5-2022

Growth Differentiation Factor 5 is a Paracrine Regulator of Sarcopenic Obesity

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Exercise Science

by

Landen W. Saling
University of Arkansas
Bachelor of Science in Exercise Science, 2020

May 2022
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

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Abstract

Sarcopenic obesity attributes to skeletal muscle loss more than sarcopenia and obesity alone. Individuals with SO suffer from the comorbidity of excess body fat and concurrent muscle mass loss due to aging. Growth differentiation factors (Gdfs) have never been recognized as playing a role in skeletal muscle maintenance in those with SO. Specifically, Gdf5, has been recognized as playing a part in Bone Morphogenic Protein signaling to activate protein synthesis and deactivate protein degradation via SMAD 1/5/8 and SMAD 4 complex. Using RNA sequencing, Gdf5 was identified as being significantly upregulated in SO mice. **Purpose:** To determine the cellular role that Gdf5 plays in sarcopenic obese skeletal muscle. **Methods:** *In vitro* experimentation was conducted by using C2C12 cell culture. Cells were grown to 70-80% confluency with 10% growth media and differentiated out for 6 days in differentiation media. Lipofectamine transfection was used to transfect myoblasts with an empty vector control plasmid or a shRNA for Gdf5 knockdown. Western blot was used to measure Gdf5 protein expression. Myotube diameter were measured for each group. Markers for myogenic regulation (Cyclin D1, MyoD, MyoG), protein degradation (Atrogin, MuRF1) and inflammatory markers (IL-6, TNF-α) were assessed using RT-PCR for mRNA abundance. **Results:** There was a 62% and 30% ($p<0.05$) decrease of Gdf5 protein expression at 28 kDa and 55 kDa in the shRNA 1 group. The shRNA 2 and 3 groups had decreases of 36% and 27% ($p<0.05$) at 55 kDa. respectively. Myotube size between groups were unchanged. Gdf5 and Dnmt3a mRNA abundance increased in the Gdf5 shRNA group when compared to control group by 51% and 14% ($p<0.05$) respectively. In the Gdf5 shRNA group, mRNA abundance of MyoD was decreased by 17% and MyoG was increased by 51% ($p<0.05$). mRNA abundance of protein degradation marker, MuRF-1, was increased by 39.2% ($p<0.05$) in the Gdf5 shRNA group.

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Chapter 1 – Introduction

Sarcopenic obesity has been recognized as a combination of two epidemics. An increasingly aged and rising obese population will be a continuous problem in healthcare for years to come. An increasingly aged and obese population creates a more detrimental condition for individuals to maintain their functional independence. For the creation of future treatment strategies, identifying and investigating the differences that sarcopenic obesity displays when compared to sarcopenia or obesity alone will be important. In this thesis, I will briefly review sarcopenia and obesity and sarcopenic obesity for current literature on their respective physiological and cellular adaptations. In addition, a brief review of current literature will be conducted on the role of growth differentiation factor 5 (Gdf5) and its mechanisms. Gdf5 has been identified as a potential therapeutic target for skeletal muscle mass loss in aging and has been recognized as being in the same family as myostatin (Gdf8). Furthermore, my thesis will discuss previous results from a sarcopenic obese mouse model and RNA sequencing data that displays differential gene expression in skeletal muscle of sarcopenic obese mice. In the end, my thesis will be to investigate the physiological role that Gdf5 plays in skeletal muscle of sarcopenic obese mice.

Chapter 2 – Literature Review

Sarcopenia and obesity as separate conditions increase a person's risk for morbidity and mortality (Beaudart et al., 2017; Flegal et al., 2013). The term, sarcopenic obesity (SO), is used to differentiate from sarcopenia and obesity alone to further define a more serious condition in aging individuals. Sarcopenia has been defined as concurrent losses of muscle mass with age that lead to physical disability and loss of muscle function (Cruz-Jentoft et al., 2019). Obesity has been defined as excess body fat and a body mass index (BMI) >30 kg/m^2 (American College of Sports Medicine. et al., 2010). Individuals with obesity can accrue detrimental effects to health through increased risks to the musculoskeletal and cardiovascular system that led to a decrease in physical function and inability to perform activities of daily living (Backholer et al., 2012; Blüher, 2019). Additionally, there is greater physical disability and muscle weakness in sarcopenic obese individuals rather than in sarcopenic or obese alone (Daskalopoulou et al., 2020; Kim et al., 2009). Sarcopenic obesity is particularly worrisome because of a steadily increasing sarcopenic obese population which is attributed to a rising aged population and increasing rate of obesity (Flegal et al., 2016; Nations, 2015). The increasingly sarcopenic obese population will lead to a greater burden to the healthcare system for years to come because of the combination of these two epidemics.

This review will cover sarcopenic obesity and its effects on skeletal muscle and the cardiovascular system so we can further extend our knowledge on the physiological changes that occur in sarcopenic obesity rather than in sarcopenia or obesity alone. Current literature on cellular and molecular adaptations will be explored to help understand the underlying physiological markers and adaptations that occur in sarcopenic obese models. This literature review will be broken into five parts. The first part will briefly cover the effects of sarcopenia

and obesity as single conditions and how they can cause skeletal muscle and cardiovascular specific changes. The second part of the review will then become more focused on sarcopenic obesity and the specific physiological adaptations that occur in skeletal muscle and the cardiovascular system. Adaptations that occur at the cellular level will also be reviewed in sarcopenic obesity since it has not been widely explored in scientific literature. The last part will then speculate on the future directions of sarcopenic obesity research, and the need for additional cellular level research in SO models rather than sarcopenic or obese models alone.

Sarcopenia

A healthcare cost of $18.5 billion was estimated for sarcopenia back in 2000 and represented 1.5% of total healthcare costs that year (Janssen et al., 2004). Since 2000, the projected rise of an aging population is expected to grow significantly while assuming a rise in healthcare costs as well. The population size of U.S. adults older than 65 is expected to double within the next three decades from 48 million to 88 million and is expected to increase 44% more by year 2030 (Sidney et al., 2019) (He et al., 2016). Sarcopenia is highly associated with decreased functional independence and increased risk of falls and fractures in aging adults (Yeung et al., 2019). Loss of force production at the skeletal muscle coincides with the overall loss of muscle strength and mass in sarcopenic individuals which largely leads decreased physical function (Cruz-Jentoft et al., 2019; Mitchell et al., 2012). Muscle phenotype and cellular level changes provide explanation for the physiological adaptations that cause a loss of force production seen in sarcopenia.

Skeletal Muscle Specific Changes

The loss of force production and muscle mass that coincides with sarcopenia can be caused by many physiological factors. Specific changes seen in skeletal muscle composition and fiber type have been shown as the few reasons for the loss of force production (Faitg et al., 2019; Mcphee et al., 2018). In sarcopenia, there is a tendency for skeletal muscle to adapt from predominantly glycolytic to oxidative fibers while also decreasing in the number and size of muscle fibers for both groups of fiber types (Elliott et al., 2016; Nilwik et al., 2013). A decrease in larger force producing glycolytic type IIA/IIB fiber types are accountable for some of the substantial losses in force production that occur in aging muscle (Lee et al., 2006).

Sarcopenic individuals and models also see changes to muscle composition by increased deposition of collagen and adipose tissue between muscle fibers (Shang et al., 2020; Zhu et al., 2019). More connective tissue surrounding muscle fibers means less area of the muscle denoted for contractility and force production. A altered state of the extracellular matrix due to fibrosis may be the cause that leads to loss of force production through impaired muscle regeneration and maintenance that is accompanied with aging (Gillies & Lieber, 2011; Mahdy, 2019). The following sections will provide a more cellular level explanation for composition and fiber type changes we see in aging muscle.

