University of Kentucky

UKnowledge

Theses and Dissertations--Biomedical Engineering | Biomedical Engineering

2023

3-DIMENSIONAL MUSCLE CONSTRUCTS: USING HYDROGELS IN ORDER TO MODEL THE EFFECTS OF EXERCISE IN DISEASE CONDITIONS

Mark McHargue
University of Kentucky, cameronmchargue2@icloud.com
Author ORCID Identifier:

Digital Object Identifier: https://doi.org/10.13023/etd.2023.238

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Recommended Citation
McHargue, Mark, "3-DIMENSIONAL MUSCLE CONSTRUCTS: USING HYDROGELS IN ORDER TO MODEL THE EFFECTS OF EXERCISE IN DISEASE CONDITIONS" (2023). *Theses and Dissertations--Biomedical Engineering*. 78.
https://uknowledge.uky.edu/cbme_etds/78

This Master's Thesis is brought to you for free and open access by the Biomedical Engineering at UKnowledge. It has been accepted for inclusion in Theses and Dissertations--Biomedical Engineering by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Mark McHargue, Student

Dr. Ramkumar T. Annamalai, Major Professor

Dr. Sridhar Sunderam, Director of Graduate Studies

3-DIMENSIONAL MUSCLE CONSTRUCTS: USING HYDROGELS IN ORDER TO MODEL THE EFFECTS OF EXERCISE IN DISEASE CONDITIONS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Biomedical Engineering in the
College of Engineering at the University of Kentucky

By
Mark Cameron McHargue
Lexington, Kentucky
Director: Dr. Ramkumar T. Annamalai, Professor of Biomedical Engineering
Lexington, Kentucky
2023

Copyright © Mark Cameron McHargue 2023
https://orcid.org/0009-0008-8995-2126

ABSTRACT OF THESIS

3-DIMENSIONAL MUSCLE CONSTRUCTS: USING HYDROGELS IN ORDER TO MODEL THE EFFECTS OF EXERCISE IN DISEASE CONDITIONS

In order to properly study the relationship between disease and muscle, a model must be created that can recapitulate the general shape and function of muscle tissue *in vivo*. Therefore, we set out to create a model that contained linearly aligned myotubes in a 3 dimensional scaffold to replicate these conditions more closely than would be possible in 2 dimensional models. Moreover, we designed our model in such a way that it would be possible to mechanically stimulate it through stretching, subjecting the cells in the muscle construct to similar conditions that they may face *in vivo*. In order to improve the quality of our model, we varied the types of 3D matrix, the components of cell culture media, and the shape of our custom made culture well. To validate its effectiveness, we have subjected the construct to mechanical stretching and placed it in a variety of conditions that simulate disease, primarily through inflammation. Given the constructs reactions to these varied conditions, we believe that we have created a powerful model of muscle that can be used to study a variety of disease states.

KEYWORDS: Muscle Construct, Tissue Culture, Exercise Mimetics, Type II Diabetes, Disease Modeling, Inflammation

Mark Cameron McHargue

(Name of Student)

04/28/2023

Date

3-DIMENSIONAL MUSCLE CONSTRUCTS:
USING HYDROGELS IN ORDER TO MODEL THE EFFECTS OF EXERCISE IN DISEASE CONDITIONS

By

Mark Cameron McHargue

Dr. Ramkumar T. Annamalai
Director of Thesis

Dr. Sridhar Sunderam
Director of Graduate Studies

04/28/2023
Date

DEDICATION

To my family – I hope this research contributes to helping you all live long, healthy lives.

ACKNOWLEDGMENTS

This thesis, while an individual work, benefitted from the insight, assistance, and encouragement from many people. My thesis committee, consisting of Dr. Annamalai, Dr. Fry, and Dr. Sunderam, were fundamental in the planning and execution of this work. It is not an exaggeration to say that without their support, this work would not have been completed. Next, I think it is important for me to acknowledge the support I received from my colleagues. Each of my lab mates in the BITE Lab, regardless of how much time our paths crossed in the lab, provided critical support that helped me to develop myself both as a researcher and a person. This also extends to members of Dr. Fry's lab, who spent many hours teaching me new methods that greatly improved my understanding.

In addition to the support I received within the lab, the support I received outside was paramount to my success. I would first like to thank my parents, who encouraged me to continue even when things were not progressing as I'd hoped. I would also like to thank my roommate, Jaxon, who helped me to stay social and meet many new people over the last two years. Finally, I would like to extend a special thanks to you, the reader. Should this work provide some sort of basis for you to make some kind of progress, I am glad that you are able to find use in it.

TABLE OF CONTENTS

ACKNOWLEDGMENTS iii

LIST OF FIGURES vii

CHAPTER 1. INTRODUCTION 1

1.1 Background 1

1.2 Muscle as a Model 2

1.2.1 Components of Muscle 2
1.2.2 Role of Muscle 3
1.2.3 Effect of Disease on Muscle 5
1.2.4 *In Vitro* Representation of Muscle 6

1.3 Significance 8

1.3.1 Drug Development 9
1.3.2 Disease Understanding 9

1.4 Goals 10

CHAPTER 2. CURRENT KNOWLEDGE AND STATE OF THE ART 11

2.1 Methods of Quantification 11

2.1.1 Gene Expression Panel 11

2.2 Current 3D in vitro muscle models 17

2.2.1 Matrix Composition 18
2.2.2 Scaffold Casting Techniques 21
2.2.3 Muscle Cell Types 23

2.3 Pathology of Type II Diabetes 27

2.3.1 Obesity 27
2.3.2 Systemic Inflammation 28
2.3.3 Metabolic Dysregulation 30

2.4 Modeling Type II Diabetes 32

2.4.1 Diabetic Induction in Rodents 32
2.4.2 Hyperglycemic Culture Conditions 35
2.4.3 Inflammatory Culture Conditions 35
2.4.4 Hyperlipidemic Culture Conditions 36

2.5 Stimulating Muscle in vitro 36

2.5.1 Electrical Stimulation 37
2.5.2 Mechanical Stimulation 38

2.6 Effects of Exercise on Muscle 39

2.6.1 Metabolic Improvement 39
2.6.2 Cellular Hypertrophy and Increased Contractility 40
2.6.3 Inflammatory Attenuation 41

CHAPTER 3. METHODOLOGIES 42

3.1 Creating a Custom Mold 42

3.1.1 Designing a Mold 42

3.1.2 3D Printing a Mold 43
3.1.3 PDMS Casting 43

3.2 Casting Hydrogels 44
3.2.1 Fibrin 44
3.2.2 Collagen 45
3.2.3 Composite 45

3.3 Cell Culture Techniques 46
3.3.1 Cell Expansion 46
3.3.2 Cell Differentiation 47

3.4 Cell Treatment 48
3.4.1 Cytokine Treatment 48
3.4.2 Conditioned Media Treatment 49

3.5 Mechanical Stimulation 49

3.6 RNA Quantification 51

3.7 Protein Synthesis Quantification 52

3.8 Fluorescent Imaging 53

3.9 Mechanical Testing 53

3.10 Statistical Analysis 54

CHAPTER 4. DATA AND RESULTS 55

4.1 Creating a Construct 55
4.1.1 Mechanical Properties 55
4.1.2 Timepoint Analyses 58

4.2 Validating as an Inflammatory Model 61
4.2.1 IFNγ Treatment 62
4.2.2 Conditioned Media Treatment 63
4.2.3 Diabetic Media Treatment 64

4.3 Effects of Mechanical Stimulation 67
4.3.1 Effects of Stimulation on Untreated Constructs 67
4.3.2 Effects of Stimulation on Conditioned Media Treated Constructs 70
4.3.3 Effects of HIIT on IFNγ Treated Constructs 72
4.3.4 Effects of Stimulation on Diabetic Treated Constructs 73

CHAPTER 5. DISCUSSION OF RESULTS 76

5.1 Muscle Constructs can be Reliably Created 76
5.1.1 Effect of Matrix Volume on Differentiation 77
5.1.2 Determining Timepoints for Construct Development 78
5.1.3 The Value of Pluronic 78
5.1.4 Improvements for the Future 79

5.2 Constructs as a Disease Model 79
5.2.1 The Problem with Co Cultures 80
5.2.2 Recapitulating Diabetes 80
5.2.3 Exercise while Inflamed 81

5.3 Mechanical Stimulation as a Tool 82
5.3.1 Common Trends in Data 83

5.3.2 How to improve Mechanical Stimulation 84
CHAPTER 6. CONCLUSION 85
BIBLIOGRAPHY 87
VITA 97

LIST OF FIGURES

FIGURE 1.1: A HIERARCHICAL DIAGRAM OF MUSCLE[4] 3
FIGURE 2: MOLD FOR CASTING CUSTOM WELLS 43
FIGURE 3: CUSTOM PDMS WELL 44
FIGURE 4: DEVELOPMENT OF CONSTRUCT OVER TIME 48
FIGURE 5: DIAGRAMS OF EXERCISE REGIMENS....50
FIGURE 6: CYTOSTRETCHER USED FOR MECHANICAL STIMULATION 51
FIGURE 7: QUANTSTUDIO USED FOR QPCR 52
FIGURE 8: INSTRON USED FOR MECHANICAL TESTING....54
FIGURE 9: MAX STRAIN PERCENTAGE OF PDMS WELLS AND 4 MG/ML FIBRIN CONSTRUCTS 56
FIGURE 10: FORCE AT BREAK (N) OF THE PINS EMBEDDED IN WELLS AND FIBRIN CONSTRUCTS 57
FIGURE 11: STIFFNESS OF EACH MATRIX 58
FIGURE 12: TIMEPOINT ANALYSIS OF COLLAGEN GELS DURING DIFFERENTIATION....59
FIGURE 13: TIMEPOINT ANALYSIS OF COMPOSITE GELS DURING DIFFERENTIATION 60
FIGURE 14: TIMEPOINT ANALYSIS OF FIBRIN GELS DURING DIFFERENTIATION 61
FIGURE 15: TRANSCRIPTIONAL EFFECT OF IFNΓ TREATMENT ON CONSTRUCTS 62
FIGURE 16: TRANSCRIPTIONAL EFFECT OF MACROPHAGE SECRETIONS ON CONSTRUCTS....63
FIGURE 17: TRANSCRIPTIONAL EFFECT OF MACROPHAGE INTRACELLULAR PROTEINS ON CONSTRUCTS...64
FIGURE 18: TRANSCRIPTIONAL EFFECT OF DIABETIC MEDIA ON CONSTRUCTS 65
FIGURE 19: PROTEIN SYNTHESIS EFFECT OF DIABETIC MEDIA ON CONSTRUCTS 66
FIGURE 20: FLUORESCENT IMAGES OF HEALTHY AND DIABETIC CONSTRUCTS 67
FIGURE 21: TRANSCRIPTIONAL EFFECTS OF EXERCISE ON HEALTHY CONSTRUCTS....68
FIGURE 22: PROTEIN SYNTHESIS EFFECT OF EXERCISE ON HEALTHY CONSTRUCTS 69
FIGURE 23: FLUORESCENT IMAGES OF CONSTRUCTS AFTER EXERCISE 70
FIGURE 24: TRANSCRIPTIONAL EFFECTS OF END EXERCISE AND MACROPHAGE INTRACELLULAR PROTEINS ON CONSTRUCTS 71
FIGURE 25: TRANSCRIPTIONAL EFFECTS OF HIIT EXERCISE AND MACROPHAGE INTRACELLULAR PROTEINS ON CONSTRUCTS 72
FIGURE 26: TRANSCRIPTIONAL EFFECTS OF IFNΓ AND HIIT EXERCISE ON CONSTRUCTS 73
FIGURE 27: TRANSCRIPTIONAL EFFECTS OF EXERCISE AND DIABETIC MEDIA ON CONSTRUCTS....74
FIGURE 28: PROTEIN SYNTHESIS EFFECTS OF EXERCISE AND DIABETIC MEDIA ON CONSTRUCTS 75
FIGURE 29: FLUORESCENT IMAGES OF CONSTRUCTS AFTER DIABETIC TREATMENT AND EXERCISE 76

CHAPTER 1. INTRODUCTION

1.1 Background

As the medical industry is forced to accommodate a population that is growing ever older, so too must it develop effective methods to treat their most prominent maladies. With around 1/3 of people aged over 65 having type II diabetes[1] and 1/5 having sarcopenia[2], disease affecting the muscles is clearly a major issue that must be addressed. Currently, animal models are the standard for observing how disease affecting the muscles progresses. This raises questions of both ethics and efficiency.

In an adult, skeletal muscle health is a strong indicator of overall health. This is because the muscles play an active role in most metabolic processes, including glucose uptake and protein synthesis[3]. There has only recently been an appreciation for how muscle pathology plays a role in many diseases that affect different locations within the body. As we begin to elucidate more and more how maintaining muscle can have long term positive downstream effects, it is vital that we improve our understanding of what has beneficial effects on the muscle.

Knowing now the importance of improving the overall understanding of muscle, it is imperative that new and more efficient means of study be developed. Muscular modeling in 2D and 2.5D is a common practice with both C2C12 immortalized myoblasts and harvested primary satellite cells. However, these models fail to recapitulate the 3D structure of *in vivo* skeletal muscle. The answer to this is to develop models that share a 3D architecture with muscle. Given that muscle in the body responds to both mechanical and electrical stimulation, so too should modeled muscle While currently there is no

standard model that accomplishes these criteria, the field of muscular tissue engineering is moving in this direction.

1.2 Muscle as a Model

Skeletal muscle is a complex tissue network with high amounts of cross talk with other systems. Therefore, it is important to understand muscle in a vacuum without the interference of confounding factors. By isolating muscle *in vitro* and performing treatments and experiments on it, one can show that the muscle itself is responding to a stimulus rather than being signaled to respond by another cell population. To that end, it is critical to understand the architecture of muscle and how conditions have already been identified to affect it.

1.2.1 Components of Muscle

Muscle is best described as a hierarchical structure. This means that microstructures compose larger structures, which in turn compose larger structures. The smallest functional units of these structures are myosin filaments and actin filaments. Through finely controlled crosslinking and release of these filaments, force can be generated in the larger macrostructure of the muscle. These filaments are connected to Z lines forming sarcomeres, the functional unit of contraction. Rows of these sarcomeres are what composes a single myofibril. While the force of a single myofibril is small, huge numbers of them organized in 3D begin to exhibit force that is greater than the sum of its parts.

Moving on from the smallest units, we can arrange these myofibrils into long, cylindrical tubes within a single muscle cell. As more of these tubes form, we arrive at a muscle fiber. A single muscle fiber is composed of many of these fibrils present in one cell, sustained by that cells mitochondria and nuclei. A complete unit of fibers will be surrounded by connective tissue, forming a bundle of muscle fibers. This is what is typically thought of when you imagine a cross section of muscle. The pinnacle of the hierarchy is when many of these bundles form around a tendon, stretching them from joint to joint. This is what defines a skeletal muscle on the macroscale.

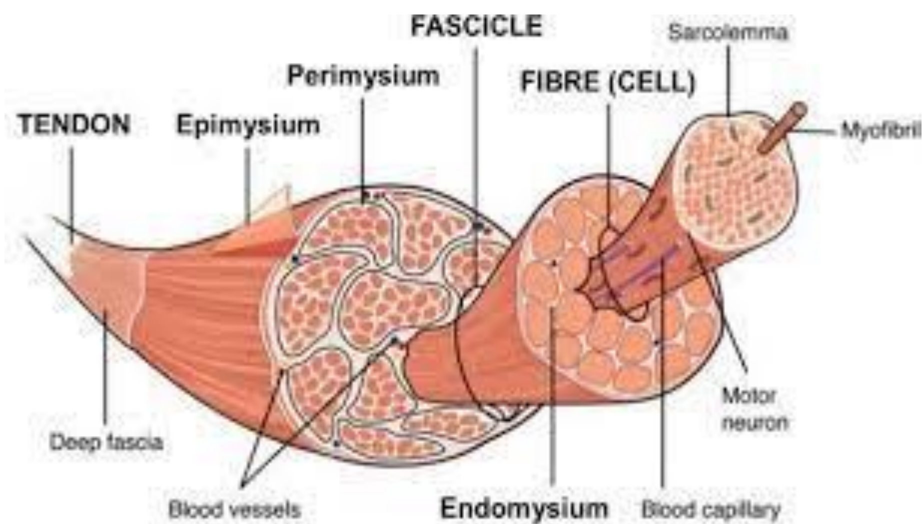

Figure 1.1: A Hierarchical Diagram of Muscle[4]

1.2.2 Role of Muscle

Muscle has two primary and two secondary roles within the body. Primarily, muscle serves to generate force and acts as a key metabolic site for glucose and proteins. Secondarily, muscle serves to generate heat and protect the organs and bones from damage. Notably, all of these roles are interconnected. This means that as muscle loses the capability to perform one of these jobs, it loses the capability to perform all of them.

Muscle generates force by forming crossbridges between myosin heads and actin filaments [5]. According to Sliding Filament Theory, the higher the surface area of the actin that is covered by myosin heads by climbing the actin filament, the more force that can be generated. This contraction and relaxation along the lengths of the bones is what allows for every movement, from running and lifting weights to moving the eyes and holding a pencil.

The muscles are also a primary regulator in whole body metabolism. Skeletal muscle is heavily involved in up to 80% of glucose uptake and removal from blood after being prompted by insulin, thereby managing blood sugar[6]. It also acts as a store of proteins and amino acids, releasing them when other bodily components need to utilize them for healing after injury[7]. These roles make healthy skeletal muscle an extremely important prerequisite for whole body health, as reduced muscle mass could lead to low protein availability or high blood glucose as a result of a lack of uptake.

The muscle also has secondary roles that are not a part of day to day physical function. Nonetheless, these are important tasks for keeping the whole body alive. The more common of these two roles would be protecting the organs from harm. When the body is impacted by an outside force, there is a risk that the force will damage critical internal organs. Increased muscular mass serves to absorb some of that force, dramatically raising the amount of force needed for major damage[8]. The muscles can also induce shivering in the event of extreme bodily cold, staving off hypothermia. This adaptation was likely crucial for our ancestors, as it may have allowed them to survive varied seasonal temperatures without issue.[9]

1.2.3 Effect of Disease on Muscle

Like any other part of the body, muscle can be negatively impacted by the effects of disease. At its end stages, essentially every disease that affects the muscles results in muscular atrophy. Atrophy is the breakdown in protein and reduction of overall volume and function of the skeletal muscle. Though similar in concept to volumetric muscle loss (VML), atrophy is not caused by a traumatic event removing muscle. Diseases can cause atrophy in multiple ways, but the primary causes are muscular inflammation and protein dysregulation. Inflammation can be brought on as a result of auto immune disorders and are known collectively as inflammatory myopathies. Protein dysregulation is more common and is primarily induced from a lack of activity. In cases such as type II diabetes, both causes can be present. This results in muscle loss related to quality of life deterioration and is a serious problem for the medical industry.

Muscle inflammation disorders, also known as inflammatory myopathies, result when inflammatory signals are sent to the muscles chronically without a normal stimulus. While inflammation can be helpful in the cases of infection and injury, chronic inflammation results in pain and weakness. Muscle inflammation also triggers upregulation of the ubiquitin ligase complex in the muscle. In healthy tissue, ubiquitin ligases remove old, damaged proteins from the cell as a form of housekeeping. In the case of diseased muscle however, this process becomes dysregulated. The ubiquitin ligases are over activated, resulting in the breakdown of healthy proteins as well. This means that muscle mass is reduced, and the muscles are weakened. This can cause dramatic quality of life issues over a long period of time if not addressed.

When ubiquitin ligases function normally but new protein is not being created to offset losses, it is known as a protein synthesis imbalance. This lack of new protein being created can be caused by something as simple as a lack of exercise but may also be indicative of a much larger problem. In healthy tissue, protein synthesis is signaled by insulin on the PI3/Akt/mTORC axis. Through mTORC's activation, new proteins can be synthesized to increase or replace the muscle tissue mass. However, if that signal from insulin is dysregulated such as in a diabetic, then there is a distinct lack of new protein being made within the cell. This becomes a much more complicated issue to address, as simply using the muscle is no longer enough to prevent its atrophy since it may take longer to heal.

1.2.4 *In Vitro* Representation of Muscle

Thanks to advancements in the cell culture and tissue engineering fields, it is no longer necessary to induce disease states in model organisms in order to observe the effects on muscle. Immortalized cells such as the C2C12 line or harvested primary myoblasts from a model organism or human can be expanded and differentiated into a model of muscle tissue, depending on the type of research being performed. Currently, the gold standard for modeling muscle is the presence of myotubes. These linearly aligned multinucleated structures are analogous to myofibrils in *in vivo* muscle. Without a means to oxygenate the engineered tissue, this is the current limit for recreating muscle. There are a number of steps and some variables to consider when designing an *in vitro* model of muscle.

Traditionally, 2 dimensional models of muscle were used as a research tool. These models involved culturing cells on a surface, typically a glass coverslip or a well plate, and allowing them to differentiate until tubes formed. While this is a reliable method for generating myotubes, it comes with some glaring problems. Practically, there is a narrow window for myotube formation and detachment from the growth surface. This is because the cells are not connected to any neural network, so they will spontaneously contract. This contraction causes their detachment from the surface and subsequent death. There is also the issue that a 2D model lacks the 3D architecture and stimuli that is present in *in vivo* tissue. This also means that a 2D model cannot be adequately stimulated without being destroyed, essentially preventing it from modeling the primary function of muscle. Thus, a 2D model is not sufficient to study the development and disease of muscle.

As a remedy to the shortcomings of the 2D model, a 3D model can more accurately replicate the dimensional orientation of the muscle. These models involve seeding cells into a hydrogel matrix and allowing them to expand, then inducing differentiation. This is accomplished by creating a cell suspension, where cells are in a liquid matrix. This suspension is then put into some kind of well where gelation takes place, creating a solid scaffold for the cells to attach and move within. The most common matrices for 3D muscle culture are fibrin, type I collagen, and gelatin. Each matrix type has its strengths and weaknesses, with stiffness and cell attachment sites being major issues of consideration.

With the cells contained within their matrix, they must then be allowed to proliferate and fill the hydrogel. This will reduce the space between cells and make the fusion process more effective. Cell proliferation can be induced by submerging the

hydrogel in growth medium. This most often consists of DMEM with 10% fetal bovine serum (FBS) and a cocktail of antibiotics. After the cells have filled their matrix, they must be differentiated. Myoblasts differentiate under events of confluence and serum starvation. Having both occur can speed the process up. By submerging the hydrogel in a differentiation medium of DMEM, 2% horse serum, and antibiotics, the cells can be induced to fuse into myotubes.

With a model formed and differentiated, there are markers that can be observed that mimic muscle function. The presence of these markers serves as a validation that the model is representative of true muscle tissue. The most functional assessment of muscle tissue formation is the generation of force. If there are myotubes present in large amounts within a construct and the construct is electrically stimulated, it should be possible to generate a twitch response. If this method is not available, it is also possible to stain for myosin heavy chain protein using a Myo-HC specific antibody. This will confirm that filaments are forming, which is an indicator of myotubes. Finally, pax7 is also an observable method for determining cell state. Pax7 is a factor responsible for keeping cells within the stem cell state, which means that they will not integrate into the myotube. If the cells within the construct are not expressing pax7, this is a clear indication that the quiescent stem cell state has been left and the cell has begun fusing into a mature myotube.

