AN IN VITRO INVESTIGATION OF THE MOLECULAR EFFECTS OF AGING AND EXERCISE SIGNALING WITHIN HUMAN SKELETAL MUSCLE CELLS

A DISSERTATION

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BY

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DEDICATION

For my parents, siblings, extended family, and friends that have been with me throughout this journey.

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ABSTRACT

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The older population is steadily increasing and is expected to double by the year 2050. With this comes the increase in prevalence of chronic disease, sarcopenia being of utmost concern in regard to skeletal muscle (SKM) health. Sarcopenia is associated with additional morbidities, including insulin resistance, resulting in the reduced quality of life in these individuals. Resistance exercise is a common form of treatment for sarcopenia to attenuate the loss of muscle mass but the quality of the muscle may still be suboptimal due to potential mitochondrial dysfunction and oxidative stress.

The purpose of the study was to invesitgate the molecular effects of aging in myoblasts related to cell homeostasis, myogenesis, and mitochondrial biogenesis and whether general exercise signaling via the β-2 adrenergic receptor (β2AR) could rescue the dysregulated signaling resulting from aging.

Human SKM myoblasts underwent an *in vitro* continued passaging protocol to represent aging *in vivo*. Formoterol (FORM), an exercise mimetic, was used to stimulate general exercise signaling. Four conditions were included in this study (n = 6), control (CON), aging (AGED), CON with FORM stimulation (CON+FORM), and AGED with FORM stimulation (AGED+FORM). Extraction of total RNA was conducted once

myoblasts reached 85% confluency and was followed by quantitative polymerase chain reaction (qPCR) analyses. Gene expression was analyzed and represented the following categories: (a) cell homeostasis, (b) myogenesis, and (c) mitochondrial biogenesis. The delta-delta cycle threshold (ΔΔCT) method was used to normalize genetic expression followed by one-way anaylysis of variance (ANOVA) for each gene with significance set at $p < .05$.

Aging resulted in a significant increase in atrophy-related gene expression only in addition to non-significant robust effects on genes associated with oxidative stress, protein synthesis, autophagy, substrate utilization, myogenesis, and mitochondrial biogenesis. Formoterol stimulation significantly increased genes related to mitochondrial biogenesis and atrophy in addition to significantly downregulated myogenesis. Additionally, FORM stimulation seems to reduce oxidative stress, though not significantly. Taking all findings into consideration, general exercise signaling representative of combined aerobic and resistance exercise may be beneficial in older individuals to reduce oxidative stress leading to improved intercellular signaling across pathways important for overall SKM health.

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CHAPTER I

INTRODUCTION

The human life expectancy in the United States has increased steadily since 1959 from 69.9 to 78.9 years old as of 2016 (Woolf & Schoomaker, 2019) as a result of advancements in healthcare and reduced exposure to risk factors (i.e., tobacco and high blood pressure; Mathers et al., 2015). As a result of these advancements, the United States population of 65-year-olds and older is predicted to double by the year 2050 in the United States (Ortman et al., 2014). The increase in life expectancy and total older population results in an increase in the incidence for chronic diseases, such as sarcopenia. Sarcopenia is the age-associated and involuntary decline in skeletal muscle mass that is associated with a subsequent decrease in strength, metabolic rate, aerobic capacity, and functionality (Lang et al., 2010). Sarcopenia is characterized by a marked decline in muscle fiber size and number, with type II muscle fibers primarily affected by these changes (Lexell, 1995). This progressive decline of muscle mass begins after the age of 30 years at a rate of 0.5-1% per year with this rate accelerating after 70 years of age to ~2% per year (Kim & Choi, 2013). These rates can amass to a 40-50% total muscle mass loss in a lifetime (Deschenes, 2004; Frontera et al., 2000; Janssen et al., 2000) along with a linear decrease in bone mineral density (Bijlsma et al., 2013). Muscular strength also becomes impaired as we age with a 1.5% decrease in strength per year between the ages of 50 and 60 years old and a 3% decrease every year afterwards (von Haehling et al., 2010).

The most widely used diagnosis criteria for sarcopenia is based on the Baumgartner criteria (Baumgartner et al., 1998). This criterion utilizes appendicular skeletal muscle mass (ASM) in the following equation to diagnose sarcopenia:

$$x = \frac{ASM(kg)}{Height(m)^2} \quad (1)$$

A clinical presence of sarcopenia is evident when the value is greater than 2 standard deviations below the mean of a young reference group. Organizations dedicated to the science and prevalence of sarcopenia have adopted a version of this equation using appendicular lean mass (ALM) in place of ASM or replaced the defining criteria (Morley et al., 2011; Muscaritoli et al., 2010). Additionally, few organizations have adopted muscle function tests to assess muscle strength or physical performance, which are subject to decline in response to reduced muscle mass. Grip strength assessment is a common tool to assess muscle strength whereas gait speed is used to assess physical performance. Subsequently, the severity of sarcopenia is also defined by the European Working Group on Sarcopenia in Older People (EWGSOP) using these defining criteria.

Dependent upon which diagnosing criteria is used, sarcopenia is estimated to be present in 25-45% of the United States older population (Cruz-Jentoft et al., 2014; Diz et al., 2015; Shafiee et al., 2017) with the highest age-related prevalence of sarcopenia being ~50% in 80-year-olds and older (Baumgartner et al., 1998). However, sarcopenia is not routinely screened for in clinical practices due to the unavailability or cost of recommended equipment with most cases remaining unidentified and untreated (Yu et al., 2016). The overall prevalence of sarcopenia is similar in both men and women

(Iannuzzi-Sucich et al., 2002; Shafiee et al., 2017). In the United States, Hispanics have a higher prevalence whereas non-Hispanic Black people have the lowest prevalence of sarcopenia (Du et al., 2018). This is due to the fat composition where non-Hispanic Black people have the lowest percent fat and Mexican Americans have the highest percent fat across ethnicities comparatively (Heymsfield et al., 2016). Briefly, adipose tissue and SKM are interconnected and distribute signals to each other that can influence the health or function of the other tissue. Adipose tissue is a major producer of inflammatory cytokines that when concentration is increased in circulation, dysfunction can occur in the individual across systems (Kim et al., 2014). The increase in percentage fat composition in addition to a reduction in skeletal muscle (SKM) mass in sarcopenic individuals is harmful and can cause dysfunction at the molecular level in SKM (Buch et al., 2016).

Individuals with sarcopenia are twice as likely to be hospitalized as those without, resulting in an estimated yearly economic cost of $40.4 billion in the United States (Goates et al., 2019). The origin of sarcopenia is multifaceted and can include the following factors: acute or chronic disease, physical inactivity, chronic low-grade inflammation, endocrine dysfunction, inflammation, nutrient deficiencies, and increased immobilization due to disease or hospitalization (Cruz-Jentoft et al., 2014; Kortebein et al., 2008). Additionally, sarcopenia is associated with decreased self-reported quality of life (QoL), poor balance, increased risk of falls, reduced activities of daily living (ADLs), and increased mortality rates (Baumgartner et al., 1998; Beaudart et al., 2018; Benjumea et al., 2018; Ferrucci et al., 1997; Landi et al., 2012; Larsson et al., 1979; Metter et al.,

2002). Furthermore, sarcopenia can contribute to the development of adverse health conditions such as insulin resistance, disability, fatigue, rheumatoid arthritis, and frailty (Dufour et al., 2013; Giles et al., 2008; Marsh et al., 2011; Xue et al., 2011). Efforts involving this population should focus on improving QoL, reduce disability and dependency, support healthy aging, improve or maintain functionality, and reduce the risk of chronic disease. Exercise is often recommended to facilitate these improvements; however, information regarding exact exercise methods and modes have not been fully elucidated to provide effective and specific recommendations to this population. Understanding underlying molecular interactions between SKM protein atrophy and synthesis in the context of concurrent exercise signaling may illuminate this issue more clearly.

Metabolic and Functional Impact of Sarcopenia

SKM is an important metabolic regulator along with providing movement as it accounts for up to 60% of body mass and any loss of either component is detrimental to overall health. The mitochondrion is an important regulator of redox homeostasis and cellular quality control and is negatively affected by aging. Reactive oxygen species (ROS), DNA damage, and denervation increases with age, resulting in increased mitochondrial stress within SKM cells (Correia-Melo et al., 2016; Spendiff et al., 2016). The mitochondrial free radical theory of aging (MFRTA) suggests oxidative damage to mitochondrial DNA (mtDNA), due to its proximal location to the electron transport chain (ETC), plays a primary role in the aging process and can result in defective ETC components leading to decreased adenosine triphosphate (ATP) production and increased

ROS generation (Miquel et al., 1980). The defection of ETC components is present in 6% of muscle fibers of the *vastus lateralis* in 49-year-olds compared to 31% in 92-year-olds, thus supporting MFRTA (Bua et al., 2006). Additionally, Short et al. (2005) described a positive correlation in mtDNA abundance and age in the *vastus lateralis* along with mitochondrial biogenesis impairment and reduced aerobic capacity. The ROS generated mitochondrial stress described above results in the opening of the mitochondrial transition permeability pore (mPTP) leading to the leakage of mitochondrial contents into the cytosol. The accumulation of mitochondrial contents in the cytosol of cells initiates apoptotic signaling resulting in controlled cell death, thus fewer SKM fibers. The accumulation of damaged mitochondria contributes to sarcopenia due to a decrease in mitochondrial clearance by mitophagy that occurs with age and results in an exacerbation of SKM loss via apoptosis (Alway et al., 2017).

Strength is the most widely associated functional component of sarcopenia that decreases with a rate three times greater than SKM mass loss due to changes of the neuromuscular system (Goodpaster et al., 2006). One such change is the irreversible, progressive neuron loss that is associated with aging. A bundle of SKM fibers innervated by a single alpha motor neuron make up a motor unit with multiple motor units occupying a single muscle. The recruitment of these motor units' function to perform movement or overcome an external load, such as weightlifting. When the alpha motor neuron is eradicated, the muscle fibers of the existing motor unit are denervated resulting in a decline in the number of neuromuscular junctions that are crucial to muscle contraction (Chai et al., 2011; Verdijk et al., 2012). *In vitro* analyses reveal an increase in

apoptosis of SKM satellite cells after muscle denervation with maintained elevated levels of caspase activity and DNA fragmentation for 10 weeks (Jejurikar et al., 2002). Similar results were repeated in an animal model of various ages (Jejurikar et al., 2006). Satellite cells are responsible for muscle growth, regeneration, and the repair of injured SKM by rapidly proliferating post-injury under normal conditions (Kuang et al., 2008). The disruption of satellite cell function as a result of denervation is a potential origin of sarcopenia by way of apoptosis and can result in impairing the regeneration process following injury.

Apoptosis/Atrophy Regulation and the Impact on SKM

Sarcopenia is the result from the disproportionate molecular increase in protein degradation and decrease in protein synthesis. Protein anabolism is stimulated through the PI3K/Akt pathway which subsequently stimulates the upregulation of mammalian target of rapamycin (mTOR), a factor essential for protein synthesis. Subsequently, FOXO3 is phosphorylated resulting in its inactivation and inhibits protein degradation by reducing the expression of atrogin-1 (ATG1; also known as MAFbx), a primary target for SKM atrophy (Clavel et al., 2006; Kandarian & Jackman, 2006). Apoptotic pathways act as a protective measure to rid damaged or stressed cells and play an important role in the regulation of the ratio of protein anabolism to catabolism along with the emergence of sarcopenia. Apoptosis increases with age (Hao et al., 2011) and contributes to the reduction in regeneration capabilities of skeletal muscle in addition to muscle atrophy (Garcia-Prat et al., 2013). The mitochondria are responsible for regulating the apoptotic pathway via p53/Bax/Bak and Bcl-2. The upregulation of the p53/Bax/Bak pathway

results in the stimulation of the apoptotic pathway while Bcl-2 inhibits the upregulation of p53/Bax/Bak in the mitochondria and prevents the release of cytochrome c from the mitochondria via mPTP openings. Damage to the DNA, which is a common consequence of aging, is a common stimulator of p53/Bax/Bak apoptotic regulation. Two apoptotic pathways occur in SKM by p53/Bax/Bak upregulation: caspase-dependent pathway and caspase-independent pathway. The caspase-dependent apoptotic pathway occurs due to the leakage of cytochrome c via the mPTP resulting in the upregulation of Apaf-1 leading to a cascading effect of the activation of pro-caspases and caspases that initiates apoptotic cell death. The caspase-independent pathway is upregulated via the release of apoptotic inducing factors (AIF) from the mitochondria that translocates to the cytosol and travels to the nucleus where it induces DNA fragmentation and chromatin condensation, resulting in apoptotic cell death (Kiraz et al., 2016). In turn, FOXO3 phosphorylation upregulates Bcl-2 expression, thus downregulating the apoptosis pathways and is a potential target in preserving SKM mass. In healthy conditions, AIF protects satellite cells from oxidative stress when housed in the intermitochondrial membrane and helps to maintain stem cell number and activation in SKM (Armand et al., 2011).

SKM atrophy is the loss of muscle mass that is regulated by specific genes and can occur as a result from apoptosis. Atrophy is a primary factor in the development of sarcopenia that is influenced by similar stressors, such as inflammation, denervation or neural inactivity, immobilization, and ROS. The molecular pathways involved in SKM atrophy are largely unexplored, with ATG1 and muscle RING finger-1 (MuRF1) being the most well-known targets involved with the process. Atrogin-1 and MuRF1 are E3

ligases expressed in all muscle tissues operating as regulators of muscle mass. The expression of MuRF1 increases after birth and the deletion of the associated gene results in the attenuation of muscle loss (Baehr et al., 2011; Labeit et al., 2010; Perera et al., 2012). These genes are required for SKM atrophy and is positively correlated with aging (Bodine & Baehr, 2014). The atrophy pathways involving ATG1 and MuRF1 are activated in response to FOXO transcription factors by way of increased glucocorticoid receptor activity under stress-related conditions. Alternatively, insulin and IGF-1 activity along with low glucocorticoid levels downregulates FOXO transcription factors thus repressing MuRF1 and ATG1 (Ebert et al., 2019). The pathway as a whole that influence the activation of MuRF1 and ATG1 is not completely understood and is of interest within the SKM field to develop therapeutic approaches to combat atrophy. One such therapeutic approach could involve the use of exercise in downregulating MuRF1 and ATG1 to preserve muscle mass.

Aging and Sarcopenic Effects on Cellular Pathways

Interconnected pathways work to maintain homeostasis and fend against unwarranted factors that work to disrupt the health of a cell (see Figure 1). Chronic exposure to factors such as exacerbated stress (i.e., radiation) and ROS reduce the integrity of these pathways, which is profound in older and sarcopenic individuals (see Figure 2). This section focuses on normal basal stimulation and altered expression seen in older and sarcopenic individuals for genes of common cellular pathways involved in SKM health.

Figure 1

Cellular pathways responsible for maintaining the health of skeletal muscle cells.

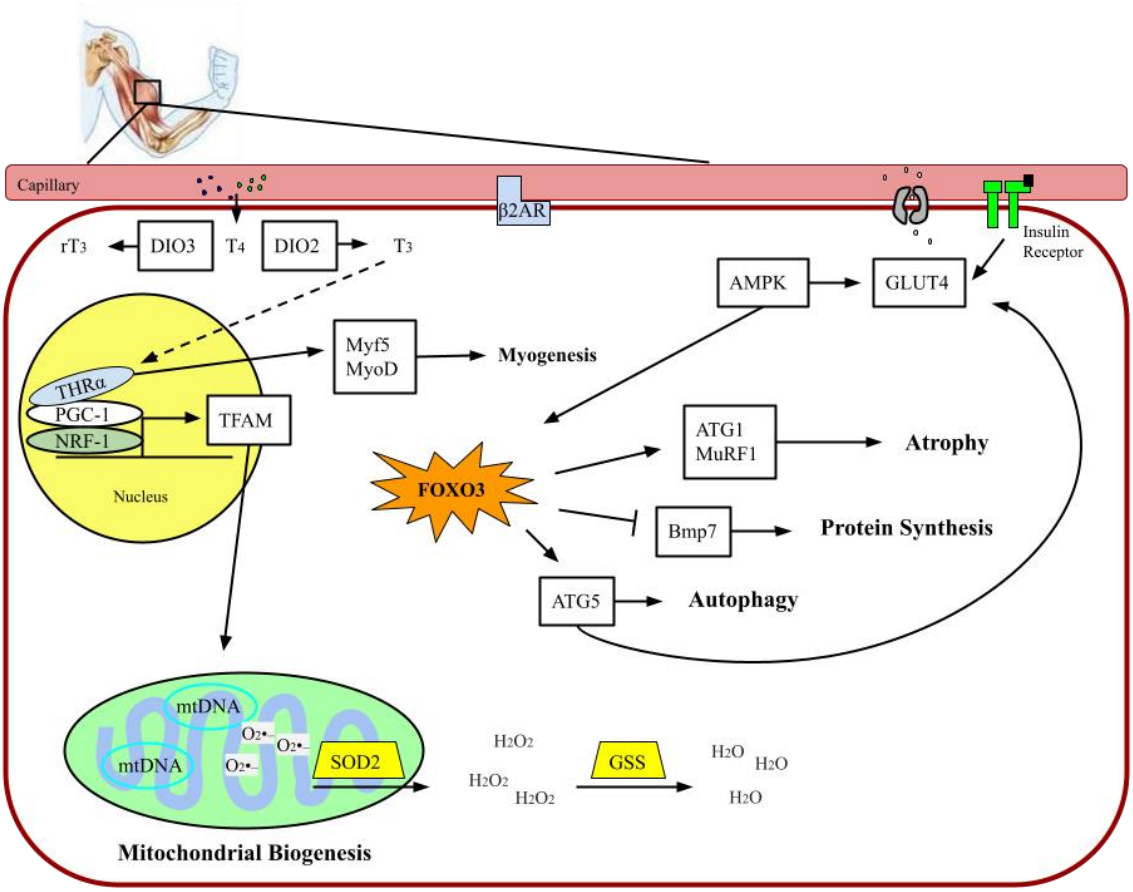
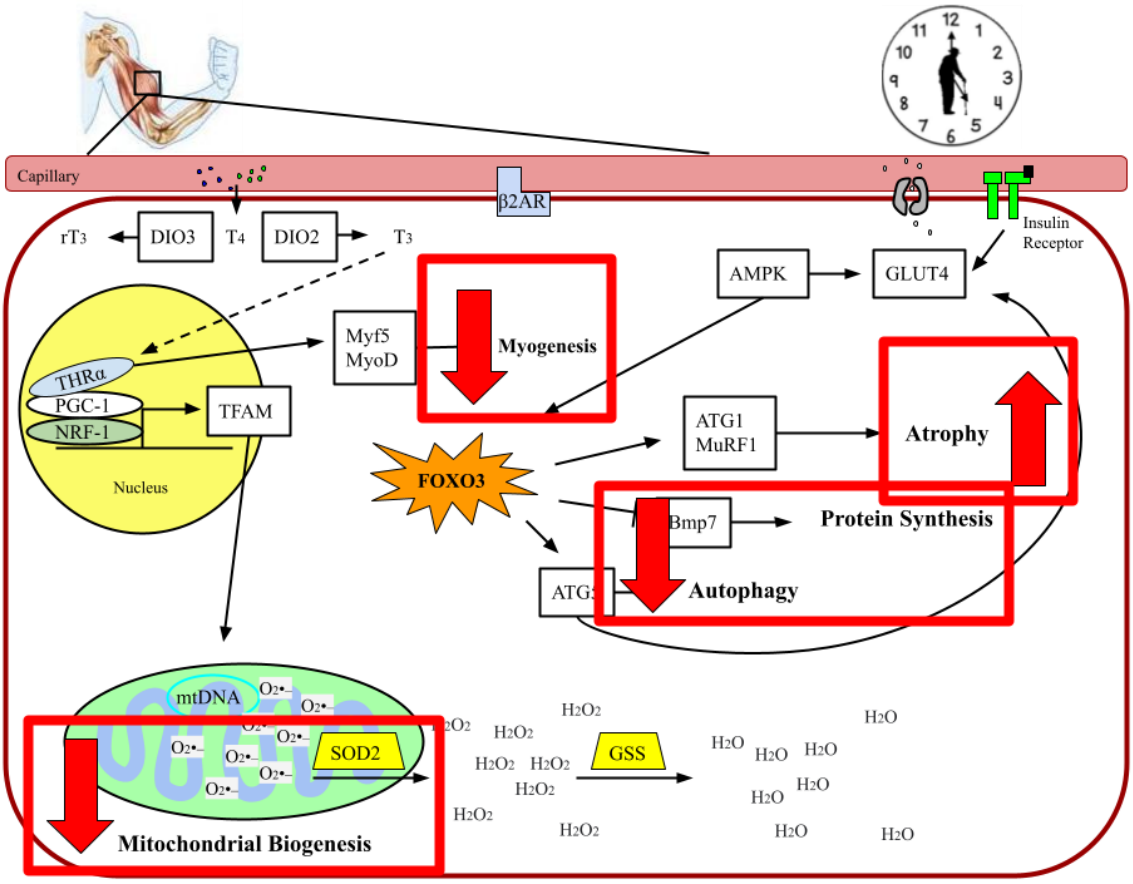


Figure 2

Aging effect on cellular pathways vital to SKM health.