Mitochondrial Dynamics

Muscle mass loss and fiber type switching could be the result of cellular level adaptations that are linked to mitochondrial dysfunction through oxidative stress. Studies have shown that oxidative stress markers, for mitochondrial damage, are significantly increased while mitochondrial biogenesis markers, like PGC-1a, are significantly decreased in aging rodents

(Kadoguchi et al., 2020; Kang et al., 2013). A shift towards more oxidative fiber types and loss of glycolytic muscle fibers could be caused by mitochondrial dysfunction at the cellular level in aging individuals. Mitofusin proteins have been proposed to be a vital candidate that contributes to mitochondrial fusion and elongation loss in oxidative fiber types and have been shown to disrupt mitochondrial area in Mfn1 and Mfn2 deficient mouse models (Chen et al., 2010; Mishra et al., 2015). Mfn1 and Mfn2 have shown trends of decreasing with age, along with other mitochondrial dynamic proteins, like OPA1, that play key roles in mitochondrial function and maintenance (Huang et al., 2019). A shift towards lower mitochondrial quality could help explain the morphological changes and fiber type shifts we see in sarcopenic muscle fibers.

Extracellular Matrix and Fibrosis

Fibrosis is an important pathological process part of the extracellular matrix (ECM) that has been linked to impaired muscle regeneration and reduced satellite cell activity (Fry et al., 2017). It has been shown that high levels of M2a macrophages are linked to increased muscle fibrosis and may contribute to muscle mass loss seen in sarcopenia (Wang et al., 2015). Wang and colleagues concluded that the transplantation of bone marrow from aged to young mice exhibited a more fibrogenic phenotype in young mice while also significantly reducing satellite cell numbers. (Wang et al., 2019). More specifically, increased WnT signaling through myogenic progenitors has been proposed as a pathway responsible for the impaired muscle regeneration we see through fibrotic changes (Brack et al., 2007). Along with satellite cell adaptations, the extracellular matrix is also subjected to increased stiffness due to the realignment of collagen fibers and decreased elastic-like ECM properties that directly affect the elasticity and mechanical properties of skeletal muscle (Stearns-Reider et al., 2017).

Cardiovascular Specific Changes

The cardiovascular system is a vital component for skeletal muscle and entire body health. Cardiovascular specific changes that occur with aging can be seen as major physiological adaptations that may be a contributing factor to sarcopenia. Lower capillarization of the muscle in aging adults showed to contribute to less muscle adaptation when resistance trained (Moro et al., 2019). A more sarcopenic state could be provided in lower capillarization of muscle in aging adults. Although lower capillarization showed less of a response to resistance training, it has also been found that there is no capillary distribution differences between young and old people (Barnouin et al., 2017). Whether aging causes a decrease in capillarization is largely unknown, but in respect to aerobic exercise and stretching, aging adults see significant angiogenic responses (Gavin et al., 2015; Hotta et al., 2018).

Cardiac muscle can succumb to an increase of extracellular matrix components in the interstitial tissue of the heart (Kong et al., 2014). An increase of ECM properties leads to a process known as cardiac fibrosis. According to the review by Kong et al, cardiac fibrosis occurs in aging adults that exhibit hypertension and increased overload pressure that results in cardiac tissue stiffness and systolic and diastolic dysfunction. Due to increase stress of the heart, ECM components accumulate in the interstitial tissue and the heart hypertrophies. This leads to a larger ventricular wall and loss of cardiac function through slower filling rates and decreased blood volumes (Dzeshka et al., 2015; Grodzicki, 1998). On the cellular level, increased TGF-B1 signaling has been associated with larger collagen synthesis and cardiomyocyte apoptosis (Khan & Sheppard, 2006). Disrupted TGF-B signaling showed to promote healthy cardiac aging in vivo of older mice when compared to uninhibited TGF-B control mice (Lyu et al., 2018). The underlying mechanism of TGF-B1 signaling could be one of the reasons why we see cardiac

fibrosis and it could be an upstream factor that exacerbates a decreased cardiovascular function and nutrient delivery to working skeletal muscle in sarcopenic individuals.

Obesity

Obesity is coupled with increased risk of cardiovascular disease and physical disability to perform activities of daily living (Bhaskaran et al., 2018; Fernandes de Souza Barbosa et al., 2018). Healthcare costs of obesity have shown to steadily rise in the past years with significantly greater healthcare costs in the 65 and older population of the U.S. (Biener et al., 2017; Kim & Basu, 2016). A BMI >30 kg/m² is highly associated with increased all-cause mortality and morbidity in young and aging adults with higher hazard ratios in those that maintain obesity through adulthood (Bhaskaran et al., 2018; Chen et al., 2019). As will be described, an absolute loss of force production is less evident in obesity, while relative and hand grip strength loss is a more common characteristic of obese individuals and will be seen as a more realistic cause for the losses we see in physical function in obesity.

Skeletal Muscle Specific Changes

Force production is vital for locomotion and performance of daily activities. Absolute strength is higher in obese individuals while relative strength remains the same between lean and obese elderly women, with the exception of handgrip strength being lower in obese (Rolland et al., 2004). A large generating force is required when performing activities of daily living and has shown consistent in studies that have investigated increased absolute strength of the lower limbs in obese individuals (Hulens et al., 2001; Tomlinson et al., 2016). Lower relative strength measurements are also seen in obese when compared to lean due to a loss of supplying sufficient

force for excess fat mass during movement (Koushyar et al., 2017). Individuals with excess weight and an insufficient relative force production lead to larger effort requirements and decreased mobility (Koushyar et al., 2020). Reasons for relative strength losses and muscle atrophy we see in obesity could be connected to inflammation and decreased insulin sensitivity at the muscle. It has been formerly reviewed that both processes have been seen to serve pathological roles in the body's ability to repair muscle and to engage in adequate protein turnover for muscle maintenance and hypertrophy (Wu & Ballantyne, 2017).

Inflammation

As mentioned by Wu and Ballantyne, cytokines, are inflammatory markers secreted by skeletal muscle and adipocytes that play vital roles of obesity-linked inflammation. It has been reviewed in literature that obese individuals see increased inflammation with markedly higher inflammatory markers being secreted from skeletal muscle and adipose tissue (Pedersen & Febbraio, 2012). The secretion of cytokines at the muscle, like IL-6, has been shown to correlate with muscle hypertrophy and to have elevated production in obese individuals (Corpeleijn et al., 2005; Mitchell et al., 2013). In contrary, IL-6 has also shown decreased protein levels in obese when compared to non-obese (Green et al., 2011). Increased presence of proinflammatory markers in the skeletal muscle presented to be abundant due to additional cytokine release from adipose tissue as well (Popko et al., 2010). The release of proinflammatory markers, like tumor necrosis factor-α (TNF-α), has been linked to the NF-κB pathway that regulates and impairs skeletal muscle by acting closely with the regulation of insulin resistance (Barma et al., 2009; Cai et al., 2005). Diet induced obese rats that exhibited TNF-α knockout showed to be protected

from insulin resistance through modulating insulin receptor signaling in muscle tissue (Uysal et al., 1997; Zeyda & Stulnig, 2009).

Cardiovascular Specific Changes

Obesity is coupled with significant risk of cardiovascular disease due to hemodynamic changes that can alter cardiac morphology (Alpert et al., 2018). A change in heart morphology due to increases in left ventricular mass was observed in coronary CT scans of obese individuals that had either normotensive or hypertensive blood pressure (Jongjirasiri et al., 2017). Cardiac hypertrophy of the left ventricular wall has been formerly reviewed as detrimental for cardiovascular health through affecting the systolic and diastolic phases of the heart (Federmann & Hess, 1994). Obesity-related cardiovascular changes could contribute towards a more complex and higher risk condition when coupled with sarcopenia.

Sarcopenic Obesity

Sarcopenic obesity (SO) is a term used that involves the pathological effects of both sarcopenia and obesity. Sarcopenic obese individuals have been studied as having significantly poorer physical function and higher cardiometabolic risk than sarcopenic or obese individuals alone (Johnson Stoklossa et al., 2017; Stephen & Janssen, 2009). As mentioned in their specific sections of the review, sarcopenia and obesity have significant roles in muscle mass and strength losses through physiological adaptations that can decrease the physical function of the persons at risk. SO combines the physiological adaptations seen with both sarcopenia and obesity and poses a more severe risk to an individual's health (Choi, 2016). Through meta-analysis, sarcopenic

obesity has shown an increase risk of all-cause mortality when compared to individuals without SO (Tian & Xu, 2016).