1.3 Significance

The muscle research ecosystem has evolved from simply research, and now has high degrees of influence in the medical and agricultural sectors. Keeping this in mind,

we see that the benefits of research that improve understanding of muscle *in vitro* and how to better cultivate and experiment with it have far reaching effects in multiple industries. As this research continues to evolve, so will the downstream effects these industries have on the economy.

1.3.1 Drug Development

Currently, the drug development approach relies on high throughput testing of many samples simultaneously. This means that simple and highly reproducible models are extremely beneficial to the pharmaceutical industry as it attempts to innovate medicine for diseases that target the skeletal muscle. Tissue engineered constructs specifically have the benefit of being observable on a cellular level throughout the treatment process. Moreover, seeing how their new drugs interact with muscles in motion provides much more data for developing a safer and more effective product. By further optimizing muscle constructs to be both cheaper and faster to produce, there is also an economic benefit that could be passed onto consumers.

1.3.2 Disease Understanding

Many of the musculoskeletal diseases that result in atrophy are poorly understood. While the mechanisms that result in the breakdown are more or less understood, how to stop them from occurring is another matter. It is well known that mechanical stimulation of the muscles can elicit a beneficial effect, increasing the size and protein production. If these effects can be replicated and optimized through exercise routines based on what generates the most positive effects, it is possible to give patients a powerful means to

manage their own health. Taking this idea further, it may also be possible to consolidate the benefits of exercise into a medication that could confer some improvement to the conditions of patients with diseases that prevent outright exercise, such as sarcopenia and muscular dystrophy.

1.4 Goals

Our primary goal in building our own muscle model is to create a simple, reliable, and economical means to research the effects of mechanical stimulation in muscle. In order to do this, we must first have the means to stimulate the muscle models effectively and consistently we build. This will be accomplished through the custom culture wells we design. Next, we must find the most conducive hydrogel composition for mechanical stimulation and myotube differentiation. If the cells are not forming proper myotubes, then the mechanical stimulation will not properly transduce, and the model will fail. Finally, we need to show that disease states can be modeled using our approach. Moreover, we need to show that the mechanical stimulation our model is centered around can generate useful data regarding these disease states. If our model can generate data that improves our understanding of the relationship between muscle exercise and disease, its goal will have been fulfilled.

CHAPTER 2. CURRENT KNOWLEDGE AND STATE OF THE ART

2.1 Methods of Quantification

In order to best understand if something is working, there must also be an understanding of the measurements being taken. There are many ways to determine the effects of a treatment on a muscle construct, and they will not always agree due to the different ways that treatment can affect the machinery of the cell. With that in mind, this section will list some of the ways that constructs can be quantified.

2.1.1 Gene Expression Panel

` mRNA expression measured through qPCR is a powerful tool to see what signals the cell is sending that it needs to produce. mRNA transcription is the first step to protein activity; therefore, it can be useful when troubleshooting where exactly dysregulation is occurring. This section will provide contexts for all of the mRNA signals that are used in this thesis in order to ascertain the current phenotype of the muscle constructs.

2.1.1.1 Igf1

Igf1 is the name of the gene that promotes production of insulin-like growth factor 1. This is a myokine that, similarly to insulin, stimulates protein production on the PI3-Akt-mTORC axis by stimulating IRS on the cell membrane surface. By activating Akt and therefore inhibiting the FoxO family, Igf1 also plays a role in inhibiting proteolysis[10]. On top of regulation of protein synthesis and degradation, Igf1 also plays a role in the stimulation of glycogen synthase in the muscle, again mediated through Akt

by inhibiting the activation of glycogen synthase kinase 3B[11]. This gives Igf1 a strong metabolic presence as well as a hypertrophic one. While the effects of Igf1 are fairly straightforward, they cannot be understated when looking at its effects on diabetes. As increasing cell mass naturally prevents atrophy and increases the area available for metabolic activity, growth through Igf1 is a strong indicator of an anti-diabetic phenotype. Therefore, increased Igf1 expression should be looked at positively through the lens of this thesis.

2.1.1.2 Fgf2

Fibroblast growth factor 2(Fgf2) is a gene that has seemingly contradictory effects within skeletal muscle. It has been shown that Fgf2 sees elevated expression at sites of regeneration in skeletal muscle[12]. This suggests a role in regeneration. Moreover, Fgf2 expression has been shown to promote satellite cell recruitment in single cell myotube models *in vitro*[13]. However, *in vivo* analysis tells a very different story. When looking at aged mice, it was found that there was an elevated level of Fgf2 in their muscles compared to adult mice[14]. It was also found that resistance exercise actually reduced these levels to a value closer to adult mice[14]. Further studies in mice of an evolutionarily conserved pathway revealed that elevated levels of Fgf2 resulted in an increase of adipose tissue in muscle, leading to sarcopenia[15]. Interestingly, despite these seemingly contradictory effects, there appears to be a relative consensus that the effects of Fgf2 are accomplished mechanistically via vascularization[16]. With all of these things in mind, it is difficult to say whether elevated Fgf2 in the model we have built is positive or negative. Given the context of an exercise model with a single cell type, it seems more likely that the effects of Fgf2 would lean more in favor of regeneration rather than adipose

infiltration, but this cannot be stated for certain based on the experiments performed herein.

2.1.1.3 Mstn

Myostatin (Mstn) is a gene that has received much attention lately due to its role in negatively regulating muscle mass. Myostatin knockouts have been shown to have an increase in muscle mass and a decrease in fat tissue[17]. Interestingly, this role is conserved across species with a wide variety of mammals all seeing the same outcomes when myostatin expression is disrupted[18]. Given that myostatin expression is increased in a variety of pathologies including obesity[19], it appears to primarily have negative outcomes for the organism. This is reinforced by the crosstalk within cells that seems to occur between Igf1 and myostatin, with the dominant protein inhibiting action of the other[20, 21]. With Akt also being affected by inhibited Igf1 activity and therefore increasing action of FoxO, this causes myostatin to also favor a proteolytic balance indirectly within the cell[22]. However, there may be some effects within muscle that are beneficial which are not readily obvious. In healthy muscle, there remains a population of quiescent satellite cells which divide asymmetrically in order to both regenerate muscle and maintain their population. Myostatin may prevent proliferation and differentiation in order to help maintain the population of satellite cells and therefore allow regeneration further down the line[23, 24], although this line of thinking is relatively new and needs more time to develop. With this considered, it can be reasonably decided that outcomes which see a decrease in myostatin are likely beneficial. However, it is worth considering that most studies observe outcomes over repeated exercise or a significant amount of time after exercise. This should be considered when interpreting results.

2.1.1.4 Fndc5

Fndc5, the precursor to irisin, is a myokine that is secreted during exercise with powerful positive effects. As part of the initial characterization of irisin, it was found that it was upregulated when myostatin is deficient[25], suggesting a role in muscle hypertrophy. Moreover, expression of irisin was linked to the browning of white adipose tissue, giving irisin a pivotal role in conferring exercise benefits such as weight loss[25]. Further characterization has also implicated irisin strongly in muscle healing and fusion, with irisin secretion being directly linked to an increase of myogenic factors, muscular strength, and muscular regeneration[26]. On top of these benefits, irisin has also been found to have beneficial effects on inflammation, neuronal health, and bone density[27, 28]. Given the wide variety of protective and restorative outcomes when irisin expression is improved, treatments which enhance the presence of fndc5 mRNA within the cell should be looked at as potentially beneficial for health, especially in regard to diabetes.

2.1.1.5 Il15

Il15 is a pleiotropic gene with immunogenic and anabolic effects. It has been shown that Il15 mRNA levels in healthy males increases several fold for a duration of time after exercise, though this does not result in an elevation of Il15 protein expression[29]. Il15 has also been shown to have beneficial effects on the muscle metabolome, prompting Glut4 translocation and enhanced glucose uptake[30]. On top of the previous benefits, Il15 also serves to prevent fat deposition within the muscle[31]. However, these benefits are not without potential caveats. Il15 has been hypothesized to potentially be a mediator for stress on the muscle, and may regulate oxidation and

fatigue[32]. Finally, research has also indicated that Il15 promotes myositis when expressed from inflamed muscle, which may serve to induce catabolic pathways[33]. With all of this in mind, it is difficult to say whether elevated Il15 expression is a good or a bad thing, and context should be closely observed. In addition, the general consensus that elevated mRNA of Il15 is often unlinked to elevated protein expression makes this a difficult trait to characterize.

2.1.1.6 Myog

Myogenin (Myog) is a gene primarily expressed in muscle during development that prompts fusion of myoblasts into multinucleated muscle filaments, the *in vivo* counterpart to the *in vitro* myotube. During myogenesis, Myog is expressed specifically in embryonic tissue where it promotes production of transcription factors that lead to muscle fusion[34]. This function is so important that mice who are double Myog knockout will quickly die[35]. This role does not appear to be as important in adult mice, as mice who lose myog expression after adulthood will still regenerate muscle tissue[36]. This may be too narrow a view however, as further research has found that myog knockouts in adulthood have heavily impacted muscle stem cell homeostasis, potentially hindering regeneration further down the line[37]. Ultimately, myog is a fairly straightforward gene that can be used in order to assess whether cells are differentiating and fusing. Elevated expression should be seen as a positive.

2.1.1.7 Myod1

MyoD1 is an early regulator of cell fate that induces mesoderm cells to a myoblast lineage. MyoD1 codes for the production of enhancers which cause histone

modification within the cell that locks it into a muscle phenotype[38]. This also causes the cells to remain in a proliferative state, potentially maintaining a satellite cell population by preventing fusion of every myoblast present within the muscle[38] MyoD1 also plays a role in muscle regeneration, as an absence of gene activity appears to lock *in vivo* cells into their proliferative phenotype without ever being able to differentiate and fuse into injured muscle[39]. Since elevated MyoD1 could indicate that differentiation is not taking place or that recovery is happening, it is difficult to determine whether elevated levels are beneficial without complete context.

2.1.1.8 Fst

Follistatin is a protein that functions primarily as an antagonist for myostatin, being a positive regulator of muscle mass as a result. Follistatin has been shown to bind to myostatin in order to prevent its activation[40]. Other follistatin related proteins that are upregulated or activated in the presence of follistatin have also been shown to complex to myostatin to prevent its function in serum, painting a clear picture of a heavy regulator of myostatin[41, 42]. In fact, this role of inhibiting myostatin is so important that follistatin deficient organisms rapidly die upon birth, with muscle mass being insufficient to promote life[43]. While most research has depicted follistatin as a simple regulator of myostatin, other research has identified function involved directly with muscle hypertrophy. Follistatin has been shown to increase mTOR activity and strength of muscle when upregulated, however these affects are attenuated when done in the presence of elevated Smad3, a potent atrogene[44]. Of note is that these experiments were performed in a myostatin knockout model, clearly defining follistatin as having function and controllers outside of its relation to myostatin[44]. Therefore, procedures which see

increased expression of follistatin should be regarded as protective for the muscle and deemed as desirable.

2.1.1.9 Glut4

Glut4 is a transporter protein that is the primary mechanism by which glucose is moved into skeletal muscle cells. An observation that has been repeatedly recapitulated in different organisms and muscles is that contraction induces an uptake of glucose within the cells[45]. In addition to this, an increase in exercise duration and intensity leads to increased glucose uptake[45, 46]. Intuitively, this means that glucose transporters, Glut4 in this case, are seeing increased expression and activity as a result of exercise[47]. On top of this increased production of Glut4 transporters induced by exercise, insulin has also been shown to induce translocation of Glut4 to the cell membrane surface in order to begin uptake of glucose[48]. Therefore, Glut4 expression and activity is a potent measure of both the level of exercise effectiveness and insulin sensitivity within the cell. When observing cell gene expression, increased Glut4 expression should be seen as a protective effect while decreased should be seen as a diabetic phenotype.

2.2 Current 3D *in vitro* muscle models

As discussed in the previous section, the idea of *in vitro* muscle constructs is not new. There have been a variety of approaches that different groups have taken in order to get the best constructs possible. These variables include altering the matrix proteins that the myotubes form in, casting the hydrogel in different manners, and harvesting cells

from different populations. In this section, we will cover each of these components and their effects on the final product.

2.2.1 Matrix Composition

When myoblasts are being seeded, the scaffold that is selected for the cells to integrate into can have major ramifications on the quality of the final muscle construct. Many different cellular and acellular scaffolds have been tested, including large numbers of different biological and synthetic biomaterials[49]. Given the scope of the research being presented here, the primary areas of discussion are going to be acellular scaffolds composed of type I collagen and fibrin proteins.

2.2.1.1 Collagen

Collagen is the most abundant protein in the body, with type I collagen composing most of the extracellular matrix in skeletal muscle[50]. Collagen is also the protein that is gives the human body a high degree of both strength and flexibility[51]. This is evidence that collagen allows for a variety of different cell types to grow within its matrix in the complex environment of a living organism. Given this, it is a clear candidate for selection when considering a scaffold for growing myotubes.

Collagen has been found to have a twisted appearance in the lattices of its microstructure, possibly due to the nature of the individual fibers to twist together into bundles[52]. This leads to large pores in the collagen, as well as some batch variability in the matrix due to twisting. This void space in the matrix makes it quite permeable to liquids, which may be convenient for treating the cells within[53]. Collagen undergoes

strain hardening at the ranges of 50-100%, but there are conflicting views about whether collagen is relatively more or less stiff than fibrin[53, 54].

It is possible that collagen takes on the aforementioned characteristics rather than its *in vivo* properties due to limitations of gel formation. *In vivo* collagen is known to have small pore sizes and minimal bundling, readily providing anchorage sites for cells[55]. However, the cooler temperatures and lower pH required to keep collagen from gelling prematurely are both known to alter this phenotype for the worse[56]. Given this major hurdle in tuning the matrix, alternatives such as fibrin are worth considering.

2.2.1.2 Fibrin

Fibrin is a large fibrillar protein that is used in the injury response. When fibrinogen in the blood is activated by thrombin, it forms together into a blood clot that prevents further blood loss or intrusion from the outside[57]. While this function may present fibrin as an odd candidate for an ECM scaffold, the fibrin matrix actually strongly resembles the environment of *in vivo* muscle tissue. This similarity is so strong in fact, that fibrin hydrogels with myobundles generated a threefold greater force than collagen counterparts[58].

Fibrin hydrogels are composed of a dense network of straight fibrillar proteins with narrow pores. The pore size is a little over half of the void space found in collagen hydrogels[13]. This reduces the permeability of the matrix, but provides more sites for adhesion for the cells within to take advantage of. Fibrin undergoes strain hardening in the 10-100% range, likely oweing to its role in the body for forming blood clots. Fibrin is also easily bound by myotubes due to an abundant expression of αV integrins that are specific to fibrin. These proteins readily allow the cells to take advantage of the

large number of attachment sites within the fibrin hydrogel[59]. In contrast, type I collagen must be bound by α2 integrins. These are not expressed by myotubes or myoblasts[60], and may make it difficult for the cells to fuse and differentiate properly.

2.2.1.3 Composites

Given the positive qualities of both gels, it is no surprise that composites which seek to capitalize on the strengths of both and the weaknesses of neither are of high interest. Many different methods for forming composite gels have been proposed, primarily focusing on changing mixing order due to the interplay between pH, temperature, and fibrillation[61]. The appearance and behavior of the proteins is quite similar to polymers, with crosslinking points denoting how the final hydrogel behaves.

The two proteins appear to form interpenetrating networks when present in comparable amounts, which results in higher mechanical property values (modulus, toughness, etc.)[61]. It is also possible for the networks to form differently, with chains forming either in parallel or in series. Since chains forming in series would cause a degree of randomness in the final physical properties, networks that are formed in parallel may be optimal when reproducibility is of the utmost importance. The primary difference between the two groups is whether individual chains contain one or both of the monomeric subunits. Gels formed in series have both, while gels formed in parallel only have one protein per chain (though chains of different proteins still may crosslink.)

Unfortunately, it does not appear that the benefits of composite gels outweigh the complications of making them. While the mechanical properties are indeed higher, this actually makes them less representative of native ECM. On top of that, the degree of randomness that comes about due to mixing order and combining two protein types

appears to make reproducibility difficult. In light of this, using singular protein hydrogels seems like the current best option.

2.2.2 Scaffold Casting Techniques

When casting a gel, equally important as the components going in are the techniques used to give it shape. Cells can be integrated into hydrogels either by encapsulation within a suspension before gelation or by seeding the cells into a preformed empty scaffold[62]. These approaches may play a substantial role in how the cells integrate with their environment, given that the matrix is the stand in for what the ECM would normally be. With this in mind, this section will seek to give insight into the different mechanistic approaches to forming a hydrogel as well as the strengths and weaknesses associated with each.

2.2.2.1 Hydrogel Suspension

Hydrogels that are seeded via suspension involve a liquid precursor with a dissolved protein inside being seeded with cells and the media necessary for cell survival[62]. This method involves crosslinking all of the protein contained within the suspension into a gel matrix, forming around and adhering to the cells in the process. This process can be done through physical crosslinking in the case of thrombin or chemical crosslinking in the case of collagen[63].

This method is the easiest to perform, requiring no specialized equipment in order to do so. Collagen and fibrin can both be dissolved in acetic acid and phosphate buffered saline respectively, with the dissolution occurring spontaneously at low and high temperatures respectively. Once the cue that induces gelation has been added to the

hydrogel suspension, the crosslinking process will begin, and the hydrogel will be formed within minutes.

2.2.2.2 Electrospinning

Hydrogel formed through electrospinning are known as hydrogel fibrous scaffolds. Electrospinning allows for the fibers to be controlled and spun into submicrometric lengths, smaller than what would be possible through normal crosslinking methods[64]. The principle of electrospinning is made possible by the reaction that liquid droplets have when they are subjected to an electrical field. The liquid will form a jet, which can then be elongated and stretched into fibers at the nanometer scale in diameter[65]. The polymeric solution can be made, as normal, then subjected to a high voltage which pulls them down a needle. They can then be collected on a mesh and used to seed cells on later[66].

Notably for muscle constructs, the fibers can be spun to have a much stronger linear alignment than a normal hydrogel suspension would have. This has been shown to promote myogenic effects on myoblasts seeded into a fibrin matrix[67]. These benefits were strong enough that even adipose derived stem cells began to exhibit an elongated and aligned morphology when seeded into the matrix[68]. This provides a strong case for electrospinning as a tool for forming hydrogels. Despite the benefits and relative ease of electrospinning, it still requires some specialized (albeit inexpensive) equipment, a means of storage, and technique in order to effectively perform.

2.2.2.3 3D Bioprinting

The most modern take on scaffolding for cells is through 3D bioprinting. This process involves using a 3D printer that has been modified to use bioinks to build a customized scaffold. This allows for specialized treatment of patient needs [69] as well as scaffolding with cells already worked in[70]. The process is also heavily tunable, with both synthetic and biological polymers being functional for the purpose of printing[70]. With that in mind, collagen is far and above the most used bioink for cellular engineering[71]. Ultimately, 3D bioprinting provides the strengths of both hydrogel suspensions and electrospun matrices, with superior design control and the ability to have cells encapsulated in the matrix during the casting process.

Despite the major benefits that 3D bioprinting has to offer, there are some clear caveats. Predictably, the new technology involved in bioprinting is fairly specialized. This leads to extremely high costs for both bioprinters and bioinks ($10,000-$150,000 for a printer alone)[71]. Moreover, bioprinting requires knowledge of specialized software and an understanding of what will work and what will not work when designing the scaffold. Therefore, while bioprinting may become the industry standard for research in the coming years, its prohibitive cost and skillset requirement relegate it primarily to the medical sector currently.

2.2.3 Muscle Cell Types

Depending on the source of the cells used for forming a muscle construct, the outcome can be very different. There are three primary sources of myoblasts, being immortalized cells, neonatal myoblasts, and adult satellite cells[72]. Cell type may affect

critical factors such as surface protein expression[73], metabolic protein expression[74], sliding filament formation[74], and growth/fusion rates[75]. Given this huge variety in potential behavior of the cells, it is essential to consider what the goal of the culture being performed is. In order to shed some light on which cell line is most useful for which purpose, this section will delve into the observed behaviors and benefits of the most common cell sources.

2.2.3.1 Immortalized Myoblasts

The immortalized cell line primarily used for muscle research is the murine C2C12 line [76]. This cell line is particularly robust and will begin to differentiate and fuse into elongated myotubes within 3-5 days of either serum starvation or confluence. This differentiation has been confirmed as multinucleated myotubes through the use of fluorescent imaging[77]. C2C12 cells also express high levels of actin and myosin, both being major indicators of proper myotube formation[74]. There is also a counterpart cell line derived from rats known as L6, however since the research presented in this thesis and the vast majority of works all use C2C12, it will remain the focus of this section.

One of the primary benefits of the C2C12 line is that it expresses proteins such as Igf1 and Glut4, both being string indicators of metabolic dysregulation when they are underrepresented in the proteome[78, 79]. C2C12 is also responsive to simulated exercise since it resembles differentiated human muscle cells, with changes being observable in the transcriptome and proteome of the cells after they are stimulated[80]. This makes C2C12 an extremely useful cell line to work with when studying metabolic disorders and how exercise can affect them. Given that C2C12 is also readily available for purchase,

relatively easy to expand, and quickly differentiates, it is an ideal choice for building muscle constructs to study metabolic disorders.

While the positives of C2C12 are readily apparent, it is worth considering some shortcomings which may convince against their use. The primary shortcoming is that C2C12, like other myotubes does not express the α2 integrin that is specific to type-1 collagen[60]. This means that collagen hydrogels will not be as bound by muscle constructs grown in them, eliminating one of the most common tools for scaffolding. C2C12 also does not have as high of expression of glut4 (and therefore a lower uptake of glucose) as rat L6 cells[74]. This somewhat undermines its role for studying metabolism. Nevertheless, it is still an extremely viable model for understanding the mechanisms of metabolic disease.

2.2.3.2 Neonatal Myoblasts

Given the highly plastic nature of cells harvested from fetal to neonatal organisms, it is no surprise that cells harvested from them may have properties that make them conducive to research. In fact, a large majority of studies on cardiac tissue grown *in vitro* were harvested from neonatal organisms rather than using immortalized cell lines[81-83]. What makes these cells attractive as a model is that they are muscle precursors, meaning that they maintain some pluripotency while also reliably differentiating into their adult states. These cells are also capable of self-renewal, making them viable for expansion in culture[84]. Finally, these neonatal cells have also been found to vascularize when implanted *in vivo*[85].

In order to acquire neonatal and fetal myoblasts, one must first acquire a population of mice to be harvested. Since the window for harvesting is relatively short (3-

5 days after birth), it makes the most sense to rear the mice in house. This does create a requirement of both the knowledge and equipment for rearing mice. There are also specific procedures in order to get the highest fidelity cells possible. Muscle from the mice must be carefully extracted, stored, minced, and trypsinized in order to build a culture[86]. If any of these steps are not performed properly, the cells may become contaminated or lyse.