Cell Homeostasis

Cells undergo stress regularly and upregulate specific genes to maintain a homeostatic balance. Once cells defer from a homeostatic balance and are unable to regain homeostasis, these unhealthy cells may undergo certain cellular functions such as apoptosis, senescence, or autophagy. Alternatively, some genes are necessary for normal cell function. The following provides examples of genes that work to maintain homeostasis and normal cell function.

The expression of FOXO3 is under the control of the PI3K/Akt pathway, as previously described, and is a regulator of insulin signaling, protein turnover, cell survival, and cell death (Stefanetti et al., 2018). During anabolic conditions FOXO3 is inhibited and in turn downregulates the age-related SKM protein degradation genes, ATG1 and MuRF1. Diseases such as muscular dystrophy and chronic obstructive pulmonary disease express high levels of FOXO3 and is a primary drive for muscle wasting in these disease states. The mRNA expression of FOXO3 has an aging effect in SKM being upregulated in older individuals (Mikkelsen et al., 2017). Alternatively, FOXO3 nuclear protein levels are decreased in old human SKM but no change in phosphorylated FOXO3 protein expression is indicated in previous studies (Drummond et al., 2014; Leger et al., 2008; Stefanetti et al., 2014), suggesting upstream regulators of FOXO3 play a major role in the prevention of muscle atrophy in older individuals (i.e., PGC-1α and the PI3K/Akt pathway).

Autophagy also plays a major role in cell health by removing damaged components of the cell and recycling functional components for proximal healthy cells. Autophagy-related gene 5 (ATG5) is an important regulator of cellular autophagy during embryonic development, cell differentiation, and homeostatic maintenance. In older individuals, ATG5 is downregulated at the molecular transcriptional level leading to a reduced propensity to manage cellular stress and potential increase in cancer incidence (Lipinski et al., 2010; Rouschop et al., 2010). Alternatively, ATG5 overexpression is correlated to prolonged lifespan in mice along with enhanced activation of autophagy (Pyo et al., 2013) suggesting a potential target in humans to prolong a quality lifespan.

Additionally, insulin resistance was improved in this study and it is suggested that the upregulation of autophagy may act as a transporter for glucose transporter type 4 (GLUT4), an important transporter for glucose uptake for energy, as both undergo similar pathways (Elhassan et al., 2018). The activation of phosphoinositide 3-kinase (PI3K) and AMP-activated protein kinase (AMPK) regulates both aforementioned processes, suggesting exercise to be a potential therapeutic approach.

Fatty-acid oxidation and mitochondrial biogenesis is regulated by AMPK to accommodate increased energy demands in SKM in response to reduced ATP stores, therefore affecting the activity of countless SKM genes. The activity of AMPK is reduced with age leading to reduced mitochondrial function and fat oxidation (Reznick et al., 2007). Additionally, there is an increase in oxidative stress, apoptotic resistance, inflammation, and hyperglycemia (Salminen & Kaarniranta, 2012). Over time, AMPK is suggested to lose its ability to respond to factors that would generally increase its activity (i.e., exercise). This in turn may be a key factor in describing the deploring nature of aging and sarcopenia as it serves as a target in response to exercise signaling within this population.

Myogenesis

Myogenesis is the process of forming muscle tissue and involves satellite cells, which remain quiescent until activated and differentiate into myofibers being highly active during embryonic development (Yan et al., 2013). Myogenesis is activated in response to damage of mature muscle, seen as a result of exercise or physical injury to

the muscle, leading to the activation of quiescent cells that proliferate and differentiate to restore homeostasis (Caldow et al., 2015; Musaro, 2014; Rudnicki et al., 2008). The transcription of paired box protein-7 (Pax7) and myogenic regulatory factors (MRF) is responsible for the regulation of myogenesis. Myogenic factor 5 (Myf5), myoblast determination protein-1 (MyoD), and myogenin (MyoG) are such MRFs involved in the processes of proliferating and differentiating myoblasts into myotubes (Bentzinger et al., 2012). Firstly, Pax7 upregulation activates satellite cells and commits these cells to the myofiber lineage. Subsequently, Myf5 is responsible for the early activation and proliferation of committed satellite cells to develop into myoblasts. The upregulation of MyoD is involved in the proliferation of committed satellite cells along with early differentiation of myoblasts into myocytes. Alternatively, MyoG is responsible for terminal differentiation of myocytes into myotubes as a result of the fusion of myocytes. Myoblasts are important in the regenerative process of damaged SKM by way of fusion with existing myofibers or the formation of new myofibers. Myomaker and Minion-Myomerger are recent discoveries that are critical in the fusion process of myogenesis with MyoD and MyoG being primary activators of these genes (Millay et al., 2014; Sampath et al., 2018). Myomaker and Minion-Myomerger are highly active during embryonic development and cease expression shortly after childbirth but are activated in response to muscle injury, such as in response to exercise.

There is an age-associated decline in $Pax7^+$ satellite cells as a result of increased apoptotic signaling (Shefer et al., 2006) and is most profound in type II fibers (Suetta et al., 2013). The number of $Pax7^+$ satellite cells per myofiber of old individuals is nearly

half those in young counterparts (Carlson et al., 2009; Day et al., 2010). Alternatively, human muscle biopsy studies have provided conflicting results on the mRNA expression of Pax7. Drummond et al. (2011) and Mikkelsen et al. (2017) reported reduced Pax7 expression in older men compared to young counterparts with an age-related effect, whereas Suetta et al. (2013) reported no difference in Pax7 expression between young (mean = 24.4-year-olds) and old men (mean = 67.3-year-olds). Bigot et al. (2008) conducted a study to determine the differences between young human myoblasts and senescent myoblasts *in vitro*. This study found that senescent myoblasts that were passaged to the end of cell cycle life were able to differentiate into myotubes but were smaller than the young cell counterparts, indicating a potential mechanism to reduced muscle size in sarcopenic adults. Additionally, the expressions for MyoD, Myf5, and MyoG were significantly lower and delayed in the senescent cells compared to the young cells during differentiation. Contradictively, Suetta et al. (2013) reported no differences in MyoD and MyoG between groups. The described differences may be partially explained as Suetta et al. (2013) did not screen participants for physical activity prior to the initiation of the study. Additionally, sarcopenia was not screened in the aforementioned studies. In a recent sarcopenia muscle biopsy study, Pax7 and MyoG were upregulated only in old sarcopenic adults compared to middle-aged counterparts with no difference in MyoD and Myf5 (Brzeszczynska et al., 2018). This was the only study involving sarcopenic adults in the analyses upon an extensive research article search uncovered. These conflicting results for both *in vivo* and *in vitro* in conjunction with a lack of research involving the aging and sarcopenic populations warrant further

investigation in addition to examining the influence of exercise stimulation on myogenesis in sarcopenic adults.

Mitochondrial Homeostasis

Mitochondria are important in regulating cellular processes involved with energy production, calcium signaling, and apoptosis. The MFRTA theory, as previously described, is suggested to play a major role in SKM aging as oxidative damage to mtDNA increases with age and results in dysfunctional mitochondria potentially leading to the development of sarcopenia (Joseph et al., 2013). Mitochondrial biogenesis involves the synthesis of new mitochondria and increase in mitochondrial mass. The driving genetic factor in activating and managing the mitochondrial biogenesis process is peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which is upregulated in response to aerobic exercise. Type I SKM fibers have a higher PGC-1α expression versus type II SKM fibers (Lin et al., 2002) and can potentially help to combat dysfunctional cellular processes involved with aging, such as apoptosis. Alternatively, peroxisome proliferator-activated receptor gamma coactivator 1-beta (PGC-1β) performs in a similar manner as and is stimulated by the same regulatory factors as PGC-1α in regulating mitochondrial biogenesis (Arany et al., 2006; Zechner et al., 2010), resulting in an alternative target for maintaining mitochondrial homeostasis and promoting mitochondrial biogenesis.

Basal PGC-1α expression is lower in older versus younger counterparts in human (Ling et al., 2004) and animal models (Baker et al., 2006; Vina et al., 2009). Subsequent

activation of mitochondrial related genes such as nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (TFAM) are in turn lower in older versus younger counterparts. Over time, this leads to a reduction in mitochondrial biogenesis and an inability to maintain an optimal number and mass of mitochondria in SKM. Consequently, this results in increased mtDNA mutation replication and accumulation, increased mitochondrial fusion, decreased mitochondrial fission, and reduced ability for mitophagy as seen in sarcopenia (Rygiel et al., 2016). Additionally, the reduced PGC-1α activity in older adults is a driving factor for age-associated insulin resistance and reduction of type I oxidative fibers (Lowell &, Shulman, 2005; Petersen et al., 2004), thus further supporting the need for treatment options in increasing mitochondrial biogenesis and improving mitochondrial health by PGC-1α/β regulation.

Thyroid Hormone Metabolism

Thyroid hormone (TH), or active T_3, exhibits palpable effects on cell signaling pathways that regulate metabolism, function, and myogenesis in SKM (Mullur et al., 2014). Unbound T_3 and its inactive form T_4 can freely enter the SKM cell, with T_3 being the only form that can bind to nuclear targets within the cell. Inactive T_4 can be converted into active T_3 by iodothyronine deiodinase 2 (DIO2) or into reverse T_3 by iodothyronine deiodinase 3 (DIO3), which is an inactive form of TH that is unable to bind to nuclear targets. The conversion of T_4 to T_3 via DIO2 is ultimately important due to the regulatory effects T_3 exhibits for SKM health, especially when circulating T_3 is in underabundance to be translocated into SKM cells. The molecular influence of TH begins with the binding of T_3 to thyroid hormone receptor alpha (THRα) at the nucleus in SKM (Brent, 2012),

which then stimulates gene transcription related to SKM metabolism, function, and myogenesis (Kupr et al., 2017). The activation of THRα stimulates PGC-1α as a result of the binding of active T_3 to the nuclear receptor, thus being a major influencer for mitochondrial biogenesis and metabolism. Additionally, sarcoendoplasmic reticulum calcium-ATPase 1 and 2 (SERCA1/2) regulates calcium homeostasis in SKM and are a prime target of T_3 for contractility, or to produce movement. The availability of T_3 directly regulates MyoD, resulting in the proliferation of committed satellite cells to produce more myoblasts for growth and repair, or myogenesis. Taking these processes into consideration, TH metabolism is important in the overall regulation of SKM health with DIO2 being a primary target to increase the concentration of T_3.

Aging is positively correlated with the circulating concentration of rT_3 and results in the low concentration of T_3 (van den Beld et al., 2005). Additionally, physical function is reduced within this population and is correlated with higher rT_3 and T_4 concentrations. High rT_3 is thought to be a result from decreased peripheral TH metabolism, as in from SKM, and is potentially indicative of catabolism. This leads to the assumption that TH metabolism plays a major role in the apoptotic signaling pathway and may be a major influencer of SKM atrophy and sarcopenia. Exercise can directly influence the metabolism of TH due to the stimulation of DIO2 via the activation of AMPK and the cAMP-PKA pathways (Egan et al., 2010; Lira et al., 2010). Thus, exercise may be a vital therapeutic approach to maintaining and promoting SKM health as a result of TH metabolism's influence on overall cellular homeostasis.

Formoterol and Dose Responses in SKM

Numerous exercise mimetics exist that mimic pathways involved in aerobic and resistance exercises seen in animal models and humans. One such exercise mimetic, Formoterol (FORM), performs in a manner similar to combined aerobic and resistance exercise, or concurrent training. Formoterol is a β_2-adrenergic receptor (β2AR) agonist that upregulates the cAMP-PKA pathway (Miura et al., 2007) with subsequent effects on PGC-1α leading to increases in mitochondrial biogenesis, cellular respiration, and reduced inflammation (Duplanty et al., 2018; Wills et al., 2012). Preliminary results from our lab based on a human SKM mature myotube cell culture model and *in vitro* FORM stimulation exhibited similar upregulation of PGC-1α and genes involved in myogenesis, mitochondrial biogenesis, and antioxidative properties as previous studies.

This study is the culmination of several projects over the last two years. Firstly, previous *in vitro* studies performed in our lab worked to optimize a human SKM cell culture model and characterize the effects of FORM on gene expression related to myogenesis, mitochondrial biogenesis, and homeostatic cell signaling in human SKM myotubes (Duplanty et al., in preparation for submission). This model involves the comparison of SKM cells during mid-myogenesis (Day 4 of myotube differentiation) and terminal differentiation (mature myotubes at Day 6) to illicit the stages of *in vivo* growth and repair in response to exercise mimetic stimulation. Secondly, our lab has explored a representative pilot model of aging and sarcopenia in human SKM myoblasts. For this pilot study, a human SKM myoblast cell line was expanded beyond what is typically considered a mature and healthy stage of cell passage (passage 7). This was done to

generate specific characteristics commonly associated with physiological aging (AGED, *n* = 2; passage 17) and were compared to healthy myoblasts (CON, *n* = 3; passage 5). The quality of cellular proliferation and passaging was calculated using validated formulas for senescence from previous literature (Bonab et al., 2006; Chen et al., 2013). Genetic analyses from this pilot study exhibited characteristics of aging SKM, such as reduction in cell homeostasis (see Figure 3), abnormal myogenic signaling (see Figure 4), reduced mitochondrial homeostasis (see Figure 5), and reduced thyroid hormone metabolism (see Figure 6). The feasibility of the current study is supported by the evidence produced from the previous studies conducted in our laboratory.

Figure 3

Preliminary Results – Cell Homeostasis.

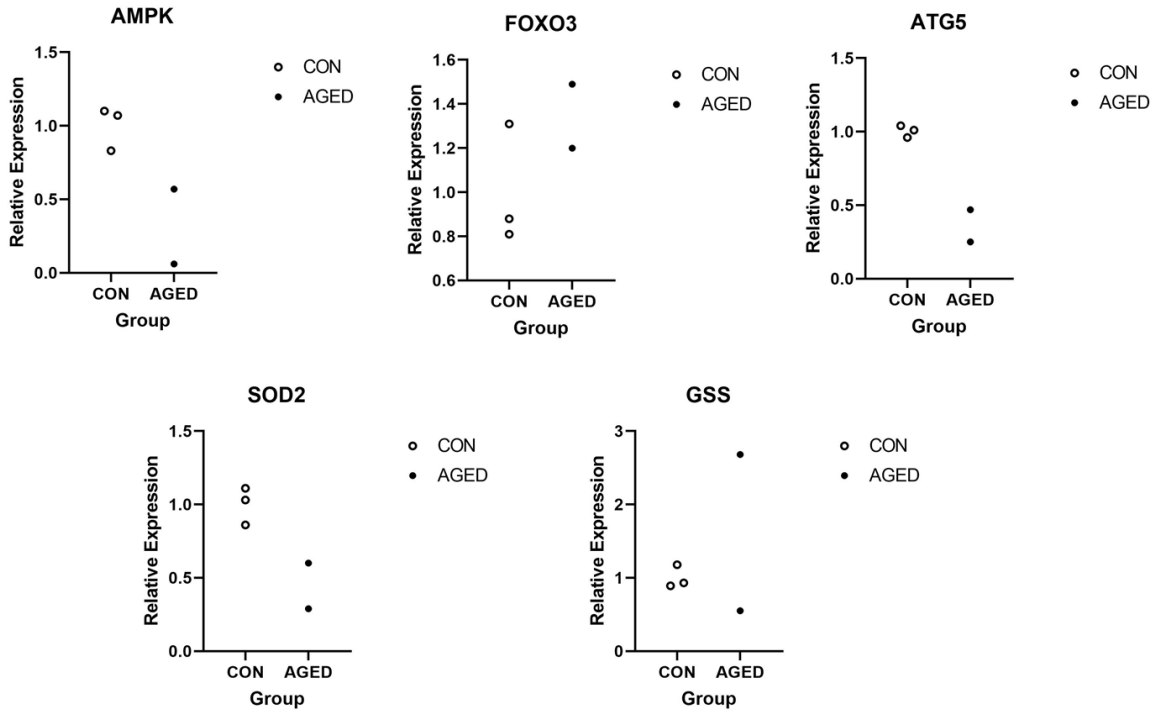


Figure 4

Preliminary Results – Myogenesis.

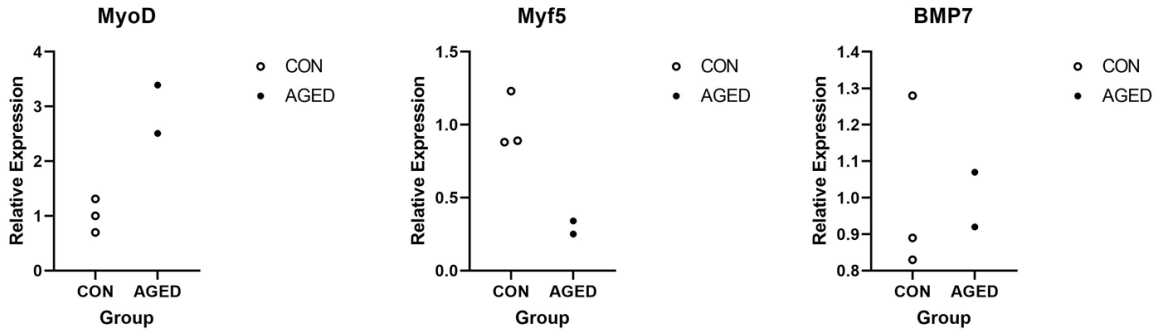


Figure 5

Preliminary Results – Mitochondrial Homeostasis.

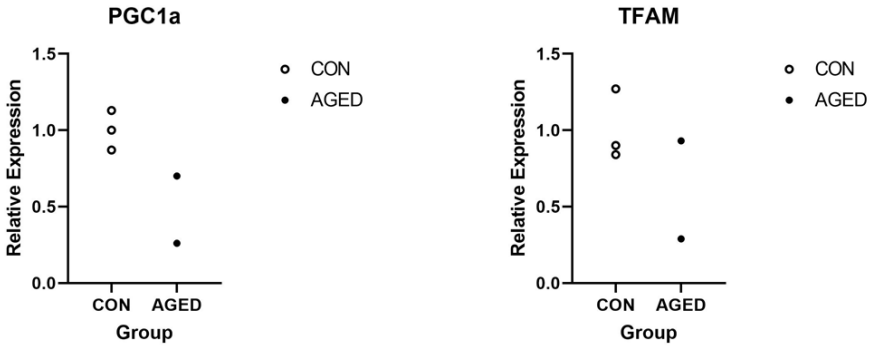
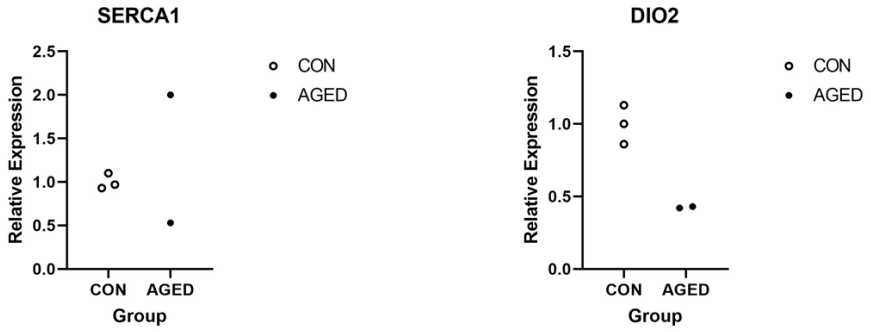


Figure 6

Preliminary Results – Thyroid Hormone Metabolism.