Defining sarcopenic obesity requires individuals to exhibit both obese and sarcopenic parameters. Sarcopenia and obesity have been previously defined using relative skeletal muscle index (RSMI) with sarcopenic values considered two standard deviations below a healthy younger person's RSMI and body fat percentages above 27% and 38% in men and women, respectively (Baumgartner, 2000). These definitions have been similarly used and combined to help define and identify sarcopenic obesity. In a study using older Korean adults, appendicular skeletal muscle mass divided by height squared (ASM/height^2) was used to identify the sarcopenic state (Kim et al., 2009). The current literature on defining sarcopenic obesity encompasses two general parameters, SO individuals exhibit two standard deviations lower skeletal muscle mass than healthy individuals and they display greater body fat percentages that place them at obese.

Skeletal Muscle Specific Changes

Accelerated increases of muscle atrophy in sarcopenic obesity have shown to be prevalent in studies that compare it to sarcopenia and obesity (Brown et al., 2021; Zhu et al., 2019). A trend towards higher inflammatory markers and significantly lower values of muscular strength and lean body mass were displayed in a study of elderly obese women identified as sarcopenic obese (Nascimento et al., 2018). Proteolysis markers, known for their role in muscle atrophy, showed an upregulation in aged mice with peri-muscular fat transplantation while downregulation occurred in young obese mice that had peri-muscular fat removed (Zhu et al., 2019). In contrary, MuRF-1 and Atrogin1 proteolysis markers showed no changes in the

sarcopenic obese model studied in Brown's paper, although significant losses in mean cross sectional area of the gastrocnemius were still observed (Brown et al., 2021). The differences seen between these papers could be due to the difference of models used.

Inflammation

Inflammation plays a significant role in skeletal muscle maintenance. TNF-α and IL-6 have been observed as elevated in sarcopenic and obese models of mice (Buford et al., 2010; Shabani et al., 2020). Greater inflammatory responses in sarcopenic obesity versus obesity mice models have been observed through larger mRNA abundance of TNF-α with IL-6 remaining unchanged in sarcopenic obese mice models (Brown et al., 2021). These findings show to be different than a human study that measured TNF-α and IL-6 as unchanged in SO versus obese individuals (Yang et al., 2015). Inflammatory responses of sarcopenic obesity need more investigation to fully determine how the differing inflammatory signaling could be modulating muscle atrophy in sarcopenic obesity versus sarcopenia and obesity alone.

Cardiovascular Specific Changes

Cardiac remodeling through cardiac hypertrophy occurs in sarcopenic obesity and may be more exacerbated than seen in sarcopenia or obesity alone. Significantly greater heart mass has been recorded in a study looking at cardiac hypertrophy in sarcopenic obese mice (Perry et al., 2018). In the study, increased inflammatory signaling and greater trends of fibrosis markers were observed and may be responsible for the cellular changes of increased cardiac remodeling observed in SO. Lower levels of mitochondrial initiation factor 2 showed to potentially contribute to cardiomyopathy in aged-obese mice (Lee et al., 2019). The different mitochondrial

changes seen in cardiac muscle of sarcopenic obesity showed to be more severe than that of the aged or obese models alone in Lee's study.

Overview of Growth Differentiation Factor 5 (Gdf5)

Growth differentiation factors (GDFs) are well known for their roles in bone and skeletal muscle development. GDFs are a subfamily of genes that are part of the transforming growth factor-beta (TGF-B) superfamily and have been additionally recognized as bone morphogenic proteins (BMPs). Growth differentiation factors have been previously studied in skeletal muscle for their effects on hypertrophy and atrophy of muscle (Egerman et al., 2015; Rodriguez et al., 2014). Gdf8, or more commonly recognized as myostatin, has been extensively studied in the past for its ability to inhibit skeletal muscle hypertrophy (Wang & Mcpherron, 2012). Basal levels of myostatin are known to lower satellite cell activation and slow the rate of hypertrophy that skeletal muscle is allowed (McCroskery et al., 2003). Due to previous studies and findings of extensively studied GDFs, it should bring attention to scientific literature that other GDFs could be responsible for skeletal muscle growth and atrophy seen in sarcopenic obese individuals.

A less prominent GDF, known as growth differentiation factor 5 (Gdf5), has been highlighted in literature as playing a role in cartilage/bone development and in skeletal muscle adaptations (Hatazawa et al., 2018; Kania et al., 2020). Gdf5 has been formerly studied in ligament healing. Administration of Gdf5 in an injury model of the medial collateral ligament (MCL) in a rat knee joint showed significant improvement in the mechanical properties and ultimate tensile strength of the knee complex (Tashiro et al., 2006). In the same study, Gdf5 increased collagen fibrils in repair tissue of the injured knee complex. Gdf5 and basic fibroblast

growth factors (bFGF) was administered in a different study for which stimulated Col1a1 expression and deposition (Saiga et al., 2010).

In addition to ligament studies, Gdf5 has also been mentioned in scientific literature regarding skeletal muscle. As mentioned above in Hatazawa et al., 2018, Gdf5 was closely regulated by Dnmt3a for which is an epigenetic factor that participates in methylation and silencing Gdf5 expression. Gdf5 has also been mentioned as an inhibitor of muscle atrophy and is an upstream regulator of several Smad family proteins including Smad1, Smad5 and Smad8 (Sartori et al., 2013). This was further verified by Hitachi et al., 2019 for where they identified *Myoparr* as a regulator of Gdf5 in denervated skeletal muscle of mice (Hitachi et al., 2019). Through RNA sequencing, Myoparr was investigated for its role in regulating several BMP signaling genes. *In vivo* Myoparr knockdown in the tibialis anterior muscle showed a significant increase of Gdf5 protein levels.

Future Directions

An investigation into the cellular and molecular mechanisms in skeletal muscle of sarcopenic obesity is needed. The physiological adaptations and mechanisms behind sarcopenic obesity and why it poses a more severe risk than sarcopenia or obesity alone needs further investigation. As talked about in this review, there is clear functional and muscle phenotype differences that is caused by cellular and molecular adaptations that only SO displays. Utilizing animal models in sarcopenic obesity research will be a vital component for identifying molecular and cellular differences for the development of future treatment strategies.

Chapter 3 – Preliminary Data

Confirmation of Sarcopenic Obese Model

In a previous study by Brown et al., 2021, they observed significant effects on body mass and gastrocnemius weights of diet induced obesity in aged mice. Aged high fat diet (HFD) mice displayed 35% greater body mass and 8% less gastrocnemius weight when compared to aged mice receiving a normal chow (NC) diet. Aged HFD mice additionally showed a 39% reduction of gastrocnemius muscle relative to body mass when compared to aged NC mice. To further confirm the model, Brown reported a 67.5% increase of gonadal fat weights in aged HFD when compared to aged NC. A 29% decrease of cross-sectional area of the gastrocnemius muscle was also observed in Brown's paper. Additionally, significant muscle loss in the plantaris was observed. The aged HFD group displayed a~9% reduction in plantaris wet weight when compared to aged NC. An effect of aging was also observed with a 12% decrease of plantaris weight between young HFD and aged HFD mice. Plantaris to body mass ratios (plant:BM) were calculated and showed significance among young HFD, aged NC and aged HFD groups. A~40% reduction in plant:BM was seen in aged HFD when compared to aged NC mice. Aged HFD also showed a~38% decrease of plant:BM when compared to the young NC group. A comparison of tibia lengths among groups was also observed with significant main effects of diet and age. Larger tibia lengths were observed in the aged group, while HFD groups exhibited smaller tibia lengths when compared to NC groups.

Gastrocnemius weight, body mass, gastrocnemius relative to body mass, gonadal fat in male C57BL/6 mice.