2.2.3.3 Adult Organism Satellite Cells

The final source of myoblasts used for muscle cell culture is satellite cells harvested from adult organisms. Satellite cells are a muscle stem cell population that is responsible for both healing the wear and tear on adult skeletal muscle and also integrating with the muscle during periods of muscular growth[87]. While also possessing the self-renewal properties of neonatal myoblasts, satellite cells are not found in as great of numbers within an adult organism as myoblasts are in a neonatal organism. However, these numbers can be bolstered thanks to the property of symmetric division of stem cells that they possess (*in vivo,* they perform asymmetric division in order to both maintain their population and heal wounds)[87]. It is due to all of these traits that adult organisms are able to grow, regenerate, and maintain their muscle mass. To that end, adult satellite cells are extremely important. This does not, however, make them the ideal cell type for muscular tissue engineering.

Satellite cells suffer from the same detriment as neonatal myoblasts in that they both require specialized knowledge and equipment to properly acquire. To make this worse, myoblasts are present in much higher quantities in neonatal organisms (about 30% of tissue) than satellite cells are in adult organisms (2-7% of tissue)[88, 89]. Given how long

it would take to acquire numbers that could be used for culture, the heterogeneity that would come from using different organisms, and the lack of immortalization, adult satellite cells are not the best option of those available.

2.3 Pathology of Type II Diabetes

Given that we are trying to characterize the effects of type II diabetes, we must first understand what type II diabetes looks like. Diabetes has been called an autoimmune disorder, an autoinflammatory disorder, and a metabolic disorder. All of these descriptions are correct, and each interplays with the others. Therefore, the best way to understand the pathology of diabetes is to follow the different effects that occur as it develops.

2.3.1 Obesity

Insulin resistance is one of the most commonly associated phenomena that occurs with obesity[90]. There are a variety of causative mechanisms for this which will be elaborated on further in the following sections. Here, a brief overview of each of the effects of obesity will be given,

One of the most well documented effects of obesity is the chronic low level systemic inflammation that occurs alongside it. Inflammatory cytokines secreted by enlarged adipocytes and macrophages that are drawn to deceased hypoxic tissue in the fat pad[91] stimulate both local tissue as well as the immune system, creating a feed forward effect of inflammation[92]. Besides these cytokines, elevated levels of free fatty acids and saturated fats begin to enter circulation, creating an even more inflammatory milieu. This

prompts the secretion of even more inflammatory cytokines, which have been shown to induce insulin resistance by dysregulating the insulin receptor pathway[93].

Obesity has also been linked with an excess of triglycerides in circulation, which can wreak havoc on the mitochondria. This is because mitochondrial genes that control lipid and glucose metabolism like PGC-1a are downregulated[94]. While this does not directly cause insulin resistance, an elevated level of triglycerides is often associated with insulin resistance. Therefore, it is not out of the question to say that mitochondrial dysregulation plays a role in further insulin resistance[95].

2.3.2 Systemic Inflammation

Inflammation is the most strongly implicated factor in the development of type ii diabetes. This is because of the sheer number of systems it can dysregulate, and that in a chronic state it appears to self-perpetuate. With this in mind, it is critical to understand each part of the inflammatory picture and how it may affect the pathology of diabetes in the muscle.

2.3.2.1 Cytokines and Chemokines

Acute phase cytokines and chemokines are present at elevated levels in patients with type 2 diabetes[96, 97]. Specifically, Il-1β, Il-6, and Tnf-α are the overexpressed cytokines, while C-Reactive Protein, fibrinogen, and haptoglobin are the overexpressed proteins[98]. Every one of the aforementioned molecules drive an inflammatory response in the body and keeping them all at elevated levels over time begins to cause metabolic dysregulation.

Tnf-α has been shown to have catabolic effects within muscle tissue. This is mediated through stimulation of the Nf-κB pathway via reactive oxygen species. Tnf receptors, especially those associated with protein loss, begin their cascade with ROS. This was proven through the finding that antioxidants actually inhibit muscle wasting in elevated Tnf-α settings[99]. These ROS then interact with and stimulate Nf-κB, beginning proteosome/ubiquitin ligase activation[100-102]. Without blunting and in events of long term Tnf-α exposure, this pathway begins indiscriminately breaking down the protein within the cell, leading to wasting[103].

Il-1β is a well-studied pro inflammatory cytokine that is also responsible for muscle atrophy[104]. Unlike Tnf-α however, Il-1β also stimulates the production of Il-6[105]. Il-6 is a pleiotropic immune cytokine that has paradoxically been shown to have both positive and negative effects. Beneficially, Il-6 has been shown to be present in overloaded muscles as part of the hypertrophy response[106]. It has also been shown to play a directive role in satellite cell integration into damaged muscle[107]. However, degrative effects have also been shown. Prolonged Il-6 exposure in the presence of Il-1β and Tnf-α has been shown to worsen muscle loss[108]. The real danger of Il-6 when combined with other cytokines, however, is its interference with the protein synthesis and insulin signaling pathway. When exposed to elevated Il-6 levels throughout the majority of their life, mice saw a decrease in Igf-1 expression and an increase in SOCS3 expression[109]. This is a major concern as not only does a lack of Igf-1 expression mean that the cell will not respond to insulin and produce new proteins, but SOCS3 is an inhibitor of Akt, the primary effector in the insulin signal cascade. Even further, exercise in the presence of elevated Il-6 did not correct this imbalance[109]. There is also a concern that Il-6 may

stimulate the release of other acute phase proteins[110]. With all of this in mind, it is easy to see the concern that this cytokine cocktail elicits.

2.3.2.2 Impact on the Pancreas

Since end stage type ii diabetes is characterized by a destruction of the pancreatic islets as a result of inflammation, these mechanisms should be understood. Elevated glucose levels have been shown to induce beta cell proliferation, however chronic exposure leads to DNA fragmentation and cell death due to glucotoxicity[111]. This also results in elevated levels of reactive oxygen species which stress the cell, especially concerning since pancreatic islets do not have a strong antioxidant response[112]. Elevated levels of fatty acids, specifically fatty acids, have also been shown to have a lipotoxic effect that results in cell apoptosis[113]. Finally, the elevated levels of Il-1β result in immune cell infiltration within the islets[114]. This causes amyloid deposition and fibrosis, decreasing cell function and ending in apoptosis.

2.3.3 Metabolic Dysregulation

Metabolic dysregulation is the primary risk factor in type II diabetes in regard to the muscle. It occurs primarily through two mechanistic pathways: insulin resistance/hyperglycemia and hyperlipidemia. While insulin resistance and hyperglycemia have similar methods of action, hyperlipidemia dysregulates different components of the cell. Therefore, these concepts will be discussed accordingly.

2.3.3.1 Hyperglycemia and Insulin Resistance

Hyperglycemia is the most well-known indicator of type II diabetes. Hyperglycemia comes about when the insulin response in the cells, especially the skeletal muscle cells, is reduced[115]. This leads to an elevated level of glucose in circulation, which stresses cell populations. Insulin resistance is the root cause of the metabolic diabetic effects, however the exact mechanism for what causes it is unknown. It is known that both lifestyle and genetic factors play a role in the onset of insulin resistance[116]. On top of this, effectively every other symptom of type II diabetes has also been shown to result in insulin resistance[117]. It is interesting to note that muscle tissue exposed to no other symptoms except hyperglycemia also developed a resistance to insulin, indicating that resistance may be a homeostatic effect similar to how nerve desensitization works[117].

Ideally, insulin would stimulate its receptor and prompt glucose uptake from the blood and protein synthesis. In a diabetic, this does not occur. Akt, the primary effector of the insulin pathway, plays many roles in both metabolism and protein synthesis. Metabolically, insulin prompts Akt to induce Glut4 translocation to the cell surface in order to begin uptake of circulating glucose[118]. It also prompts the synthesis of glycogen by inhibiting the primary glycogen synthase inhibitor, GSK-3[119]. It also begins a signaling cascade to activate mTORC1 and prompt protein synthesis[120]. When insulin resistance begins occurring, these functions are disrupted. Moreover, in the systemic inflammation caused by obesity, elevated Il6 levels cause Akt to become inhibited through the phosphorylation of STAT3 which leads to transcription of SOCS3, both potent negative effectors on Akt's pathway[121, 122].

2.3.3.2 Hyperlipidemia

The other major metabolic dysregulation that occurs in type II diabetes is hyperlipidemia. In the context of obesity, the circulating levels of fatty acids and lipids are dramatically increased[123]. While properly processed lipids are not a problem, muscle lipid intermediates such as DAGs and ceramides which build up as a result of the elevated lipid circulation begin to interfere with Akt function through inhibition and worsen the effects of insulin resistance[124]. DAG buildup results in phosphorylation of serine kinases on insulin receptors, preventing signal transduction[125]. Ceramides disturb Akt, preventing proper uptake of glucose and activation of mTORC[126].

2.4 Modeling Type II Diabetes

Knowing now about the pathology of diabetes, we must begin to delve into recreating diabetic conditions in an experimental setting. The primary factors of type II diabetes in the muscles are inflammatory milieu, hyperglycemia, and hyperlipidemia. Depending on the model in question, the approach to causing these conditions could change quite dramatically. Therefore, this section will set about discussing the different symptoms of type II diabetes in the context of their induction.

2.4.1 Diabetic Induction in Rodents

Original models of diabetes did not use cell culture, but rather rodents that were treated in order to cause a disease state. This allows for a holistic view of diabetes and allows researchers to observe the interplay within different biological systems throughout

the body in response to the mass dysregulation of processes associated with diabetes. Since the start point for when an organism has type II diabetes is unclear, two primary methods for generating a disease state are favored. These are obesity through a high feeding diet and beta cell insufficiency through destruction.

2.4.1.1 Obesity Induced Hyperglycemia

Given that obesity is the primary cause of diabetes in humans, using obesity as a tool to study diabetes in rodents makes logical sense. When mice and rats are fed diets that are high in fat, they begin to spontaneously develop type II diabetes[127]. While there is a degree of genetic variation in mouse lines in how they respond to this diet[128], lines like B6 mice are useful in mimicking the human expression of metabolic disorders[129]. These mice see the same progression of hyperlipidemia, hyperglycemia, and inflammation that their human counterparts see as their weight gain progresses. There are also changes along the Akt/mTORC axis that have implications for protein synthesis.

Since mice will follow the same disease progression as humans given the same prompt of obesity, they are an extremely powerful tool for understanding diabetes. With this in mind, there are considerations to make when using this model. As mentioned before, line selection is crucial. Certain mouse lines like the AKR/J and the DBA/2J groups will not show a strong response to feeding induced obesity[130]. Gender also plays a major role, with female mice not responding as strongly to feeding due to differences in leptin expression[131]. Finally, obese mice have a much higher stress response from cues in their environment. This means that special care must be taken to prevent the mice from experiencing negative effects of stress that may confound experimental results[132]. If all of these cares are taken, obese mice can make for excellent models.

2.4.1.2 Beta cell insufficiency

A more direct if less accurate to the source model for diabetes is destroying the beta cell population within mice. Diabetes is characterized by a lack of insulin response in the cell, either due to insulin resistance and inability of the beta cells to keep up with demand or by lack of production in the first place (Type II and Type I diabetes, respectively). Since the root cause of the lack of an insulin response is the same however, simply removing the beta cells which produce insulin in the pancreas is a viable means of getting a diabetic phenotype.

Beta cell destruction is most often accomplished through the use of a toxin known as streptozocin. This is a compound that preferentially targets and kills beta cells[133]. Through this, the mice are induced into a state of hypoinsulinemia. This isolates the effects of a lack of insulin signaling in the cell while removing the potentially confounding factors of hyperglycemia, hyperlipidemia, and inflammation. Therefore, if the goal of the study is to explicitly see how a lack of insulin affects a population of a certain cell type, this method is quite useful.

Unfortunately, the removal of so many confounding factors removes major components involved in diabetes. This method is particularly ineffective for holistically studying type II diabetes. Type II diabetes is associated with an entire group of maladies that cause dysregulation throughout the cell. To remove stresses associated with chronic inflammation, chronic elevated blood glucose, and chronic elevation of circulating free fatty acid, all of which source different dysregulations within systemic cell populations[134-136], is a case of missing the forest for the trees. Therefore, unless the goal is studying the specific mechanism of how low insulin is impacting a specific pathway, this

approach does not seem to be particularly suited to understanding the larger picture of diabetes.

2.4.2 Hyperglycemic Culture Conditions

One of the primary tools to induce diabetes *in vitro* is by exposing the cells to elevated levels of glucose[137]. During the differentiation process, the cells will see reduced levels of myogenesis. This results in fewer tubes, creating a more phenotypically diseased construct. Insulin sensitivity will also decrease dramatically. As a direct downstream effect of this, Akt stimulation will be reduced, and protein synthesis will be severely impacted. Finally, the mitochondria will begin to breakdown and fragment, along with elevated levels of reactive oxygen species (ROS)[138, 139]. All of these symptoms are in line with what is observed *in vivo* as diabetes develops within the muscles. This suggests that *in vitro* models have a high enough degree of fidelity in replicating an organism that they may be used to study the mechanistic effects of type II diabetes.

2.4.3 Inflammatory Culture Conditions

Inflammation plays a massive component in the pathology of type ii diabetes, contributing to both its onset and its symptoms[98]. Therefore, models must incorporate this property when they are being developed. One of the keyways to accomplish this is to subject the constructs to inflammatory cytokines *in vitro*. The primary candidates for the effects of inflammation in diabetes are Tnf-α, Il-6, and Il-1β. Many studies have already been performed on the effects of Tnf-α on muscle constructs *in vitro*. The observed effects appear to be that insulin sensitivity and Akt activity are both decreased, similar to the effects of hyperglycemia[140, 141]. This may not be the whole picture however as Tnf-α

is also known to upregulate ubiquitin ligases that breakdown proteins. Therefore, further studies that quantify protein content should be performed. Il-1β and Il-6 are also major contributors to the systemic inflammation which results in type II diabetes[142]. Both of these cytokines have been found in elevated levels of serum in prediabetic patients and mice and contribute to cellular stresses and dysregulation that eventually boil over into fully developed type II diabetes[142]. Therefore, they should be included when attempting to recreate the diabetic inflammasome.

2.4.4 Hyperlipidemic Culture Conditions

The final major contributor to type II diabetes that can be added to *in vitro* culture that will be discussed here is hyperlipidemia. Hyperlipidemia occurs *in vivo* when lipid content within the blood serum becomes so elevated that it begins to dysregulate and stress cells. When this has been replicated *in vitro,* the effects have been in line with the previously discussed culture conditions which simulate diabetes. First, insulin signaling and Akt function within the cell are both reduced, preventing the cell from synthesizing new proteins[143, 144]. A decrease in hypertrophic irisin and an increase in cellular apoptosis were also observed, highlighting how intensely that hyperlipidemia affects cells[145]. With all of these factors in mind, lipid content must be considered when designing a model.

2.5 Stimulating Muscle *in vitro*

Given the role of muscle as a contractile tissue, it logically follows that research focusing on the contraction of muscle would be of interest. Both electrical stimulation and mechanical stimulation can be performed on the cells, causing them to contract and

stretch respectively. Both of these processes occur in the normal function of muscle, and therefore may provide worthwhile insights into the transcriptome and proteome of muscle in action. With this in mind, the cells behave differently in response to different stimuli. Despite this variation in response, both can be used to show the effects of simulated exercise on muscle constructs. This section will focus on how these techniques work and differences in the responses of the muscle constructs to them.

2.5.1 Electrical Stimulation

Far and above the most common tool for muscle stimulation *in vitro* is electrical stimulation. This process involves exposing the muscle to 1-10 V of electricity in order to recapitulate the effects of the neuromuscular junction in native muscle. When done properly, this causes the cells inside of the construct to contract. If the cells have differentiated and fused into myotubes, it is possible to measure an emergent force from the construct. Force transduction can be an extremely powerful tool for measuring the effects of treatments on muscle, and has been used in the past to see how treatments affect the strength of the tissue[146]. Electrical stimulation can also be used to simulate exercise, given that weighted muscular contraction is the primary tool for muscle hypertrophy *in vivo*[146].

It has been found that electrical stimulation of constructs has extremely beneficial effects to their continued development. Electrical stimulation causes an increase in myosin formation as well as force generation of constructs when it is used during the differentiation process[147, 148]. Electrical stimulation also boosts metabolic function, upregulating Igf1 to prompt more insulin sensitivity and therefore more glucose uptake and protein synthesis[149]. This is extremely promising, since it suggests that contraction

via electrical stimulation confers some of the same benefits as native muscle tissue experiences when it is exercised.

While electrical stimulation has clear benefits, issues arise when the subject of optimization is brought up. Some studies have found that a variety of frequency and voltage combinations did not result in any significant change[150]. This indicates that optimization may need to be done for each different construct model. This could significantly lengthen the experimental process, especially if there are a variety of matrices being used. In spite of this, electrical stimulation remains a powerful and easily quantifiable tool of the effects of exercise and other treatments.

2.5.2 Mechanical Stimulation

Mechanical stimulation is the process of externally stimulating the cells with movement in order to generate a response. The cells detect this movement through the stretching of the integrins they place on their scaffold matrix. This process of mechanotransduction is possible due to focal adhesion sites that connect the integrins bound to the ECM to the internal actin cytoskeletal network[151]. This allows mechanical forces to not only be detected by the cell but responded to through upregulation of different genetic pathways. This also prompts the re organization of the cytoskeletal network, which is essential for proper muscle growth and development[152]

Since exercise involves rhythmic stretching and contraction of muscle against resistance, it is not surprising that the act of stretching also induces changes within the cell. Indeed, mechanical stretching could be seen as the other half of the stimulation puzzle when considering how muscle behaves *in vivo*. While it is considerably less

common as a research tool than electrical stimulation, it is still a valuable resource in understanding how muscle changes and restructures in a construct when exercised.

2.6 Effects of Exercise on Muscle

Exercise is known to be healthy when performed correctly. It increases the volume of the muscle as well as the contractile force it can generate. Beyond that, it also makes the muscle more metabolically active. Since improving the health of constructs through exercise is the primary goal of this project, the mechanisms that enable these processes should be understood. This will allow the identification of methods that do or do not work.

2.6.1 Metabolic Improvement

The skeletal muscle system is some of the most metabolically active tissue in the body, owing to the fact that it requires a huge amount of energy to function[153]. In order to accommodate this massive need for energy, the muscle is the primary storage site for glycogen in order to guarantee that there is always energy available[154]. This glucose can then be put through the electron transport chain, providing readily usable ATP.

The process of metabolism is heavily attenable by the skeletal muscle, with glucose transport and mitochondrial count both able to be increased[155, 156]. This way, as metabolic demands increase, so do metabolic resources. This conveniently also reduces glucose in the blood, reducing effects of long term high glucose. This increase of energy uptake also makes exercise easier the next time it is performed. The methods for how these metabolic events occur depend on the type of exercise. Resistance training

accomplishes this by increasing the size and strength of the muscle, thereby increasing the energy demand. Endurance exercise improves metabolism by the upregulation of PGC-1a, an up regulator of mitochondrial genes and a co-activator of PPARg[157]. Endurance also upregulates genes that encourage the oxidation of circulating free fatty acids by the mitochondria, thereby reducing the total lipid content, and preventing the risk of hyperlipidemia induced mitochondria fragmentation.

2.6.2 Cellular Hypertrophy and Increased Contractility

Muscular hypertrophy is characterized by the enlargement of the contractile elements (actin and myosin filaments) and the associated increase of cell size to accommodate[158]. As a result, the number of sarcomeres and myofibers in parallel within the muscle also increase[159]. As the machinery that enables contraction becomes larger and more numerous, so does the ability for the muscle to contract against resistance and generate force.

As insulin sensitivity within the cell increases in response to exercise, the Akt/mTORC pathway is triggered. This pathway controls protein synthesis and inhibits protein degradation[160]. At the same time, insulin growth factors may be spliced by mechanical stimulation into an isoform directly associated with muscular hypertrophy, known conveniently as mechano growth factor (MGF)[161]. Insulin content is also known to be responsible for an increase in protein synthesis. Likewise, insulin levels within recently exercised muscle remain elevated for several days[161].

Muscular hypoxia has also been shown to have major benefits on muscle hypertrophy, even in the absence of exercise[162, 163]. While it remains unconfirmed, one theory for why this occurs is the effects of ROS within the muscle[164]. ROS have been

shown to have hypertrophic effects in both cardiac and skeletal muscle, as well as signaling satellite cell proliferation and MAPK (a hypertrophic protein) upregulation[161]. These ROS are produced as a result of increased metabolism and hypoxia[165].

2.6.3 Inflammatory Attenuation

There is a large amount of evidence linking frequent physical activity with a decrease in inflammatory signaling within the body[166]. This appears to be due to decrease signaling from inflammatory cytokines like Il-6 and Tnf-α, but especially a decrease in the circulating level of CRP[167]. As these signaling molecules are reduced, so too are the negative effects associated with chronic inflammation on the body.

The primary mechanism for why exercise appears to reduce inflammation likely lies in the reduction of adipose tissue. While both high and low BMI groups see improvement in circulating inflammatory biomarkers, high BMI groups see significantly more. When BMI is taken into account, this significance disappears[168]. Therefore, one anti-inflammatory result that exercise mediates is the reduction of adipose tissue mass. This is not the only adaptation that occurs, however. More directly, aerobic exercise training resulted in a decrease of inflammatory factors like Tnf-α being produced in the cell, as well as reduced the number of immunologically active CD14+CD16+ monocytes present in the body[169, 170].

Despite all the anti-inflammatory impacts of exercise on the muscle, there is an acute inflammatory response that immediately follows exercise. However, this response is necessary for coordination of the benefits of exercise and is actually anti-inflammatory

in nature. Exercise results in a temporary dramatic upregulation of Il-6 within the muscle, however a decrease in the levels of Il-1β and Tnf-α[171, 172]. This increase in Il-6 may actually inhibit the expression of Tnf-α, so long as the Il-6 levels remain acute and do not become chronic[173]. Given all these differences, this acute inflammation as a result of exercise appears to be completely different than chronic inflammation, both providing benefits and mitigating the event of long term inflammation occurring.

CHAPTER 3. METHODOLOGIES

3.1 Creating a Custom Mold

In order to properly address our area of interest, a well that helped induce proper differentiation was necessary. It must also be flexible in order to mechanically stimulate the myotubes inside. This section will cover the methodologies of designing this well.

3.1.1 Designing a Mold

Molds were designed to recapitulate the rough femur length of a mouse at 12.375 mm. In order to allow proper settling of hydrogel matrix, the channel between both "heads" of the dumbbell shape was made 3mm, with extra volume in the heads to account for the pins. These properties were all factored into the blind of the mold. Parallel holes were also inserted into the mold behind the blind. This was to allow bars to be inserted through the mold, providing a means to mechanically stimulate the cells.

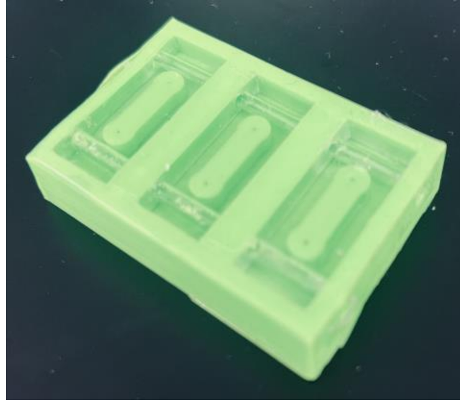

Figure 2: Mold for Casting Custom Wells

3.1.2 3D Printing a Mold

The GCode for the custom mold was made using PRUSASlicer software. Molds were 3D printed using PLA filament on a PRUSA Slicer mini.