Statement of the Problem

Sarcopenia increases one's vulnerability to unpropitious outcomes such as frailty, insulin resistance, disability, and mortality (Dufour et al., 2013; Giles et al., 2008; Marsh et al., 2011; Xue et al., 2011). Clinical testing for sarcopenia is not routine due to the high costs of the equipment required for accurate diagnosis. Biological markers found in circulation or within SKM can take the place of diagnosing sarcopenia, but more studies are warranted to determine what biological markers can be validated to diagnose sarcopenia. Additionally, aging and sarcopenia alter physiological pathways such as myogenesis, mitochondrial homeostasis, apoptosis, and metabolism and warrant treatment options to slow down the progression of or reverse these adverse effects. Physical inactivity exacerbates these abnormal mechanisms and contributes to sarcopenia and accelerated aging.

Hypotheses

This study aims to utilize an *in vitro* approach to investigate the hypothesized age-related dysregulation of gene expression systems related to SKM health, and to test the hypothesis that stimulation of exercise signaling pathways may mitigate this degradation. To test these hypotheses, the specific aims for this study are:

AIM 1: Using a novel *in vitro* model for investigating aging human SKM myoblasts, changes to genes related to mitochondrial homeostasis, myogenesis, and cell homeostasis were assessed.

AIM 2: The effects of exercise signaling stimulation were tested on genes related to mitochondrial homeostasis, myogenesis, and cell homeostasis using *in vitro* aged human SKM myoblasts.

Significance

Previous literature has predominantly been conducted using *in vivo* and *in vitro* rodent models on aging and sarcopenia with contradictory results when compared to the limited *in vivo* human model research. Additionally, these research studies have focused on specific aspects of cellular targets related to the aging process and warrant further investigation of the effect of mitochondrial health on other cellular pathways. Furthermore, the effects of specific modes of exercise in the sarcopenic population is unclear. The proposed study will focus on making connections between factors that drive sarcopenia and the effect of exercise stimulation on these processes. The results from this

study will inform future investigators on the overall effects of aging on SKM health and provide understanding on the potential therapeutic benefits of exercise for sarcopenia.

CHAPTER II

LITERATURE REVIEW

Aging is a natural process that affects all aspects of life with the rate of aging being different from organism to organism. In humans, lifestyle over the accumulation of years greatly influences this rate with physical activity status and nutrition being primary contributors. Sarcopenia is a common disease developed as a result of aging and is characteristic of SKM mass loss, impaired functionality, and reduced strength. Older individuals with sarcopenia report increased fatigue, less energy to perform tasks, poor mental health, reduced ADLs, poor balance, reduced locomotion, and decreased QoL (Baumgartner et al., 1998; Beaudart et al., 2017; Beaudart et al., 2018; Benjumea et al., 2018; Ferrucci et al., 1997; Landi et al., 2012; Larsson et al., 1979; Metter et al., 2002). Additionally, sarcopenia is commonly associated with insulin resistance, disability, frailty, arthritis, and cardiovascular disease (Dufour et al., 2013; Giles et al., 2008; Marsh et al., 2011; Xia et al., 2020; Xue et al., 2011).

SKM is an important tissue within the human body as it accounts for approximately half of the body mass while functioning as a metabolic regulator for glucose and fat oxidation, providing stability, and producing power to overcome an external force. Biological aging impacts one's health beginning at the molecular level that progresses to influence the physiological behavior of an organism. SKM is not spared from the destruction this process exhibits. Over time, there is an accumulation of

damage at the cellular level that directly contributes to physiological aging (Lopez-Otin et al., 2013). The damage that occurs is reactive to the environment of the targeted cells and potentially serves as a protective mechanism against toxic or harmful traits within the environment in order to maintain or re-establish homeostasis. Damage to the DNA is accumulated over a lifespan as a result of exogenous (i.e., radiation) or endogenous (i.e., ROS) factors. The alteration of the genetic coding can affect essential genes and transcriptional pathways necessary for the health of the cell resulting in cellular dysfunction that ultimately contributes to tissue and organismal homeostatic imbalance. Telomeres, which serve as a protective shield for chromosomes where the genetic code is stored, are susceptible to age-associated deterioration. As cells replicate, the telomeres shorten and gradually lose their protective characteristics resulting in limited proliferative capacity of cells, also known as the Hayflick limit (Hayflick & Moorhead, 1961). The process of telomere shortening is present in humans as a normal response to biological aging (Blasco, 2007) and results in cellular apoptosis or senescence.

In SKM, it is evident that DNA damage and telomere shortening occur as a consequence of aging resulting in the upregulation of apoptosis and the loss of SKM mass (Ahmad et al., 2012). Men with sarcopenia have shorter telomere length compared to age-matched non-sarcopenic men, with no association of telomere length to mortality (Rippberger et al., 2018). While the telomere length within this population is not associated with mortality, it plays a direct role in the quality of the SKM. A seminal study in relation to this dissertation by Bigot et al. (2008) passaged SKM cells of the quadriceps muscle from human donors ranging in age from an unborn fetus to 17 years old until

senescence to determine the MRF capabilities as SKM cells begin to lose proliferative properties. One key finding is these senescent cells were still able to differentiate from myoblasts to myotubes but were smaller compared to young differentiated myotubes. Senescent cells were found to have delayed upregulation of p57, a protein responsible for signaling cells to exit the proliferative cell cycle, along with being 2.4-fold lower in concentration compared to young SKM cells. Myogenin was also found to have delayed upregulation along with a 7-fold decrease in protein concentration compared to young SKM cells. Alternatively, MyoD upregulation was not delayed but was 3.3-fold lower in protein concentration. Additionally, the DNA-binding properties of MyoD and Myf5 were delayed and lower in senescent SKM cells versus young cells. It is suggested that MyoD is a primary regulator of the myogenic regulatory process leading to the subsequent delayed upregulation and reduced expression of MyoG and p57. This study provides basis that as SKM cells reach senescence, the proliferative and differentiative properties of these cells are impaired and may contribute to the overall homeostatic imbalance of SKM as a result of aging.

Telomere length in SKM seems to be influenced by physical activity status. Rae et al. (2010) set to determine the relationship between chronic endurance training and SKM telomere length in young to middle-aged adults. The endurance trained group reported an average of 14.2 years of racing and 4,424 hours of training over the course of their competitive career. Although telomere length in endurance trained adults was the same compared to sedentary adults, telomere length for the trained group was shorter in individuals with greater number of years and hours of training compared to lesser number

of years and hours of training. This is potentially due to the continued proliferation of SKM satellite cells responsible for repair in response to the damage accumulated with training. Additionally, Kadi et al. (2008) reported longer SKM telomere length in competitive powerlifters compared to non-lifting participants. The powerlifting athletes trained three to four sessions per week for approximately 7 hours and had been training and competing for 8 years on average. Alternatively, the greater weight lifted for each powerlifting movement was inversely related to telomere length. Comparatively, Osthus et al. (2012) conducted a pilot study to examine the relationship of telomere length and long-term endurance exercise in young and older adults who competed in cross-country skiing. The results of this study found telomere length to be longer in older athletes (> 65-year-olds) compared to older non-athletes with no differences found between young athletes (20- to 30-year-olds) and young non-athletes. VO_{2max} was also found to be positively correlated to telomere length in athletes only. Additionally, SKM telomere length was impaired in moderately trained older adults (70- to 83-year-olds) compared to young adults (20- to 31-year-olds) in a study conducted by Ponsot et al. (2008). These results indicate a potential negative effect of high-volume training compared to a moderate training volume. The preserved telomere length in old healthy endurance athletes may be a result from improved mitochondrial function, increased upregulation of telomerase, and the suppression of apoptosis and senescence proteins as a result from endurance training (Ludlow & Roth, 2011; Rae et al., 2010; Werner et al., 2009). Mechanisms responsible for potential telomere length preservation in resistance trained individuals is unknown. Taking this evidence into account, exercise intervention at a

moderate volume could potentially attenuate telomere shortening along with secondary effects associated with aging and sarcopenia.

The mitochondria play a major role in the age-associated decline of SKM health while also serving as a target to attenuate this decline. The mitochondria are organelles most abundant in Type I SKM that provide energy for the cell in the form of ATP to maintain order and function within the tissue. Aerobic exercise increases the number and size of the mitochondria to accommodate the increased oxidative and energy demand associated with aerobic exercise. In turn, the increase in mitochondria makes these organelles more efficient antioxidant facilitators. Alternatively, the mitochondria are main contributors to cellular homeostasis while also facilitating many pathways associated with aging. The mitochondria are additionally responsible for regulating ROS production, inflammation, senescence, and apoptotic pathways. Reactive oxygen species are generated by the mitochondria as byproducts to ATP production in the form of superoxides ($O_2^{-\bullet}$) that can be dismutased into less harmful hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD2; Powers & Jackson, 2008). ROS overproduction characteristic of aging is harmful to the cell and can lead to telomere shortening, DNA damage, cell toxicity, and apoptosis via opening of mPTP (Nilsson & Tarnopolsky, 2019). Zhou et al. (2011) demonstrated such evidence of ROS overproduction resulting from dysfunctional mitochondria that activates the inflammasome NLRP3, which subsequently increases systemic proinflammatory cytokine circulation and exacerbates the inflammatory response. The upregulation of apoptotic signaling, impaired stimulation of autophagic pathways, and inflammaging in turn contributes to muscle atrophy,

providing evidence of mitochondrial dysfunction being an important contributor to sarcopenia.

Exercise is a beneficial therapeutic approach to reducing oxidative stress that is potentially damaging to the mitochondria and inflammation concomitant with mitochondrial dysfunction. In turn, the delay of pathogenesis, optimal health extension, and added longevity are additional benefits to the reduction in ROS generation and improved mitochondrial health. Acute aerobic exercise provides benefits to cell health by stimulating mitochondrial biogenesis, antioxidant pathways, autophagy, and the immune response (Garatachea et al., 2015; Nilsson & Tarnopolsky, 2019; Powers & Jackson, 2008). Alternatively, acute resistance exercise stimulates the protein synthesis Akt-mTOR pathway responsible for building muscle mass and remains elevated up to 48-hr post-exercise in older men (Bell et al., 2015). The repetition of exercise, both aerobic and resistance exercise, exhibits a hormesis response with improvements in stress resistance, mitochondrial health, and organismal functional properties (Nilsson et al., 2019; Ristow & Zarse, 2010; Tapia, 2006). Thus, increased exercise volume over time is beneficial in improving overall tissue health and function while reducing an adaptation effect.

Chronic aerobic exercise has been found to stimulate mitochondrial biogenesis along with improving mitochondrial function via upregulation of mtDNA copying, increased transcription of mitochondrial related genes, improved oxidative enzyme function, increased ATP synthesis, and increased total mitochondrial volume (Broskey et al., 2014; Menshikova et al., 2006; Short et al., 2003). To demonstrate these effects, Short et al. (2003) recruited men and women ranging in age from 21 to 87 year olds who were

otherwise healthy and did not participate in exercise for more than 30 min twice per week. Participants were randomized into a control group or 16-week aerobic exercise training group consisting of 20-40 min cycling three to four times per week at 70-80% HR max. In general, VO_{2peak}, weight (kg), waist circumference (cm), BMI (kg/m^2), fat distribution, triglyceride level, and insulin sensitivity improved in the exercise group. Within this study population, COX4 and ND4 were the only genes influenced by age for mRNA concentration while GLUT4, PGC-1α, NRF-1, and TFAM were not affected. Exercise, however, increased mRNA expression of all genes after 16 weeks of aerobic exercise training with no significant interaction with age or sex, thus providing evidence aerobic exercise may help to reduce mitochondrial dysfunction associated with sarcopenia by increasing mitochondrial volume and improving individual mitochondrial function (Jacobs & Lundby, 2013; Jubrias et al., 2001). Aerobic exercise increases PGC-1α expression in young human and animal models, but its effect on PGC-1α levels and mitochondrial biogenesis in older models remains debatable (Vina et al., 2009). The activation of AMPK as a result of reduced energy availability induces PGC-1α, increasing mitochondrial biogenesis and fat oxidation. However, AMPK activation is blunted with age in rats (Reznick et al., 2007) but mitochondrial biogenesis can be promoted in older adult humans via exercise (Menshikova et al., 2006; Short et al., 2003), suggesting an alternate activator of PGC-1α is responsible such as MAPK (Cherry & Piantadosi, 2015).

Chronic resistance exercise training does not stimulate mitochondrial biogenesis to the same effect as aerobic exercise in healthy individuals but does add beneficial

effects to the overall health of the mitochondria by improving mtDNA transcription, enhancing antioxidant properties, and reducing oxidative damage in older SKM (Ji & Zhang, 2014; Melov et al., 2007; Parise, Brose et al., 2005; Parise, Phillips et al., 2005; Tarnopolsky, 2009). Jubrias et al. (2001) reported improved mitochondrial quantity following resistance exercise training in older individuals compared to aerobic exercise training, contradictory to established pathways influenced by either exercise type. Similarly, Pesta et al. (2011) reported no difference between aerobic and resistance exercise training in comparison of mitochondrial enhancement. These studies included older individuals who participated in normal daily activities but were otherwise sedentary. It is suggested that the improved mitochondrial density and health is a result of the activation of satellite cells leading to the fusion of mature myofibers, thus resulting in the dilution of mutated mtDNA in dysfunctional mitochondrial by mitochondrial fusion (Tarnopolsky, 2009). One aspect to consider with older individuals is the quality of mTOR regulation. Protein synthesis is regulated by the activation of mTORC1 resulting in the increase in contractile protein content and overall cell growth to promote hypertrophy (Baar & Esser, 1999; Bodine et al., 2001; Kimball et al., 2002; Nader, 2005; Rennie et al., 2004; Terzis et al., 2008). Resistance exercise and amino acid intake are primary stimulators of the muscle protein synthesis pathway and provide robust responses individually or in combination. Older individuals have been found to have a blunted response in muscle protein synthesis in response to acute resistance training at 70% intensity compared to young adults (Fry et al., 2011). The blunted anabolic response to resistance exercise in older adults is evident initially and up to 24-hours after the cease

of exercise in older compared to young individuals (Fry et al., 2011; Kumar et al., 2009; Mayhew et al., 2009). It is suggested that acute resistance exercise is not sufficient enough to stimulate protein synthesis in older individuals and repeated bouts of exercise is needed to accrue enough muscle proteins for a hypertrophic effect. The effect of chronic exercise, both aerobic and resistance training, is of importance to examine the exacerbating or rescuing genome-wide molecular effect exercise may have in young and old sedentary adults. Additionally, the lifelong effect of exercise on the genome is unclear.

Taking this evidence into account, concurrent training may be beneficial to individuals with sarcopenia due to the reduction in mtDNA mutations as a result of resistance training and the mitochondrial biogenesis stimulation effect of aerobic exercise. Additionally, increases in physical activity, whether aerobic, resistance, or combined exercise, increases the antioxidant effects of SKM due to a shift in muscle fiber type from fast to intermediate, making the tissue slightly more oxidative (Powers & Jackson, 2008). The effects of concurrent exercise on molecular pathways integral to overall SKM health and the functional capacity of SKM is less known within this population. Recent evidence suggests 8 weeks of concurrent exercise 3 sessions per week increases VO_{2max} along with the expression of myogenesis proteins Pax7, Myf5, and MyoG in blood circulation regardless of exercise order in sarcopenic men resulting in increased SKM mass and power (Moghadam et al., 2020). The direct effect on these myogenic markers within SKM comparatively to circulation in response to concurrent exercise warrants further investigation as circulatory markers may not be completely

indicative of tissue response. Alternatively, the effect concurrent exercise exhibits on molecular pathways involved in SKM mitochondrial biogenesis and cell homeostasis within the aging and sarcopenic population are unknown.

The age-associated increase in atrophic genes ATG1 and MuRF1 contribute to the reduction in SKM mass. Increased ROS generation, inflammation, immobilization, and denervation of the muscle contribute to the upregulation of these genes. The pathway stimulation of ATG1 and MuRF1 is largely unknown and warrants further research on examining potential interconnected pathways in regulating these atrophy genes. Research involving the influence of exercise on expression levels of ATG1 and MuRF1 offer conflicting results. Rat models have reported reduced activities of ATG1 and MuRF1 in response to both aerobic and resistance exercise training (Al-Nassan et al., 2012; Zanchi et al., 2009). In both rat models, the rats were of adult age and not considered old but were considered sedentary or underwent hindlimb suspension to induce SKM atrophy. Raue et al. (2007) had old women (average 85 years old) and young women (average 23 years old) perform lower body resistance exercise at 70% 1-repetition maximum with muscle biopsies taken pre-exercise and 4 hours post-exercise. Results indicated higher mRNA expression of MuRF1 and FOXO3 pre-exercise in the older women compared to the young women. In response to resistance exercise, the older women had a 2.5-fold increase in ATG1 mRNA expression with no change in the young women. Additionally, there was an increase in MuRF1 expression in both groups with no difference between groups. The increased expression of MuRF1 and ATG1 mRNA provide evidence of contribution to the breakdown of SKM following injury, or exercise, with a greater role

to this process in older individuals. Additionally, 12-week resistance training in older women (average 85 years old) resulted in no changes to ATG1, FOXO3, and MuRF1 mRNA expression (Williamson et al., 2010). Myostatin increased in older women in response to resistance exercise training resulting in reduced protein synthesis. The mRNA expression of FOXO3, myostatin, and MuRF1 were elevated compared to young women (average 24 years old) post-training. Alternatively, aerobic exercise training may not have an effect on the atrophy pathway. Older women (average 70 years old) performed 12 weeks of aerobic exercise 20-45 minutes three to four sessions per week at 60-80% heart rate reserve in a study by Konopka et al. (2010). Muscle biopsies of the vastus lateralis were taken pre- and post-exercise intervention. Results indicated reduced mRNA expression of myostatin, FOXO3, and Mrf4 in response to the 12-week aerobic exercise intervention. No changes in MuRF-1, ATG1, MyoG, and MyoD were reported. The regulation of ATG1 and MuRF1 expression is facilitated by FOXO3. Even though the mRNA expression of FOXO3 decreased, the protein concentration and phosphorylation of FOXO3 remained unchanged and thus did not alter the expression of ATG1 and MuRF1.

Further investigation of the influence exercise has on ATG1 and MuRF1 should include men and focus on larger sample sizes as the sample size in each of the aforementioned studies were low. Additionally, the timing of muscle biopsies may be critical in obtaining accurate results on the influence exercise has on the atrophy pathways. Exercise exhibits as a catabolic process and thus results in the breakdown of SKM in response to injury and stimulation of repair. The atrophy genes play a role in this

process and would be directly influenced by exercise to aid in the restorative process shortly following. Lastly, the influence exercise exhibits on the atrophic genes within the sarcopenic population is unknown. The expression of these genes would be greater in sarcopenic adults compared to age-matched controls due to the catabolic nature of the disease. Exercise, whether aerobic, resistance, or concurrent, could provide beneficial to this pathway and reduce muscle wasting initiated by ATG1 and MuRF1. Additionally, the presence of ATG1 and MuRF1 genetic expression in myoblasts are currently unknown and warrant further investigation as to the integrity of the regenerative process myoblasts are primary contributors to.