Treatment	Gastrocnemius (mg)	Body mass (g)	Gonadal Fat (mg)	Gastrocnemius/BM (mg g $^{-1}$)
Young (3–4 mo)				
NC	149.33 ± 3.32	28.91 ± 0.45	358.2 ± 100.8	5.16 ± 0.01
HFD	162.38 ± 2.49$^{\#}$	38.14 ± 0.64$^{\#}$	1491.1 ± 259.9$^{\#}$	4.34 ± 0.11$^{\#}$
Aged (22–24 mo)				
NC	152.44 ± 4.99	35.14 ± 2.09	997.96 ± 297.2	4.39 ± 0.14$^{\#}$
HFD	140.51 ± 3.82$^{\$}$	53.70 ± 2.48$^{\$}$	1671.7 ± 205.0$^{\$}$	2.66 ± 0.10$^{\$}$

Values are means ± SEM. *Note.* BM = body mass. Significant difference from the young NC distinguished by $^{\#}$. Significant difference from the old NC distinguished by $^{\$}$, $P \leq 0.05$.

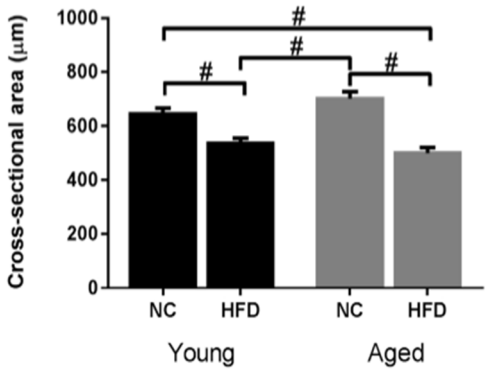

Table & Figure from Brown et al., 2021. Gastrocnemius, body mass, gonadal fat weights and mean CSA for gastrocnemius for Young NC, Young HFD, Aged NC and Aged HFD mice.

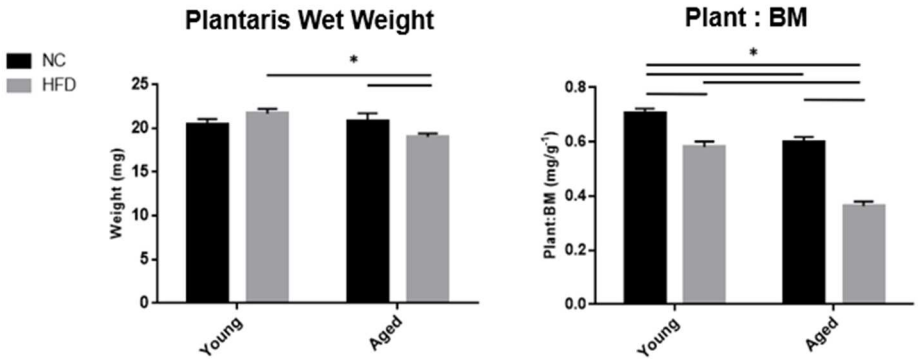

Figure 1. Confirmation of Sarcopenic Obese Model in Plantaris. (A) Plantaris wet weights among experimental groups. (B) Plantaris weight to body mass ratios for experimental groups. (C) Pre-planned comparison of tibia lengths between young and aged groups. Bar values are means ± SEM. Significance is denoted with "*" above bar. Lines above groups represent interference among group means. The p value is set at $P \leq 0.05$.

The preliminary data of the gastrocnemius, plantaris and gonadal fat displays significant muscle mass loss in the aged HFD group when compared to young HFD and aged NC groups. Larger gonadal fat weights and smaller muscle mass weights were observed in aged HFD mice. In addition, a smaller CSA was previously published and observed in aged HFD mice. Smaller muscle CSA and less muscle mass is representative of less tetanic force and muscle degradation, respectively. Significance was not found between young NC and aged HFD plantaris muscle wet weights, but tibia lengths still showed a significant increase in length with aging. The average plantaris wet weight stayed the same with an increase of age and body mass. These findings suggest that aged mice, receiving a high fat diet, are representative of sarcopenic obesity since they display a more severe state of muscle wasting.

RNA Sequencing of Aged NC versus Aged HFD Mice

After completion of the study, isolated RNA from the plantaris muscle were shipped off for RNA sequencing analysis. We decided to investigate differential gene expression differences in the plantaris muscles of aged lean and aged obese mice. The plantaris was chosen because it contains majority of fast-twitch fibers and because the plantaris muscle weights showed to be significantly lower in aged HFD mice when compared to the aged NC group. Once RNA sequencing data completed and data was received, differentially expressed (DE) genes of the plantaris muscle were analyzed using Ingenuity Pathway Analysis (IPA) in aged NC versus aged HFD mice. Originally, data was processed with more stringent measures at a Log2FC>1.0 that accounted for only 30 upregulated and 16 downregulated DE genes. Differentially expressed genes were adapted to less restricted measures to Log2FC>0.6 for which accounted for 100 upregulated and 60 down regulated DE genes.

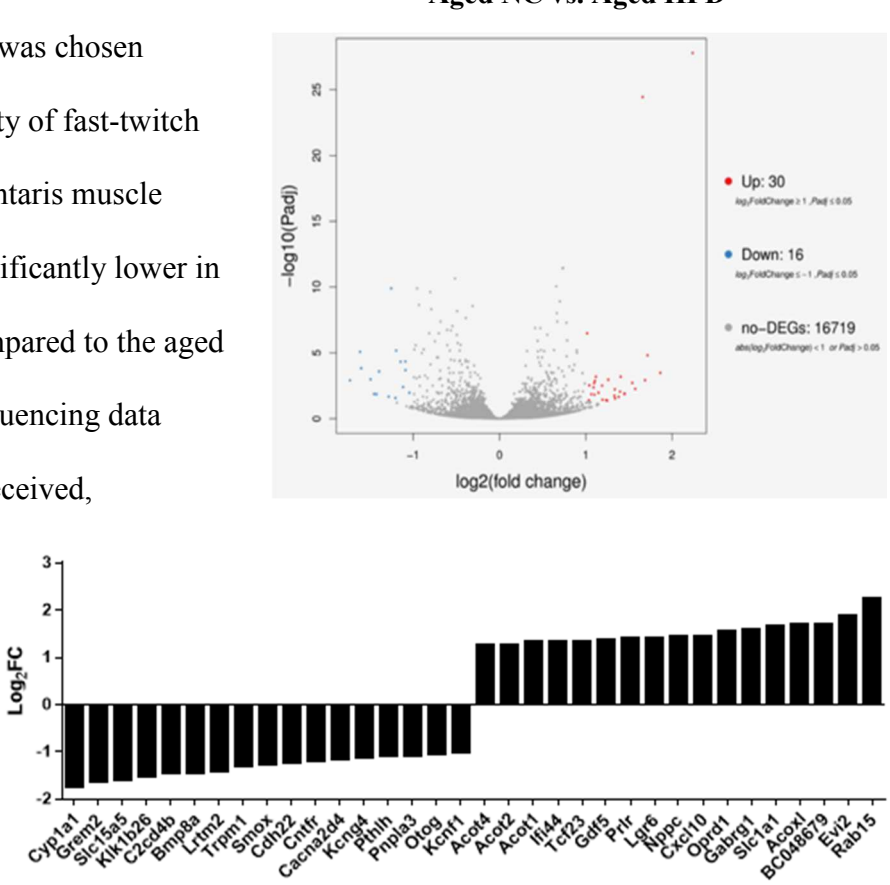

Figure 2. RNA Sequencing Data and Analysis from Ingenuity Pathway Analysis software that is representative of aged NC and aged HFD comparison. (A) Volcano plot displaying upregulated and downregulated genes in aged NC versus aged HFD groups. (B) The top 17 upregulated and downregulated genes.

We then chose five gene targets from the top upregulated and downregulated genes to identify and verify altered mRNA expression via real-time polymerase chain reaction (RT-PCR) in aged obese mice. In total, five genes were analyzed and verified using RT-PCR. The five genes included Cyp1a1, Rab15, Cdh22, Smox and Gdf5. Cyp1a1, Rab15 and Cdh22 showed significant main effects due to diet. Smox showed no significance between aged NC and aged HFD mice but showed significant interference among the other groups. Although Smox showed significant interference and could be further analyzed for its potential effects in sarcopenic obesity, it still did not display a clear significant difference between the aged groups. A notable~6-fold decrease of expression was found in aged HFD when compared to young HFD. Further investigation of Smox in the future could be interesting to pursue.