3.1.3 PDMS Casting

Custom wells were created by casting PDMS in our 3D printed molds. The PDMS was Sylgard 184 (FisherScientific, NC9285739), and was mixed at a 10:1 ratio with the curing agent. The mixture was then cured in an oven at 60°C for 2 hours. After curing, bars were removed from the mold and a razor was used to remove the cast from the mold. Following this, the sides of the well were trimmed in order to preserve the motor of the CytoStretcher. Following this, NiTi pins were inserted into the center points of the dumbbell shape with a distance of 12.375 mm between them. Extra PDMS was then coated around the base of the pin in order to prevent fluid leaking.

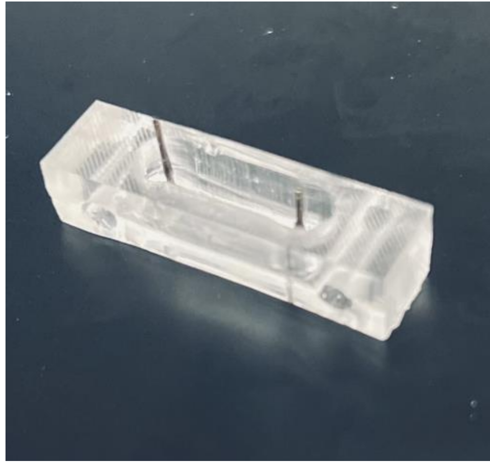

Figure 3: Custom PDMS Well

3.2 Casting Hydrogels

Hydrogels were cast with three distinct mixtures and therefore received different casting protocols. Those protocols are outlined here.

3.2.1 Fibrin

In order to cast fibrin hydrogels, 10 mg/mL of fibrinogen protein from bovine plasma (Sigma, F8630) were dissolved in 1x PBS by incubating at 37°C. The fibrinogen solution was filter sterilized in order to prevent contamination of samples. Dissolved fibrinogen was then mixed with cells in a media suspension to a concentration of 4 mg/mL. After proper mixing, thrombin (Sigma, T6634) at 50 units per mL of mixture. All reagents were kept cold during casting. Once mixed, the matrix-cell suspension mixture was cast into the PDMS well around the pins. Gelation was allowed to occur for 15 minutes at 37°C before growth media was added to the hydrogel. This process was repeated multiple times per experiment in order to prevent premature gelation.

3.2.2 Collagen

Type I Collagen from Calf Skin (MPBiomedicals, 215002683) was dissolved in filter sterilized and autoclaved .02N acetic acid to a concentration of 5 mg/mL. The collagen was then slowly mixed using a magnetized stirrer at 4°C for 3 days, when it was then aliquoted into multiple tubes in order to prevent contamination risk. When the time came to make the hydrogel, 10x PBS and .025 NaOH were mixed (in order to reach biological osmolarity and neutral pH, respectively), followed by the collagen solution. Before the new solution could gel, cells in media suspension were added to the solution and mixed thoroughly. The complete collagen – cell solution was then cast into the well and allowed to gel for 45 minutes at 37°C. Following this period, growth media was added. This process was repeated multiple times per experiment to prevent premature gelation.

3.2.3 Composite

Composite gels were cast in a mixture of methods from fibrin and collagen approaches. Depending on the specific concentration of the matrix proteins, the values of fibrinogen and collagen are flexible. First, fibrinogen prepared as described previously, .025 M NaOH, and 10x PBS were mixed. Next, collagen as prepared previously was mixed. Following this, cells in media suspension were added to the mixture. Finally, thrombin (50 units/mL) was added to the mixture. After rapid but thorough mixing, gels were cast in the same manner as previously described. Gels were allowed to incubate at 37°C for 45 minutes before the addition of growth media.

3.3 Cell Culture Techniques

This section will cover the methods used in order to expand cells and differentiate them into myotubes. This will include media formulations, timelines, and detachment methods.

3.3.1 Cell Expansion

C2C12 myoblasts were thawed for 2 minutes in a water bath at 37°C after removal from liquid nitrogen storage. Cells were then immediately mixed into growth media which was made of 10% fetal bovine serum (Thermofisher, 26140079), 1% stabilized antibiotic antimycotic solution (100x) (Fisher, A5955-100ML), and 89% DMEM High Glucose + Glutamax (Thermofisher ,10566-016). A stock solution of 6-aminocaproic acid (50mg/mL) was then added to a final concentration of 2 mg/mL order to prevent fibrinolysis during expansion in gels. The cell suspension was then added to a T175 flask and dispersed. Myoblasts were then cultured to 75-90% in 2D before extraction with TrypLE express (Gibco, 12604039). TrypLE was added to cells washed in PBS for 5 minutes at 37°C before being neutralized by an equal volume of growth media. Following, cells were pelleted via centrifuge while the TrypLE/growth media mixture was aspirated off of them. Cells were then mixed into serum free DMEM (same as prior) before being cast into hydrogels. Once in gels, cells were expanded for two days prior to differentiation, with media being refreshed daily.

IC-21 macrophages were thawed in the same manner as previously described. Their growth media was identical except for the media used, which was RPMI 1640 (ATCC Modification) (Thermo, A1049101). Cells were expanded to 90% confluency

using the same methods as previously described. If detachment was necessary, growth media was removed and replaced with PBS. The flask was then placed on ice for 20 minutes. At this point, cells were conducive to being removed. If difficulties occurred, mild agitation through tapping the flask could be used. If this was insufficient, a cell scraper was used to force detachment.

3.3.2 Cell Differentiation

Myoblast differentiation was performed through cell starvation. After the two day expansion in growth media, differentiation media was added for a standard of five days (This varied in time point experiments). Differentiation media consisted of the same ingredients as growth media, except the 10% FBS volume was replaced by 2% heat inactivated horse serum (MilliPoreSigma, H1138-100ML). 1% of ITS solution (100x) (Invitrogen, 4140045) was also added to help the differentiation process. A stock solution of 6-aminocaproic acid (50mg/mL) was then added to a final concentration of 1.5 mg/mL order to prevent fibrinolysis during differentiation. During differentiation, cells had media refreshed daily.

Figure 4: Development of Construct over Time

Macrophage polarization occurred by adding cytokines to their serum free media. In order to induce an M1 phenotype, 100 ng/mL of LPS (Sigma, L6529-1MG) and 50 ng/mL of IFNγ (Perprotech/Fisher, 315-05) were added. In order to induce an M2 phenotype, 40 ng/mL of Il-4 (Perprotech/Fisher, 214-14) and 20 ng/mL of Il-13 (Perprotech/Fisher, 210-13) were added. Cells were polarized for 24 hours.

3.4 Cell Treatment

In order to properly understand how cells were treated in order to replicate disease states, the following sections will explain concentrations and additions of additives.

3.4.1 Cytokine Treatment

Cells were treated with cytokines in two distinct fashions, diabetic media and IFNγ treatment. In both cases, cells received a fresh dose of reconstituted cytokines daily, in tandem with their daily media changes. Cytokines were added into the differentiation media of the cells and mixed before media was added to the culture well. Murine IFNγ was added at a concentration of 20 ng/mL. For diabetic media, murine Il-1β (Perprotech/Fisher, 211-11B), Tnf-α (Perprotech/Fisher, 315-01A), and Il-6(Thermo,

RMIL6I) were added into media at a concentration of 5 ng/mL. Treatments for both groups occurred for two days before either mechanical stimulation or harvesting.

3.4.2 Conditioned Media Treatment

Cells were also treated using conditioned media from macrophages. There were two approaches for this: taking cellular secretions and intracellular proteins. To take secretions, macrophages were cultured until confluent and then polarized. The flask was rinsed with PBS and serum free media was added. After two days, the media was removed and centrifuged in order to pellet any cell debris. Following this, the media was added to the muscle constructs. In order to get the intracellular proteins, cells were detached using ice and PBS. Following agitation to bolster cell numbers, cells in suspension were freeze-thawed using liquid nitrogen and a 37°C water bath for 5 cycles. After, the mixture was spun down to remove any cell debris. The freeze thawed media was then added to muscle constructs.

3.5 Mechanical Stimulation

Cells were mechanically stimulated using a CuriBio Cytostretcher. The cytostretcher was placed inside of a cell incubator during treatment. The Cytostretcher was controlled by the CuriBio proprietary software NaOMI. Cells were loaded into the stretcher and run through either a high intensity interval training (HIIT) regimen or an END (endurance)

regimen.

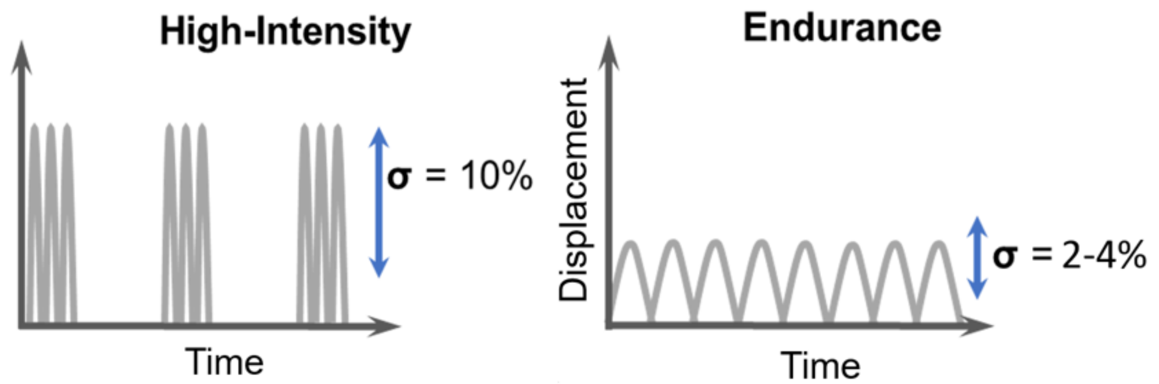

Figure 5: Diagrams of Exercise Regimens

The HIIT regimen stretched the cells at 10% strain for one hour for six cycles with a one hour break between cycles. The endurance regimen stretched cells at 4% strain for one six hour cycle with no breaks.

Figure 6: Cytostretcher used for Mechanical Stimulation

3.6 RNA Quantification

Samples were placed in Trizol (Thermo, 15596018) and were used as intended in the manual. Chloroform was added into the Trizol in order to isolate the RNA. Following, the aqueous phase was removed and placed into isopropanol with glycogen as a carrier in order to precipitate the RNA into a pellet. Once the RNA was precipitated, the isopropanol was replaced with ethanol in order to further stabilize the pellet. Finally, all ethanol was removed and distilled, RNAse free water was placed onto the pellet before being heated to 60°C for 10-15 minutes to facilitate RNA unwinding. The RNA was then thoroughly mixed and quantified, with all samples being diluted to the same concentration.

Once samples were equalized, one step real time PCR was performed, providing CT data for RNA content within the cells. RNA from each sample was placed with an appropriate taqman probe and superscript III reverse transcriptase (ThermoFisher,

11732088). The values from this protocol were based on the superscript manual recommendations. Once samples were prepared, a protocol was made on QuantStudio software and loaded into the QuantStudio PCR machine.

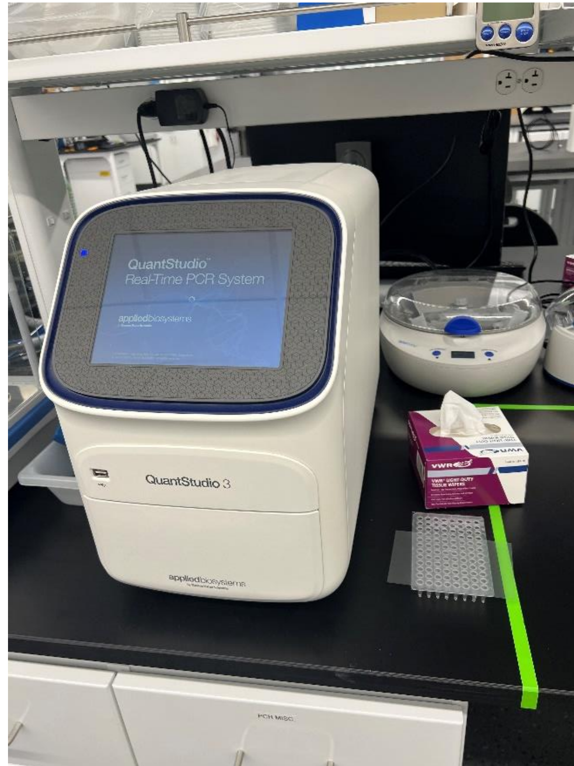

Figure 7: QuantStudio used for qPCR.

3.7 Protein Synthesis Quantification

Protein synthesis was quantified using the Click-it OPP kit for protein synthesis. After treatment period, constructs were pulsed in media for thirty minutes with a 1:1000 diluted Click-It reagent. After this, the constructs were collected and fixed normally. Before staining but after permeabilization, constructs were treated with the Click-It reaction cocktail for one hour in darkness. From this point, samples were stained as previously described.

3.8 Fluorescent Imaging

Samples were fixed in 10% formalin for between one and twenty four hours before processing. Cells were then washed with PBS before being permeabilized with .5% Triton X-100 (Sigma, X100) for 15 minutes. Samples were then washed again, and if applicable the Click-It OPP (ThermoFisher, C10428) protein synthesis cocktail added. This step was only performed when samples were treated with the Click-It OPP reaction agent before fixation. Following Click-It reaction, cells were again washed with PBS. Phalloidin and DAPI (Thermofisher, D3571) stains were added at a concentration of 1:200 and 1:500 respectively for one hour. Depending on the presence of Click-It reaction agent, the actin stain varied between phalloidin 594 (ThermoFisher, A12381) and phalloidin 488 (ThermoFisher, A12379). Actin and DAPI were added within a 1% BSA mixture. Following this stain, cells were washed with PBS before being hydrated with PBS and wrapped in foil, then being stored at 4°C.

3.9 Mechanical Testing

Mechanical testing was conducted using an Instron 68SC-05. Protocols were designed in such a manner that the object of interest (well, thread, or construct) would be placed between two clamps and then pulled to the breaking point. Once samples were broken, data of their displacement and the force they exhibited were collected. This allowed for a measurement of the max force and stiffness. When compared against one

another, such as in the stiffness chart, these values were normalized such that they had the displacement input for their force generated output.

Figure 8: Instron used for Mechanical Testing

3.10 Statistical Analysis

Statistical analysis was performed using One Way ANOVA tests on SigmaPlot 15. If differences in samples were found to be significant, significance was verified using Holms-Sidak testing. If samples failed the equal variance test, one way analysis on ranks and Dunn’s test were used in order to verify significance.

CHAPTER 4. DATA AND RESULTS

4.1 Creating a Construct

The first sets of experiments that were performed were based around building a reliable model to test our hypotheses. This took the vast majority of the time and resources spent working on this project. As this section took the longest to complete, other sections will have data that was collected using inferior methods as the superior approaches had not been developed yet. This data will be clearly indicated as such.

4.1.1 Mechanical Properties

In order to begin characterizing the muscle constructs, mechanical properties were measured using an Instron. Specifically, these tests were performed in order to guarantee

that the custom components would not fail when subjected to mechanical stimulation.

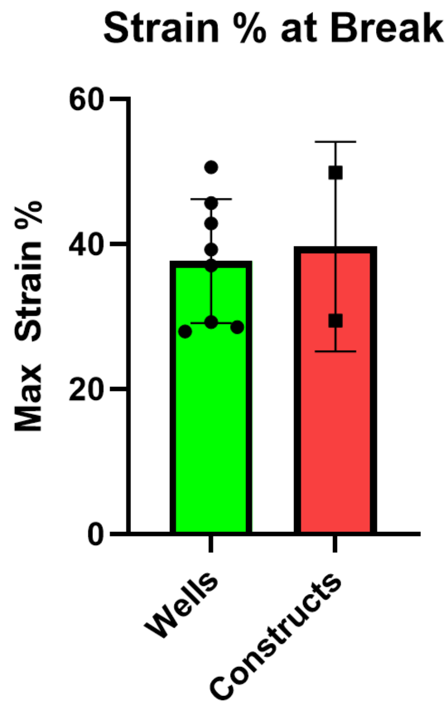

Figure 9: Max Strain Percentage of PDMS Wells and 4 mg/mL fibrin constructs

The data pictured in figure 9 shows that both the final construct model and the wells that they would be being stretched in were both easily able to undergo strains above the max value of 10% strain that they would be being subjected to during stimulation. The wells had an average max strain of 37.67±8.531%. The fibrin constructs had an average max strain of 39.68±14.44%. Given the low sample number of fibrin constructs that were tested, it is possible this value may shift up or down. Regardless, it is firmly above the minimum necessary strain for mechanical stimulation.

Following this experiment, the values for the max force the pins could withstand before breaking was compared to the max force the constructs could withstand. This test was necessary in order to validate that the force from stretching during mechanical

stimulation would not cause the gels to pull the pins lose from the well, resulting in a loss of uniaxial force.

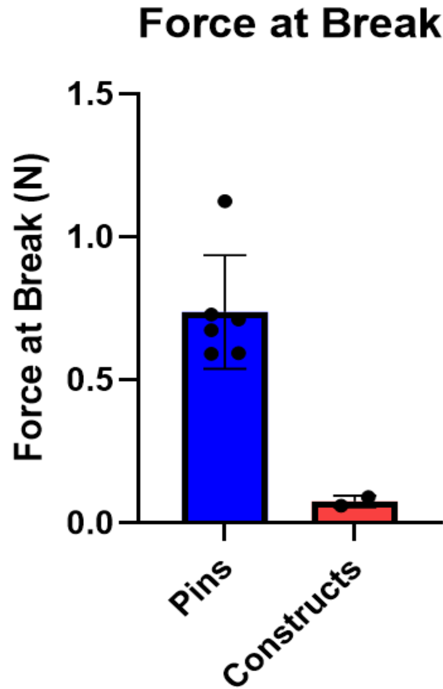

Figure 10: Force at break (N) of the pins embedded in wells and fibrin constructs.

The data gathered from this experiment (figure 10) once again clearly show that the custom wells are more than sufficient to mechanically stimulate the muscle constructs. The pins were able to withstand an average of .7380±.1986N before being pulled out of the well, while the constructs were only able to withstand .0750±.0212N before being torn. Therefore, the pins would not be able to be pulled out by the constructs during stretching, maintaining uniaxial force.

In order to better characterize constructs, stiffness from representative samples was also calculated. This was done to provide more information on why cells may respond differently in different matrices.

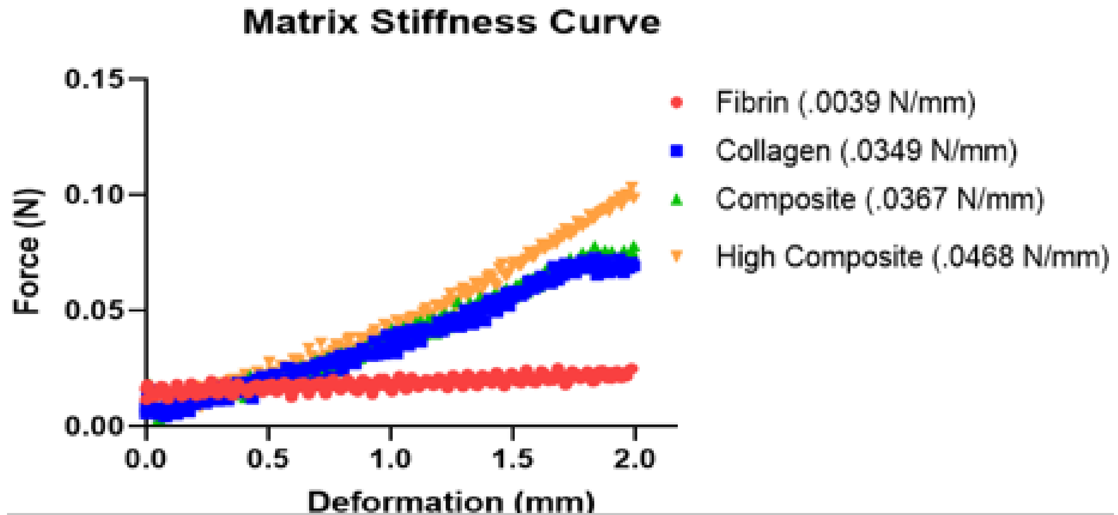

Figure 11: Stiffness of each matrix

The stiffness of each matrix is listed above in figure 11, and clearly shows that the addition of collagen dramatically increases the stiffness of the construct. Interestingly, the high composite group had an extra 2 mg/mL of fibrin (4 mg/mL fibrin, 2 mg/mL collagen total) and still retained this property.

4.1.2 Timepoint Analyses

Gene expression analysis of the cells within the muscle constructs was also performed at different timepoints to determine how the cells responded to each type of matrix. Given that the matrices had different protein compositions, porosities, and stiffnesses, it was

possible that this affected the signal transduction within the cells.

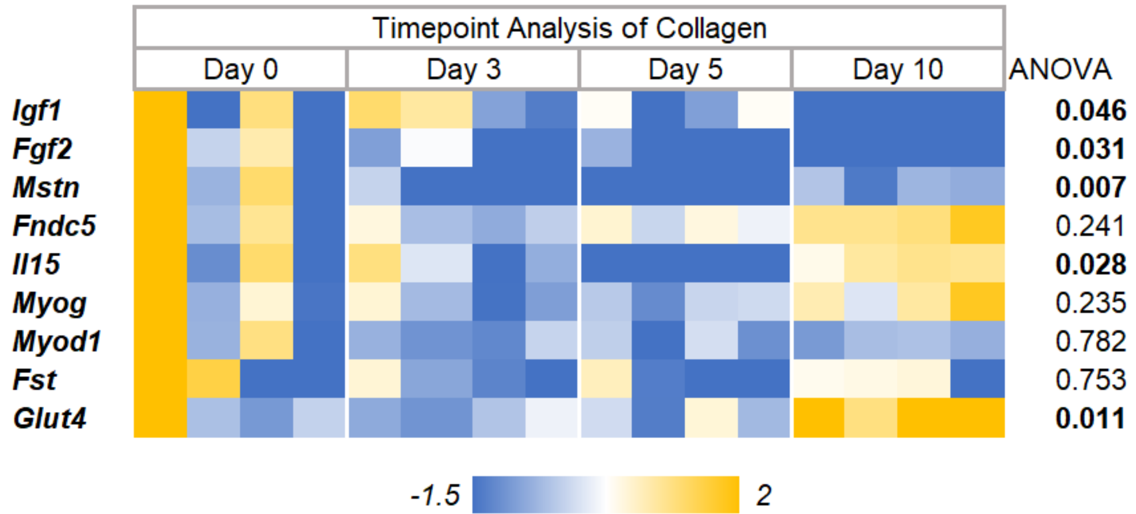

Figure 12: Timepoint analysis of collagen gels during differentiation

It was found that cells seeded in collagen gels responded fairly poorly to increased duration within the construct. The construct used here was the non-optimized version of collagen. Reductions in Igf1 and Glut4 expression suggest that the cells were not particularly metabolically active, either through protein production or glucose uptake. Fgf2 was also strongly downregulated over time, suggesting that myoblast renewal was not occurring within the construct. Il15 also showed strong downregulation, but the pleiotropic nature of Il15 makes it difficult to discern how or why this may have been occurring. Interestingly, both Il15 and Glut4 both see marked upregulation by the tenth day of differentiation. This may suggest that the cells were differentiating very slowly, but this cannot be confirmed.