In vitro models may be beneficial for sarcopenia due to the impaired regeneration properties of SKM within this population (Bigot et al., 2008; Lorenzon et al., 2004). Additionally, potential underlying conditions that influence SKM health and exacerbates impaired cellular pathways within this population call for *in vitro* models on the exploration of the influence of aging and sarcopenia only. Exercise mimetics are common *in vitro* pharmacological treatments to upregulate pathways associated with aerobic or resistance exercise. A common target for such exercise mimetics is the β2AR, which is influenced by neurotransmitters epinephrine and norepinephrine *in vivo* in response to exercise in SKM. The exercise mimetic, FORM, stimulates β2AR with subsequent upregulation of the cAMP-PKA pathway in SKM (Duplanty et al., 2018; Wills et al., 2012) and acts in a manner similar to *in vivo* concurrent training, or the combination of aerobic and resistance exercise. Formoterol has been successful in improving SKM health in rodent models associated with aging, cancer cachexia, and muscular dystrophy by

increasing overall body mass, SKM mass and strength, and type II muscle fiber quantity (Lynch & Ryall, 2008). Formoterol increased the phosphorylation of mTOR resulting in the increase in SKM protein synthesis, cross-sectional area, and force production along with decreased inflammation in aged rats undergoing post-injury SKM regeneration (Conte et al., 2012). Myogenesis is also upregulated in response to FORM stimulation in muscle wasting rat models via MyoD and MyoG resulting in SKM regeneration and satellite cell activation along with increased SKM mass (Ametller et al., 2011). Data from our lab adds to the evidence of the influence FORM has on SKM cellular pathways *in vitro* comparatively between mid-myogenesis and end-myogenesis. Genes associated with mitochondrial homeostasis, cell homeostasis, and myogenesis were upregulated in healthy human SKM cells (Duplanty et al., in preparation for submission). Taking these results into account, FORM is an acceptable exercise mimetic to investigate the role exercise stimulation plays in regulating cellular pathways influenced by aging and pathologies in relation to SKM myogenesis, metabolism, cell homeostasis, and mitochondrial homeostasis.

Summary

In summary, aging negatively influences molecular pathways vital to overall SKM health resulting in tissue dysregulation and declined overall organismal health. Regular physical activity beginning early in life and continuing into older adulthood is beneficial to maintaining overall tissue health. Sarcopenia is associated with co-morbidities such as insulin resistance, depression, cardiovascular disease, and frailty (Dufour et al., 2013; Giles et al., 2008; Marsh et al., 2011; Xia et al., 2020; Xue et al.,

2011) contributing approximately $40 billion in direct and indirect economic cost (Goates et al., 2019), thus increasing the need for therapeutic approaches to combat the disease. Mitochondrial health plays a major role in maintaining SKM health vital to preventing sarcopenia development as the organelle directly influences pathways associated with apoptosis, senescence, and autophagy. The increased stimulation of apoptosis and senescent pathways with concomitant reduced stimulation of the autophagy pathway are directly attributed to sarcopenia. The improvement in mitochondrial quality and quantity within SKM is a targeted approach to reducing the deleterious effects of aging and combating sarcopenia. Previous *in vivo* human and rodent models offer incomplete and contradictory results in the role cellular pathways are influenced by aging and sarcopenia in addition to exercise stimulation. Due to the comorbidity occurrence associated with sarcopenia along with the impaired ability of SKM rejuvenation following injury, an *in vitro* model of aging and sarcopenia is feasible to further explore molecular pathway connections associated with these processes along with examining the role exercise stimulation plays with these pathways. The significance and novel approach of the current study proposal emphasizes the experimental *in vitro* use of human SKM cells may shed light on the behaviors of interconnecting pathways in response to aging and sarcopenia. Additionally, the exercise stimulation of β2AR indicative of concurrent exercise via the exercise mimetic FORM will deliver insight to the performance of these impaired pathways in response to exercise and provide direction to appropriate exercise prescriptions within this population. Previous research has mainly focused on serial exercise interventions with concurrent exercise interventions being largely unexplored

within the aging and sarcopenic population and warrants further investigation to elucidate the effects on interconnecting pathways associated with overall SKM health within this population.

CHAPTER III

METHODOLOGY

Experimental Approach to the Problem

This study aimed to utilize a previously established *in vitro* model of aging SKM cells and to compare the expression of genes related to cellular health with non-aged control cells. Additionally, this study aimed to investigate the role exercise-signaling played in the regulation of the proposed cellular pathways in an *in vitro* model of aging SKM cells. Data collected from each experimental group included the expression of genes related to cell homeostasis (Cell), mitochondrial homeostasis (Mito), and myogenesis (Myo; see Table 1).

Table 1

Target genes related to cell homeostasis (Cell), myogenesis (Myo), and mitochondrial homeostasis (Mito).

Gene	Related Cellular Pathway	Primary Function
AMPK	Cell	Activate glucose and fatty acid uptake in response to low energy
ATG5	Cell	Regulate autophagy; rids damaged cells
ATG1	Cell	Skeletal muscle atrophy
Bmp7	Cell	Regulates protein synthesis
β2AR	Cell	Binds epinephrine and stimulates exercise-related pathways
DIO2	Cell	Conversion and activation of $T_4 \rightarrow T_3$ (thyroid hormone)
FOXO3	Cell	Regulates protein synthesis and cell death
GLUT4	Cell	Mediates glucose uptake for energy
GSS	Cell	Antioxidant properties
MyoD	Myo	Regulates proliferation of myoblasts
Myf5	Myo	Regulates proliferation of myoblasts
MuRF1	Cell	Skeletal muscle atrophy
NRF1	Mito	Regulates mitochondrial biogenesis
PGC-1α/β	Mito	Regulates mitochondrial biogenesis
SOD2	Mito	Antioxidant properties
TFAM	Mito	Regulates mitochondrial biogenesis

Study Design

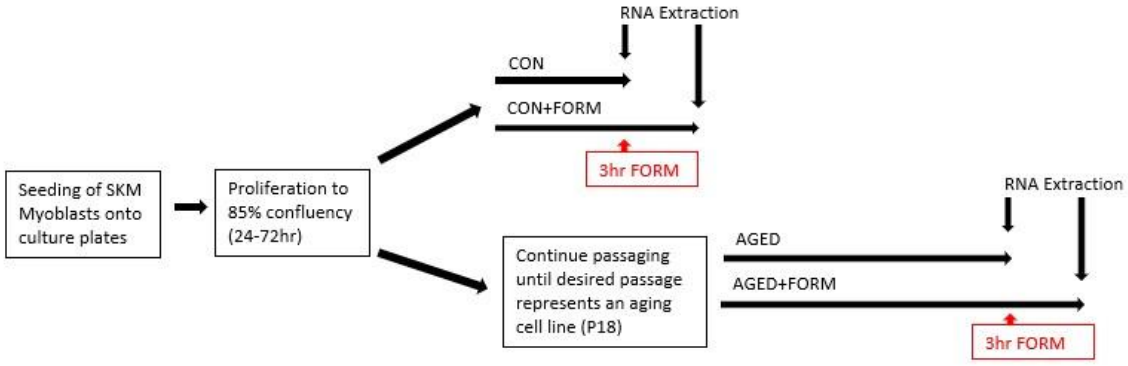
The study was approved by the Texas Woman's University Institutional Review Board. Figure 7 depicts the overall design for the current study. Commercially available (Millipore Sigma, St. Louis, MO, USA), primary human SKM myoblasts obtained from healthy adult donors (passage 2) and de-identified of personal information were used for this study. Each condition was composed of a sample size of six ($n = 6$).

The cells were housed in a Thermo Fisher Midi40 incubator (Thermo Fisher Scientific, Asheville, NC) under controlled temperature (37 °C), humidity, and 5% CO2 (representing physiological conditions). The myoblasts were cultured in 35 mm, six-well collagen-coated plates with Human Skeletal Muscle Growth Media (Cell Applications, San Diego, CA), which includes specific nutrients and hormones that mimic in vivo conditions for healthy SKM cell growth. Myoblasts for the control group (CON) were plated at passage 5 at a density of 70K cells per well and proliferated for approximately 72 hours in the collagen-coated plates until they reached ~85% confluency (i.e., covering 85% of the collagen-coated plate). Similarly, the aged cell culture group (AGED) were plated in two 10 cm plates at passage 5 and proliferated to ~85% confluency, extracted, and passaged repeatedly until passage 17 at a density of 300K cells per plate. At passage 18, the AGED cells were seeded at the same density as CON in six-well plates, as previously established. The AGED cells proliferated approximately 72 to 96 hours to reach confluency. The AGED cells needed extended time to reach optimal confluency due to the gradual loss of proliferative properties as a result of repeated passaging, an effect that is comparable to an *in vivo* phenotype. Data was collected for both experimental groups (CON and AGED) to further assess how the aging process influences cellular pathways in myoblasts. Both groups underwent two experimental conditions: 1) control conditions (CON) and 2) acute Formoterol stimulation (FORM; Sigma-Aldrich, St. Louis, MO). The FORM condition involved the treatment of FORM to the respected myoblasts for 3hr prior to extraction on the final day of proliferation to

desired confluency. Cells were maintained and checked daily under uniform incubation conditions.

Figure 7

Overview of Study Design.



RNA Extraction

The Qiagen miRNeasy Kit (Qiagen, Germantown, MD) was used to extract RNA from all conditions. The extracted RNA was stored at -80°C until all samples were ready for analysis.

qPCR for Gene Expression

Complementary DNA (cDNA) was synthesized from 1µg of total RNA extracted via reverse transcriptase (Qiagen, Germantown, MD). Primers for each gene (forward and reverse) was used to span exon-exon junctions (IDT, Integrated DNA Technologies, Skokie, IL). A 20µL total volume reaction consisting of PowerUp SYBR Green Master

Mix (Applied Biosystems, Carlsbad, CA) and 10ng cDNA was used for qPCR analyses for each gene. All qPCR reactions was analyzed in duplicate for each target gene using the QuantStudio 3 RealTime PCR System (Applied Biosystems, San Francisco, CA). Data was analyzed via the comparative CT method (ΔΔCT) with all target genes normalized to an endogenous control, ribosomal protein S13 (RPS13).

Statistical Analysis

The Grubb's test was used to determine any outliers for each condition per gene prior to data analysis. Any outliers were excluded from statistical analyses. Data was analyzed via SPSS v25.0 (IBM, Armonk, NY). A one-way repeated measures analysis of variance (ANOVA) was used to determine significant differences between conditions for each gene. A Welch correction was used if homogeneity of variance was violated followed by the Games-Howell post hoc test for post hoc comparisons with statistical significance set at $p < .05$.

CHAPTER IV

RESULTS

This study aimed to investigate the physiological effects of aging and exercise stimulation via the exercise mimetic, FORM, on over encompassing gene expression in an *in vitro* human SKM myoblast model. The results for this study are organized into categories representative of genes performing in a similar manner or in conjunction with each other for interpretation: a) cell homeostasis (see Figure 8), b) myogenesis (see Figure 9), and c) mitochondrial biogenesis (see Figure 10).

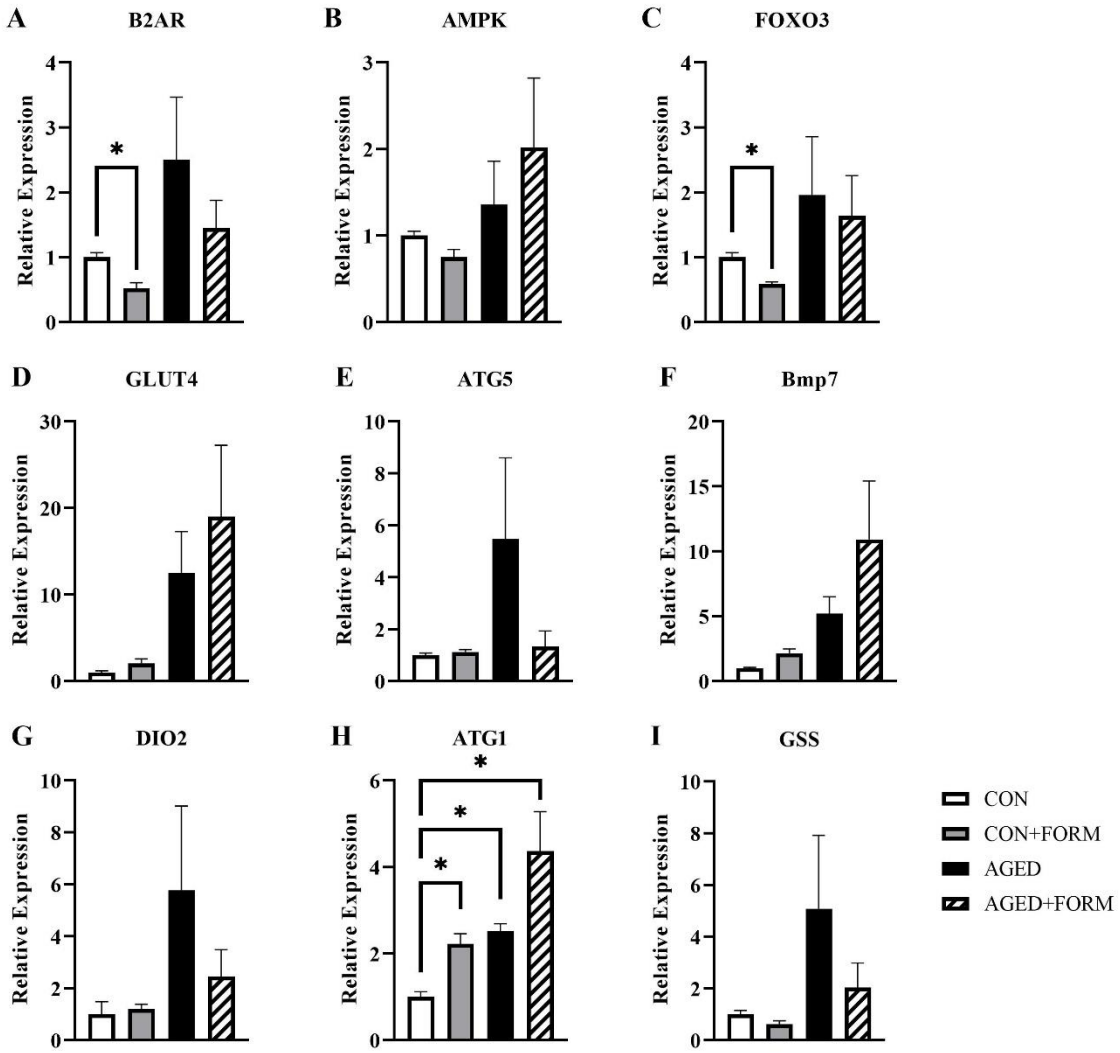
Genes Related to Cell Homeostasis

Interconnected genes that are seemingly unrelated work to maintain a homeostatic environment in cells and are important in the overall health of SKM. The following illustrates the statistical influence of AGED and FORM individually and in combination within myoblasts on genes related to cellular homeostasis. Formoterol stimulation decreased β2AR [$F(3, 9.95) = 6.46$, $p = 0.01$] ($p = 0.01$; see Figure 8A) and FOXO3 [$F(3, 9.28) = 9.39$, $p < 0.01$] ($p = 0.01$; see Figure 8C) expression in CON + FORM (0.52 ± 0.09 and 0.59 ± 0.03, respectively) compared to CON (1.00 ± 0.07 and 1.00 ± 0.07, respectively). The genetic expression of ATG1 [$F(3, 9.13) = 22.44$, $p < 0.01$] (see Figure 8H) increased within the CON + FORM (2.22 ± 0.24, $p = 0.02$), AGED (2.53 ± 0.16, $p < 0.01$), and AGED + FORM (4.37 ± 0.91, $p = 0.04$) conditions compared to CON (1.00 ± 0.12) only, suggesting a potential AGED and FORM effect on ATG1 expression. The

changes in genetic expression for AMPK [$F(3, 9.56) = 2.44$, $p = 0.13$], GLUT4 [$F(3, 9.12) = 4.18$, $p = 0.04$], ATG5 [$F(3, 10.04) = 1.01$, $p = 0.43$], Bmp7 [$F(3, 8.58) = 7.73$, $p < 0.01$], DIO2 [$F(3, 9.15) = 1.03$, $p = 0.42$], and GSS [$F(3, 9.93) = 2.28$, $p = 0.14$] were not significantly different between conditions via post hoc comparisons, though an effect of condition may be likely for GLUT4 and Bmp7 according to ANOVA analyses.

Figure 8

*Cellular homeostasis-related genetic expression. * = significance between conditions, p < 0.05. Data expressed as mean ± SEM.*



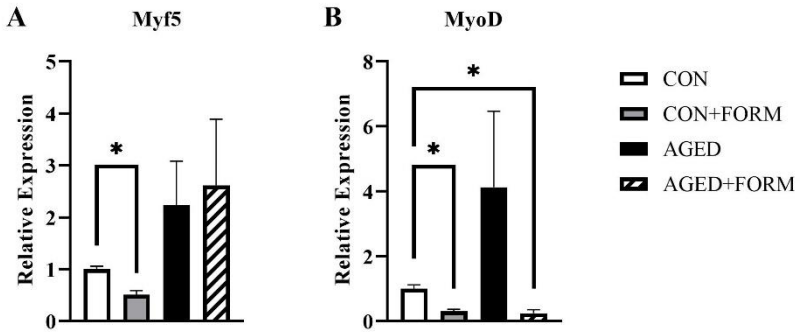
Genes Related to Early Myogenesis

Myoblasts are important in the regeneration process as a result of injury to replenish the damaged cells in SKM with the health of the proliferating myoblasts

playing a large role in the overall physiological function of SKM once fused into existing myofibers. The following illustrates the statistical influence of AGED and FORM individually and in combination within myoblasts on genes related to early myogenesis, Myf5 [$F(3, 9.84) = 8.48$, $p < 0.01$] and MyoD [$F(3, 10.13) = 9.44$, $p < 0.01$]. The AGED condition did not influence the genetic expression of Myf5 (see Figure 9A) nor MyoD (see Figure 9B). Formoterol stimulation reduced the expression of Myf5 for the CON + FORM condition (0.51 ± 0.08, $p < 0.01$) compared to CON only (1.00 ± 0.06; see Figure 9A), whereas FORM stimulation reduced the expression of MyoD in both CON + FORM (0.31 ± 0.06, $p < 0.01$) and AGED + FORM (0.25 ± 0.11, $p < 0.01$) conditions compared to CON expression (1.00 ± 0.12; see Figure 9B).

Figure 9

*Myogenesis-related genetic expression. * = significance between conditions, $p < 0.05$. Data expressed as mean ± SEM.*



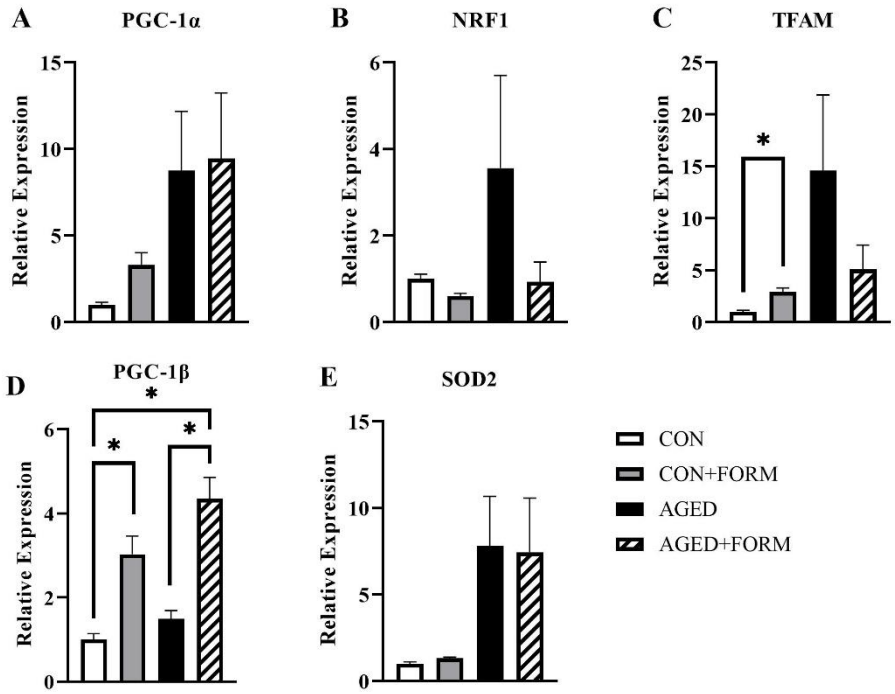
Genes Related to Mitochondrial Homeostasis

Mitochondrial health is important for combating free radicals and producing energy to perform movement and bodily functions. The following illustrates the statistical

influence of AGED and FORM conditions individually and in combination within myoblasts on genes related to mitochondrial homeostasis. Post hoc comparisons revealed no differences between conditions on the genetic expression for PGC-1α [$F(3, 8.60) = 5.71$, $p = 0.02$] (see Figure 10A), NRF1 [$F(3, 9.67) = 3.29$, $p = 0.07$] (see Figure 10B), and SOD2 [$F(3, 9.33) = 4.31$, $p = 0.04$] (see Figure 10E), though an effect of condition may be likely according to ANOVA analyses for PGC-1α and SOD2. Formoterol stimulation increased the genetic expression for CON + FORM (2.92 ± 0.38) compared to CON only in TFAM [$F(3, 9.10) = 7.97$, $p < 0.01$] (1.00 ± 0.16, $p = 0.01$; see Figure 10C). Additionally, FORM stimulation increased the genetic expression for CON + FORM (3.03 ± 0.43) compared to CON only (1.00 ± 0.14, $p = 0.02$), whereas AGED + FORM expression (4.35 ± 0.51) was higher compared to CON (1.00 ± 0.14, $p < 0.01$) and AGED (1.49 ± 0.20, $p = 0.01$) in PGC-1β [$F(3, 18) = 18.28$, $p < 0.01$] (see Figure 10D).