Of the five chosen, growth differentiation factor 5 (Gdf5) showed a~26-fold increase of expression in the aged HFD group when compared to the aged NC group.

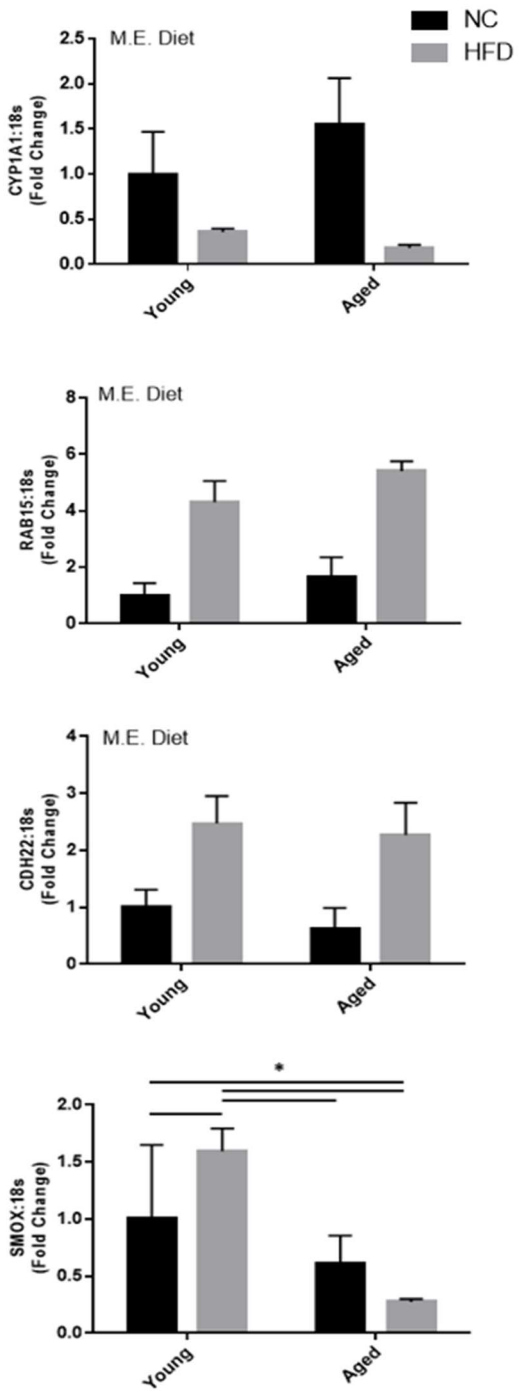

Figure 3. Four of the five gene targets chosen for validation from RNA sequencing of the plantaris muscle in aged NC vs aged HFD mice.

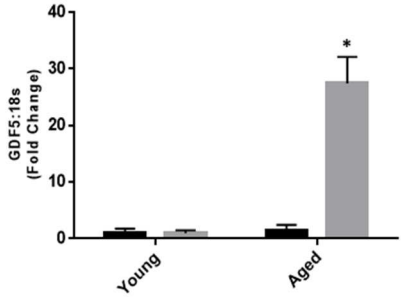

Figure 4. mRNA abundance of growth differentiation factor 5 among experimental groups

In conclusion, these preliminary results display a confirmation of a sarcopenic obese mouse model and the effect it has on muscle mass loss in the plantaris muscle. RNA sequencing of the plantaris muscle in aged HFD mice showed differential gene expression for over a hundred genes that are either upregulated or downregulated when compared to aged NC mice. Five gene targets were chosen for substantial difference of gene expression and further verified using real time polymerase chain reaction. Gdf5 was one of the five gene targets that showed significantly altered gene expression in aged HFD mice. This remarkable increase of Gdf expression requires further investigation for its role in sarcopenic obese skeletal muscle and if it can be targeted as a therapeutic for future treatment strategies.

Chapter 4 – Specific Aims

GROWTH DIFFERENTIATION FACTOR 5 IS A PARACRINE REGULATOR OF SARCOPENIC OBESITY

Sarcopenia and obesity are two conditions consistently on the rise in developed countries (Sidney et al., 2019). The increasing rise of the aged population coincides with increasing rates of obesity and contributes to a specific comorbid condition that is more detrimental to overall health than sarcopenia or obesity alone.

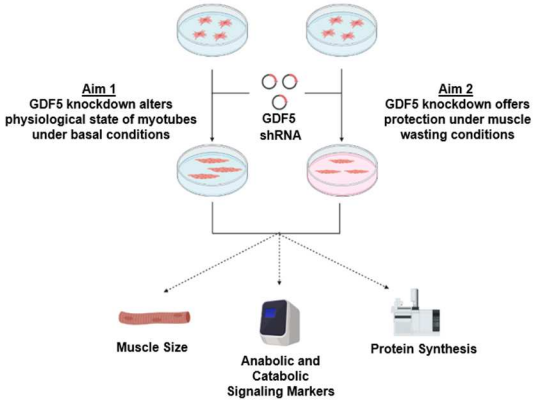

Figure 5. Schematic of Proposal

Sarcopenic obesity (SO) associates with less mobility, greater risk of cardiovascular disease and greater losses of skeletal muscle mass when compared to sarcopenia alone (Daskalopoulou et al., 2020; Johnson Stoklossa et al., 2017). However, there has been a lack of studies to investigate cellular level alterations that contribute to the poor state of skeletal muscle in SO. Prior research done in our laboratory demonstrated altered phenotypes through lower gastrocnemius muscle mass and cross-sectional area in a SO mouse model (Brown et al., 2021). Through previous RNA sequencing, growth differentiation factor 5 (Gdf5) was notably upregulated in the skeletal muscle of sarcopenic obese mice. GDF5 has been known as a gene that encodes for ligands of the transforming growth factor-beta (TGF-B) family of proteins. Myostatin (Gdf8) is a more commonly known growth differentiation factor notable for its ability to inhibit hypertrophy and in the same subfamily as Gdf5. The current literature for Gdf5, also known as bone morphogenic protein 14 (BMP14), is contentious and reports both advantages and disadvantages with upregulation in skeletal muscle of aging mice models (Hatazawa et al., 2018; Traoré et al., 2019). Gdf5 has been closely investigated in ligament healing and in aged muscle, but it has not

been investigated closely on its physiological effects in skeletal muscle of SO. Identifying Gdf5's physiological role in skeletal muscle of SO could give crucial insight on its role in skeletal muscle and its potential use for future treatment strategies. Therefore, the **OVERALL GOAL** of the proposal is to determine the physiological role of Gdf5 plays in sarcopenic obese skeletal muscle. In addition, the **CENTRAL HYPOTHESIS** is that *in vitro* Gdf5 knockdown will increase myotube size and induce an increase in protein synthesis rates under basal conditions. In addition, we hypothesize Gdf5 knockdown will mitigate the deleterious effects of an SO micro-environment *in vitro*.

AIM 1. DETERMINE IF *IN VITRO* GDF5 KNOCKDOWN ALTERS PHYSIOLOGICAL STATE OF MYOTUBES UNDER BASAL CONDITIONS

I hypothesize that Gdf5 knockdown will increase myotube growth under basal conditions. C2C12 myoblasts will be cultured and differentiated into myotubes for 24 and 48 hours at confluence for 24 and 48 hours while simultaneous being administered Gdf5-shRNA via lipofectamine transfection to knockdown the expression of Gdf5 inside muscle cells. A scrambled shRNA will be used as a control for the experiment. Myotube diameters, protein synthesis rates and muscle cell markers such as Igf-1, MyoG, MyoD, MuRF-1 and Atrogin will be used to assess effects that Gdf5 knockdown has on catabolic and anabolic signaling in muscle cells.