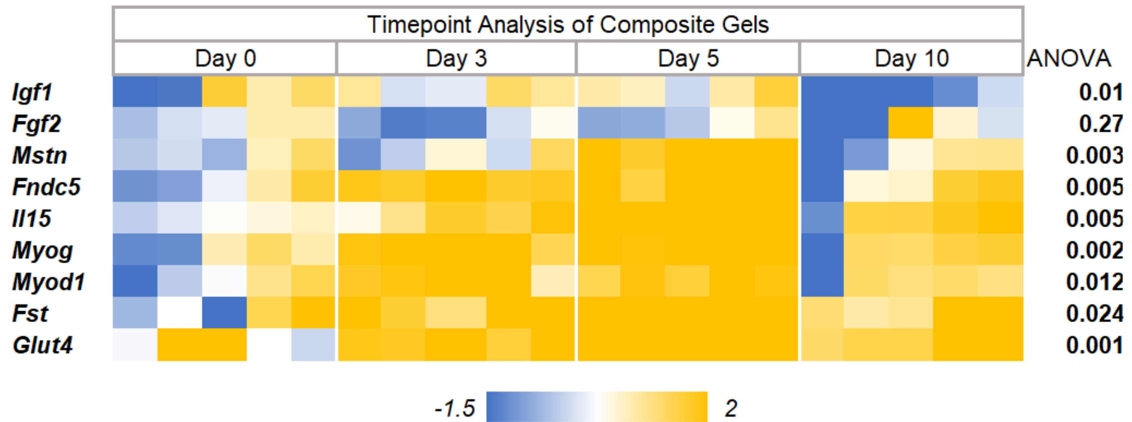

Figure 13: Timepoint analysis of composite gels during differentiation

Analysis was also performed on composite gels (also not yet optimized) to observe the effects of matrix on differentiation over time. The results were dramatically different. Glut4 was highly upregulated, but Igf1 saw some variance throughout the differentiation period, and was ultimately downregulated by day ten. Mstn was also heavily upregulated when compared against the collagen matrix. However, so too was the myostatin antagonist Fst, so the effects may have been minimal overall. Positively, known hypertrophic and obesity fighting gene Fndc5 was upregulated, signaling that the cells were actively growing and synthesizing proteins. Somewhat curiously, both Myog and Myod1 were both upregulated. These genes are known to have counter effects, promoting and inhibiting myotube differentiation respectively. It is possible that there were distinct populations of cells in the constructs involved in differentiating and replenishing the population, though this cannot be confirmed.

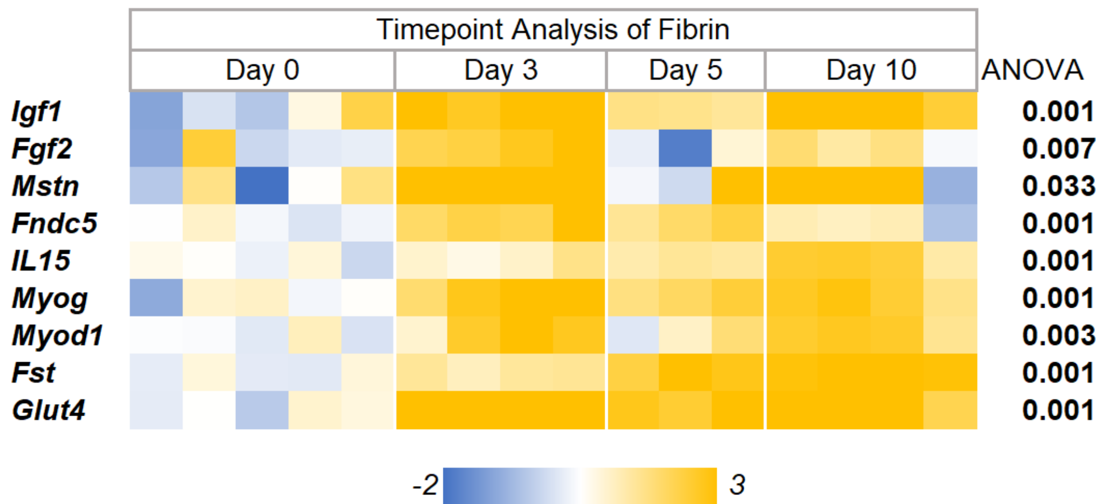

Figure 14: Timepoint analysis of fibrin gels during differentiation

Finally, we performed a timepoint analysis on fibrin matrix constructs. Outcomes here were very similar to the composite, with strong upregulation occurring as time progressed in every gene. The difference between the two is that fibrin did not see a down regulatory trend on day 10. Upregulation was also strong enough that it was necessary to increase the scale seen in the figure. This provides evidence that the myoblasts are actively hindered by the presence of collagen, rather than needing some combinatorial approach. This is convenient, as fibrin is also the easiest of the three matrix options to work with. Given all these considerations, it appears fairly clearly that fibrin matrices are the most conducive for cell growth.

4.2 Validating as an Inflammatory Model

Testing was also performed in order to validate the muscle constructs as a model for inflammation. The results from those tests are presented in this section. Testing here was focused on the effects of inflammatory stimuli, and therefore does not contain a mechanical stimulus.

4.2.1 IFNγ Treatment

Cells were treated with differentiation media containing 20 ng/mL of IFNγ cytokine. They were exposed for two days, with the media being refreshed each day. The effects on the gene expression of the cells are depicted in figure 15.

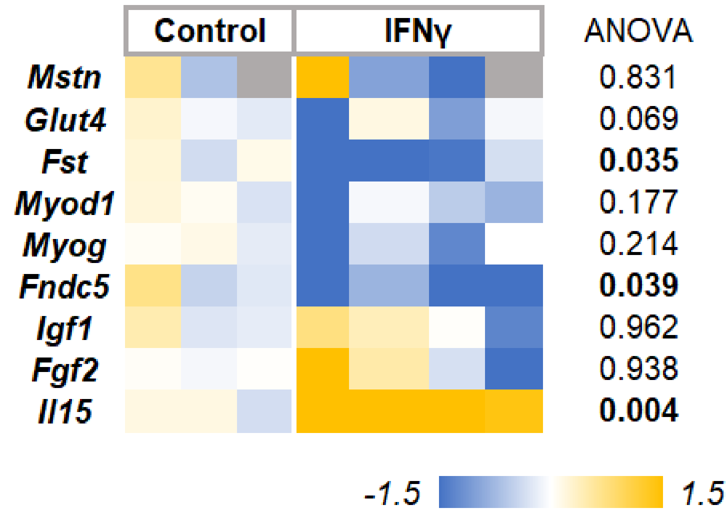

Figure 15: Transcriptional Effect of IFNγ Treatment on Constructs

Constructs saw significant downregulation in Fndc5, a known hypertrophic and pro metabolic gene. This indicates that IFNγ is causing an extremely pro inflammatory phenotype in the muscle. It is possible that it is not directly acting on Fndc5, but instead PPARG and PGC-1a, both of which promoted the upregulation of Fndc5. IFNγ treatment also saw the significant upregulation of Il15 expression in the sample, showing that the cells were having an extreme immunological response. While other effects were not significant, there are clear trends in the downregulation of beneficial genes for muscle across the sample, such as Myog and Fst. Interestingly, Mstn also appears to trend toward downregulation. This could imply that its role as a muscle hypertrophy regulator is only activated in instances where hypertrophy is likely, and not a passive function.

4.2.2 Conditioned Media Treatment

As an attempt in more accurately recapitulating the effects of inflammation *in vivo*, conditioned media was taken from cells. Specifically, secretions from extracellular and intracellular sources. While these experiments were not performed with the finalized construct model, the effects do indicate how macrophage interaction may affect myoblasts and myotubes. The results are pictured in figures 16 and 17. In figure 16,

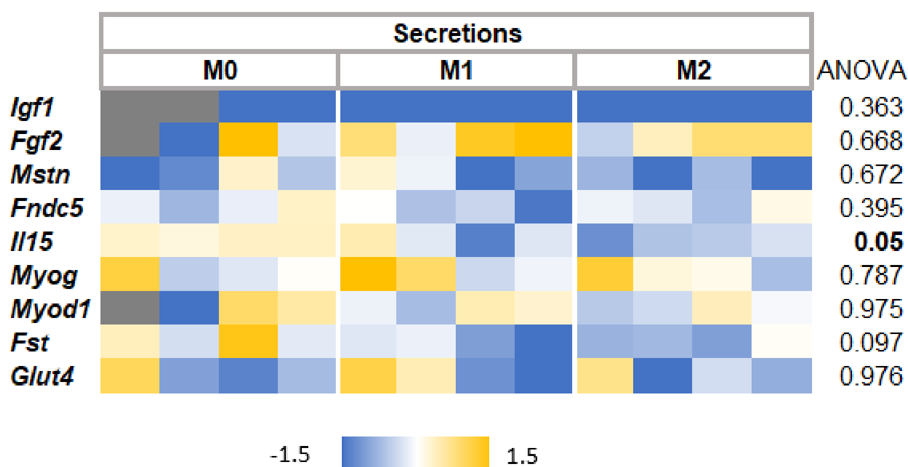

Figure 16: Transcriptional Effect of Macrophage Secretions on Constructs

we see that the only significant change in expression is in Il15. Interestingly, it is a downregulation rather than an upregulation in both groups. Despite this, the response from the myocytes was not particularly strong. In order to generate a stronger response, macrophages were freeze thawed in order to burst the membrane and harvest the proteins inside.

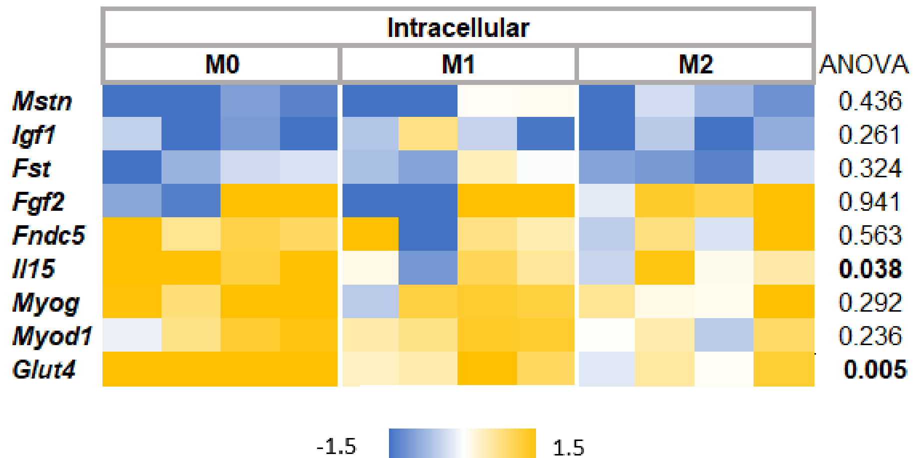

Figure 17: Transcriptional Effect of Macrophage Intracellular Proteins on Constructs

While there still was not considerable significance, trends are much more apparent than when compared to cells exposed to secretions. Significantly, Glut4 appears to be downregulated when exposed to proteins from either macrophage phenotype compared to when it is exposed to nonpolarized macrophage proteins. Also of note, the Il15 response was actually significantly higher in these same samples than when compared against the Il15 expression of polarized samples. This possibly indicates that increased immunoactivity can prompt increased activity of glucose transporters.

4.2.3 Diabetic Media Treatment

As a means of assessing whether our construct could recapitulate diabetes, we created a diabetic media treatment that contained the cytokines most upregulated during type II diabetes. This treatment was then applied to static constructs, and their protein synthesis and gene expression values were quantified. The results of the gene expression analysis are pictured in figure 18.

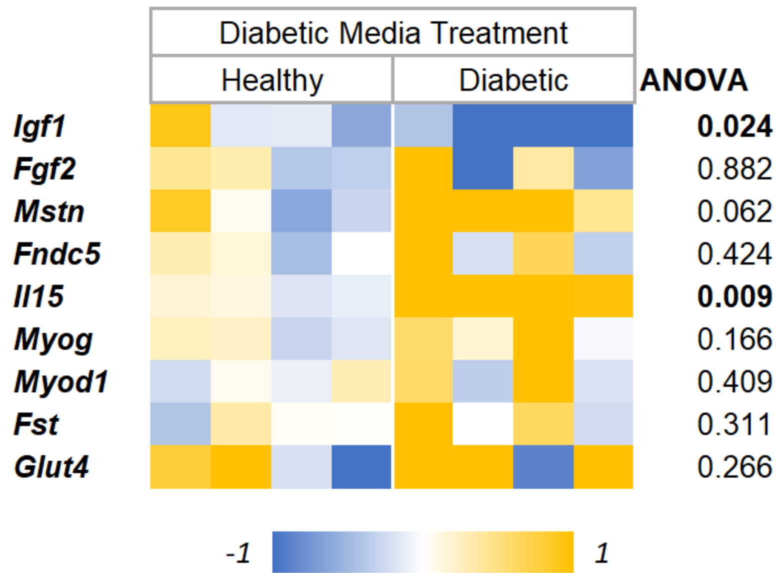

Figure 18: Transcriptional Effect of Diabetic Media on Constructs

The data collected is interesting, as it suggests relatively minor changes from the transcription side of the cell. Since Il15 upregulation is likely the consistent immune response seen in every other treatment, the primary change of interest here is Igf1 expression. Igf1 is responsible for cellular hypertrophy, but it is not the only hypertrophic gene on the panel. Therefore, the specific downregulation that it sees may not be the entire picture of what is happening within the cell. Knowing this, we interpreted the protein synthesis data seen in figure 19.

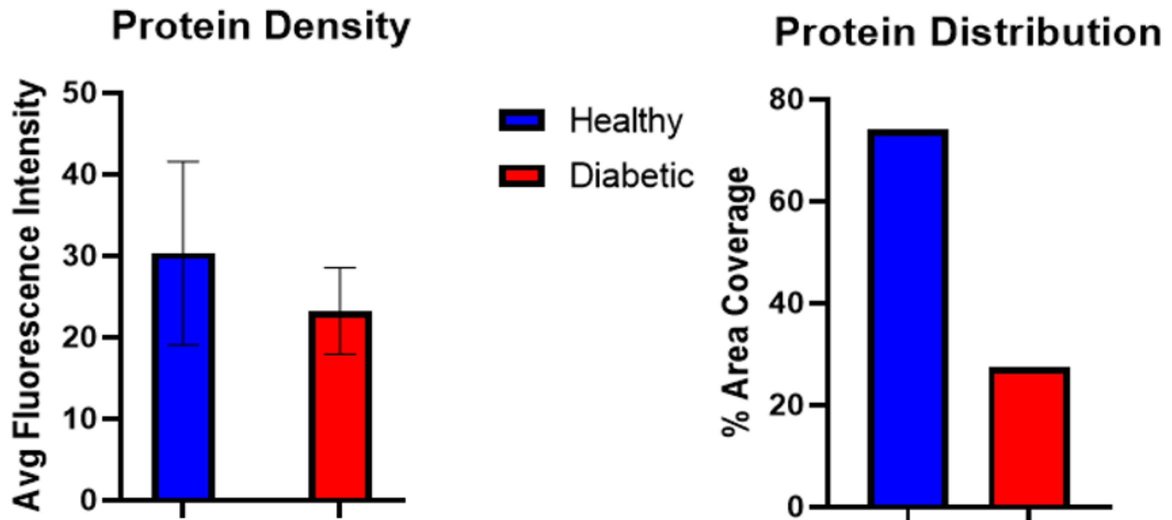

Figure 19: Protein Synthesis Effect of Diabetic Media on Constructs

This data is much more consistent with what one would expect to see out of a type II diabetic. The average density of protein in any one part of the cell is lower, indicated by a less intense average fluorescence from the protein. Moreover, the amount of protein actually distributed within the cell is dramatically lower. Only around 25% of the cell body has newly synthesized protein after diabetic treatment, while almost 75% of the healthy cell is filled with newly synthesized protein. This, combined with the gene expression data, is a clear sign that diabetes affects primarily the machinery of protein production. While Igf1 reduction likely contributes to this dramatic loss of protein synthesis, it is important to consider that there are other hypertrophic genes like Fndc5 that remained unaffected. With such a dramatic shift however, it stands to reason that, like observed in diabetics, the Akt-mTORC axis has been dysregulated and protein production slowed. This is solid grounds that the diabetic media that was formulated is effective in recapitulating the diabetic phenotype and muscle atrophy *in vitro*.

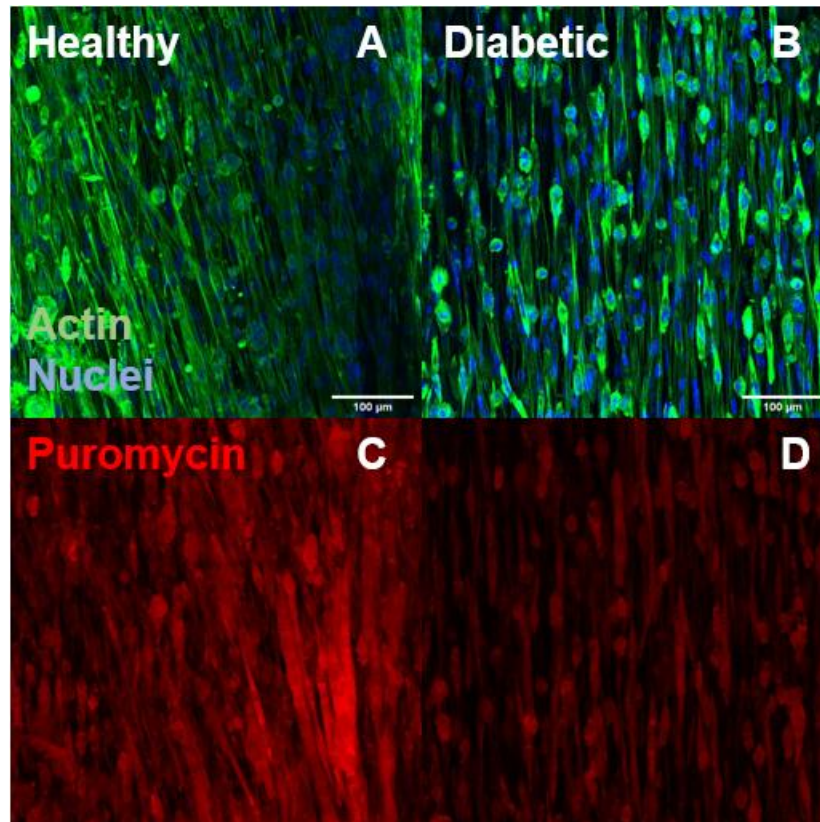

Figure 20: Fluorescent Images of Healthy and Diabetic Constructs

4.3 Effects of Mechanical Stimulation

Mechanical stimulation from exercise is known to have benefits for protein synthesis, strength, and metabolic disease protection. However, the exact changes that occur in the muscle during exercise are unknown. In this section, we will seek to identify specific changes that occur when a construct is mechanically stimulated.

4.3.1 Effects of Stimulation on Untreated Constructs

In order to get the best possible understanding of how mechanical stimulation may affect muscles in disease state, we first set out to see how mechanical stimulation affected muscles in a normal state. Depicted in figure 21 is the gene expression heatmap

for optimized constructs that were exercised in both a HIIT and END regimen. The results are fairly clear, with HIIT having strong benefits in both hypertrophic and metabolic protective genes. Significant activity of Fndc5 and Fst suggest that the cells are increasing protein synthesis and fusion activity. Il15 is also upregulated in HIIT, notably without any pro inflammatory stimuli. Il15 is known to have hypertrophic effects as a part of its pleiotropy, which could explain this. It is also known that exercise is also associated with an acute phase of inflammation post training, which may also explain the upregulation. This could even suggest a causative link between the immune and hypertrophic actions of Il15, meaning that the acute inflammatory response would be

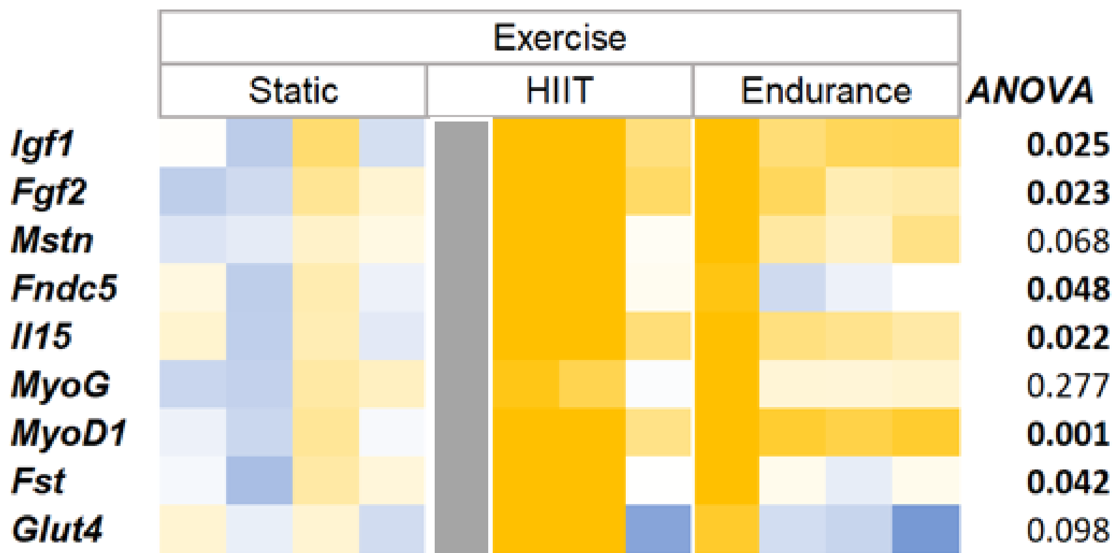

Figure 21: Transcriptional Effects of Exercise on Healthy Constructs

the cue that is causing hypertrophy. There is also significant upregulation in Igf1, though only a trend in Glut4. This is intriguing, as in previous experiments with inflammatory treatment this behavior was inverted. The upregulation of Myod1 and Fgf2 also may show that the cells are actively maintaining an active myoblast population in order to recover from the effects of stimulation, although this cannot be confirmed. Overall, the

same trends that occurred in HIIT exercise are also observable in the endurance regimen. This indicates that either the higher strain or the rest period during exercise is important for receiving the full host of beneficial effects from exercise.