Figure 10

*Mitochondrial homeostasis-related genetic expression. * = significance between conditions, $p < 0.05$. Data expressed as mean ± SEM.*



CHAPTER V

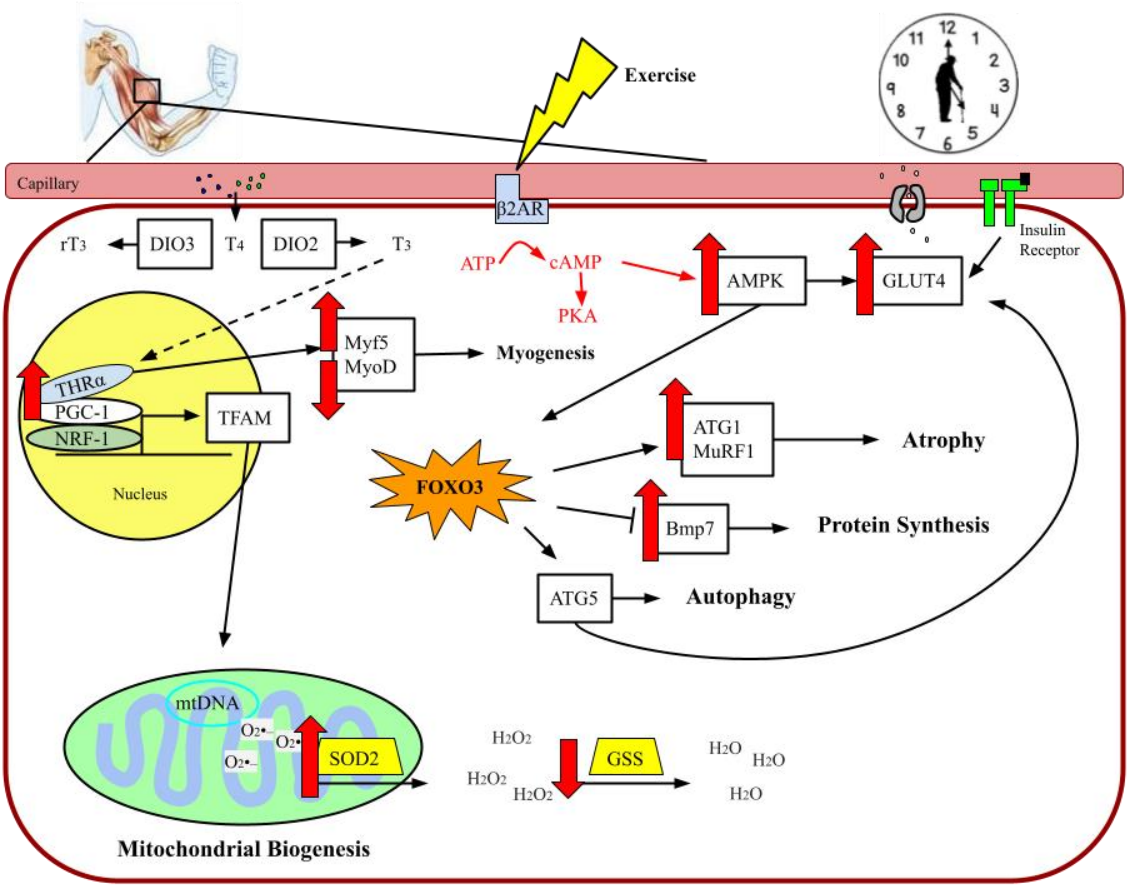
IMPLICATIONS, RECOMMENDATIONS, AND CONCLUSIONS

The purpose of this study was to elaborate on the molecular pathway interactions and changes influenced by aging and by combined exercise stimulation in an *in vitro* skeletal muscle model using primary myoblasts. Differential expression of genes across pathways is present in AGED myoblasts within this study. Additionally, FORM stimulation exacerbated or rescued the genetic expression within these myoblasts. While these myoblasts are not a complete representation of intact skeletal muscle, they give perspective on the potential genetic activity of satellite cells *in vivo*. Further, this study provides insight into the dysregulation of molecular signaling as a consequence of aging and the genetic viability of these cells in preparation for fusing with existing myofibers. Secondarily, this study provides insight into the potential benefits exercise stimulation bestows upon molecularly challenged myoblasts resulting in improved molecular function which may be beneficial when fusing with existing myofibers. Previous studies from our lab focused on establishing FORM as an exercise mimetic for studying the molecular effects of exercise stimulation during the differentiation process of myogenesis (Duplanty et al., in preparation for submission). While these studies involved the influence of exercise in healthy myotubes, the results are of importance for the current study for accurate interpretation and pathway interactions in myoblasts.

As previously mentioned, this study focuses on the molecular influence of aging and FORM stimulation during early myogenesis in myoblasts. Therefore, the data may not be representative of the genetic expression expected in mature myotubes, but rather focuses on the stages of proliferation and cell survival during satellite cell activation. It is also important to note that the metabolic needs for myoblasts may be in contrast to mature myotubes as the promotion of cell-turnover is generally the favorable pathway of myoblasts (Wakelam, 1985) and is possibly exacerbated or opposed to in response to aging and FORM stimulation. This is further exacerbated by lower mitochondrial density in myoblasts versus myotubes, which yields less ability to produce ATP and mitigate cellular ROS (Wagatsuma & Sakuma, 2013). Significant findings, in contrast to the highly visible differences between groups, within the current study were limited due to the high variability in AGED and AGED+FORM conditions. This high variability in AGED cells can be interpreted as an increase in genetic dysregulation, which is a meaningful indicator of a valid aging cell model. This is reinforced by the fact that CON cells displayed much lower variability for each gene, even though they were from the same cell line. Thus, the data from the present study will be presented in a way that both highlights significant findings as well as provides explanations of obviously different, yet non-significant (absolute), differences in gene expression. Additionally, the interpretation of the data will be presented within subsections representative of genes grouped in specific cellular pathways as outlined in Table 1. Figure 11 displays an overview of the genetic responses for genes analyzed in this study for AGED + FORM.

Figure 11

Aging and FORM effect on cellular pathways vital to SKM health.



Genes Related to Cell Homeostasis

A combination of genes with varying individual responsibilities work to maintain a healthy cellular environment for normal processes to occur including, but not limited to substrate utilization, protein synthesis and degradation, and cell clearance. The regulation of β2AR results in cascading events, especially as a response to exercise. Epinephrine and norepinephrine bind to β2AR resulting in the dephosphorylation of ATP into cAMP

with subsequent upregulation of protein kinase A (PKA) and AMPK, representing a low-energy state and increasing signals for substrate metabolism. Formoterol binds to β2AR in a similar manner to epinephrine and norepinephrine with the same subsequent processes that follow (Wannenes et al., 2012). Interestingly, β2AR expression statistically decreased in response to FORM stimulation in myoblasts. Additionally, aging increased absolute basal expression of β2AR with a subsequent absolute decrease following FORM stimulation. Similar patterns of β2AR expression have occurred in previous studies conducted in our lab (Duplanty et al., in preparation for submission). An explanation for these abnormal responses of β2AR following FORM stimulation and aging could be related to decreased receptor sensitivity and a negative feedback loop. As cells increase in age, so does oxidative stress (Baumann et al., 2016). This is mirrored in the present study by an upregulation of SOD2 and GSS. Accumulation of oxidative stress is harmful to the integrity of DNA sequence and integrity of the cell (Barzilai & Yamamoto, 2004). The sensitivity of β2AR may have been increased in response to the accumulated oxidative stress in AGED myoblasts to attempt to maintain the integrity of the myoblasts or stimulated the myogenic process to form new cells. Evidence for this potential response to aging lies with the upregulation of antioxidant and myogenesis-related genes. Additionally, there may be a negative feedback loop or limit to the amount of receptor stimulation for β2AR, as evidenced by the decreased expression in response to FORM stimulation in CON and AGED myoblasts. Once β2AR has reached its threshold for stimulation, it seems to decrease in expression to below basal levels.

When the cell experiences a low energy state, AMPK is upregulated to stimulate substrate utilization with a subsequent increase in GLUT4 expression in myoblasts (Kurth-Kraczek et al., 1999). In response to aging, there seems to be a mild absolute increase in AMPK expression indicative of low energy availability in the form of ATP with a subsequent absolute increase in GLUT4. The increase in GLUT4 expression stimulates the translocation of the GLUT4 vesicle to the sarcolemma to promote glucose uptake from the capillaries (Kurth-Kraczek et al., 1999). The translocation of this transporter is primarily stimulated by the binding of insulin to the insulin receptor in response to increased blood glucose levels in healthy individuals (Kanai et al., 1993). With exercise or in low-energy states, GLUT4 can be translocated to the sarcolemma for glucose uptake from the capillaries also (Messina et al., 2015). Though not significant, this process seems to be occurring in the aging group with an increase in absolute expression of GLUT4 in response to FORM stimulation. The expression of AMPK has previously reported to be reduced in old SKM (Reznick et al., 2007). It is important when interpreting the data that the mRNA expression of AMPK from Reznick et al. (2007) was from complete intact muscle and involved a combination of myoblasts and myofibers. Myofibers have higher numbers of nuclei and are more efficient with the transcription process compared to myoblasts (Cadot et al., 2015). As a result, myofibers may be the predominant source of mRNA expression. Therefore, the energy sensing and substrate utilization abilities of AGED myoblasts may still be viable and can potentially rescue the same signaling pathways in intact muscle.

A secondary regulator of glucose uptake is the increase in DIO2 expression to promote increased T_3 concentration within the cell (Weinstein et al., 1994). The increase in T_3 concentration results in the increase in substrate metabolism and myogenesis (Kupr et al., 2017). The absolute expression of DIO2 was increased in AGED cells, indicating potential low ATP stores leading to the associated increase in GLUT4 expression. The regulation of DIO2 is suggested to be under the positive control of AMPK expression (Ojuka et al., 2002), but rather DIO2 absolute expression decreased in conjunction with AMPK upregulation in response to exercise stimulation in AGED myoblasts in the current study. This would suggest that, particularly with myoblasts, DIO2 and T_3 intercellular concentration may be influenced by other factors and is potentially a direct regulator of GLUT4 expression during resting conditions with AMPK being the prime regulator in response to stress, such as exercise, in response to the increase in cAMP levels. Secondarily, the potential decrease in oxidative stress within AGED myoblasts in response to FORM stimulation may be a contributor to the decrease in DIO2 expression.

The expression of GLUT4 is also suggested to be stimulated by the upregulation of the autophagy gene, ATG5 (Elhassan et al., 2018). Autophagy is an important action in the homeostatic maintenance process and involves the recycling of cellular components. Autophagy is highly stimulated during embryonic development, cellular differentiation, and during cellular stress and is regulated by AMPK and FOXO3 stimulation. *In vivo* studies report a marked decrease in ATG5 transcription in older individuals suggesting a reduced ability to manage cellular stress and damage (Lipinski et al., 2010; Rouschop et

al., 2010). However, absolute expression of ATG5 was increased in the AGED myoblasts in response to AMPK stimulation. Similar to the rationale for AMPK upregulation in the present study, ATG5 transcription may still be viable in myoblasts in older individuals and provides a functional autophagic pathway for rescuing cellular function for SKM. With FORM stimulation, ATG5 absolute expression decreased in AGED myoblasts. This could be a result of the potential decrease in oxidative stress, as evidenced by the increased expression of mitochondrial SOD2 and decreased expression of intercellular GSS. As a result, FORM stimulation may lead to reduced oxidative stress and improve the integrity of AGED myoblasts.

The regulation of overall SKM cellular homeostasis is controlled by the interconnecting pathways of autophagy, apoptosis, protein synthesis, and atrophy. Of importance to this study, atrophy by way of ATG1 seems to have both an aging and acute exercise significant effect. The expression of ATG1 in myoblasts increased in response to both aging and FORM stimulation (see Figure 8H). Interestingly, MuRF1 was undetectable in myoblasts suggesting MuRF1 may play a larger role in atrophy regulation in matured myofibers while ATG1 can be thought of as being primarily responsible for atrophy regulation in myoblasts. The overall influence of ATG1 is relatively unknown but is suggested to be regulated by FOXO1/3 activation (Bodine & Baehr, 2014). The expression of ATG1 is in line with the existing literature suggesting that there is a positive correlation with aging and an increase in expression following acute exercise stimulation (Bodine & Baehr, 2014; Raue et al., 2007). The increase in oxidative stress

exhibited in the AGED myoblasts may be a direct stimulator of ATG1 expression in conjunction with the increase in FOXO3 expression. Interestingly, FORM stimulation significantly decreased FOXO3 expression in the young, healthy myoblasts of the present study with mild decreases in the AGED myoblasts. With the associated reduction in oxidative stress, as evidenced by SOD2 and GSS regulation, other factors currently unknown may be contributing to the increase in ATG1 expression in response to FORM stimulation. The increase in ATG1 expression following exercise seems to be an acute response to promote cellular clearance and protein degradation as a result of damage that is common following exercise and is more profound in older individuals. Exercise training may be beneficial in reducing basal levels of ATG1 and MuRF1 along with reducing the response levels to acute exercise (Al-Nassan et al., 2012; Zanchi et al., 2009), but more research is needed in older individuals. Previous *in vivo* studies involving 12 weeks of resistance or aerobic training in older individuals did not show a reduction in basal expression of ATG1 and MuRF1 (Konopka et al., 2010; Williamson et al., 2010). This may be due to the nature of the "training" protocols used lacking a true manipulation of volume and intensity for resistance training and intensity for aerobic training. Future *in vivo* training studies involving older individuals should implement a progressive training regimen involving alternating muscle groups with the manipulation of volume and intensity for resistance training and the daily changes in intensity and time for aerobic exercise along with the personal choice of mode of exercise (i.e., cycle ergometer, rowing machine, treadmill, Nu-Step). The chronic stimulation of high-intensity exercise, whether resistance or aerobic training, without the addition of low-

intensity days throughout a training program may result in negative physiological responses and increases in injury (Cheng et al., 2020). Additionally, concurrent exercise training may be beneficial for the aging population due to the stimulation of both the mitochondrial biogenesis and protein synthesis pathways and may potentially mitigate the basal atrophic effect. Long-term exercise training with a minimum of 6 months may also be key in implementing beneficial effects to the atrophy pathways as detrimental effects in SKM as a result of aging may be present and warrant extended treatment time to induce a positive effect (Churchward-Venne et al., 2015).

Counterintuitively, protein synthesis signaling may be increased as evidenced by the observed absolute stepwise increases of Bmp7 expression. This, in conjunction with the increase in atrophy signaling by way of ATG1 in the current study, would suggest that atrophy signaling may have a direct influence on protein synthesis and protein turnover. Though not significant, Bmp7 expression of myoblasts increased markedly in a stepwise fashion similar to ATG1 in response to both aging and FORM stimulation.

Genes Related to Myogenesis

Myogenesis begins with the activation of quiescent satellite cells that then commit to the SKM lineage by proliferating as myoblasts and differentiate into myotubes with MRFs regulating specific segments of the process (Bentzinger et al., 2012). This process is responsible for maintaining normal cell turnover and is enhanced in response to damage, or exercise. In healthy adult SKM, it is suggested that a myonuclei turnover rate upwards of 1-2% occurs per week (Schmalbruch & Lewis, 2000). This occurs by the

proliferation of myoblasts and differentiation into myotubes that are then fused with existing myofibers resulting in replenishing or increasing the myonuclei pool.

Of importance to this study, Myf5 and MyoD are responsible for the proliferation process during early myogenesis (Bentzinger et al., 2012). Myogenesis is a high-energy consuming pathway that is potentially downregulated as part of a heirarchy of needs system in SKM, in response to cellular stressors, such as exercise (Wagatsuma & Sakuma, 2013). Both Myf5 and MyoD expression decreased in response to FORM stimulation within the CON group, suggesting a shift in metabolic needs away from myoblast proliferation. Though not significant, aging seems to increase proliferative properties within these cells for both Myf5 and MyoD expression. This suggests that cells lose homeostatic properties in response to aging and are attempting to replenish the stores of viable myoblasts, rather than prepare for differentiation, and thus reduce the potential for intracellular damage. Additionally, FORM maintained the absolute expression of Myf5 but significantly decreased MyoD expression in AGED myoblasts. Similar patterns of expression in response to FORM stimulation in healthy mature myotubes were present in previous work from our lab (Duplanty et al., in preparation for submission) indicating there is a loss in signaling at the Myf5-MyoD junction of proliferation and early differentiation for MyoD transcription.

The expression of MyoD is regulated by several factors including the upregulation of THRα, FOXO3 stimulation, and increased Akt expression within the protein synthesis pathway. Though not analyzed in the current study, Akt is a potential

target responsible for the genetic expression of MyoD. The increased expression of Bmp7 upregulates Akt expression by way of SMAD 1/5/8 transcription factors leading to the upregulation of protein synthesis (Tsuchida et al., 2008). The expression of Bmp7 along with MyoD were both increased in absolute terms in AGED myoblasts, suggesting Akt expression may have been stimulated to promote protein synthesis to counteract the stimulation of atrophy. Consequently, SMAD 6/7 are inhibitors of SMAD 1/5/8 (Sartori et al., 2014) and may play a role in blocking the expression of Akt/mTOR in specific cellular environments. Taking this into consideration, MyoD expression was significantly lower in response to FORM stimulation for both CON and AGED myoblasts suggesting the influence of SMAD 6/7 on the protein synthesis pathway. The etiology of SMAD 6/7 upregulation is currently unknown but warrants investigation, especially as it relates to aging and exercise in SKM. Evidence in the current study related to potential Akt and SMAD 6/7 regulation with consequential influence on MyoD expression includes the increased absolute expression of Bmp7 in response to FORM stimulation and aging shown in Figure 8F. This increased expression may be a result of potential blocked stimulation of downstream protein synthesis targets, such as Akt, in order to attempt to rescue this downstream effect and stimulated protein synthesis. A second potential hypothesis of what is occuring with MyoD regulation in response to aging and FORM would be its connection to FOXO3 expression. The upregulation of FOXO3 increases the expression of MyoD, stimulating the myogenesis pathway (Dentice et al., 2010). In the current study, FOXO3 expression seems to increase in AGED myoblasts and mildly decrease with FORM stimulation. In conjunction, the expression of MyoD follows in a

similar pattern with seemingly being stimulated in AGED cells and downregualted in response to FORM stimulation. This suggests that FORM may block the autophagy signaling of FOXO3 leading to the downregulation of MyoD and subsequent myogenesis.