AIM 2. DETERMINE IF *IN VITRO* GDF5 KNOCKDOWN OFFERS PROTECTIVE STATE UNDER MUSCLE WASTING CONDITIONS

The goal for this aim is to determine if Gdf5 knockdown will offer protective benefits against myotube size losses under sarcopenic obese conditions through utilizing previously harvested

sarcopenic obese mice plasma. C2C12 cells will be cultured and differentiated into myotubes prior to administering SO plasma and Gdf5-shRNA. The effect of Gdf5 knockdown on myotubes under muscle wasting conditions will be measured by using the same measures described in Aim 1. The methods will be performed to ensure healthy myotube size and physiological state is maintained.

Chapter 5 – Methodology

C2C12 Culture

C2C12 myoblasts was plated in six-well plates with DMEM media (10% growth media) containing 20% Fetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin for a total volume of 2 mL as previously described (Brown et al., 2018). After plating the cells and administering 10% growth media, the cells were incubated in 5% CO2 at 37C with DMEM media being changed every 48 hours. When cell confluence was reached, the media was changed for cell differentiation. Differentiation media contained 2% horse serum, 1% Penicillin/Streptomycin, 5% HEPES. 0.75% transferrin and 0.75% insulin to promote differentiation of cells as previously shown by Brown et al., 2021.

DNA Plasmid Isolation

Bacterial glycerol stocks of Gdf5 shRNA and empty vector controls were previously purchased from Millipore Sigma. Gdf5 shRNA bacterial stocks were amplified in 250 mL of LB broth overnight before isolated. Isolating DNA plasmids was conducted via a PureLink HiPure Plasmid Filter Maxiprep kit (K211017, Life Technologies, Carlsbad, CA, USA).

Cell Transfection

Cells were transfected with previously isolated DNA plasmid containing a Gdf5 shRNA. Using lipofectamine transfection, a total of 4 µL of Lipofectamine 2000 and 1 µg of plasmid Gdf5 shRNA (Millipore Sigma, Clone IDs: NM_008109.1-969s1c1, NM_008109.1-2028s1c1, NM_008109.1-1641s1c1, and NM_008109.2-1942s21c1) was diluted and incubated for 20 minutes in 50 µL of Opti-mem media for the formation of lipid and DNA complexes. The Opti-

mem media containing lipid and DNA complexes was administered to myoblasts at 70% confluence for 5 hours as previously described (Lee et al., 2016).

Myotube Diameters

Myotube diameters were measured using a Nikon NIS Elements BR software (Melville, NY, USA). Images used to analyze myotube diameters were taken at 10X magnification using a phase contrast microscope (Olympus CKX41, Tokyo, Japan). The pictures were captured using a Nikon Camera Sight DS-Vil. A total of 125 myotube diameters were measured per well in a 6 well plate. The average of the 125 diameters was calculated and used as data for graphical representation. This was previously described by (Brown et al., 2021).

RNA Isolation, cDNA Synthesis, Real-Time Polymerase Chain Reaction (RT-PCR)

Post-differentiated myotubes were briefly harvested and extracted of RNA using 1 mL of Trizol reagent. RNA was isolated using a common RNA isolation kit (Thermo Fisher, PureLink™RNA Mini Kit, Cat#12183025). After isolating, RNA was measured using a BioTek Take3 micro-volume microplate with a BioTek PowerWave XS microplate reader (BioTek Instruments Inc., Winooski, VT) to determine sample concentration. After RNA isolation, cDNA was synthesized by reverse transcription from 1μg of total RNA using a Superscript Vilo cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA). Thereafter, synthesized cDNA was aliquoted into 1:10 and 1:100 dilutions for adequate storage and future analysis. Gene amplification using the Step One Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) was used to measure mRNA abundance of the following TaqMan probe targets: Atrogin-1, MURF1, CyclinD1, MyoD, Myogenin, Gdf5, Dnmt3a, IL-6 and TNF-α. The cDNA was

amplified for the previously mentioned targets using 5 ng of cDNA per fluorescent TaqMan probe in a total volume of 25uL reaction tube that contains TaqMan Universal Master mix (Applied Bio-Systems). Samples were incubated at 95ºC for 4 min before undergoing denaturation, annealing and extension for 45 cycles.

Western Blot

Cell were harvested and prepared as previously described (Brown et al., 2021). Cells were placed in protein loading buffer that contains phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO), 0.01% bromophenol blue with protease inhibitors, 50 mM pH 6.8 Tris HCl, 1% sodium dodecyl sulfate, 10% glycerol, 20 mM dithiothreitol and 127 mM 2-mercaptoethanol. Cells were harvested by using a cell scraper from their respective wells to sterile microcentrifuge tubes. To denature the proteins, the cells were placed in loading buffer and heated at 95C for 5 minutes and centrifuged for an additional 5 minutes at 13000 RPM. To determine protein concentration, an RC/DC assay kit was used. The amount of loaded homogenate protein was determined based on concentration of sample protein and calculated to 40 ug prior to loading into 10% SDS-polyacrylamide gels. Once protein was run through the gels to differentiate the bands of proteins, gels were transferred to polyvinylidene difluoride (PVDF) membranes. Equal loading of proteins was verified by Ponceau S staining. Membranes were then blocked with 5% milk in Tris-buffered saline (TBS) for 2 hours. Primary antibody for anti-Gdf5 (Abcam, ab93855) was used and diluted with 5% milk in TBS. After overnight incubation with primary antibodies, secondary antibodies were diluted at 1:10000 with 5% milk in TBS and membranes were incubated for an additional 50 minutes. The actin band at 45 kDa was used for

normalization and analysis of Gdf5 protein. All membranes were imaged using a LiCor Odyssey FC as previously described (Brown et al., 2021; Lim et al., 2021; Rosa-Caldwell et al., 2020).

Statistical Analysis

An unpaired student's T-test set at an alpha value of 0.05 was used to determine statistical significance. A two-way ANOVA was used to identify main effects of age and diet and whether interactions are present between dependent variables in the performed experiments. If a significant interaction is present, the difference among means will be assessed using Fisher's LSD post-hoc analysis. A two-way ANOVA will be used to analyze pre-planned comparisons of young and aged tibia lengths. Statistical significance will be set at $p \leq 0.05$.

Chapter 6 – Results

In Vitro Gdf5 Knockdown in C2C12 Myoblasts

To determine the most efficient shRNA to knockdown Gdf5, three different short hairpin RNAs were transfected in myoblasts and assessed for Gdf5 protein content. Gdf5 protein content using shRNA 1 was significantly lower with a 62% ($p<0.05$) and 30% ($p<0.05$) decrease in Gdf5 protein expression at the 28 and 55 kDa bands, respectively. In shRNA 2 and shRNA 3, protein content was significantly reduced at 55 kDa by 36% ($p<0.05$) and 27% ($p<0.05$), respectively, when compared to the control group. Additionally, protein content measured at 28 kDa in shRNA 2 and 3 were unchanged. Due to these results and current literature, shRNA 1 was used for the following experiments for its ability to knockdown Gdf5 at 28 kDa and 55 kDa.

Myotube Size and Gdf5 mRNA Abundance in Gdf5 Knockdown Myotubes

Myotube diameters were measured in both experimental groups. There was not a significant difference of myotube size between groups. In the shRNA group, Gdf5 mRNA abundance showed to have increased by 51% ($p<0.05$) when compared to the empty vector control group. Dnmt3a was also measured for its role in regulating Gdf5 gene expression, as previously shown by Hatazawa et al., 2018. Dnmt3a displayed a ~14% ($p<0.05$) increase in mRNA abundance in the shRNA group when compared to the control group.

mRNA Abundance of Cell Cycle, Protein Degradation and Inflammatory Markers in Gdf5 Knockdown Myotubes

CyclinD1, MyoD and Myogenin (MyoG) were measured for mRNA abundance due to their role cell cycle progression. Among experimental groups, Cyclin D1 was not different

between groups. For MyoD, a 17% ($p<0.05$) decrease was observed in the shRNA group when compared to the control group. Conversely, the shRNA group exhibited a 21% increase of MyoG mRNA abundance when compared to control group. Protein degradation markers, MuRF-1 and Atrogin displayed different effects during in vitro Gdf5 knockdown. MuRF-1 showed a 39.2% ($p<0.05$) increase in mRNA abundance in the shRNA group when compared to the control group. Atrogin showed no difference in mRNA abundance between experimental groups. Additionally, inflammatory markers TNF-α and IL-6 were additionally measured and showed no statistical difference between groups.

