tumor-free body weight (13% at 16 days and 14% at 22 days) over C26/control mice (Figure 4.4D). Tumor sizes were similar between groups at both time points (Figure 4.4E). At 16 days after injection, C26/ACVR2B mice showed increased gastrocnemius mass (+27.1%) and quadriceps mass (+23.6%) versus C26/controls (Fig. 4.4F). At 22 days after injection, C26/ACVR2B mice showed even more increased gastrocnemius mass (+40.6%) with quadriceps mass also increased (+23.1%) (Figure 4.4G). Although not measured in this trial, in separate trials, C26/ACVR2B-Fc mice also showed increased adipose mass versus C26/controls. These data showing muscle preservation by ACVR2B-Fc administration in two models indicates that blocking the myostatin signaling pathway is a potent means of reducing muscle loss in cancer cachexia.
Figure 4.4 ACVR2B-Fc Inhibits Muscle Wasting in Cancer Cachexia

(A-C) Lewis lung carcinoma (LLC), N=12 per group. A) Changes in body weight, B) LLC and CHO tumor size, and D) muscle and abdominal fat pad mass in mice with LLC and CHO-ACVR2B-Fc cells or LLC and CHO-control cells. (D-G) C26 adenocarcinoma, N=5-10 per group. D) Tumor-free body weight, E) C26 and CHO tumor mass, F) gastrocnemius (gast) and quadriceps (quad) mass on day 16 and H) day 22 after injection. *P<0.05, **P<0.01, ***P<0.001.
Discussion

Conclusions

These studies provide encouraging evidence for inhibiting myostatin-family proteins in order to treat cancer cachexia in mice and potentially as a method for therapeutic strategy in cancer-induced muscle wasting. It has been shown previously that myostatin administration was sufficient to induce wasting [123]. Others have reported evidence of increased myostatin signaling in cancer cachexia [139]. This is the first evidence that acute inhibition of myostatin family ligands is a potent approach for reducing muscle wasting in cancer cachexia. Using the soluble ACVR2B-Fc in two models of cancer cachexia, we observed 23-40% increased muscle mass versus controls. Such a marked increase in muscle mass in cancer cachexia has never been published before, however studies have been performed since the publication of these studies to accompany these results.

Myostatin deletion and muscle hypertrophy is protective in certain models of muscle disease and atrophy, including Duchenne’s muscular dystrophy [140], amyotrophic lateral sclerosis [141], limb-girdle muscular dystrophy [142], glucocorticoid administration [143], and sarcopenia [144]. However, myostatin null mice actually show enhanced atrophy after hind limb suspension [145]. Similarly, we observe that myostatin knockout mice are more susceptible to muscle wasting in two models of cancer, including one model which did not induce cachexia in wild-type mice. These results indicate first that although myostatin is capable of inducing cachexia [123], muscle-derived myostatin is clearly not the sole mediator of cachexia. Moreover, the results suggest that a lack of myostatin signaling from the earliest stages of development might render muscle
susceptible to wasting. The mechanism is unknown, but it might involve compensatory changes in other growth regulatory pathways. As well, the reportedly altered muscle fiber phenotype in myostatin null mice, including loss of oxidative characteristics and reduced force generation, might predispose them to cancer cachexia [146].

TSA increases muscle size and function by increasing follistatin [125, 126]. Although TSA increased follistatin expression in tumor-bearing mice, it did not preserve or increase muscle mass in cachexia. (Bonetto, et al. also report negative results with TSA on muscle size, but failed to observe increased follistatin expression [133]). TSA is not specific for follistatin; indeed, it globally affects the expression of potentially thousands of genes, both in muscle and other organs. The failure of TSA to inhibit wasting, therefore, does not indicate that modulation of the myostatin/follistatin axis is insufficient to protect muscle in cancer cachexia.