Genes Related to Mitochondrial Homeostasis

The health of the mitochondria are important in regulating the overall health of SKM. Importantly, SKM mitochondria are responsible for a major fraction of whole-body metabolism with mitochondrial dysregulation within this tissue potentially impacting overall organismal health (Hwang et al., 2012). When mitochondrial health is compromised, there is an increase in oxidative stress that negatively affects the genetic expression and physiological functions of SKM that requires increased energetic demand to counteract the increased oxidative stress, promote mitophagy, and generate new healthy mitochondria.

Mitochondrial biogenesis related genes PGC-1α/β, NRF1, and TFAM are important for generating new mitochondria to replace or fuse with existing mitochondria to sufficiently keep up with energy demand and reduce oxidative stress (Popov, 2020). Formoterol signifantly increased PGC-1β and TFAM expression in the CON group, while there was an absolute increase in PGC-1α. The genetic expression of NRF1 and SOD2 were not affected by FORM stimulation in CON cells. The lack of influence on SOD2 expression may be indicative of low levels of oxidative stress in young, healthy myoblasts. Consequently, AGED cells reportedly had marked increase in absolute expression of PGC-1α, NRF1, TFAM, and SOD2 suggesting marked mitochondrial

dysfuction and high oxidative stress in old myoblasts. The potential presence of marked oxidative stress and mitochondrial dysfunction in AGED cells could promote an energy shift to a mitochondrial biogenesis focus away from a myogenesis focus, as described previously, to combat the oxidative stress and reduce mitochondrial dysfunction by producing new mitochondria (Lee & Wei, 2005). Formoterol stimulation does not seem to have an effect on PGC-1α, NRF1, TFAM, and SOD2 in AGED myoblasts. Alternatively, PGC-1β expression was significantly increased in response to FORM in AGED cells, suggesting that PGC-1β plays a stronger role than PGC-1α in myoblasts (compared to previous literature reporting on PGC-1α/β in mature myotubes; Mortensen et al., 2006). This suggests PGC-1β can be thought of as a primary regulator of mitochondrial biogenesis in myoblasts via exercise stimulation and should be investigated in conjunction with PGC-1α as many studies have not done.

The potential presence of oxidative stress in AGED cells is evident with the increase in GSS absolute expression in conjunction with SOD2. During ATP production, $O_2^{-\bullet}$ is a highly reactive oxidative byproduct at complex III of the electron transport chain and is reduced by SOD2 into H_2O_2 (Fridovich, 1995; Powers & Jackson, 2008). Hydrogen peroxide is a mildly reactive oxidative stress and is reduced by GSS into water (H_2O) and single oxygen ions. Due to the increased absolute expressions of GSS and SOD2 in the AGED myoblasts, it is suggested that high concentrations of both $O_2^{-\bullet}$ and H_2O_2 are present. Interestingly, GSS absolute expression reduced in response to FORM stimulation in AGED myoblasts but no change in SOD2 absolute expression was present.

This potentially indicates SOD2 was efficient in suppressing the accumulation of $O_2^{-\bullet}$ and H_2O_2 production, thus likely reducing oxidative stress in AGED myoblasts. Importantly, the reduction in oxidative stress would reduce the overall cellular and physiological dysfunction in SKM.

Limtations

An *in vitro* model is useful for examining molecular interactions in response to stimulators or treatment to represent *in vivo* conditions. In the case of the present study, the aging of myoblasts via a passaging protocol was used to illicit similar molecular responses and pathway interactions you would see in old and sarcopenic SKM. While FORM stimulation was used to mimic *in vivo* exercise stimulation by interacting with β2AR, it is unknown if similar cascading events occur proportionally *in vivo*. Secondly, it is not possible to compare the use of FORM to stimulate exercise-related pathways with the amount of exercise conducted by an individual. Additionally, it is unknown if the use of a passaging protocol to elicit an aging effect on myoblasts is representative of the molecular and physiological changes that occur *in vivo*. One way to help validate the passaging process to represent an aging model of SKM would be to measure telomere length in older individuals and the cells of a similar *in vitro* model. Another factor to take into consideration is the threat of external validity when using an *in vitro* model. *In vitro* models lack the signaling and hormonal influence that occurs extrinsically to the targeted tissue and threatens the external validity for generalizability. An *in vitro* model examines a specific aspect of an issue and does not take into consideration of the external factors

that may be occuring *in vivo*. Lastly, the interpretation of the data should be approached with caution as an increase in Type I error may be evident as a result of multiple statistical analyses.

Conclusions

This is the first study to the investigator's knowledge to examine the changes of molecular targets in myoblasts in response to aging and exercise stimulation and provides meaningful inferences for future research related to the field of geroscience. Aging of the SKM is detrimental to the molecular processes that occur to maintain cell and organismal health. General exercise stimulation of β2AR activation may be beneficial in counteracting or restoring the effects of aging in SKM. Taking this evidence into account, molecular pathways related to cell homeostasis, myogenesis, and mitochondrial biogenesis may still be viable in maintaining cell health in AGED myoblasts. This is evidenced in the maintained expression of SOD2 and decreased expression of GSS suggesting improvement in oxidative stress harmful to the integrity of cellular and organismal function along with potential improved mitochondrial function in response to acute exercise stimulation via FORM. This study was conducted to investigate the molecular changes and pathway interactions related to cell homeostasis, myogenesis, and mitochondrial biogenesis as a result of aging in SKM myoblasts. Additionally, this study examined the influence acute exercise stimulation exhibited on the expression of genes and pathway interactions aforementioned in aged myoblasts. Based on the overall findings in the present study, we can speculate that exercise stimulation may be able to

reduce overall oxidative stress present in aged myoblasts and potentially rescue the dysregulation of genes directly influenced by oxidative stress.

Future Studies

The interpretation of the data is speculative, in part, due to our discussion of robust, yet non-significant, changes in gene expression found in this study. Solely investigating gene expression certainly provides important data, but the inclusion of protein expression analysis in future studies will be important for revealing more of the overall picture. Further investigation involving measurements for protein expression, mitochondrial function, oxidative stress, and other aging-related factors, such as telomere shortening, are warranted. Future measurements should be conducted in both *in vitro* and *in vivo* studies to elaborate on the aging effects at a molecular level in myoblasts, mature myotubes, and SKM biopsies. Additionally, the prescription of exercise, whether acute or chronic, would be beneficial to explore in the field of geroscience on the molecular and functional properties aforementioned in SKM.

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APPENDIX A

Raw Data & Calculations

Table A1

Raw Data for β2AR Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**B2AR**	**B2AR**	**RPS13**	**B2AR**	**RPS13**	**B2AR**	**DCt**
P5_CON_01	1.00	21.972	21.978	27.598	27.546	0.02	0.13	21.98	27.57	5.60
P5_CON_02	2.00	21.967	21.959	28.014	27.895	0.03	0.30	21.96	27.95	5.99
P5_CON_03	3.00	21.989	21.930	28.177	28.131	0.19	0.12	21.96	28.15	6.19
P5_CON_04	4.00	21.680	21.471	27.346	27.305	0.68	0.11	21.58	27.33	5.75
P5_CON_05	5.00	21.795	21.775	27.693	27.475	0.07	0.56	21.78	27.58	5.80
P5_CON_06	6.00	22.088	22.068	27.543	27.577	0.06	0.09	22.08	27.56	5.48
P5_CON+FORM_07	7.00	23.887	23.834	30.188	30.438	0.16	0.58	23.86	30.31	6.45
P5_CON+FORM_08	8.00	23.196	23.278	29.444	29.527	0.25	0.20	23.24	29.49	6.25
P5_CON+FORM_09	9.00	23.159	23.409	29.720	29.854	0.76	0.32	23.28	29.79	6.50
P5_CON+FORM_10	10.00	23.467	23.527	30.341	29.285	0.18	2.51	23.50	29.81	6.32
P5_CON+FORM_11	11.00	24.360	24.252	31.923	31.798	0.31	0.28	24.31	31.86	7.55
P5_CON+FORM_12	12.00	23.536	23.573	31.949	31.623	0.11	0.72	23.55	31.79	8.23
P18_AGED_13	13.00	23.823	23.645	30.650	30.823	0.53	0.40	23.73	30.74	7.00
P18_AGED_14	14.00	23.355	23.344	29.988	29.588	0.03	0.95	23.35	29.79	6.44
P18_AGED_15	15.00	23.728	23.752	30.549	30.717	0.07	0.39	23.74	30.63	6.89
P18_AGED_16	16.00	23.650	27.126	29.381	29.077	9.68	0.74	25.39	29.23	3.84
P18_AGED_17	17.00	26.145	26.969	29.810	29.686	2.19	0.30	26.56	29.75	3.19
P18_AGED_18	18.00	24.824	23.054	27.746	27.975	5.23	0.58	23.94	27.86	3.92
P18_AGED+FORM_19	19.00	23.216	23.048	30.352	30.740	0.51	0.90	23.13	30.55	7.41
P18_AGED+FORM_20	20.00	23.734	23.779	29.994	29.720	0.14	0.65	23.76	29.86	6.10
P18_AGED+FORM_21	21.00	24.394	24.611	31.271	30.466	0.63	1.84	24.50	30.87	6.37
P18_AGED+FORM_22	22.00	26.320	29.900	32.763	31.854	9.00	1.99	28.11	32.31	4.20
P18_AGED+FORM_23	23.00	28.670	25.487	31.376	31.840	8.31	1.04	27.08	31.61	4.53
P18_AGED+FORM_24	24.00	26.355	26.328	31.275	31.747	0.07	1.06	26.34	31.51	5.17

Table A2

Calculations for β2AR Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	5.60	-0.21	1.15	1.14
	5.99	0.19	0.88	0.87
	6.19	0.39	0.76	0.75
	5.75	-0.05	1.04	1.02
	5.80	0.00	1.00	0.99
	5.48	-0.32	1.25	1.23
AVE	**5.80**		**1.01**	**1.00**
			SEM	***0.07***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	6.45	0.65	0.64	0.63
	6.25	0.45	0.73	0.72
	6.50	0.70	0.62	0.61
	6.32	0.51	0.70	0.69
	7.55	1.75	0.30	0.29
	8.23	2.43	0.19	0.18
			AVE	**0.52**
			SEM	***0.09***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	7.00	1.20	0.44	0.43
	6.44	0.64	0.64	0.63
	6.89	1.09	0.47	0.46
	3.84	-1.96	3.89	3.84
	3.19	-2.61	6.11	6.03
	3.92	-1.88	3.68	3.63
			AVE	**2.51**
			SEM	***0.96***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	7.41	1.61	0.33	0.32
	6.10	0.30	0.81	0.80
	6.37	0.56	0.68	0.67
	4.20	-1.60	3.04	3.00
	4.53	-1.27	2.42	2.38
	5.17	-0.63	1.55	1.53
			AVE	**1.45**
			SEM	***0.43***

Table A3

Raw Data for PGC-1α Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**PGC1a**	**PGC1a**	**RPS13**	**PGC1a**	**RPS13**	**PGC1a**	**DCt**
P5_CON_01	1.00	21.972	21.978	32.882	31.505	0.02	3.02	21.98	32.19	10.22
P5_CON_02	2.00	21.967	21.959	31.314	31.568	0.03	0.57	21.96	31.44	9.48
P5_CON_03	3.00	21.989	21.930	31.881	32.036	0.19	0.34	21.96	31.96	10.00
P5_CON_04	4.00	21.680	21.471	31.680	31.639	0.68	0.09	21.58	31.66	10.08
P5_CON_05	5.00	21.795	21.775	31.916	31.586	0.07	0.74	21.78	31.75	9.97
P5_CON_06	6.00	22.088	22.068	30.841	31.188	0.06	0.79	22.08	31.01	8.94
P5_CON+FORM_07	7.00	23.887	23.834	32.325	31.940	0.16	0.85	23.86	32.13	8.27
P5_CON+FORM_08	8.00	23.196	23.278	31.940	31.645	0.25	0.65	23.24	31.79	8.56
P5_CON+FORM_09	9.00	23.159	23.409	31.676	31.615	0.76	0.14	23.28	31.65	8.36
P5_CON+FORM_10	10.00	23.467	23.527	31.143	31.201	0.18	0.13	23.50	31.17	7.67
P5_CON+FORM_11	11.00	24.360	24.252	33.219	33.128	0.31	0.20	24.31	33.17	8.87
P5_CON+FORM_12	12.00	26.503	24.188	32.233	32.466	6.46	0.51	25.35	32.35	7.00
P18_AGED_13	13.00	23.823	23.645	32.888	32.380	0.53	1.10	23.73	32.63	8.90
P18_AGED_14	14.00	23.355	23.344	31.090	31.065	0.03	0.06	23.35	31.08	7.73
P18_AGED_15	15.00	23.728	23.752	31.842	32.115	0.07	0.60	23.74	31.98	8.24
P18_AGED_16	16.00	23.650	27.126	31.415	31.243	9.68	0.39	25.39	31.33	5.94
P18_AGED_17	17.00	26.145	26.969	31.694	31.723	2.19	0.07	26.56	31.71	5.15
P18_AGED_18	18.00	24.824	23.054	30.730	30.969	5.23	0.55	23.94	30.85	6.91
P18_AGED+FORM_19	19.00	23.216	23.048	31.841	31.668	0.51	0.39	23.13	31.75	8.62
P18_AGED+FORM_20	20.00	23.734	23.779	31.672	31.629	0.14	0.09	23.76	31.65	7.89
P18_AGED+FORM_21	21.00	24.394	24.611	32.407	32.851	0.63	0.96	24.50	32.63	8.13
P18_AGED+FORM_22	22.00	26.320	29.900	33.334	32.963	9.00	0.79	28.11	33.15	5.04
P18_AGED+FORM_23	23.00	28.670	26.385	33.122	33.411	5.87	0.62	27.53	33.27	5.74
P18_AGED+FORM_24	24.00	26.355	26.328	33.441	32.999	0.07	0.94	26.34	33.22	6.88

Table A4

Calculations for PGC-1α Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	10.22	0.44	0.74	0.70
	9.48	-0.30	1.23	1.17
	10.00	0.22	0.86	0.82
	10.08	0.30	0.81	0.77
	9.97	0.19	0.88	0.84
	8.94	-0.84	1.79	1.70
AVE	**9.78**		**1.05**	**1.00**
			SEM	***0.16***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	8.27	-1.51	2.84	2.70
	8.56	-1.22	2.34	2.22
	8.36	-1.42	2.67	2.54
	7.67	-2.11	4.30	4.09
	8.87	-0.91	1.88	1.79
	7.00	-2.78	6.85	6.51
			AVE	**3.31**
			SEM	***0.71***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	8.90	-0.88	1.84	1.75
	7.73	-2.05	4.15	3.94
	8.24	-1.54	2.91	2.77
	5.94	-3.84	14.31	13.60
	5.15	-4.63	24.74	23.50
	6.91	-2.87	7.31	6.95
			AVE	**8.75**
			SEM	***3.43***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	8.62	-1.16	2.23	2.12
	7.89	-1.89	3.70	3.51
	8.13	-1.65	3.15	2.99
	5.04	-4.74	26.75	25.42
	5.74	-4.04	16.47	15.65
	6.88	-2.90	7.47	7.10
			AVE	**9.46**
			SEM	***3.78***

Table A5

Raw Data for AMPK Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**AMPK**	**AMPK**	**RPS13**	**AMPK**	**RPS13**	**AMPK**	**DCt**
P5_CON_01	1.00	21.972	21.978	25.707	25.709	0.02	0.01	21.98	25.71	3.73
P5_CON_02	2.00	21.967	21.959	25.968	25.952	0.03	0.04	21.96	25.96	4.00
P5_CON_03	3.00	21.989	21.930	26.090	26.132	0.19	0.11	21.96	26.11	4.15
P5_CON_04	4.00	21.680	21.471	25.586	25.791	0.68	0.56	21.58	25.69	4.11
P5_CON_05	5.00	21.795	21.775	25.828	25.770	0.07	0.16	21.78	25.80	4.01
P5_CON_06	6.00	22.088	22.068	25.944	25.742	0.06	0.55	22.08	25.84	3.77
P5_CON+FORM_07	7.00	23.887	23.834	27.838	27.803	0.16	0.09	23.86	27.82	3.96
P5_CON+FORM_08	8.00	23.196	23.278	27.645	27.881	0.25	0.60	23.24	27.76	4.53
P5_CON+FORM_09	9.00	23.159	23.409	27.587	27.555	0.76	0.08	23.28	27.57	4.29
P5_CON+FORM_10	10.00	23.467	23.527	27.658	27.230	0.18	1.10	23.50	27.44	3.95
P5_CON+FORM_11	11.00	24.360	24.252	29.221	28.930	0.31	0.71	24.31	29.08	4.77
P5_CON+FORM_12	12.00	24.831	24.793	29.760	29.845	0.11	0.20	24.81	29.80	4.99
P18_AGED_13	13.00	23.823	23.645	28.775	28.812	0.53	0.09	23.73	28.79	5.06
P18_AGED_14	14.00	23.355	23.344	27.670	27.781	0.03	0.28	23.35	27.73	4.38
P18_AGED_15	15.00	23.728	23.752	28.530	28.781	0.07	0.62	23.74	28.66	4.92
P18_AGED_16	16.00	23.650	27.126	27.844	27.766	9.68	0.20	25.39	27.81	2.42
P18_AGED_17	17.00	23.041	23.022	27.825	27.790	0.06	0.09	23.03	27.81	4.78
P18_AGED_18	18.00	24.824	23.054	26.345	26.305	5.23	0.11	23.94	26.32	2.39
P18_AGED+FORM_19	19.00	23.216	23.048	27.788	27.759	0.51	0.07	23.13	27.77	4.64
P18_AGED+FORM_20	20.00	23.734	23.779	28.096	28.133	0.14	0.09	23.76	28.11	4.36
P18_AGED+FORM_21	21.00	24.394	24.611	29.034	28.846	0.63	0.46	24.50	28.94	4.44
P18_AGED+FORM_22	22.00	26.320	29.900	29.645	30.071	9.00	1.01	28.11	29.86	1.75
P18_AGED+FORM_23	23.00	28.670	26.385	29.316	29.298	5.87	0.04	27.53	29.31	1.78
P18_AGED+FORM_24	24.00	26.355	26.328	30.124	30.666	0.07	1.26	26.34	30.40	4.05

Table A6

Calculations for AMPK Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	3.73	-0.23	1.17	1.16
	4.00	0.03	0.98	0.97
	4.15	0.19	0.88	0.87
	4.11	0.15	0.90	0.89
	4.01	0.05	0.96	0.96
	3.77	-0.20	1.15	1.14
AVE	**3.96**		**1.01**	**1.00**
			SEM	***0.05***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	3.96	0.00	1.00	1.00
	4.53	0.56	0.68	0.67
	4.29	0.32	0.80	0.79
	3.95	-0.02	1.01	1.00
	4.77	0.81	0.57	0.57
	4.99	1.03	0.49	0.49
			AVE	**0.75**
			SEM	***0.09***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	5.06	1.10	0.47	0.46
	4.38	0.41	0.75	0.75
	4.92	0.95	0.52	0.51
	2.42	-1.55	2.92	2.90
	4.78	0.81	0.57	0.57
	2.39	-1.58	2.98	2.96
			AVE	**1.36**
			SEM	***0.50***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	4.64	0.68	0.62	0.62
	4.36	0.40	0.76	0.76
	4.44	0.48	0.72	0.71
	1.75	-2.21	4.64	4.61
	1.78	-2.18	4.54	4.51
	4.05	0.09	0.94	0.93
			AVE	**2.02**
			SEM	***0.80***