Chapter 7 - Discussion

During this study, I investigated the role of Gdf5 through in vitro experimentation. A DNA plasmid that encoded for a specific short hairpin RNA that knocks down protein expression of Gdf5 was successfully administered in vitro. Although cell phenotype was not altered, the results still showed Gdf5's ability to alter BMP signaling through measuring relevant gene targets. Additionally, Gdf5's role in sarcopenic obesity has yet to be investigated by present studies. Through measuring Gdf5 mRNA abundance and BMP signaling markers, we observed cellular alterations that offered new insight, while also confirming previous literature on Gdf5. The present findings of this study provided additional evidence and importance for further investigation of Gdf5's interaction in skeletal muscle of sarcopenic obesity.

Identifying a Molecular Weight for Quantifying Gdf5 Protein Content

While investigating Gdf5 protein content for in vitro Gdf5 knockdown, previous literature has shown to measure Gdf5 at varying molecular weights. Previous literature shows Gdf5 protein has been measured at 13 and 28 kDa. The present study used an antibody that was intended to be measured at 55 kDa. The varying molecular weights that Gdf5 protein has been observed at is inconsistent among the literature. In previous literature, contradicting molecular weights of Gdf5 protein seem to be chosen based on the model and tissue being used. A previous publication on long noncoding Myoparr showed to measure Gdf5 protein content at 28 kDA in the tibialis anterior muscle of mice (Hitachi et al., 2019). Other literature measured Gdf5 protein at 13 kDa in thoracic ligamentum flavum cells of humans (Qu et al., 2016). In the present study, Gdf5 was measured at 55 and 28 kDa. These two molecular weights were chosen due to their

combined quantitative difference of Gdf5 protein content when compared to control groups. The study observed significant less Gdf5 content at both 55 and 28 kDa in harvested myoblasts. Mole

Role of MyoD and Myogenin during Gdf5 Knockdown

In the present study, MyoD and Myogenin (MyoG) mRNA abundance showed to be altered when GDF5 expression was knocked down. These transcription factors are myogenic regulatory proteins that play an important role in early differentiation of skeletal muscle. MyoG and MyoD have been previously shown to regulate genes that are required for myoblast fusion via myomaker and myomerger (Adhikari et al., 2021). It can be suggested that these transcription factors work together to maintain their respective expression. As previously studied, MyoG has been shown to inefficiently bind without the presence of MyoD and could therefore have consequential effects on overall myomaker transcription (Cao et al., 2006; Luo et al., 2015). Our results showed MyoD abundance to be significantly decreased while MyoG was increased. In a previous study by Goodman and Hornberger., 2014, they displayed how the presence of GDFs in BMP Signaling play an important part via the inhibition of HDAC4, myogenin and E3 ligases (MuRF1, Atrogin-1, MUSA1) to prevent protein degradation in the muscle. Due to GDF5 mRNA abundance being decreased by knockdown of shRNA, the proposed mechanism of BMP signaling was followed with the present study. A lower presence of GDF5 would allow for intercellular activation of HDAC4 to proceed with the activation of myogenin and E3 ligases to promote protein degradation. An increase of myogenin availability in the cell could be acting in accordance with MyoD expression. The downstream effects of the miscommunication between MyoG and MyoD binding could be negatively affecting myomaker transcription and muscle growth. Further investigation is needed to observe whether myomaker

transcription is affected by GDF5 knockdown. It would be advantageous to see the effects of in vivo GDF5 knockdown to observe the long-term and the intensity of the effect that miscommunication of MyoG and MyoD poses for myomaker transcription.

Gdf5's Role in Sarcopenic Obesity – What is it doing?

Gdf5's role in sarcopenic obesity has still yet to be determined. As previously discussed, the absence of Gdf5 presents favorable data for disrupting BMP signaling and a negative impact on muscle growth. In our preliminary data, the SO mouse showed to have a 26-fold increase in Gdf5 mRNA abundance. The greater mRNA abundance of GDF5 could possibly be acting in a positive role that is mitigating the effects of SO in skeletal muscle. BMP signaling has been mentioned as creating a more "anti-atrophic" effect in skeletal muscle (Sartori et al., 2013; Wang et al., 2014). The compounding effects that sarcopenic obesity poses could be inducing BMP signaling and its anti-atrophic role via the increased gene expression of Gdf5. This anti-atrophic effect needs further investigation to determine if it changes muscle phenotype and what causes its activation in the sarcopenic obese state.

Furthermore, a previous study conducted by Hatazawa et al., 2018, observed increased Gdf5 expression as contributing to atrophy through suppressing satellite cell differentiation and regeneration. Their study's model used denervation and Dnmt3a as upstream factors that are responsible for increased Gdf5 expression in an injured model of mice. A complete knockout of Dnmt3a showed to increase Gdf5 expression. Conversely, in our in vitro model, Gdf5 protein expression was only knock downed post-translationally. Our results indicated that Gdf5 and Dnmt3a mRNA abundance increased. This explains a mechanism that compensates for ineffective translation of Gdf5 protein by the shRNA used in our study. The possible mechanism

of Gdf5 and Dnmt3a's role in muscle regeneration still requires further investigation in sarcopenic obese skeletal muscle. The models being used could help explain Gdf5's differing role among varying muscle wasting models.

Appendix

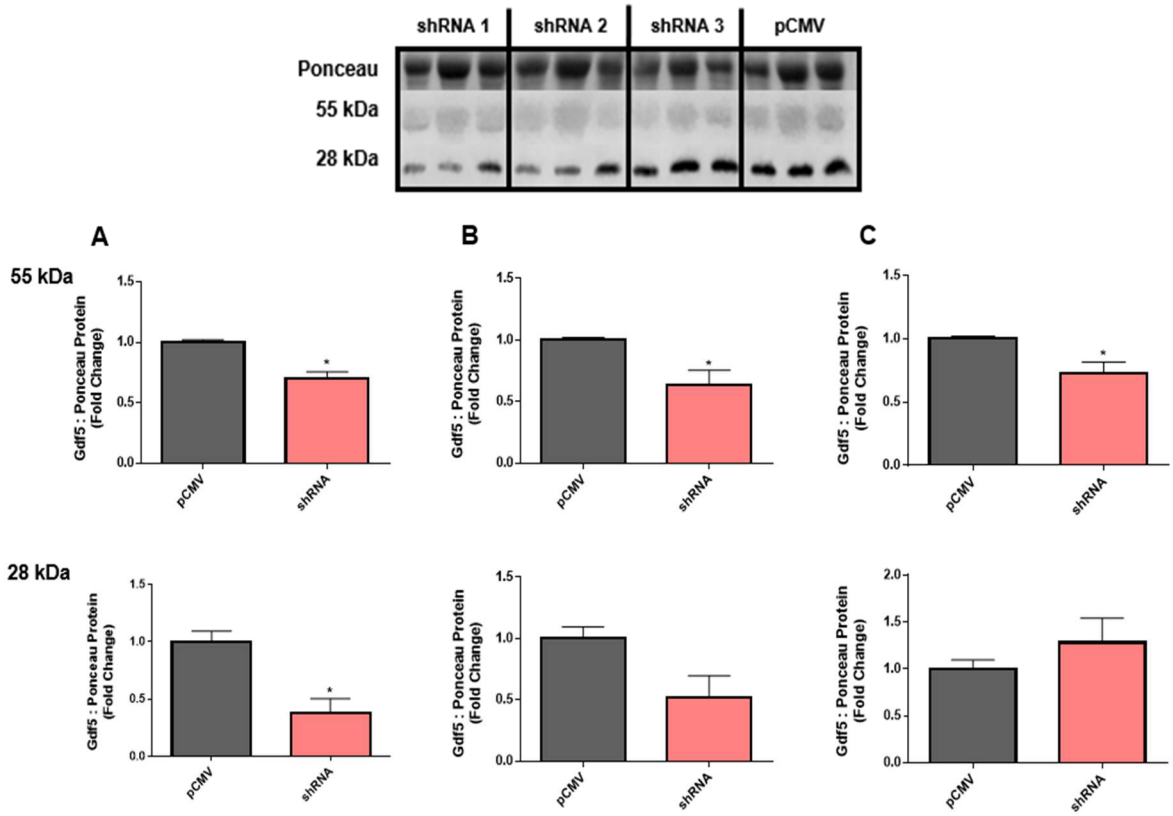

Figure 1. In Vitro Gdf5 Knockdown in C2C12 Myoblasts. A) Gdf5 protein expression measured at 55 kDa and 28 kDa using shRNA 1. B) Gdf5 protein expression measured at 55 kDa and 28 kDa using shRNA 2. C) Gdf5 protein expression measured at 55 kDa and 28 kDA using shRNA 3. Statistical significance between groups denoted by "*" and set at a p=0.05.