Indeed, inhibition of myostatin family ligands using continuous administration of ACVR2B-Fc potently promotes muscle preservation in cancer cachexia. ACVR2B-Fc is not specific for myostatin and other known ACVR2B ligands include GDF-11 and the Activins [124]. Thus the protective effect of ACVR2B-Fc may be due to inhibition of these other targets, alone or with myostatin. Indeed, ACVR2B-Fc also induces muscle growth in myostatin knockout mice, indicating that other ligands also tonically inhibit muscle size [124]. ACVR2B-Fc has been shown to improve muscle mass and function in amyotrophic lateral sclerosis [147], hypoxia-induced muscle dysfunction [148] and obesity [149], but not in severe spinal muscular atrophy [150]. These contrasting results indicate that the efficacy of myostatin inhibition on muscle mass, function and survival may be time, dose and context specific.
The protective effects of ACVR2B-Fc in two distinct models of cancer cachexia suggest that myostatin family ligand inhibition might be a general approach to preserving muscle in cancer. The C26 line used herein secretes high levels of IL-6 and neutralization of IL-6 has been shown to be protective in this model [151, 152]. With no known crosstalk between myostatin/Activin receptor/SMAD signaling and IL-6/gp130/STAT signaling, targeting both pathways simultaneously might attain more effects. In contrast, the LLC line used in this study results in no detectable plasma IL-6 in host mice, suggesting that ACVR2B-Fc can inhibit wasting produced by IL-6-independent mechanisms. This seems to indicate broad applicability of myostatin family inhibition for muscle preservation in cancer cachexia.

By preventing cachexia we can enhance the quality of life for many patients and it will provide opportunities for surgical intervention and therapies where they were previously not good candidates. Since the publication of these studies, several other studies have been published to compliment the methods used here and the data here has been reviewed extensively [112, 114, 153].

**Future Directions**

To accompany these studies, it would be interesting to examine signaling pathways related to myostatin that are affected in muscles from mice treated with CHO-ACVR2b or CHO-MSTN compared to CHO-Control. In order to understand the molecular differences between cancer-induced and myostatin-induced cachexia, it would be interesting to determine the pathways that are affected similarly or differently in each
type of muscle wasting. Smad2/3 phosphorylation would be expected to increase with CHO-MSTN or cancer-cachexia models.

Some of the molecular targets to be examined would include:

**Pathways related to decreasing cell proliferation**

- p21 (would be expected to increase with CHO-MSTN or cancer-cachexia models)
- Cdk2 (would be expected to decrease with CHO-MSTN or cancer-cachexia models)

**Pathways related to myogenic differentiation**

- MyoD, myogenin and Myf-5 (would be expected to decrease with CHO-MSTN or cancer-cachexia model).

Additionally, since inflammation plays a key role in the development and progression of cachexia it would be interesting to target an inflammatory cytokine like IL-6 (since this has been shown to be activated in serum of patients with muscle wasting and can induce cachexia by systemic administration) in addition to using an inhibitor of myostatin or myostatin-family ligands to determine if there is even more protection of the muscle fibers.
Chapter 5: Concluding Remarks

The studies outlined throughout this thesis represent two types of diseases that even with current tools in medicine do not have proper treatments. Quality of life for these patients is diminished and prevents them from receiving proper medical care. These diseases are and will continue to be even more costly and clinically relevant in the future with increases in the aging population. There have been significant advances in these fields towards understanding the molecular mechanisms responsible for the disease etiologies and this project outlines several novel players in both heart valve development and disease and cancer-induced muscle wasting. Most scientists discover as they perform research to answer hypotheses, more questions arise and the mechanisms unravel to become even more complex than they may have originally thought. Given the complexity of the development and diseases involved with heart valves and skeletal muscle this is certainly true. Hopefully, the tools used to determine the critical pathways involved will evolve as quickly as scientists can question. William Harvey may have said it best when he stated in On the Motion of the Heart and Blood in Animals,

“Very many maintain that all we know is still infinitely less than all that still remains unknown.”
Citations