Table A7

Raw Data for FOXO3 Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**FOXO3**	**FOXO3**	**RPS13**	**FOXO3**	**RPS13**	**FOXO3**	**DCt**
P5_CON_01	1.00	21.972	21.978	27.067	27.081	0.02	0.04	21.98	27.07	5.10
P5_CON_02	2.00	21.967	21.959	27.514	27.590	0.03	0.20	21.96	27.55	5.59
P5_CON_03	3.00	21.989	21.930	27.571	27.593	0.19	0.06	21.96	27.58	5.62
P5_CON_04	4.00	21.680	21.471	27.031	26.969	0.68	0.16	21.58	27.00	5.42
P5_CON_05	5.00	21.795	21.775	27.199	27.133	0.07	0.17	21.78	27.17	5.38
P5_CON_06	6.00	22.088	22.068	27.130	27.110	0.06	0.05	22.08	27.12	5.04
P5_CON+FORM_07	7.00	23.887	23.834	29.921	29.734	0.16	0.44	23.86	29.83	5.97
P5_CON+FORM_08	8.00	23.196	23.278	29.318	29.273	0.25	0.11	23.24	29.30	6.06
P5_CON+FORM_09	9.00	23.159	23.409	29.152	29.215	0.76	0.15	23.28	29.18	5.90
P5_CON+FORM_10	10.00	23.720	24.211	29.610	31.298	1.45	3.92	23.97	30.45	6.49
P5_CON+FORM_11	11.00	24.360	24.252	30.580	30.426	0.31	0.36	24.31	30.50	6.20
P5_CON+FORM_12	12.00	24.831	24.793	31.008	30.831	0.11	0.41	24.81	30.92	6.11
P18_AGED_13	13.00	23.823	23.645	31.680	31.304	0.53	0.85	23.73	31.49	7.76
P18_AGED_14	14.00	23.355	23.344	30.157	30.160	0.03	0.01	23.35	30.16	6.81
P18_AGED_15	15.00	23.728	23.752	30.969	31.461	0.07	1.11	23.74	31.22	7.48
P18_AGED_16	16.00	23.650	27.126	29.980	29.584	9.68	0.94	25.39	29.78	4.39
P18_AGED_17	17.00	26.145	26.969	29.353	29.468	2.19	0.28	26.56	29.41	2.85
P18_AGED_18	18.00	24.824	23.054	27.600	27.382	5.23	0.56	23.94	27.49	3.55
P18_AGED+FORM_19	19.00	23.216	23.048	28.730	28.558	0.51	0.43	23.13	28.64	5.51
P18_AGED+FORM_20	20.00	23.734	23.779	28.992	29.245	0.14	0.62	23.76	29.12	5.36
P18_AGED+FORM_21	21.00	24.394	24.611	31.102	30.162	0.63	2.17	24.50	30.63	6.13
P18_AGED+FORM_22	22.00	26.320	29.900	32.213	32.170	9.00	0.09	28.11	32.19	4.08
P18_AGED+FORM_23	23.00	28.670	26.385	30.706	30.742	5.87	0.08	27.53	30.72	3.20
P18_AGED+FORM_24	24.00	26.355	26.328	32.462	32.411	0.07	0.11	26.34	32.44	6.10

Table A8

Calculations for FOXO3 Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	5.10	-0.26	1.20	1.18
	5.59	0.23	0.85	0.84
	5.62	0.26	0.83	0.82
	5.42	0.06	0.96	0.94
	5.38	0.02	0.99	0.97
	5.04	-0.32	1.25	1.23
AVE	**5.36**		**1.01**	**1.00**
			SEM	***0.07***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	5.97	0.61	0.66	0.65
	6.06	0.70	0.62	0.61
	5.90	0.54	0.69	0.68
	6.49	1.13	0.46	0.45
	6.20	0.84	0.56	0.55
	6.11	0.75	0.60	0.59
			AVE	**0.59**
			SEM	***0.03***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	7.76	2.40	0.19	0.19
	6.81	1.45	0.37	0.36
	7.48	2.12	0.23	0.23
	4.39	-0.97	1.95	1.93
	2.85	-2.51	5.68	5.61
	3.55	-1.81	3.50	3.46
			AVE	**1.96**
			SEM	***0.90***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	5.51	0.15	0.90	0.89
	5.36	0.00	1.00	0.99
	6.13	0.77	0.59	0.58
	4.08	-1.28	2.42	2.40
	3.20	-2.16	4.48	4.43
	6.10	0.74	0.60	0.59
			AVE	**1.64**
			SEM	***0.62***

Table A9

Raw Data for SOD2 Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**SOD2**	**SOD2**	**RPS13**	**SOD2**	**RPS13**	**SOD2**	**DCt**
P5_CON_01	1.00	21.972	21.978	25.976	26.010	0.02	0.09	21.98	25.99	4.02
P5_CON_02	2.00	21.967	21.959	26.048	26.016	0.03	0.09	21.96	26.03	4.07
P5_CON_03	3.00	21.989	21.930	26.367	26.341	0.19	0.07	21.96	26.35	4.39
P5_CON_04	4.00	21.680	21.471	25.944	25.932	0.68	0.03	21.58	25.94	4.36
P5_CON_05	5.00	21.795	21.775	26.179	26.385	0.07	0.55	21.78	26.28	4.50
P5_CON_06	6.00	22.088	22.068	25.552	25.471	0.06	0.23	22.08	25.51	3.43
P5_CON+FORM_07	7.00	23.887	23.834	27.239	27.395	0.16	0.40	23.86	27.32	3.46
P5_CON+FORM_08	8.00	23.196	23.278	26.984	26.971	0.25	0.04	23.24	26.98	3.74
P5_CON+FORM_09	9.00	23.159	23.409	26.928	26.979	0.76	0.13	23.28	26.95	3.67
P5_CON+FORM_10	10.00	23.720	24.211	31.030	33.902	1.45	6.26	23.97	32.47	8.50
P5_CON+FORM_11	11.00	24.360	24.252	27.992	27.888	0.31	0.26	24.31	27.94	3.63
P5_CON+FORM_12	12.00	23.536	23.573	27.466	27.395	0.11	0.18	23.55	27.43	3.88
P18_AGED_13	13.00	23.823	23.645	27.064	26.957	0.53	0.28	23.73	27.01	3.28
P18_AGED_14	14.00	23.355	23.344	25.696	25.668	0.03	0.08	23.35	25.68	2.33
P18_AGED_15	15.00	23.728	23.752	26.637	26.530	0.07	0.28	23.74	26.58	2.84
P18_AGED_16	16.00	23.650	27.126	25.999	26.046	9.68	0.13	25.39	26.02	0.63
P18_AGED_17	17.00	26.145	26.969	26.305	26.330	2.19	0.07	26.56	26.32	-0.24
P18_AGED_18	18.00	24.824	23.054	24.938	24.918	5.23	0.06	23.94	24.93	0.99
P18_AGED+FORM_19	19.00	23.216	23.048	27.348	27.250	0.51	0.25	23.13	27.30	4.17
P18_AGED+FORM_20	20.00	23.734	23.779	26.505	26.572	0.14	0.18	23.76	26.54	2.78
P18_AGED+FORM_21	21.00	24.394	24.611	27.175	27.167	0.63	0.02	24.50	27.17	2.67
P18_AGED+FORM_22	22.00	26.320	29.900	28.232	27.778	9.00	1.15	28.11	28.00	-0.11
P18_AGED+FORM_23	23.00	28.670	26.385	27.613	27.572	5.87	0.11	27.53	27.59	0.07
P18_AGED+FORM_24	24.00	26.355	26.328	28.305	28.355	0.07	0.13	26.34	28.33	1.99

Table A10

Calculations for SOD2 Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	4.02	-0.11	1.08	1.05
	4.07	-0.06	1.04	1.01
	4.39	0.27	0.83	0.81
	4.36	0.23	0.85	0.82
	4.50	0.37	0.77	0.75
	3.43	-0.70	1.62	1.57
AVE	**4.13**		**1.03**	**1.00**
			SEM	***0.12***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	3.46	-0.67	1.59	1.54
	3.74	-0.39	1.31	1.27
	3.67	-0.46	1.38	1.33
	8.50	4.37	0.05	
	3.63	-0.50	1.41	1.36
	3.88	-0.25	1.19	1.15
			AVE	**1.33**
			SEM	***0.06***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	3.28	-0.85	1.81	1.75
	2.33	-1.80	3.47	3.36
	2.84	-1.29	2.44	2.36
	0.63	-3.49	11.27	10.91
	-0.24	-4.37	20.65	19.99
	0.99	-3.14	8.82	8.54
			AVE	**7.82**
			SEM	***2.86***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	4.17	0.04	0.97	0.94
	2.78	-1.35	2.54	2.46
	2.67	-1.46	2.75	2.66
	-0.11	-4.23	18.82	18.22
	0.07	-4.06	16.73	16.19
	1.99	-2.14	4.41	4.27
			AVE	**7.46**
			SEM	***3.12***

Table A11

Raw Data for Myf5 Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**Myf5**	**Myf5**	**RPS13**	**Myf5**	**RPS13**	**Myf5**	**DCt**
P5_CON_01	1.00	21.972	21.978	22.272	22.296	0.02	0.08	21.98	22.28	0.31
P5_CON_02	2.00	21.967	21.959	22.802	22.792	0.03	0.03	21.96	22.80	0.83
P5_CON_03	3.00	21.989	21.930	22.798	22.786	0.19	0.04	21.96	22.79	0.83
P5_CON_04	4.00	21.680	21.471	22.143	22.137	0.68	0.02	21.58	22.14	0.56
P5_CON_05	5.00	21.795	21.775	22.485	22.470	0.07	0.05	21.78	22.48	0.69
P5_CON_06	6.00	22.088	22.068	22.625	22.528	0.06	0.30	22.08	22.58	0.50
P5_CON+FORM_07	7.00	23.887	23.834	24.891	24.848	0.16	0.12	23.86	24.87	1.01
P5_CON+FORM_08	8.00	23.196	23.278	24.780	24.955	0.25	0.50	23.24	24.87	1.63
P5_CON+FORM_09	9.00	23.159	23.409	24.691	24.730	0.76	0.11	23.28	24.71	1.43
P5_CON+FORM_10	10.00	23.467	23.527	24.771	24.874	0.18	0.29	23.50	24.82	1.33
P5_CON+FORM_11	11.00	24.360	24.252	27.387	27.581	0.31	0.50	24.31	27.48	3.18
P5_CON+FORM_12	12.00	23.536	23.573	25.260	25.229	0.11	0.09	23.55	25.24	1.69
P18_AGED_13	13.00	23.823	23.645	24.241	24.289	0.53	0.14	23.73	24.27	0.53
P18_AGED_14	14.00	23.355	23.344	23.845	24.218	0.03	1.10	23.35	24.03	0.68
P18_AGED_15	15.00	23.728	23.752	24.353	24.339	0.07	0.04	23.74	24.35	0.61
P18_AGED_16	16.00	23.650	27.126	23.372	23.460	9.68	0.27	25.39	23.42	-1.97
P18_AGED_17	17.00	23.041	23.022	23.599	23.523	0.06	0.23	23.03	23.56	0.53
P18_AGED_18	18.00	24.824	23.054	22.762	22.814	5.23	0.16	23.94	22.79	-1.15
P18_AGED+FORM_19	19.00	23.216	23.048	24.660	24.597	0.51	0.18	23.13	24.63	1.50
P18_AGED+FORM_20	20.00	23.734	23.779	24.988	24.997	0.14	0.03	23.76	24.99	1.24
P18_AGED+FORM_21	21.00	24.394	24.611	25.257	25.249	0.63	0.02	24.50	25.25	0.75
P18_AGED+FORM_22	22.00	26.320	29.900	26.196	26.178	9.00	0.05	28.11	26.19	-1.92
P18_AGED+FORM_23	23.00	28.670	26.385	25.280	25.236	5.87	0.12	27.53	25.26	-2.27
P18_AGED+FORM_24	24.00	26.355	26.328	27.884	28.081	0.07	0.50	26.34	27.98	1.64

Table A12

Calculations for Myf5 Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	0.31	-0.31	1.24	1.23
	0.83	0.21	0.86	0.86
	0.83	0.21	0.86	0.86
	0.56	-0.06	1.04	1.03
	0.69	0.07	0.95	0.94
	0.50	-0.12	1.09	1.08
AVE	**0.62**		**1.01**	**1.00**
			SEM	***0.06***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	1.01	0.39	0.76	0.76
	1.63	1.01	0.50	0.49
	1.43	0.80	0.57	0.57
	1.33	0.70	0.61	0.61
	3.18	2.56	0.17	0.17
	1.69	1.07	0.48	0.47
			AVE	**0.51**
			SEM	***0.08***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	0.53	-0.09	1.07	1.06
	0.68	0.06	0.96	0.95
	0.61	-0.02	1.01	1.00
	-1.97	-2.59	6.04	5.99
	0.53	-0.09	1.07	1.06
	-1.15	-1.77	3.42	3.39
			AVE	**2.24**
			SEM	***0.84***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	1.50	0.87	0.55	0.54
	1.24	0.61	0.65	0.65
	0.75	0.13	0.91	0.91
	-1.92	-2.54	5.83	5.78
	-2.27	-2.89	7.42	7.36
	1.64	1.02	0.49	0.49
			AVE	**2.62**
			SEM	***1.27***

Table A13

Raw Data for GLUT4 Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**GLUT4**	**GLUT4**	**RPS13**	**GLUT4**	**RPS13**	**GLUT4**	**DCt**
P5_CON_01	1.00	21.972	21.978	32.331	31.758	0.02	1.26	21.98	32.04	10.07
P5_CON_02	2.00	21.967	21.959	32.111	32.382	0.03	0.59	21.96	32.25	10.28
P5_CON_03	3.00	21.989	21.930	31.767	32.282	0.19	1.14	21.96	32.02	10.06
P5_CON_04	4.00	21.680	21.471	31.419	31.275	0.68	0.32	21.58	31.35	9.77
P5_CON_05	5.00	21.795	21.775	30.463	31.199	0.07	1.69	21.78	30.83	9.05
P5_CON_06	6.00	22.088	22.068	30.622	30.722	0.06	0.23	22.08	30.67	8.59
P5_CON+FORM_07	7.00	23.887	23.834	32.096	31.905	0.16	0.42	23.86	32.00	8.14
P5_CON+FORM_08	8.00	23.196	23.278	31.666	32.040	0.25	0.83	23.24	31.85	8.62
P5_CON+FORM_09	9.00	23.159	23.409	32.226	31.617	0.76	1.35	23.28	31.92	8.64
P5_CON+FORM_10	10.00	23.467	23.527	30.815	31.023	0.18	0.48	23.50	30.92	7.42
P5_CON+FORM_11	11.00	24.360	24.252	32.880	33.997	0.31	2.36	24.31	33.44	9.13
P5_CON+FORM_12	12.00	24.831	24.793	34.070	35.480	0.11	2.87	24.81	34.78	9.96
P18_AGED_13	13.00	23.823	23.645	31.430	31.194	0.53	0.53	23.73	31.31	7.58
P18_AGED_14	14.00	23.355	23.344	31.290	31.283	0.03	0.02	23.35	31.29	7.94
P18_AGED_15	15.00	23.728	23.752	31.994	31.529	0.07	1.03	23.74	31.76	8.02
P18_AGED_16	16.00	23.650	27.126	31.454	30.905	9.68	1.24	25.39	31.18	5.79
P18_AGED_17	17.00	26.145	26.969	31.408	30.847	2.19	1.27	26.56	31.13	4.57
P18_AGED_18	18.00	24.824	23.054	28.953	28.979	5.23	0.06	23.94	28.97	5.03
P18_AGED+FORM_19	19.00	23.216	23.048	32.105	32.172	0.51	0.15	23.13	32.14	9.01
P18_AGED+FORM_20	20.00	23.734	23.779	30.938	30.566	0.14	0.86	23.76	30.75	7.00
P18_AGED+FORM_21	21.00	24.394	24.611	31.212	30.785	0.63	0.97	24.50	31.00	6.50
P18_AGED+FORM_22	22.00	26.320	29.900	32.211	32.467	9.00	0.56	28.11	32.34	4.23
P18_AGED+FORM_23	23.00	28.670	26.385	31.397	31.355	5.87	0.09	27.53	31.38	3.85
P18_AGED+FORM_24	24.00	26.355	26.328	32.585	32.429	0.07	0.34	26.34	32.51	6.17

Table A14

Calculations for GLUT4 Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	10.07	0.43	0.74	0.67
	10.28	0.64	0.64	0.58
	10.06	0.43	0.74	0.68
	9.77	0.13	0.91	0.83
	9.05	-0.59	1.51	1.37
	8.59	-1.04	2.06	1.87
AVE	**9.64**		**1.10**	**1.00**
			SEM	***0.21***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	8.14	-1.50	2.82	2.57
	8.62	-1.02	2.03	1.84
	8.64	-1.00	2.00	1.82
	7.42	-2.22	4.65	4.22
	9.13	-0.51	1.42	1.29
	9.96	0.33	0.80	0.72
			AVE	**2.08**
			SEM	***0.50***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	7.58	-2.06	4.17	3.79
	7.94	-1.70	3.25	2.95
	8.02	-1.62	3.07	2.79
	5.79	-3.85	14.39	13.07
	4.57	-5.07	33.52	30.45
	5.03	-4.61	24.45	22.20
			AVE	**12.54**
			SEM	***4.75***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	9.01	-0.63	1.55	1.41
	7.00	-2.64	6.24	5.67
	6.50	-3.14	8.83	8.02
	4.23	-5.41	42.49	38.59
	3.85	-5.79	55.32	50.24
	6.17	-3.47	11.10	10.08
			AVE	**19.00**
			SEM	***8.26***

Table A15

Raw Data for ATG5 Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**ATG5**	**ATG5**	**RPS13**	**ATG5**	**RPS13**	**ATG5**	**DCt**
P5_CON_01	1.00	21.729	21.559	27.741	27.437	0.56	0.78	21.64	27.59	5.94
P5_CON_02	2.00	21.901	21.898	27.926	27.969	0.01	0.11	21.90	27.95	6.05
P5_CON_03	3.00	21.763	21.963	27.734	27.715	0.65	0.05	21.86	27.72	5.86
P5_CON_04	4.00	21.860	22.092	27.243	27.608	0.75	0.94	21.98	27.43	5.45
P5_CON_05	5.00	21.950	21.716	28.183	28.209	0.76	0.07	21.83	28.20	6.36
P5_CON_06	6.00	22.054	22.011	27.934	27.883	0.14	0.13	22.03	27.91	5.88
P5_CON+FORM_07	7.00	24.017	24.032	29.471	29.652	0.04	0.43	24.02	29.56	5.54
P5_CON+FORM_08	8.00	23.486	23.546	29.959	29.641	0.18	0.75	23.52	29.80	6.28
P5_CON+FORM_09	9.00	23.502	23.691	29.289	29.212	0.57	0.19	23.60	29.25	5.65
P5_CON+FORM_10	10.00	23.730	23.458	29.222	29.398	0.81	0.43	23.59	29.31	5.72
P5_CON+FORM_11	11.00	24.360	24.252	30.053	30.166	0.31	0.27	24.31	30.11	5.80
P5_CON+FORM_12	12.00	23.918	25.031	29.753	30.080	3.21	0.77	24.47	29.92	5.44
P18_AGED_13	13.00	24.212	24.096	30.652	30.119	0.34	1.24	24.15	30.39	6.23
P18_AGED_14	14.00	23.813	23.743	29.461	29.445	0.21	0.04	23.78	29.45	5.68
P18_AGED_15	15.00	24.002	24.183	29.944	30.044	0.53	0.24	24.09	29.99	5.90
P18_AGED_16	16.00	24.165	23.900	29.645	29.823	0.78	0.42	24.03	29.73	5.70
P18_AGED_17	17.00	27.419	27.601	29.031	29.208	0.47	0.43	27.51	29.12	1.61
P18_AGED_18	18.00	26.200	25.482	28.389	28.677	1.97	0.71	25.84	28.53	2.69
P18_AGED+FORM_19	19.00	20.979	21.104	29.376	29.378	0.42	0.01	21.04	29.38	8.34
P18_AGED+FORM_20	20.00	21.860	21.942	29.565	29.819	0.27	0.61	21.90	29.69	7.79
P18_AGED+FORM_21	21.00	22.338	21.229	30.571	29.981	3.60	1.38	21.78	30.28	8.49
P18_AGED+FORM_22	22.00	23.893	26.934	31.031	30.997	8.46	0.08	25.41	31.01	5.60
P18_AGED+FORM_23	23.00	26.165	26.385	30.809	31.062	0.59	0.58	26.28	30.94	4.66
P18_AGED+FORM_24	24.00	28.093	26.385	31.072	31.348	4.43	0.62	27.24	31.21	3.97