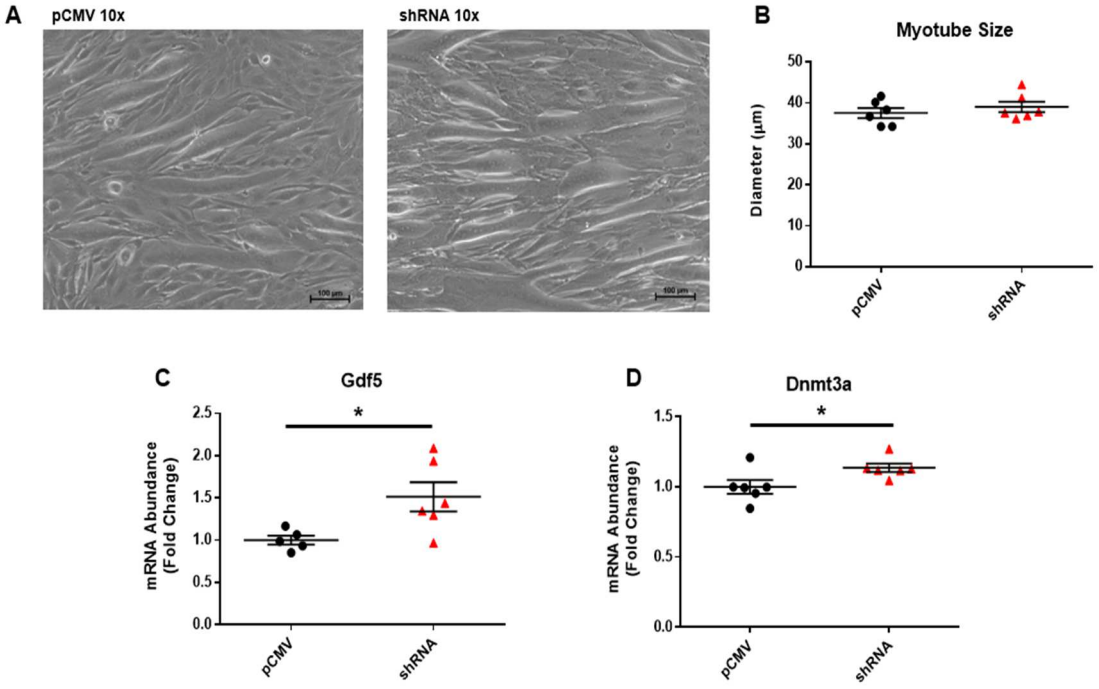

Figure 2. Myotube Size and Gdf5 mRNA Abundance in Gdf5 Knockdown **Myotubes** A) Representative images for pCMV (control) and shRNA groups at 10x magnification. B) Graphical representation of myotube size assessed by measuring myotube diameters between experimental groups. C) mRNA abundance of Gdf5 between control and shRNA group. D) mRNA abundance of Dnmt3a between control and shRNA group. Statistical significance denoted by "*" and set at a p=0.05.

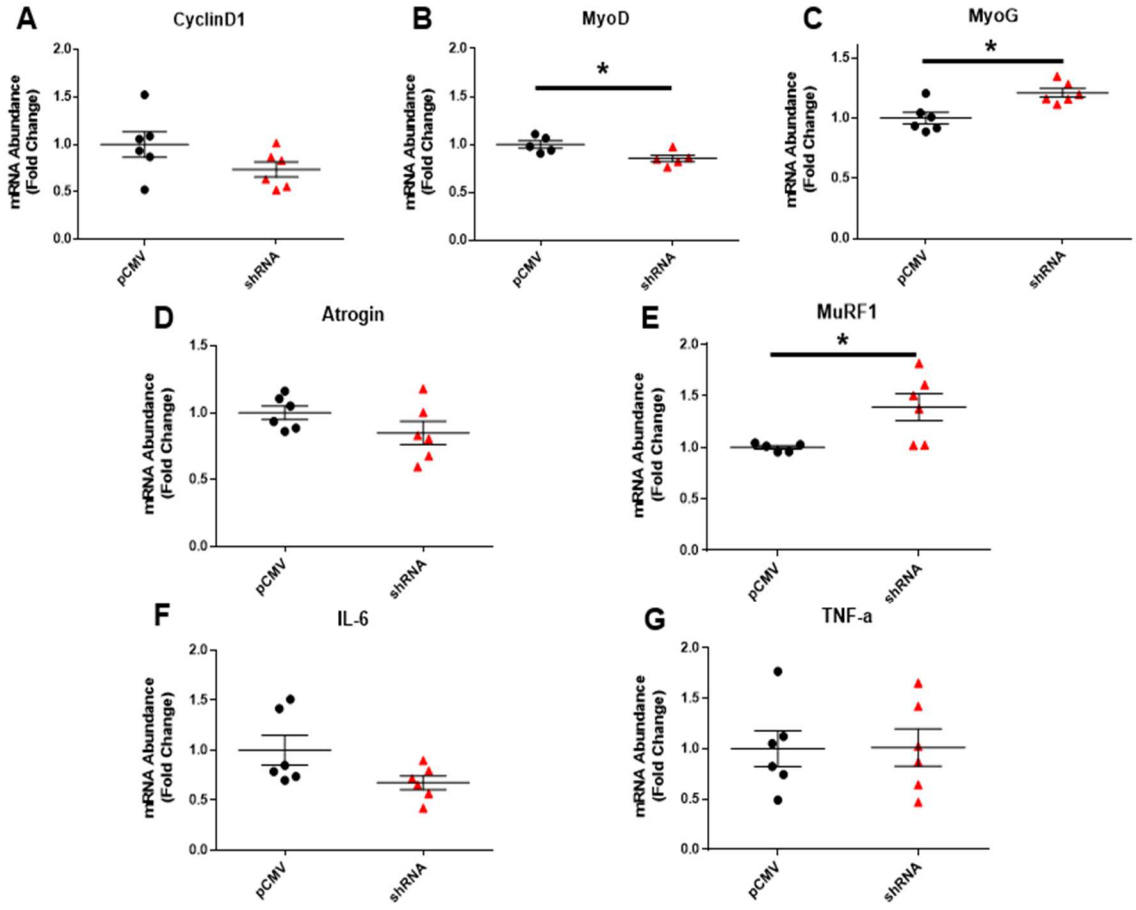

Figure 3. mRNA Abundance of Cell Cycle, Protein Degradation, and Inflammatory Markers in Gdf5 Knockdown Myotubes A) mRNA abundance of CyclinD1 between control and shRNA groups. B) mRNA abundance of MyoD between control and shRNA groups. C) mRNA abundance of MyoG between control and shRNA groups. D) mRNA abundance of E3 ligase, Atrogin, among experimental groups. E) mRNA abundance of E3 ligase, MuRF1, among experimental groups. F) mRNA abundance of inflammatory marker, IL-6, among experimental groups. G) mRNA abundance of inflammatory, TNF-α, among experimental groups. Statistical significance denoted by "*" and set at a p=0.05.

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