VITA

Margaret Elizabeth Benny Klimek was born and raised in northeastern Pennsylvania along with two older siblings, Karen Marie and Joseph Christopher Jr. Her parents, Joseph Christopher Sr. and Shirley Benny, highly valued education and upon graduation from Tamaqua Area High School in 2001 supported Margaret through her undergraduate studies at Moravian College in Bethlehem, Pennsylvania. Here Margaret excelled in her studies and graduated with a Bachelor of Science in Biology, Cum Laude, with Honors in Biology. Margaret’s love for science first became evident during high school AP biology class where she loved learning about life and asking questions about how cellular processes worked. This interest was furthered while at Moravian College, where she immersed herself in biology courses and had experiences working in several laboratories in the biology department. Margaret also served as president of the school’s Tri-Beta Biological Honor Society chapter and captain of the Track and Field Team. After receiving her undergraduate degree, Margaret continued to work with Dr. David Vasily, a dermatologist, with whom she did volunteer work with while a student at Moravian. Here Margaret was involved with clinical research involving pattern baldness and assisted with several other IRB studies in dermatology.

While visiting her sister on trip to Mobile, Alabama she met the love of her life, Bradley James Klimek. Margaret decided to move to Alabama after spending a year in a long distance relationship. Prior to moving, Margaret accepted a job as a Research Technician at the University of South Alabama to perform post-baccalaureate research in pulmonary biology in the lab of Dr. Brian Fouty in the Center for Lung Biology. It was here that Margaret learned she truly enjoyed performing research at the bench and asking questions
about her research. June 7, 2008, Margaret and Brad were married in a small ceremony in Connecticut surrounded by family and close friends. Upon their return to Mobile they packed and began their journey together as husband and wife and moved to Miami Beach.

Margaret began graduate school at the University of Miami Miller School of Medicine as an IBS student in August 2008 and decided to join the department of Molecular, Cell and Developmental Biology early in her first year. While in graduate school at the University of Miami, she was able to gain intellectual independence and learn to use many practical techniques. She utilized skills of organization and preparing ahead to balance critical time in the laboratory while excelling in graduate classes. Margaret started her research by studying muscle wasting and specifically examining myostatin inhibition in cancer cachexia under Dr. Teresa Zimmers. Margaret then continued her work in heart valve development and disease with Dr. Joy Lincoln and Dr. Christian Faul examining miRNA regulation of heart valve development and effects of phosphate on valve calcification. Although her path was unique through graduate school, it provided a diverse background and expanded her technical knowledge beyond the topics she was researching. She learned to be self-motivated and very independent scientist able to prioritize and structure her own experiments. Margaret has presented posters at international meetings, received travel awards and grown as a scientist by interacting with researchers from around the world.

Margaret has not only has expanded her research and experience in science but also has two additions to her family since starting graduate school. Her children, Niko Parker (3 years) and Alexandra Elizabeth (11 months) are truly the center of her life. She
especially enjoys watching her children grow and learn every day. Niko is a very inquisitive little boy that loves racecars, the U and “Miami Heat Basketball” especially #6 (LeBron James). Alex is a sweet, well natured little girl and loves following her brother around and making airplane sounds when she isn’t smiling or laughing at her older brother. Margaret’s husband Brad has been an amazing support for her throughout graduate school and is a Commander in the United States Coast Guard. He is a graduate of Navy Flight School and qualified as both a pilot and flight instructor. Currently, he is stationed at the USGC Headquarters in D.C. working in the Office of Aviation Forces and finishing his Masters in Sustainability and Environmental Management through Harvard Extension School. Margaret resides in Arlington, VA with her family where they all enjoy participating in local running events and will be seen jogging around Washington-Lee High School Track or on the National Mall with the kids in tow.

Graduate school has prepared Margaret intellectually and technically to perform high quality research at the post-doctoral level. Recently, Margaret has accepted a position as a post-doctoral researcher under the mentorship of Kanneboyina Nagaraju, DVM, PhD, and a principal investigator at Children's National Medical Center Children's Research Institute in the Center for Genetic Medicine Research (CGMR) in Washington, DC. The focus of her project there will be on muscle biology, specifically in the context of utilizing exon-skipping in order to restore function of the dystrophin protein and ultimately ameliorate the symptoms of Duchenne’s muscular dystrophy. In this lab Margaret will grow intellectually as a scientist and further develop her skills to be an independent researcher. Margaret completed her course of studies in the Graduate School of the University of Miami in August 2008, where she was granted a PhD degree.
in the department of Molecular, Cell and Developmental Biology in December 2013.