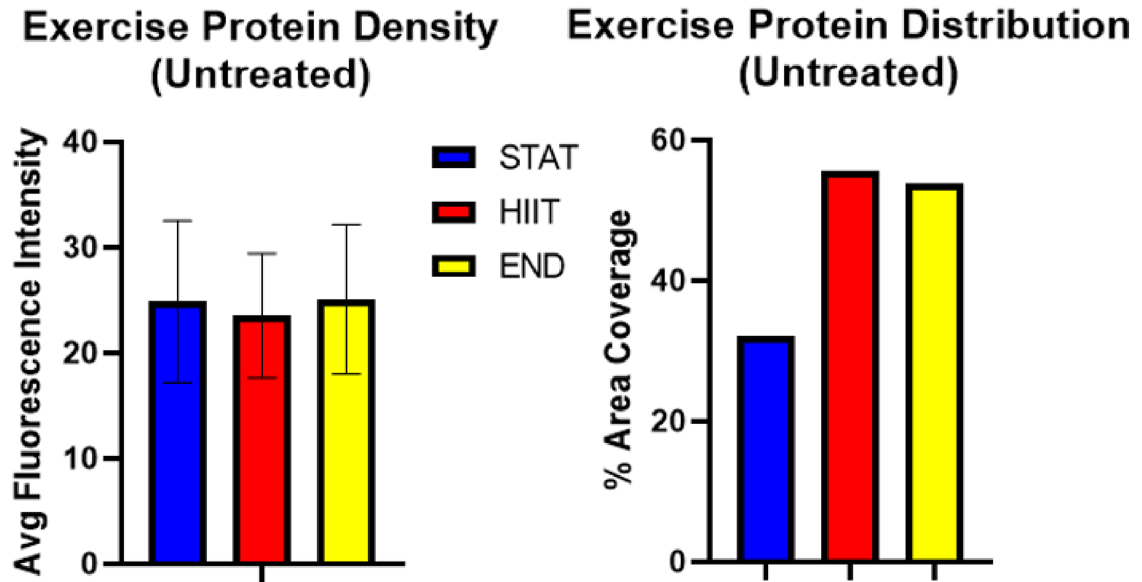

Figure 22: Protein Synthesis Effect of Exercise on Healthy Constructs

In line with the upregulation of genes in both exercise regimens is the increase in protein synthesis after exercise. HIIT and END subjected constructs were both synthesizing proteins more than the static, showing that they are hypertrophic in nature when not being given any other treatment. It is interesting that their increase in synthesis is comparable, as only the mRNA upregulation seen in HIIT was high enough to be significant. This could indicate that not all of the mRNA that is being differentially expressed in HIIT is being transcribed.

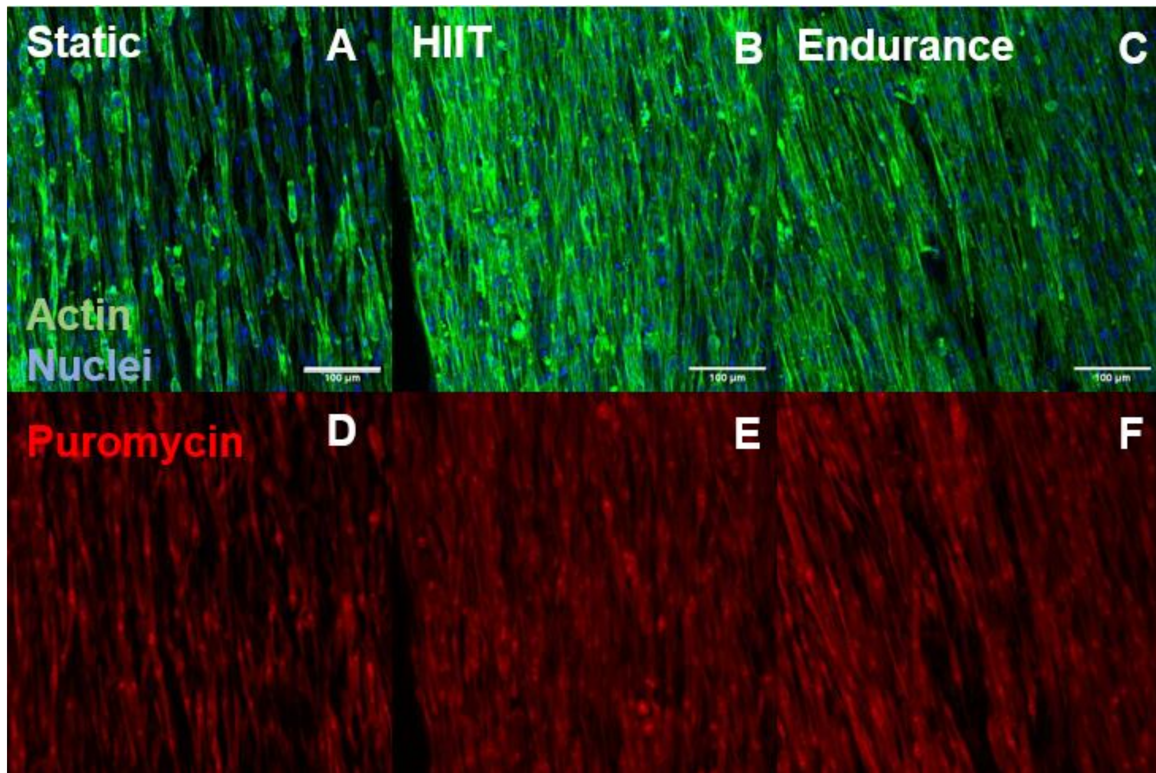

Figure 23: Fluorescent Images of Constructs after Exercise

4.3.2 Effects of Stimulation on Conditioned Media Treated Constructs

Despite the mild effects of the macrophage proteins on constructs, it was still a point of interest whether or not exercise would cause some level of differential expression. In order to test this, we utilized our existing exercise model and treated our constructs with secretions from M0, M1, and M2 macrophages.

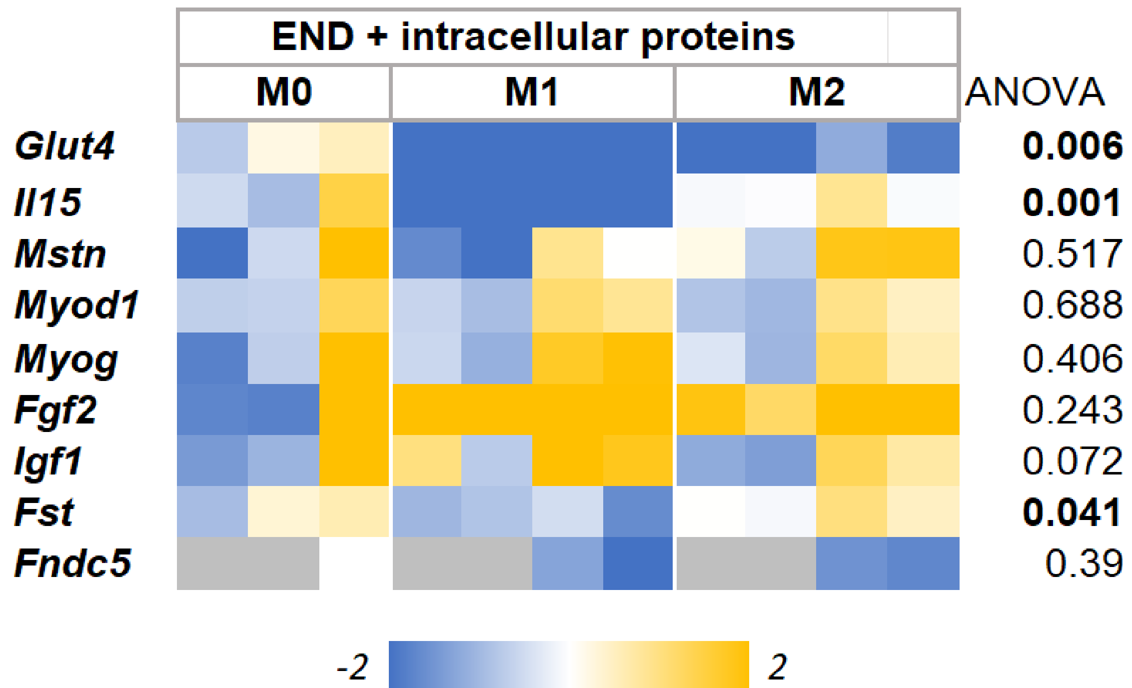

Figure 24: Transcriptional Effects of END Exercise and Macrophage Intracellular Proteins on Constructs

Shown in figure 24, constructs treated and given endurance exercise saw downregulation of several genes. Glut4 was heavily downregulated in both the M1 and M2 exposure groups, suggesting some metabolic dysregulation was occurring. Il15 was downregulated oddly, given that it usually is upregulated as a result of immunological events. Fst transcription went down in the M1 group and up in the M2 group, but without any other trends of hypertrophic genes it is difficult to make an assessment from this.

Pictured in figure 25, the same experiment performed with HIIT exercise showed no significant change in any gene. It is possible that this is an indication that HIIT has a restorative effect on muscle when it is under this kind of inflammation that endurance lacks. It is also possible however that the responses were simply not strong enough to generate a significant response, given previous experiments. Therefore, like the

endurance and macrophage proteins experiment, any conclusion drawn from this data should be taken with caution.

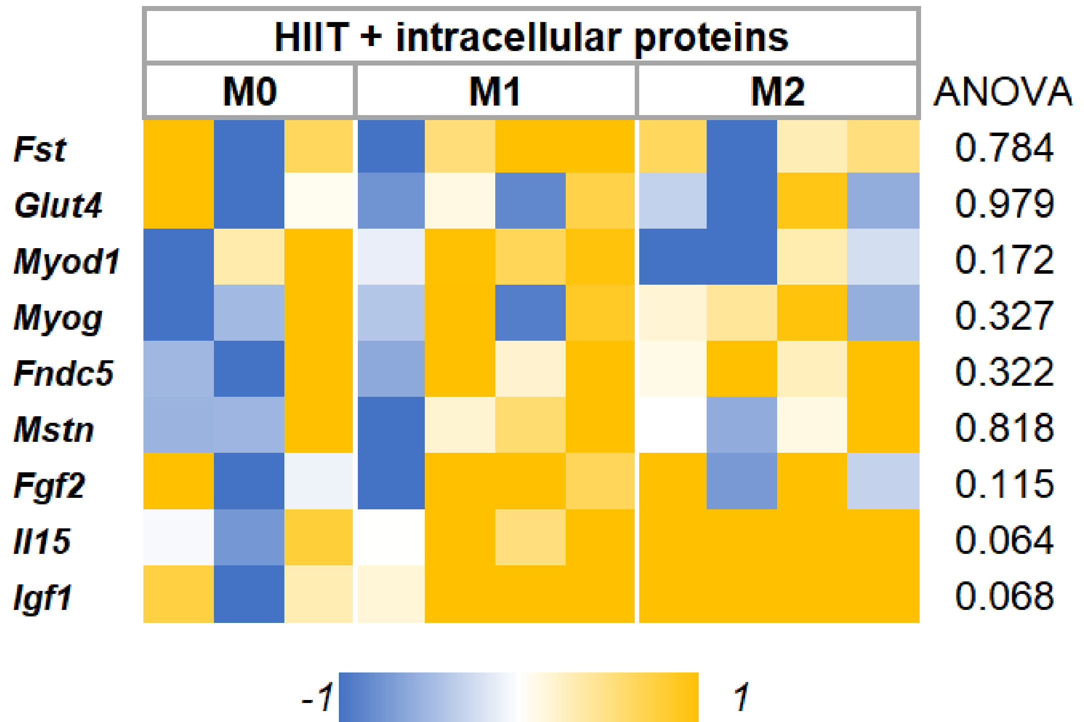

Figure 25: Transcriptional Effects of HIIT Exercise and Macrophage Intracellular Proteins on Constructs

4.3.3 Effects of HIIT on IFNγ Treated Constructs

Constructs were also exercised with a HIIT in the presence of 20ng/mL of IFNγ. The heatmap of the effects on gene expression is shown in figure 26. Predictably, IFNγ caused major downregulation of genes deemed beneficial in both the static and HIIT

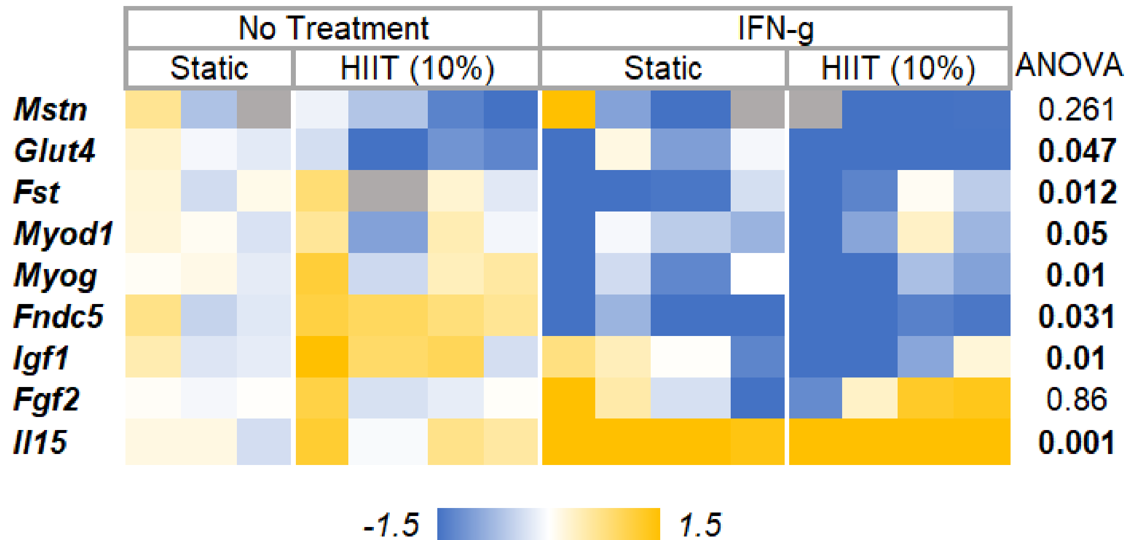

Figure 26: Transcriptional Effects of IFNγ and HIIT Exercise on Constructs

groups. Notably, the effects were more pronounced between the HIIT groups where treatment was or was not given, rather than a combination of the HIIT and static groups. What this means is that there is actually a larger difference in outcome of a healthy construct which performs HIIT and an inflamed construct that performs HIIT, rather than a healthy exercised construct and a construct that does not engage in any exercise. This implies that HIIT in inflammatory conditions is actually harmful for muscle, rather than restorative. This paints a picture of exercise as a protective tool, rather than one that can solve health problems.

4.3.4 Effects of Stimulation on Diabetic Treated Constructs

Knowing now how IFNγ impacts constructs, we decided to test how the cytokine cocktail found in diabetics changed gene expression and protein synthesis in our more reliable optimized fibrin construct. What we found was interesting, as it had the same overall effect regardless of the cytokines used. Even with the overall concentration

volume of cytokines, constructs still seemed to maintain the trend of being worse off after mechanical stimulation rather than better.

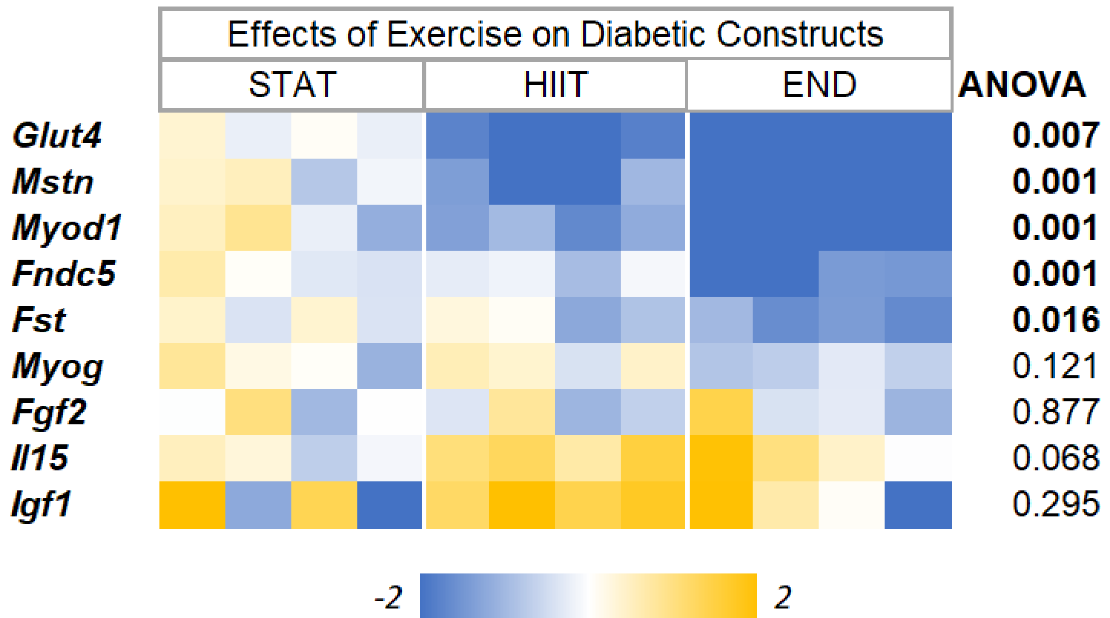

Figure 27: Transcriptional Effects of Exercise and Diabetic Media on Constructs

Metabolic behavior seemed to be significantly downregulated, as shown in figure 17, with Glut4 being heavily downregulated the same as when treated with IFNγ. Hypertrophy also seems to be taking a hit, with Fndc5 also being downregulated. An interesting trend that this data presents is that inflammatory factors seem to inhibit Mstn, which is odd considering that Mstn has an antagonistic role to muscle growth. This could represent a potential homeostatic mechanism for the body to maintain mass, or it could suggest that the body only deems Mstn production necessary when the potential for muscle growth is available. This also tracks with roles of Fst as a Mstn inhibitor, and Fst remains downregulated.

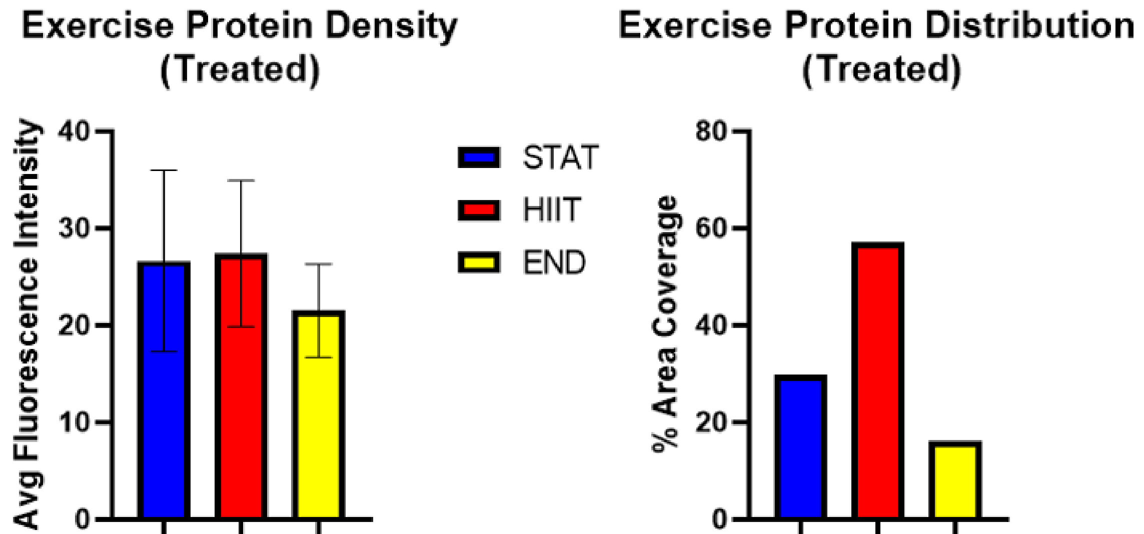

Figure 28: Protein Synthesis Effects of Exercise and Diabetic Media on Constructs

Figure 28 also seems to continue the trend with diabetic conditions affecting the machinery of protein synthesis, as static and endurance regimen constructs had significantly reduced synthesis compared to their healthy counterparts. Very interesting is that HIIT appears to somewhat resolve the issue of reduced protein synthesis, seeing a dramatic increase compared to the other two regimens. This could indicate that high levels of strain with adequate rest are enough to prompt more protein expression in spite of the reduced transcription of mRNA. It is possible that mechanotransducers are playing a larger role in this synthesis that there is simply a gene not on this panel being upregulated, but neither can be confirmed from this data alone.

Overall, this data continues the trend that exercise while inflamed is worse than rest. Attention catching is that endurance is actually significantly worse than HIIT, though both have negative trends. As HIIT has rest in between bouts of stimulation, the problem with exercise may be the lack of rest rather than the strain itself. More data would be necessary to validate this conclusion, however. Moreover, this data serves to

validate the previous IFNγ model which utilized an inferior model. Despite this, outcomes remained predictable.

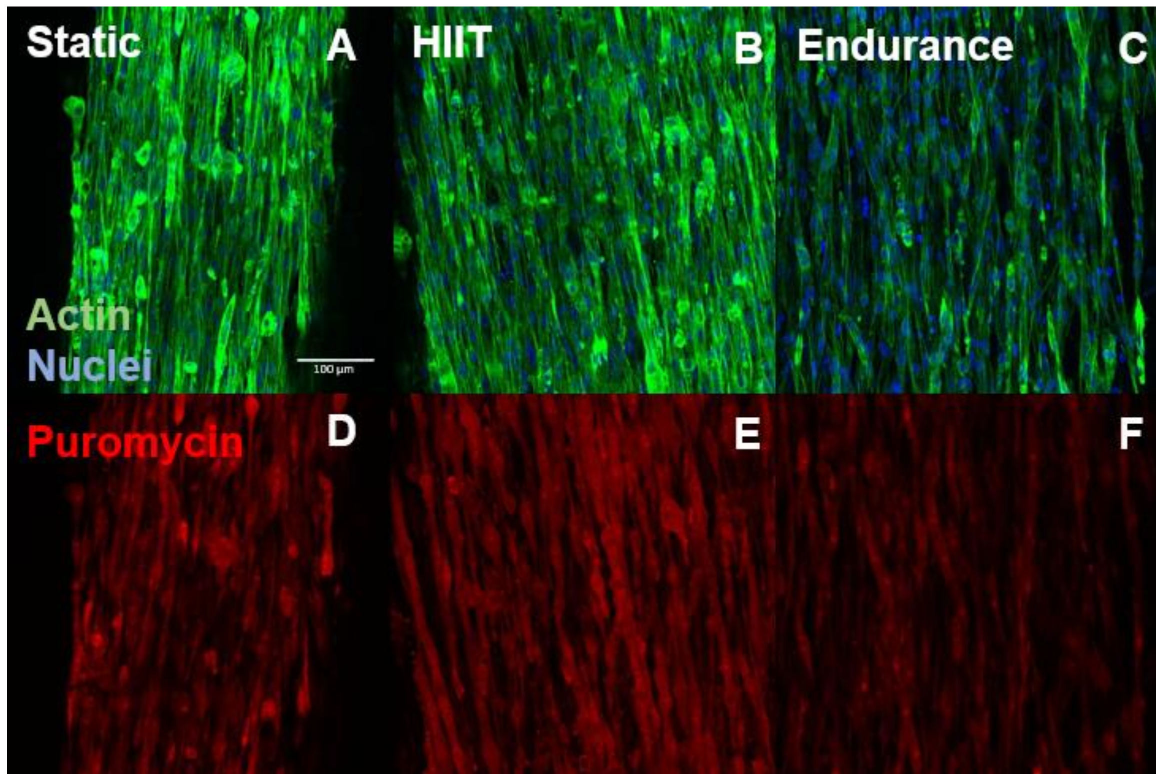

Figure 29: Fluorescent Images of Constructs After Diabetic Treatment and Exercise

CHAPTER 5. DISCUSSION OF RESULTS

5.1 Muscle Constructs can be Reliably Created

Fulfilling one of the major aims of this project, we have created muscle constructs that reliably fuse into myotubes. As of their latest iteration, the 4 mg/mL fibrin construct at low relative volumes provides consistent data that may be used to generate conclusions. This is because the cells inside are behaving predictably, consistently forming into multinucleated myotubes. With this accomplished, this model can now effectively be replicated by anybody with access to a 3D printer, an oven, and pluronic.

5.1.1 Effect of Matrix Volume on Differentiation

When going through different iterations of the construct, a common theme was that the myoblasts in the construct did not fuse. This presented the largest roadblock for moving forward, as myoblasts and myotubes have different genetic profiles. This caused major issues when performing qPCR, as even a single unfused sample could cause the experiment to fail the equal variance test. Moreover, exercise will not affect myotubes properly as force will not be transduced along the body of the cell. This makes it impossible to gather useful data.

While performing experiments with fibrin, it was necessary to reduce the matrix volume for a time in order to maintain cell density when cell count was low. This was the first time that every sample in a sample group fully fused. With further testing, it was found that it was necessary for the matrix volume to be much lower than the volume of the well in order to provide uniaxial tension between the pins. This was proven when, as a result of repairs, the volume of the well decreased. Thus, the matrix took up a higher ratio of volume than it should have, and the myoblasts did not fuse.

What these pieces of information suggest is that there is a minimum amount of force before cells fuse. Moreover, it should be possible to calculate the exact ratio of how much matrix is necessary for each well volume. These values could also change as the distance between pins changes. These values are important as larger and more biomimetic constructs are developed.

5.1.2 Determining Timepoints for Construct Development

When determining the timepoints for the differentiation experiment, points were chosen that would hopefully give a gradient view of the genetic profile of the constructs as they developed. This does not seem to be the case unfortunately, as the difference between days three and five are functionally identical in both analyses. This suggests that the majority of differentiation, at least from a genetic standpoint, occurs between days 1 and three of differentiation. This is interesting, as similar models differentiate for much longer periods of time. While it would be reasonable to say that our model is simply still differentiating by day five, this is not consistent with the fact that there are multinucleated myotubes. Myotubes are also observable by day three through light microscopy, though they appear to be less aligned. This presents the possibility that the differentiation and fusion in our model occurs early on, and the remaining time is spent organizing within the architecture.

5.1.3 The Value of Pluronic

Even after the constructs had been optimized, a problem that would sometimes occur was the construct sticking to the side of the well. This would disrupt uniaxial force, preventing fusion. At this point, we attempted to integrate a common technique used when casting hydrogels by treating the wells with pluronic f-127. Pluronic is a tri-block copolymer that prevents adhesion of the hydrogel to the well surface. On top of this, it also causes a much more even spreading of the hydrogel suspension within the well. This makes the casting process with low volumes much easier. For these reasons, all future experiments using this model should make use of a pluronic treatment on the well.

5.1.4 Improvements for the Future

Even as we complete the project, there is room to further improve the construct. For starters, reducing the volume of construct to its absolute minimum will make the constructs more economical to run in parallel, as it will use both less protein and require fewer cells. Furthermore, reducing the volume also serves to increase the uniaxial force. This may be beneficial for improving the rate of fusion even further. This could be measured by measuring the average length of the tubes in matrix.

Beyond volume modifications, the specific cell types used could also be changed. Neonatal mouse myoblasts have the potential to also be a powerful model for simulating disease since they are not immortalized. Expanding on this, seeding human cells could provide an even more accurate model. As medicine becomes more personalized, this model could potentially be made using cells from an individual in order to determine how their body would react to treatment. With all of these things in mind, there are still plenty of areas where the model could be made more useful.

5.2 Constructs as a Disease Model

The second major aim of this project was to show that our muscle constructs responded to disease states in a similar enough way to an organism that they could be used as a model. To that end, we treated our constructs with a variety of cytokines, reconstituted and directly harvested alike. What we found is that our constructs were responsive at high concentrations, but less so when the exact concentration could not be controlled, such as with the cytokines secreted from macrophages. Despite this, exposing

constructs to cytokines at levels normally seen as a part of *in vitro* experimentation did provide reliable and predictable data, showing that the constructs that we developed can stand on even ground with constructs designed by different groups. To that end, what we set out to do has been fulfilled.

5.2.1 The Problem with Co Cultures

When first designing our model, one of the key things we wanted to include was the ability to mimic the immune dysregulation more properly in type II diabetes by utilizing cocultures with macrophages. While the idea would have been interesting had it worked, it also forfeited one of the key advantages of our system. As a tool for studying muscle, our model is powerful because it removes the confounding factors that other systems introduce. We can selectively re add these systems through the introduction of cell signaling molecules. When these other cell types are integrated into the construct however, data collection becomes much more difficult. When measuring RNA expression, PCR has no means to identify whether RNA comes from a myoblast or an immune cell. Therefore, new variables are inserted into the data. While something like protein synthesis could be observed using click it technology, this ultimately provides less data than just adding cytokines into the media. Therefore, we decided not to include cocultures in the final model.

5.2.2 Recapitulating Diabetes

As diabetes was the primary disease we were attempting to model, it received the most attention when developing our tools for inducing disease state. Type II diabetes is an extremely complex inflammatory and metabolic disorder, with multiple parts that feed

forward and induce their own upregulation. To that end, there is no real perfect way to create a perfect diabetic *in vitro* model. Such a thing would require a slow and study uptick of proinflammatory cytokines from adipose tissue, an ever increasing insulin supply from pancreatic tissue, and muscle tissue that would slowly respond to both. This also does not factor in the numerous elevated molecules that are not specifically signal molecules, namely things like CRP and LPS.

By understanding how complex diabetes is, a critical approach can be taken that breaks it into its component parts. For instance, while elevating free fatty acids certainly cause a large degree of damage to the upkeep of muscle, so too do inflammatory cytokines. These cytokines are also more readily uptaken by the cell, making them very useful for generating an observable response. This is all to say that while diabetes cannot be perfectly replicated in a model, seeing how the component pieces (inflammatory cytokines, inflammatory chemokines, hyperglycemia, etc.) all function independently and with each other improves our understanding mechanistically of what is happening in diabetics without having so many moving parts as to be a black box.

5.2.3 Exercise while Inflamed

A consistent trend in the data is that exercise under inflammatory conditions loses its protective value and becomes harmful. This does make a degree of intuitive sense. It is known that the cytokines the cells were exposed to are inhibitory to protein synthesis. This does not explain, however, why exercise worsens the state of the cell. Given that mechanical strain is a form of stress, it is possible that the cell is not even attempting to recover and is rather becoming more dysregulated. This presents an interesting target for

future research on the mechanisms of exercise and could potentially offer avenues for exercise mimetics.

An attention grabbing piece of data from the exercised while inflamed experiments is that Mstn and Fst were both downregulated. Mstn is known as a regulator of muscle growth, preventing excess hypertrophy. It is possible however that this effect of mstn only occurs when the muscle is in a position to grow, and that is certainly not under conditions of high inflammation. Backing this up is that Fst is also downregulated, and Fst is a known inhibitor of Mstn. Identifying what prompts the production of Mstn could also reveal a controlling factor of muscle growth.

5.3 Mechanical Stimulation as a Tool

The final aim of this project was to validate that not only our constructs could be mechanically stimulated, but that the act of stimulation causes differential effects in both healthy and diseased cells. In the results section, we see clearly that stimulation does cause observable trends in constructs, especially the expression of genes associated with metabolic protection and hypertrophy. While it may seem intuitive to say that stimulation replicating exercise is producing these results, this actually shows that the constructs are responding as they should. If exercise caused major downregulation in the genes we described, that would suggest that something necessary was lost in the simplification from *in vivo* to *in vitro*. Since the constructs behave as expected, however, we can firmly call this aim fulfilled.

5.3.1 Common Trends in Data

Consistently, we see that the result of mechanical stimulation in a healthy sample is an improvement in the overall expression of metabolic and hypertrophic positive genes. Specifically, Fndc5, Glut4, and Igf1 are all significantly upregulated in HIIT exercise, with up regulatory trends occurring in endurance exercise. These are the same benefits that we see in organisms *in vivo*, which is solid grounds to say that our construct is recapitulating the effects of exercise through mechanical stimulation. Notably, we see that the effects of HIIT are more pronounced than the effects of endurance. There are two possible answers for this. One is that higher strain stimulation generates a stronger response than lower strain. The other is that rest between bouts is extremely important for properly getting the benefits of exercise. Both of these hypotheses can be tested with new regimens, and results can be used to further optimize our model.

Another result we repeatedly see is that exercise under inflammatory conditions causes a decrease in the expression of hypertrophy genes, rather than an increase. This means that an inflamed construct that does no exercise is actually transcribing more hypertrophy controllers than one that does exercise. We also see that this trend results in a downregulation of Glut4, but no effect on Igf1. Given how the function of these two genes is critical for healthy metabolism, it is odd that only one would be affected. It is also known that inflammation causes inhibition of the insulin signaling pathway, which Igf1 plays a primary role in near the beginning while Glut4 action is near the end. This supports data that shows inflammatory factors dysregulate the pathway near Akt, near the middle of the pathway. Interestingly, Fndc5, another hypertrophic gene on a different pathway, is also downregulated. Fndc5, like Glut4, is an effector molecule rather than a

signal transducer. Therefore, even if the pathway is still being activated, its effects cannot be conferred if there is a break somewhere in the chain. If these two pathways have a similar molecule involved in transduction somewhere that is being interfered with, this could represent a strong therapeutic target.

Besides only the mRNA expression, protein synthesis also had trends in both exercise and diabetes. When constructs were only treated with diabetic media, they saw dramatically reduced protein synthesis in spite of only minor changes in the mRNA expression. In contrast, constructs which were exercised without any kind of treatment saw elevated mRNA expression and protein synthesis. Consistent with this, constructs exercised while under diabetic stimulation saw downregulation in gene expression and changes in protein synthesis. Interestingly, these results do not appear to be necessarily correlated. For example, when treated with diabetes and subjected to HIIT training, constructs see a wave of downregulation in beneficial genes yet still have the benefit of elevated protein synthesis. What is prompting this synthesis, given that there is less mRNA to read, is unknown.

5.3.2 How to improve Mechanical Stimulation

Though a useful tool, mechanical stimulation is still not a perfect replication of exercise. *In vivo* exercise has components of stretching and contraction. Mechanical stimulation only recapitulates the stretch. Fortunately, electrical stimulation is capable of eliciting a contractile response when properly performed. While it does require more optimization than mechanical stimulation to generate a measurable response, the effects have also been found to be comparable to *in vivo* exercise. With all of this in mind, a combinatorial approach could provide the answer to perfected exercise simulation. A

system that mechanically stretches constructs, then electrically stimulates them at the end of the movement. If the correct timing is performed, this could mimic the same stretch-contract rhythm that occurs during aerobic training. While this technology does not currently exist yet, all of the component technologies have already been designed and marketed. Therefore, this represents a gap in the market that somebody with a reasonable level of design could capitalize on.

CHAPTER 6. CONCLUSION

In conclusion, we have developed a novel model for studying the effects of disease on muscle constructs that consistently recapitulates the effects of both exercise and disease. In our system, HIIT replicating stimulation resulted in a phenotype that saw significant upregulation of genes that are hypertrophic and metabolically protective. We also saw that as a result of treatment with inflammatory molecules, the constructs lose these benefits of exercise. Even more interesting than this, exercise becomes a net negative, rather than a positive. This presents a fascinating prospect for new research, and verification of these effects *in vivo* could be vital to improving our understanding of exercise as both a protective measure and a means for treating disease

The system still has more room for flexibility, including research on the effects of greater strains and different levels of rest. With multiple variables that can be tuned to different degrees, it could take a significant amount of time to isolate and optimize the best results in muscle health. As a future direction, researchers who follow up on this project should focus on what the most important factor is for muscle reactivity: strain or rest. They should also introduce the idea of exercising constructs first, then prompting

with inflammation. This could paint a clearer picture of whether or not exercise is truly protective for the muscle, or only beneficial in times of good health.

Finally, coculture experiments that elucidate how exercise benefits systems in tandem rather than in isolation could illustrate the systemic nature of the body. While our current research has shown that specific mechanisms of muscle can be identified, we have not shown if those same results would occur when crosstalk with immunocytes, adipocytes, or osteocytes is occurring. Therefore, this research is far from complete. However, this marks a good start on understanding how type II diabetes directly impacts muscle, and what approaches can be developed to combat its adverse effects.

1. Kalyani, R.R., Golden, S.H. & Cefalu, W.T. Diabetes and Aging: Unique Considerations and Goals of Care. *Diabetes Care* **40**, 440-443 (2017).
2. Tagliafico, A.S., Bignotti, B., Torri, L. & Rossi, F. Sarcopenia: how to measure, when and why. *La radiologia medica* **127**, 228-237 (2022).
3. Wolfe, R.R. The underappreciated role of muscle in health and disease. *The American journal of clinical nutrition* **84**, 475-482 (2006).
4. Tsang, H., Tse, K., Chan, K., Lu, G. & Lau, A.K. Energy absorption of muscle-inspired hierarchical structure: Experimental investigation. *Composite Structures* **226**, 111250 (2019).
5. Bershitsky, S.Y. et al. Muscle force is generated by myosin heads stereospecifically attached to actin. *Nature* **388**, 186-190 (1997).
6. Merz, K.E. & Thurmond, D.C. Role of skeletal muscle in insulin resistance and glucose uptake. *Comprehensive Physiology* **10**, 785-809 (2011).
7. Argilés, J.M., Campos, N., Lopez-Pedrosa, J.M., Rueda, R. & Rodriguez-Mañas, L. Skeletal muscle regulates metabolism via interorgan crosstalk: roles in health and disease. *Journal of the American Medical Directors Association* **17**, 789-796 (2016).
8. DiGirolamo, D.J., Kiel, D.P. & Esser, K.A. Bone and skeletal muscle: neighbors with close ties. *Journal of bone and mineral research* **28**, 1509-1518 (2013).
9. Block, B.A. Thermogenesis in muscle. *Annual review of physiology* **56**, 535-577 (1994).
10. Schiaffino, S. & Mammucari, C. Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. *Skeletal Muscle* **1**, 4 (2011).
11. Desbois-Mouthon, C. et al. Insulin and IGF-1 stimulate the beta-catenin pathway through two signalling cascades involving GSK-3beta inhibition and Ras activation. *Oncogene* **20**, 252-259 (2001).
12. Guthridge, M., Wilson, M., Cowling, J., Bertolini, J. & Hearn, M.T. The role of basic fibroblast growth factor in skeletal muscle regeneration. *Growth Factors* **6**, 53-63 (1992).
13. Yablonka-Reuveni, Z., Seger, R. & Rivera, A.J. Fibroblast growth factor promotes recruitment of skeletal muscle satellite cells in young and old rats. *J Histochem Cytochem* **47**, 23-42 (1999).
14. Kim, J.S., Yoon, D.H., Kim, H.J., Choi, M.J. & Song, W. Resistance exercise reduced the expression of fibroblast growth factor-2 in skeletal muscle of aged mice. *Integr Med Res* **5**, 230-235 (2016).
15. Mathes, S. et al. FGF-2-dependent signaling activated in aged human skeletal muscle promotes intramuscular adipogenesis. *Proc Natl Acad Sci U S A* **118** (2021).
16. Lefaucheur, J.P., Gjata, B., Lafont, H. & Sebille, A. Angiogenic and inflammatory responses following skeletal muscle injury are altered by immune neutralization of endogenous basic fibroblast growth factor, insulin-like growth factor-1 and transforming growth factor-beta 1. *J Neuroimmunol* **70**, 37-44 (1996).
17. Hamrick, M.W., Pennington, C., Webb, C.N. & Isales, C.M. Resistance to body fat gain in 'double-muscled' mice fed a high-fat diet. *Int J Obes (Lond)* **30**, 868-870 (2006).

18. McPherron, A.C., Lawler, A.M. & Lee, S.J. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* **387**, 83-90 (1997).
19. Allen, D.L., Hittel, D.S. & McPherron, A.C. Expression and function of myostatin in obesity, diabetes, and exercise adaptation. *Med Sci Sports Exerc* **43**, 1828-1835 (2011).
20. Trendelenburg, A.U. et al. Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size. *Am J Physiol Cell Physiol* **296**, C1258-1270 (2009).
21. Amirouche, A. et al. Down-regulation of Akt/mammalian target of rapamycin signaling pathway in response to myostatin overexpression in skeletal muscle. *Endocrinology* **150**, 286-294 (2009).
22. McFarlane, C. et al. Myostatin induces cachexia by activating the ubiquitin proteolytic system through an NF-kappaB-independent, FoxO1-dependent mechanism. *J Cell Physiol* **209**, 501-514 (2006).
23. McCroskery, S., Thomas, M., Maxwell, L., Sharma, M. & Kambadur, R. Myostatin negatively regulates satellite cell activation and self-renewal. *J Cell Biol* **162**, 1135-1147 (2003).
24. McFarlane, C. et al. Myostatin signals through Pax7 to regulate satellite cell self-renewal. *Exp Cell Res* **314**, 317-329 (2008).
25. Huh, J.Y., Dincer, F., Mesfum, E. & Mantzoros, C.S. Irisin stimulates muscle growth-related genes and regulates adipocyte differentiation and metabolism in humans. *Int J Obes (Lond)* **38**, 1538-1544 (2014).
26. Reza, M.M. et al. Irisin is a pro-myogenic factor that induces skeletal muscle hypertrophy and rescues denervation-induced atrophy. *Nature Communications* **8** (2017).
27. Liu, L. et al. The Role of Irisin in Exercise-Mediated Bone Health. *Front Cell Dev Biol* **9**, 668759 (2021).
28. Rabiee, F. et al. New insights into the cellular activities of Fndc5/Irisin and its signaling pathways. *Cell & Bioscience* **10** (2020).
29. Nielsen, A.R. et al. Expression of interleukin-15 in human skeletal muscle effect of exercise and muscle fibre type composition. *J Physiol* **584**, 305-312 (2007).
30. Krolopp, J.E., Thornton, S.M. & Abbott, M.J. IL-15 Activates the Jak3/STAT3 Signaling Pathway to Mediate Glucose Uptake in Skeletal Muscle Cells. *Front Physiol* **7**, 626 (2016).
31. Quinn, L.S., Anderson, B.G., Strait-Bodey, L., Stroud, A.M. & Argilés, J.M. Oversecretion of interleukin-15 from skeletal muscle reduces adiposity. *Am J Physiol Endocrinol Metab* **296**, E191-202 (2009).
32. Pistilli, E.E. & Quinn, L.S. From anabolic to oxidative: reconsidering the roles of IL-15 and IL-15Rα in skeletal muscle. *Exerc Sport Sci Rev* **41**, 100-106 (2013).
33. Huang, P.L. et al. Skeletal muscle interleukin 15 promotes CD8(+) T-cell function and autoimmune myositis. *Skelet Muscle* **5**, 33 (2015).
34. Faralli, H. & Dilworth, F.J. Turning on myogenin in muscle: a paradigm for understanding mechanisms of tissue-specific gene expression. *Comp Funct Genomics* **2012**, 836374 (2012).
35. Hasty, P. et al. Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature* **364**, 501-506 (1993).

36. Meadows, E., Flynn, J.M. & Klein, W.H. Myogenin regulates exercise capacity but is dispensable for skeletal muscle regeneration in adult mdx mice. *PLoS One* **6**, e16184 (2011).
37. Ganassi, M., Badodi, S., Wanders, K., Zammit, P.S. & Hughes, S.M. Myogenin is an essential regulator of adult myofibre growth and muscle stem cell homeostasis. *Elife* **9** (2020).
38. Blum, R. & Dynlacht, B.D. The role of MyoD1 and histone modifications in the activation of muscle enhancers. *Epigenetics* **8**, 778-784 (2013).
39. Megeney, L.A., Kablar, B., Garrett, K., Anderson, J.E. & Rudnicki, M.A. MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes & Development* **10**, 1173-1183 (1996).
40. Amthor, H. et al. Follistatin complexes Myostatin and antagonises Myostatin-mediated inhibition of myogenesis. *Dev Biol* **270**, 19-30 (2004).
41. Hill, J.J. et al. The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. *J Biol Chem* **277**, 40735-40741 (2002).
42. Hill, J.J., Qiu, Y., Hewick, R.M. & Wolfman, N.M. Regulation of myostatin in vivo by growth and differentiation factor-associated serum protein-1: a novel protein with protease inhibitor and follistatin domains. *Mol Endocrinol* **17**, 1144-1154 (2003).
43. Lee, S.J. et al. Regulation of muscle mass by follistatin and activins. *Mol Endocrinol* **24**, 1998-2008 (2010).
44. Winbanks, C.E. et al. Follistatin-mediated skeletal muscle hypertrophy is regulated by Smad3 and mTOR independently of myostatin. *J Cell Biol* **197**, 997-1008 (2012).
45. Ahlborg, G., Felig, P., Hagenfeldt, L., Hendler, R. & Wahren, J. Substrate turnover during prolonged exercise in man. Splanchnic and leg metabolism of glucose, free fatty acids, and amino acids. *J Clin Invest* **53**, 1080-1090 (1974).
46. Coyle, E. et al. Carbohydrate feeding during prolonged strenuous exercise can delay fatigue. *Journal of Applied Physiology* **55**, 230-235 (1983).
47. Richter, E.A. & Hargreaves, M. Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiol Rev* **93**, 993-1017 (2013).
48. Huang, J., Imamura, T. & Olefsky, J.M. Insulin can regulate GLUT4 internalization by signaling to Rab5 and the motor protein dynein. *Proceedings of the National Academy of Sciences* **98**, 13084-13089 (2001).
49. Mulbauer, G.D. & Matthew, H.W.T. Biomimetic Scaffolds in Skeletal Muscle Regeneration. *Discoveries (Craiova)* **7**, e90 (2019).
50. Vandenburgh, H.H., Karlisch, P. & Farr, L. Maintenance of highly contractile tissue-cultured avian skeletal myotubes in collagen gel. *In vitro cellular & developmental biology* **24**, 166-174 (1988).
51. Ricard-Blum, S. The collagen family. *Cold Spring Harb Perspect Biol* **3**, a004978 (2011).
52. Lai, V.K. et al. Microstructural and mechanical differences between digested collagen–fibrin co-gels and pure collagen and fibrin gels. *Acta biomaterialia* **8**, 4031-4042 (2012).

53. Moreno-Arotzena, O., Meier, J.G., Del Amo, C. & García-Aznar, J.M. Characterization of Fibrin and Collagen Gels for Engineering Wound Healing Models. *Materials (Basel)* **8**, 1636-1651 (2015).
54. Lai, V.K., Lake, S.P., Frey, C.R., Tranquillo, R.T. & Barocas, V.H. Mechanical behavior of collagen-fibrin co-gels reflects transition from series to parallel interactions with increasing collagen content. *J Biomech Eng* **134**, 011004 (2012).
55. Doyle, A.D. Generation of 3D collagen gels with controlled diverse architectures. *Current protocols in cell biology* **72**, 10.20. 11-10.20. 16 (2016).
56. Sung, K.E. et al. Control of 3-dimensional collagen matrix polymerization for reproducible human mammary fibroblast cell culture in microfluidic devices. *Biomaterials* **30**, 4833-4841 (2009).
57. Pieters, M. & Wolberg, A.S. Fibrinogen and fibrin: An illustrated review. *Res Pract Thromb Haemost* **3**, 161-172 (2019).
58. Hinds, S., Bian, W., Dennis, R.G. & Bursac, N. The role of extracellular matrix composition in structure and function of bioengineered skeletal muscle. *Biomaterials* **32**, 3575-3583 (2011).
59. Katagiri, Y. et al. Involvement of α6β3 Integrin in Mediating Fibrin Gel Retraction (∗). *Journal of Biological Chemistry* **270**, 1785-1790 (1995).
60. Mayer, U. Integrins: redundant or important players in skeletal muscle? *Journal of Biological Chemistry* **278**, 14587-14590 (2003).
61. Coradin, T., Wang, K., Law, T. & Trichet, L. Type I Collagen-Fibrin Mixed Hydrogels: Preparation, Properties and Biomedical Applications. *Gels* **6**, 36 (2020).
62. Caliari, S.R. & Burdick, J.A. A practical guide to hydrogels for cell culture. *Nat Methods* **13**, 405-414 (2016).
63. Akther, F., Little, P., Li, Z., Nguyen, N.-T. & Ta, H.T. Hydrogels as artificial matrices for cell seeding in microfluidic devices. *RSC advances* **10**, 43682-43703 (2020).
64. Khan, N. Applications of electrospun nanofibers in the biomedical field. *SURG Journal* **5**, 63-73 (2012).
65. Xue, J., Xie, J., Liu, W. & Xia, Y. Electrospun nanofibers: new concepts, materials, and applications. *Accounts of chemical research* **50**, 1976-1987 (2017).
66. Azimi, B. et al. Bio-Based Electrospun Fibers for Wound Healing. *J Funct Biomater* **11** (2020).
67. Gilbert-Honick, J. et al. Engineering functional and histological regeneration of vascularized skeletal muscle. *Biomaterials* **164**, 70-79 (2018).
68. Gilbert-Honick, J. et al. Adipose-derived Stem/Stromal Cells on Electrospun Fibrin Microfiber Bundles Enable Moderate Muscle Reconstruction in a Volumetric Muscle Loss Model. *Cell Transplant* **27**, 1644-1656 (2018).
69. Nagarajan, N., Dupret-Bories, A., Karabulut, E., Zorlutuna, P. & Vrana, N.E. Enabling personalized implant and controllable biosystem development through 3D printing. *Biotechnology advances* **36**, 521-533 (2018).
70. Hospodiuk, M., Dey, M., Sosnoski, D. & Ozbolat, I.T. The bioink: A comprehensive review on bioprintable materials. *Biotechnology advances* **35**, 217-239 (2017).

71. Antoine, E.E., Vlachos, P.P. & Rylander, M.N. Review of collagen I hydrogels for bioengineered tissue microenvironments: characterization of mechanics, structure, and transport. *Tissue Engineering Part B: Reviews* **20**, 683-696 (2014).
72. Cittadella Vigodarzere, G. & Mantero, S. Skeletal muscle tissue engineering: strategies for volumetric constructs. *Front Physiol* **5**, 362 (2014).
73. Grabowska, I., Szeliga, A., Moraczewski, J., Czaplicka, I. & Brzóska, E. Comparison of satellite cell-derived myoblasts and C2C12 differentiation in two- and three-dimensional cultures: changes in adhesion protein expression. *Cell Biol Int* **35**, 125-133 (2011).
74. Abdelmoez, A.M. et al. Comparative profiling of skeletal muscle models reveals heterogeneity of transcriptome and metabolism. *Am J Physiol Cell Physiol* **318**, C615-c626 (2020).
75. Jang, M., Scheffold, J., Røst, L.M., Cheon, H. & Bruheim, P. Serum-free cultures of C2C12 cells show different muscle phenotypes which can be estimated by metabolic profiling. *Scientific Reports* **12**, 1-15 (2022).
76. Wong, C.Y., Al-Salami, H. & Dass, C.R. C2C12 cell model: its role in understanding of insulin resistance at the molecular level and pharmaceutical development at the preclinical stage. *J Pharm Pharmacol* **72**, 1667-1693 (2020).
77. Ding, H. et al. Chronic reactive oxygen species exposure inhibits glucose uptake and causes insulin resistance in C2C12 myotubes. *Biochemical and Biophysical Research Communications* **478**, 798-803 (2016).
78. Aguirre, G.A., De Ita, J.R., de la Garza, R.G. & Castilla-Cortazar, I. Insulin-like growth factor-1 deficiency and metabolic syndrome. *J Transl Med* **14**, 3 (2016).
79. Wang, T., Wang, J., Hu, X., Huang, X.J. & Chen, G.X. Current understanding of glucose transporter 4 expression and functional mechanisms. *World J Biol Chem* **11**, 76-98 (2020).
80. Carter, S. & Solomon, T.P. In vitro experimental models for examining the skeletal muscle cell biology of exercise: the possibilities, challenges and future developments. *Pflügers Archiv-European Journal of Physiology* **471**, 413-429 (2019).
81. Rau, T. et al. Overexpression of wild-type Gαi-2 suppresses β-adrenergic signaling in cardiac myocytes. *The FASEB journal* **17**, 1-23 (2003).
82. Bursac, N. et al. Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies. *American Journal of Physiology-Heart and Circulatory Physiology* **277**, H433-H444 (1999).
83. Yost, M.J. et al. A novel tubular scaffold for cardiovascular tissue engineering. *Tissue engineering* **10**, 273-284 (2004).
84. Wang, Y.X., Dumont, N.A. & Rudnicki, M.A. Muscle stem cells at a glance. *J Cell Sci* **127**, 4543-4548 (2014).
85. Saxena, A.K., Marler, J., Benvenuto, M., Willital, G.H. & Vacanti, J.P. Skeletal muscle tissue engineering using isolated myoblasts on synthetic biodegradable polymers: preliminary studies. *Tissue engineering* **5**, 525-531 (1999).
86. van der Schaft, D.W. et al. Engineering skeletal muscle tissues from murine myoblast progenitor cells and application of electrical stimulation. *J Vis Exp*, e4267 (2013).

87. Collins, C.A. et al. Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell* **122**, 289-301 (2005).
88. Hawke, T.J. & Garry, D.J. Myogenic satellite cells: physiology to molecular biology. *Journal of applied physiology* (2001).
89. Halevy, O. et al. Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal. *Developmental dynamics: an official publication of the American Association of Anatomists* **231**, 489-502 (2004).
90. Ginsberg, H.N. Insulin resistance and cardiovascular disease. *The Journal of clinical investigation* **106**, 453-458 (2000).
91. Donath, M.Y. & Shoelson, S.E. Type 2 diabetes as an inflammatory disease. *Nature Reviews Immunology* **11**, 98-107 (2011).
92. Ota, T. Obesity-induced inflammation and insulin resistance. (Frontiers Media SA, 2015).
93. Orr, J.S. et al. Toll-like receptor 4 deficiency promotes the alternative activation of adipose tissue macrophages. *Diabetes* **61**, 2718-2727 (2012).
94. Petersen, K.F. & Shulman, G.I. Etiology of insulin resistance. *The American journal of medicine* **119**, S10-S16 (2006).
95. Wondmkun, Y.T. Obesity, Insulin Resistance, and Type 2 Diabetes: Associations and Therapeutic Implications. *Diabetes Metab Syndr Obes* **13**, 3611-3616 (2020).
96. Spranger, J. et al. Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Diabetes* **52**, 812-817 (2003).
97. Pickup, J.C., Mattock, M.B., Chusney, G.D. & Burt, D. NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. *Diabetologia* **40**, 1286-1292 (1997).
98. Donath, M.Y. & Shoelson, S.E. Type 2 diabetes as an inflammatory disease. *Nat Rev Immunol* **11**, 98-107 (2011).
99. Sen, C.K., Khanna, S., Reznick, A.Z., Roy, S. & Packer, L. Glutathione regulation of tumor necrosis factor-α-induced NF-κB activation in skeletal muscle-derived L6 cells. *Biochemical and biophysical research communications* **237**, 645-649 (1997).
100. Llovera, M., García-Martínez, C., Agell, N., López-Soriano, F.J. & Argilés, J.M. TNF can directly induce the expression of ubiquitin-dependent proteolytic system in rat soleus muscles. *Biochemical and biophysical research communications* **230**, 238-241 (1997).
101. Garciamartinez, C., Llovera, M., Agell, N., Lopezsoriano, F.J. & Argiles, J.M. Ubiquitin gene expression in skeletal muscle is increased during sepsis: involvement of TNF-α but not IL-1. *Biochemical and biophysical research communications* **217**, 839-844 (1995).
102. García-Martínez, C., Agell, N., Llovera, M., López-Soriano, F.J. & Argilés, J.M. Tumour necrosis factor-α increases the ubiquitinization of rat skeletal muscle proteins. *FEBS letters* **323**, 211-214 (1993).
103. Lecker, S.H., Solomon, V., Mitch, W.E. & Goldberg, A.L. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. *The Journal of nutrition* **129**, 227S-237S (1999).

104. Li, W., Moylan, J.S., Chambers, M.A., Smith, J. & Reid, M.B. Interleukin-1 stimulates catabolism in C2C12 myotubes. *Am J Physiol Cell Physiol* **297**, C706-714 (2009).
105. Luo, G., Hershko, D.D., Robb, B.W., Wray, C.J. & Hasselgren, P.O. IL-1beta stimulates IL-6 production in cultured skeletal muscle cells through activation of MAP kinase signaling pathway and NF-kappa B. *Am J Physiol Regul Integr Comp Physiol* **284**, R1249-1254 (2003).
106. Spangenburg, E.E. & Booth, F.W. Leukemia inhibitory factor restores the hypertrophic response to increased loading in the LIF (−/−) mouse. *Cytokine* **34**, 125-130 (2006).
107. Serrano, A.L., Baeza-Raja, B., Perdiguero, E., Jardí, M. & Muñoz-Cánoves, P. Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. *Cell metabolism* **7**, 33-44 (2008).
108. Soda, K., Kawakami, M., Kashii, A. & Miyata, M. Characterization of mice bearing subclones of colon 26 adenocarcinoma disqualifies interleukin-6 as the sole inducer of cachexia. *Japanese journal of cancer research* **85**, 1124-1130 (1994).
109. De Benedetti, F. et al. Interleukin 6 causes growth impairment in transgenic mice through a decrease in insulin-like growth factor-I. A model for stunted growth in children with chronic inflammation. *The Journal of clinical investigation* **99**, 643-650 (1997).
110. Muñoz-Cánoves, P., Scheele, C., Pedersen, B.K. & Serrano, A.L. Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword? *Febs j* **280**, 4131-4148 (2013).
111. Donath, M.Y., Gross, D.J., Cerasi, E. & Kaiser, N. Hyperglycemia-induced beta-cell apoptosis in pancreatic islets of Psammomys obesus during development of diabetes. *Diabetes* **48**, 738-744 (1999).
112. Poitout, V. & Robertson, R.P. Glucolipotoxicity: fuel excess and beta-cell dysfunction. *Endocr Rev* **29**, 351-366 (2008).
113. Maedler, K., Oberholzer, J., Bucher, P., Spinas, G.A. & Donath, M.Y. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. *Diabetes* **52**, 726-733 (2003).
114. Ehses, J.A. et al. Increased number of islet-associated macrophages in type 2 diabetes. *Diabetes* **56**, 2356-2370 (2007).
115. Stumvoll, M., Goldstein, B.J. & Van Haeften, T.W. Type 2 diabetes: principles of pathogenesis and therapy. *The Lancet* **365**, 1333-1346 (2005).
116. Hossan, T., Kundu, S., Alam, S.S. & Nagarajan, S. Epigenetic Modifications Associated with the Pathogenesis of Type 2 Diabetes Mellitus. *Endocr Metab Immune Disord Drug Targets* **19**, 775-786 (2019).
117. Tomás, E. et al. Hyperglycemia and Insulin Resistance: Possible Mechanisms. *Annals of the New York Academy of Sciences* **967**, 43-51 (2006).
118. Ng, Y., Ramm, G., Lopez, J.A. & James, D.E. Rapid activation of Akt2 is sufficient to stimulate GLUT4 translocation in 3T3-L1 adipocytes. *Cell Metab* **7**, 348-356 (2008).
119. Wan, M. et al. A noncanonical, GSK3-independent pathway controls postprandial hepatic glycogen deposition. *Cell Metab* **18**, 99-105 (2013).

120. Liu, Y., Vertommen, D., Rider, M.H. & Lai, Y.C. Mammalian target of rapamycin-independent S6K1 and 4E-BP1 phosphorylation during contraction in rat skeletal muscle. *Cell Signal* **25**, 1877-1886 (2013).
121. Jorgensen, S.B. et al. Deletion of skeletal muscle SOCS3 prevents insulin resistance in obesity. *Diabetes* **62**, 56-64 (2013).
122. Guadagnin, E., Mázala, D. & Chen, Y.W. STAT3 in Skeletal Muscle Function and Disorders. *Int J Mol Sci* **19** (2018).
123. Holland, W.L. et al. Lipid mediators of insulin resistance. *Nutr Rev* **65**, S39-46 (2007).
124. Kraegen, E.W. & Cooney, G.J. Free fatty acids and skeletal muscle insulin resistance. *Curr Opin Lipidol* **19**, 235-241 (2008).
125. Lowell, B.B. & Shulman, G.I. Mitochondrial dysfunction and type 2 diabetes. *Science* **307**, 384-387 (2005).
126. Holland, W.L. et al. Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. *Cell Metab* **5**, 167-179 (2007).
127. Schemmel, R. & Sclafani, A. Animal models of obesity: classification and characaterization. *Int J Obes* **8**, 491-508 (1984).
128. Johnson, P., Greenwood, M., Horwitz, B. & Stern, J.S. Animal models of obesity: genetic aspects. *Annual review of nutrition* **11**, 325-353 (1991).
129. Collins, S., Martin, T.L., Surwit, R.S. & Robidoux, J. Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. *Physiology & behavior* **81**, 243-248 (2004).
130. Friedman, J., Leibel, R., Bahary, N., Siegel, D. & Truett, G. Genetic analysis of complex disorders. *Annals of the New York Academy of Sciences* **630**, 100-115 (1991).
131. Bégin-Heick, N. Of mice and women: the β3-adrenergic receptor leptin and obesity. *Biochemistry and cell biology* **74**, 615-622 (1996).
132. Coleman, D.L. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. *Diabetologia* **14**, 141-148 (1978).
133. Graham, M.L., Janecek, J.L., Kittredge, J.A., Hering, B.J. & Schuurman, H.J. The streptozotocin-induced diabetic nude mouse model: differences between animals from different sources. *Comp Med* **61**, 356-360 (2011).
134. Tsalamandris, S. et al. The Role of Inflammation in Diabetes: Current Concepts and Future Perspectives. *Eur Cardiol* **14**, 50-59 (2019).
135. Grarup, N., Sparsø, T. & Hansen, T. Physiologic characterization of type 2 diabetes-related loci. *Curr Diab Rep* **10**, 485-497 (2010).
136. Schofield, J.D., Liu, Y., Rao-Balakrishna, P., Malik, R.A. & Soran, H. Diabetes Dyslipidemia. *Diabetes Ther* **7**, 203-219 (2016).
137. Sheng, C.Y., Son, Y.H., Jang, J. & Park, S.J. In vitro skeletal muscle models for type 2 diabetes. *Biophys Rev (Melville)* **3**, 031306 (2022).
138. Luo, W., Ai, L., Wang, B.F. & Zhou, Y. High glucose inhibits myogenesis and induces insulin resistance by down-regulating AKT signaling. *Biomed Pharmacother* **120**, 109498 (2019).
139. Mangnall, D., Bruce, C. & Fraser, R.B. Insulin-stimulated glucose uptake in C2C12 myoblasts. *Biochem Soc Trans* **21**, 438s (1993).

140. del Aguila, L.F., Claffey, K.P. & Kirwan, J.P. TNF-alpha impairs insulin signaling and insulin stimulation of glucose uptake in C2C12 muscle cells. *Am J Physiol* **276**, E849-855 (1999).
141. Dzamko, N. et al. AMPK β1 Deletion Reduces Appetite, Preventing Obesity and Hepatic Insulin Resistance. *Journal of Biological Chemistry* **285**, 115-122 (2010).
142. O'Neill, C.M. et al. Circulating levels of IL-1B+IL-6 cause ER stress and dysfunction in islets from prediabetic male mice. *Endocrinology* **154**, 3077-3088 (2013).
143. Yang, M. et al. Saturated fatty acid palmitate-induced insulin resistance is accompanied with myotube loss and the impaired expression of health benefit myokine genes in C2C12 myotubes. *Lipids in Health and Disease* **12**, 104 (2013).
144. Hirabara, S.M., Curi, R. & Maechler, P. Saturated fatty acid-induced insulin resistance is associated with mitochondrial dysfunction in skeletal muscle cells. *J Cell Physiol* **222**, 187-194 (2010).
145. Yano, N., Zhao, Y.T. & Zhao, T.C. The Physiological Role of Irisin in the Regulation of Muscle Glucose Homeostasis. *Endocrines* **2**, 266-283 (2021).
146. Chen, Z., Li, B., Zhan, R.Z., Rao, L. & Bursac, N. Exercise mimetics and JAK inhibition attenuate IFN-γ-induced wasting in engineered human skeletal muscle. *Sci Adv* **7** (2021).
147. De Deyne, P.G. Formation of sarcomeres in developing myotubes: role of mechanical stretch and contractile activation. *American journal of physiology-Cell physiology* **279**, C1801-C1811 (2000).
148. Brevet, A., Pinto, E., Peacock, J. & Stockdale, F.E. Myosin synthesis increased by electrical stimulation of skeletal muscle cell cultures. *Science* **193**, 1152-1154 (1976).
149. Aas, V., Torblå, S., Andersen, M.H., Jensen, J. & Rustan, A.C. Electrical stimulation improves insulin responses in a human skeletal muscle cell model of hyperglycemia. *Annals of the New York Academy of Sciences* **967**, 506-515 (2002).
150. Park, H. et al. Effects of electrical stimulation in C2C12 muscle constructs. *J Tissue Eng Regen Med* **2**, 279-287 (2008).
151. Humphrey, J.D., Dufresne, E.R. & Schwartz, M.A. Mechanotransduction and extracellular matrix homeostasis. *Nature reviews Molecular cell biology* **15**, 802-812 (2014).
152. Damm, T.B. & Egli, M. Calcium's role in mechanotransduction during muscle development. *Cellular Physiology and Biochemistry* **33**, 249-272 (2014).
153. Gaitanos, G.C., Williams, C., Boobis, L.H. & Brooks, S. Human muscle metabolism during intermittent maximal exercise. *J Appl Physiol (1985)* **75**, 712-719 (1993).
154. Ørtenblad, N., Westerblad, H. & Nielsen, J. Muscle glycogen stores and fatigue. *J Physiol* **591**, 4405-4413 (2013).
155. Long, Y.C. et al. Role of AMP-activated protein kinase in the coordinated expression of genes controlling glucose and lipid metabolism in mouse white skeletal muscle. *Diabetologia* **48**, 2354-2364 (2005).
156. Lira, V.A., Benton, C.R., Yan, Z. & Bonen, A. PGC-1alpha regulation by exercise training and its influences on muscle function and insulin sensitivity. *Am J Physiol Endocrinol Metab* **299**, E145-161 (2010).

157. Ferraro, E., Giammarioli, A.M., Chiandotto, S., Spoletini, I. & Rosano, G. Exercise-induced skeletal muscle remodeling and metabolic adaptation: redox signaling and role of autophagy. *Antioxid Redox Signal* **21**, 154-176 (2014).
158. Vierck, J. et al. Satellite cell regulation following myotrauma caused by resistance exercise. *Cell Biol Int* **24**, 263-272 (2000).
159. Tesch, P.A. & Larsson, L. Muscle hypertrophy in bodybuilders. *Eur J Appl Physiol Occup Physiol* **49**, 301-306 (1982).
160. Thomas, G. & Hall, M.N. TOR signalling and control of cell growth. *Curr Opin Cell Biol* **9**, 782-787 (1997).
161. Schoenfeld, B.J. The mechanisms of muscle hypertrophy and their application to resistance training. *J Strength Cond Res* **24**, 2857-2872 (2010).
162. Kubota, A., Sakuraba, K., Sawaki, K., Sumide, T. & Tamura, Y. Prevention of disuse muscular weakness by restriction of blood flow. *Med Sci Sports Exerc* **40**, 529-534 (2008).
163. Takarada, Y., Takazawa, H. & Ishii, N. Applications of vascular occlusion diminish disuse atrophy of knee extensor muscles. *Med Sci Sports Exerc* **32**, 2035-2039 (2000).
164. Takarada, Y. et al. Rapid increase in plasma growth hormone after low-intensity resistance exercise with vascular occlusion. *J Appl Physiol (1985)* **88**, 61-65 (2000).
165. He, F. et al. Redox Mechanism of Reactive Oxygen Species in Exercise. *Front Physiol* **7**, 486 (2016).
166. Hsu, F.C. et al. Association between inflammatory components and physical function in the health, aging, and body composition study: a principal component analysis approach. *J Gerontol A Biol Sci Med Sci* **64**, 581-589 (2009).
167. Beavers, K.M., Brinkley, T.E. & Nicklas, B.J. Effect of exercise training on chronic inflammation. *Clin Chim Acta* **411**, 785-793 (2010).
168. Pischon, T., Hankinson, S.E., Hotamisligil, G.S., Rifai, N. & Rimm, E.B. Leisure-time physical activity and reduced plasma levels of obesity-related inflammatory markers. *Obesity research* **11**, 1055-1064 (2003).
169. Smith, J.K., Dykes, R., Douglas, J.E., Krishnaswamy, G. & Berk, S. Long-term exercise and atherogenic activity of blood mononuclear cells in persons at risk of developing ischemic heart disease. *Jama* **281**, 1722-1727 (1999).
170. Timmerman, K.L., Flynn, M.G., Coen, P.M., Markofski, M.M. & Pence, B.D. Exercise training-induced lowering of inflammatory (CD14+CD16+) monocytes: a role in the anti-inflammatory influence of exercise? *J Leukoc Biol* **84**, 1271-1278 (2008).
171. Pedersen, M. et al. Circulating levels of TNF-alpha and IL-6-relation to truncal fat mass and muscle mass in healthy elderly individuals and in patients with type-2 diabetes. *Mech Ageing Dev* **124**, 495-502 (2003).
172. Febbraio, M.A. & Pedersen, B.K. Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. *Faseb j* **16**, 1335-1347 (2002).
173. Starkie, R., Ostrowski, S.R., Jauffred, S., Febbraio, M. & Pedersen, B.K. Exercise and IL-6 infusion inhibit endotoxin-induced TNF-alpha production in humans. *Faseb j* **17**, 884-886 (2003).

VITA

Education:
Bachelor of Science Degree
School: University of Kentucky
Major: Biology
Status: Lewis Honor's College

Experience:
Research Technician, Department of Biomedical Engineering, University of Kentucky, August 2021-May 2023
Lab Technician, Department of Biology, University of Kentucky, August 2019-August 2021

Abstracts & Presentations:
McHargue MC, Reardon K, and Annamalai RT. "Effects of Mechanical Stimulation on Muscle in a Novel Type II Diabetic Environment", Biomedical Engineering Society Annual Meeting, October 2022, San Antonio, Texas.

McHargue MC, and Annamalai RT. "The effect of exercise mimetics on inflammation-induced muscle atrophy in Type II Diabetes", Biomaterials Day, October 2022, Nashville, Tennessee.

Mark Cameron McHargue