Table A16

Calculations for ATG5 Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	5.94	0.02	0.99	0.97
	6.05	0.12	0.92	0.90
	5.86	-0.06	1.04	1.03
	5.45	-0.47	1.39	1.36
	6.36	0.44	0.74	0.72
	5.88	-0.05	1.03	1.02
AVE	**5.92**		**1.02**	**1.00**
			SEM	***0.09***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	5.54	-0.39	1.31	1.28
	6.28	0.36	0.78	0.77
	5.65	-0.27	1.21	1.18
	5.72	-0.21	1.15	1.13
	5.80	-0.12	1.09	1.07
	5.44	-0.48	1.40	1.37
			AVE	**1.13**
			SEM	***0.09***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	6.23	0.31	0.81	0.79
	5.68	-0.25	1.19	1.17
	5.90	-0.02	1.02	1.00
	5.70	-0.22	1.17	1.15
	1.61	-4.31	19.90	19.55
	2.69	-3.23	9.40	9.23
			AVE	**5.48**
			SEM	***3.12***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	8.34	2.41	0.19	0.18
	7.79	1.87	0.27	0.27
	8.49	2.57	0.17	0.17
	5.60	-0.32	1.25	1.23
	4.66	-1.26	2.40	2.36
	3.97	-1.95	3.87	3.80
			AVE	**1.34**
			SEM	***0.60***

Table A17

Raw Data for Bmp7 Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**Bmp7**	**Bmp7**	**RPS13**	**Bmp7**	**RPS13**	**Bmp7**	**DCt**
P5_CON_01	1.00	21.729	21.559	31.236	30.952	0.56	0.65	21.64	31.09	9.45
P5_CON_02	2.00	21.901	21.898	31.535	31.468	0.01	0.15	21.90	31.50	9.60
P5_CON_03	3.00	21.763	21.963	31.281	30.926	0.65	0.81	21.86	31.10	9.24
P5_CON_04	4.00	21.860	22.092	31.803	31.971	0.75	0.37	21.98	31.89	9.91
P5_CON_05	5.00	21.950	21.716	30.733	31.479	0.76	1.70	21.83	31.11	9.27
P5_CON_06	6.00	22.054	22.011	31.628	31.694	0.14	0.15	22.03	31.66	9.63
P5_CON+FORM_07	7.00	24.017	24.032	32.612	32.609	0.04	0.01	24.02	32.61	8.59
P5_CON+FORM_08	8.00	23.486	23.546	32.262	33.329	0.18	2.30	23.52	32.80	9.28
P5_CON+FORM_09	9.00	23.502	23.691	32.571	32.262	0.57	0.67	23.60	32.42	8.82
P5_CON+FORM_10	10.00	23.730	23.458	31.224	31.779	0.81	1.25	23.59	31.50	7.91
P5_CON+FORM_11	11.00	24.360	24.252	33.028	32.544	0.31	1.04	24.31	32.79	8.48
P5_CON+FORM_12	12.00	24.733	25.031	32.936	32.430	0.85	1.09	24.88	32.68	7.80
P18_AGED_13	13.00	24.212	24.096	30.259	30.429	0.34	0.39	24.15	30.34	6.19
P18_AGED_14	14.00	23.813	23.743	31.805	31.599	0.21	0.46	23.78	31.70	7.92
P18_AGED_15	15.00	24.002	24.183	31.475	32.124	0.53	1.44	24.09	31.80	7.71
P18_AGED_16	16.00	24.165	23.900	30.899	30.926	0.78	0.06	24.03	30.91	6.88
P18_AGED_17	17.00	23.041	23.022	31.990	32.073	0.06	0.18	23.03	32.03	9.00
P18_AGED_18	18.00	22.334	22.342	29.026	28.901	0.03	0.30	22.34	28.96	6.63
P18_AGED+FORM_19	19.00	20.979	21.104	31.161	30.804	0.42	0.82	21.04	30.98	9.94
P18_AGED+FORM_20	20.00	21.860	21.942	29.515	29.663	0.27	0.35	21.90	29.59	7.69
P18_AGED+FORM_21	21.00	22.338	21.229	29.522	30.114	3.60	1.40	21.78	29.82	8.03
P18_AGED+FORM_22	22.00	23.893	26.934	31.501	31.904	8.46	0.90	25.41	31.70	6.29
P18_AGED+FORM_23	23.00	26.165	26.385	31.105	31.361	0.59	0.58	26.28	31.23	4.96
P18_AGED+FORM_24	24.00	28.093	26.385	32.204	31.876	4.43	0.72	27.24	32.04	4.80

Table A18

Calculations for Bmp7 Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	9.45	-0.07	1.05	1.04
	9.60	0.08	0.94	0.93
	9.24	-0.28	1.21	1.20
	9.91	0.39	0.76	0.75
	9.27	-0.24	1.18	1.17
	9.63	0.11	0.93	0.91
AVE	**9.52**		**1.01**	**1.00**
			SEM	***0.07***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	8.59	-0.93	1.91	1.88
	9.28	-0.24	1.18	1.17
	8.82	-0.70	1.62	1.60
	7.91	-1.61	3.05	3.02
	8.48	-1.04	2.05	2.03
	7.80	-1.72	3.29	3.25
			AVE	**2.16**
			SEM	***0.33***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	6.19	-3.33	10.04	9.92
	7.92	-1.59	3.02	2.98
	7.71	-1.81	3.51	3.47
	6.88	-2.64	6.23	6.15
	9.00	-0.52	1.43	1.41
	6.63	-2.89	7.42	7.33
			AVE	**5.21**
			SEM	***1.29***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	9.94	0.42	0.75	0.74
	7.69	-1.83	3.55	3.51
	8.03	-1.48	2.80	2.76
	6.29	-3.23	9.37	9.26
	4.96	-4.56	23.60	23.31
	4.80	-4.72	26.30	25.98
			AVE	**10.92**
			SEM	***4.50***

Table A19

Raw Data for DIO2 Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**DIO2**	**DIO2**	**RPS13**	**DIO2**	**RPS13**	**DIO2**	**DCt**
P5_CON_01	1.00	21.729	21.559	28.757	28.325	0.56	1.07	21.64	28.54	6.90
P5_CON_02	2.00	21.901	21.898	29.123	29.064	0.01	0.14	21.90	29.09	7.19
P5_CON_03	3.00	21.763	21.963	29.116	28.934	0.65	0.44	21.86	29.02	7.16
P5_CON_04	4.00	21.860	22.092	28.973	29.216	0.75	0.59	21.98	29.09	7.12
P5_CON_05	5.00	26.230	25.267	29.781	29.075	2.65	1.70	25.75	29.43	3.68
P5_CON_06	6.00	25.238	22.759	28.633	28.813	7.30	0.44	24.00	28.72	4.72
P5_CON+FORM_07	7.00	24.017	24.032	29.413	29.439	0.04	0.06	24.02	29.43	5.40
P5_CON+FORM_08	8.00	23.486	23.546	29.076	29.386	0.18	0.75	23.52	29.23	5.71
P5_CON+FORM_09	9.00	23.502	23.691	28.831	28.925	0.57	0.23	23.60	28.88	5.28
P5_CON+FORM_10	10.00	23.730	23.458	28.420	28.505	0.81	0.21	23.59	28.46	4.87
P5_CON+FORM_11	11.00	24.360	24.252	29.792	29.716	0.31	0.18	24.31	29.75	5.45
P5_CON+FORM_12	12.00	24.733	25.031	29.202	29.245	0.85	0.10	24.88	29.22	4.34
P18_AGED_13	13.00	24.212	24.096	30.028	29.962	0.34	0.16	24.15	30.00	5.84
P18_AGED_14	14.00	23.813	23.743	29.071	29.227	0.21	0.38	23.78	29.15	5.37
P18_AGED_15	15.00	24.002	24.183	29.550	29.597	0.53	0.11	24.09	29.57	5.48
P18_AGED_16	16.00	24.165	23.900	29.365	29.386	0.78	0.05	24.03	29.38	5.34
P18_AGED_17	17.00	27.419	27.601	28.500	28.680	0.47	0.45	27.51	28.59	1.08
P18_AGED_18	18.00	26.200	25.482	27.635	27.751	1.97	0.30	25.84	27.69	1.85
P18_AGED+FORM_19	19.00	20.979	21.104	27.628	27.741	0.42	0.29	21.04	27.68	6.64
P18_AGED+FORM_20	20.00	21.860	21.942	28.048	28.178	0.27	0.33	21.90	28.11	6.21
P18_AGED+FORM_21	21.00	22.338	21.229	28.974	28.763	3.60	0.52	21.78	28.87	7.08
P18_AGED+FORM_22	22.00	23.893	26.934	29.668	29.695	8.46	0.07	25.41	29.68	4.27
P18_AGED+FORM_23	23.00	26.165	26.385	29.095	29.247	0.59	0.37	26.28	29.17	2.90
P18_AGED+FORM_24	24.00	28.093	26.385	30.206	29.978	4.43	0.54	27.24	30.09	2.85

Table A20

Calculations for DIO2 Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	6.90	0.77	0.59	0.35
	7.19	1.06	0.48	0.28
	7.16	1.03	0.49	0.29
	7.12	0.99	0.50	0.30
	3.68	-2.45	5.46	3.22
	4.72	-1.40	2.65	1.56
AVE	**6.13**		**1.69**	**1.00**
			SEM	***0.49***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	5.40	-0.73	1.66	0.98
	5.71	-0.41	1.33	0.79
	5.28	-0.85	1.80	1.06
	4.87	-1.26	2.40	1.41
	5.45	-0.68	1.60	0.95
	4.34	-1.79	3.45	2.04
			AVE	**1.20**
			SEM	***0.19***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	5.84	-0.29	1.22	0.72
	5.37	-0.76	1.69	1.00
	5.48	-0.65	1.57	0.92
	5.34	-0.79	1.73	1.02
	1.08	-5.05	33.12	19.54
	1.85	-4.28	19.39	11.44
			AVE	**5.77**
			SEM	***3.25***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	6.64	0.51	0.70	0.41
	6.21	0.08	0.94	0.56
	7.08	0.96	0.52	0.30
	4.27	-1.86	3.63	2.14
	2.90	-3.23	9.41	5.55
	2.85	-3.28	9.69	5.72
			AVE	**2.45**
			SEM	***1.04***

Table A21

Raw Data for GSS Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**GSS**	**GSS**	**RPS13**	**GSS**	**RPS13**	**GSS**	**DCt**
P5_CON_01	1.00	21.729	21.559	29.164	29.195	0.56	0.08	21.64	29.18	7.54
P5_CON_02	2.00	21.901	21.898	29.116	29.114	0.01	0.00	21.90	29.12	7.22
P5_CON_03	3.00	21.763	21.963	29.587	29.437	0.65	0.36	21.86	29.51	7.65
P5_CON_04	4.00	21.860	22.092	28.361	28.379	0.75	0.05	21.98	28.37	6.39
P5_CON_05	5.00	21.950	21.716	28.130	28.344	0.76	0.53	21.83	28.24	6.40
P5_CON_06	6.00	22.054	22.011	28.601	28.631	0.14	0.07	22.03	28.62	6.58
P5_CON+FORM_07	7.00	24.017	24.032	32.074	31.994	0.04	0.18	24.02	32.03	8.01
P5_CON+FORM_08	8.00	23.486	23.546	31.694	31.790	0.18	0.21	23.52	31.74	8.23
P5_CON+FORM_09	9.00	23.502	23.691	31.432	31.731	0.57	0.67	23.60	31.58	7.98
P5_CON+FORM_10	10.00	23.730	23.458	30.203	30.264	0.81	0.14	23.59	30.23	6.64
P5_CON+FORM_11	11.00	24.360	24.252	31.943	32.185	0.31	0.53	24.31	32.06	7.76
P5_CON+FORM_12	12.00	24.733	25.031	32.427	31.908	0.85	1.14	24.88	32.17	7.29
P18_AGED_13	13.00	24.212	24.096	31.749	31.792	0.34	0.10	24.15	31.77	7.62
P18_AGED_14	14.00	23.813	23.743	31.515	31.609	0.21	0.21	23.78	31.56	7.78
P18_AGED_15	15.00	24.002	24.183	31.808	32.153	0.53	0.76	24.09	31.98	7.89
P18_AGED_16	16.00	24.165	23.900	31.006	31.164	0.78	0.36	24.03	31.08	7.05
P18_AGED_17	17.00	27.419	27.601	30.611	30.669	0.47	0.13	27.51	30.64	3.13
P18_AGED_18	18.00	26.200	25.482	28.874	28.805	1.97	0.17	25.84	28.84	3.00
P18_AGED+FORM_19	19.00	20.979	21.104	30.085	30.029	0.42	0.13	21.04	30.06	9.02
P18_AGED+FORM_20	20.00	21.860	21.942	29.945	29.785	0.27	0.38	21.90	29.86	7.96
P18_AGED+FORM_21	21.00	22.338	21.229	30.580	30.696	3.60	0.27	21.78	30.64	8.85
P18_AGED+FORM_22	22.00	23.893	26.934	31.503	32.308	8.46	1.78	25.41	31.91	6.49
P18_AGED+FORM_23	23.00	26.165	26.385	30.891	30.757	0.59	0.31	26.28	30.82	4.55
P18_AGED+FORM_24	24.00	28.093	26.385	31.636	31.948	4.43	0.69	27.24	31.79	4.55

Table A22

Calculations for GSS Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	7.54	0.57	0.67	0.63
	7.22	0.25	0.84	0.79
	7.65	0.69	0.62	0.58
	6.39	-0.57	1.48	1.39
	6.40	-0.56	1.47	1.38
	6.58	-0.38	1.30	1.22
AVE	**6.96**		**1.07**	**1.00**
			SEM	***0.15***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	8.01	1.05	0.48	0.45
	8.23	1.26	0.42	0.39
	7.98	1.02	0.49	0.46
	6.64	-0.32	1.25	1.17
	7.76	0.79	0.58	0.54
	7.29	0.32	0.80	0.75
			AVE	**0.63**
			SEM	***0.12***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	7.62	0.65	0.64	0.60
	7.78	0.82	0.57	0.53
	7.89	0.92	0.53	0.49
	7.05	0.09	0.94	0.88
	3.13	-3.83	14.26	13.38
	3.00	-3.97	15.62	14.66
			AVE	**5.09**
			SEM	***2.83***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	9.02	2.05	0.24	0.23
	7.96	1.00	0.50	0.47
	8.85	1.89	0.27	0.25
	6.49	-0.47	1.39	1.30
	4.55	-2.42	5.33	5.01
	4.55	-2.41	5.32	4.99
			AVE	**2.04**
			SEM	***0.95***

Table A23

Raw Data for MyoD Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**MyoD**	**MyoD**	**RPS13**	**MyoD**	**RPS13**	**MyoD**	**DCt**
P5_CON_01	1.00	21.729	21.559	26.238	26.237	0.56	0.00	21.64	26.24	4.59
P5_CON_02	2.00	21.901	21.898	26.339	26.473	0.01	0.36	21.90	26.41	4.51
P5_CON_03	3.00	21.763	21.963	26.581	26.570	0.65	0.03	21.86	26.58	4.71
P5_CON_04	4.00	21.855	21.614	25.953	25.952	0.78	0.00	21.73	25.95	4.22
P5_CON_05	5.00	21.950	21.716	25.582	25.564	0.76	0.05	21.83	25.57	3.74
P5_CON_06	6.00	22.054	22.011	25.820	25.877	0.14	0.16	22.03	25.85	3.82
P5_CON+FORM_07	7.00	24.017	24.032	30.435	30.802	0.04	0.85	24.02	30.62	6.59
P5_CON+FORM_08	8.00	23.486	23.546	29.530	29.721	0.18	0.46	23.52	29.63	6.11
P5_CON+FORM_09	9.00	23.502	23.691	29.959	30.416	0.57	1.07	23.60	30.19	6.59
P5_CON+FORM_10	10.00	23.730	23.458	28.565	28.562	0.81	0.01	23.59	28.56	4.97
P5_CON+FORM_11	11.00	24.360	24.252	30.306	30.900	0.31	1.37	24.31	30.60	6.30
P5_CON+FORM_12	12.00	24.733	25.031	30.729	30.448	0.85	0.65	24.88	30.59	5.71
P18_AGED_13	13.00	24.212	24.096	29.680	29.489	0.34	0.46	24.15	29.58	5.43
P18_AGED_14	14.00	23.813	23.743	29.032	28.906	0.21	0.31	23.78	28.97	5.19
P18_AGED_15	15.00	24.002	24.183	29.724	29.828	0.53	0.25	24.09	29.78	5.68
P18_AGED_16	16.00	24.165	23.900	28.866	28.699	0.78	0.41	24.03	28.78	4.75
P18_AGED_17	17.00	27.419	27.601	28.053	27.982	0.47	0.18	27.51	28.02	0.51
P18_AGED_18	18.00	26.200	25.482	26.795	26.748	1.97	0.13	25.84	26.77	0.93
P18_AGED+FORM_19	19.00	20.979	21.104	29.144	29.268	0.42	0.30	21.04	29.21	8.16
P18_AGED+FORM_20	20.00	21.860	21.942	28.958	29.134	0.27	0.43	21.90	29.05	7.15
P18_AGED+FORM_21	21.00	22.338	21.229	30.705	30.498	3.60	0.48	21.78	30.60	8.82
P18_AGED+FORM_22	22.00	23.893	26.934	32.352	32.624	8.46	0.59	25.41	32.49	7.07
P18_AGED+FORM_23	23.00	26.165	26.385	30.902	30.884	0.59	0.04	26.28	30.89	4.62
P18_AGED+FORM_24	24.00	28.093	26.385	33.250	32.493	4.43	1.63	27.24	32.87	5.63

Table A24

Calculations for MyoD Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	4.59	0.33	0.80	0.77
	4.51	0.24	0.85	0.82
	4.71	0.45	0.73	0.71
	4.22	-0.05	1.03	1.00
	3.74	-0.52	1.44	1.39
	3.82	-0.45	1.36	1.32
AVE	**4.26**		**1.04**	**1.00**
			SEM	***0.12***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	6.59	2.33	0.20	0.19
	6.11	1.85	0.28	0.27
	6.59	2.33	0.20	0.19
	4.97	0.71	0.61	0.59
	6.30	2.03	0.24	0.24
	5.71	1.44	0.37	0.36
			AVE	**0.31**
			SEM	***0.06***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	5.43	1.17	0.45	0.43
	5.19	0.93	0.53	0.51
	5.68	1.42	0.37	0.36
	4.75	0.49	0.71	0.69
	0.51	-3.76	13.52	13.06
	0.93	-3.33	10.09	9.75
			AVE	**4.13**
			SEM	***2.34***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	8.16	3.90	0.07	0.06
	7.15	2.88	0.14	0.13
	8.82	4.55	0.04	0.04
	7.07	2.81	0.14	0.14
	4.62	0.35	0.78	0.76
	5.63	1.37	0.39	0.37
			AVE	**0.25**
			SEM	***0.11***

Table A25

Raw Data for NRF1 Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**NRF1**	**NRF1**	**RPS13**	**NRF1**	**RPS13**	**NRF1**	**DCt**
P5_CON_01	1.00	21.729	21.559	27.966	27.989	0.56	0.06	21.64	27.98	6.33
P5_CON_02	2.00	21.901	21.898	28.462	28.302	0.01	0.40	21.90	28.38	6.48
P5_CON_03	3.00	21.763	21.963	28.312	28.456	0.65	0.36	21.86	28.38	6.52
P5_CON_04	4.00	21.860	22.092	27.480	27.610	0.75	0.33	21.98	27.54	5.57
P5_CON_05	5.00	21.950	21.716	28.203	28.023	0.76	0.45	21.83	28.11	6.28
P5_CON_06	6.00	22.054	22.011	27.986	28.038	0.14	0.13	22.03	28.01	5.98
P5_CON+FORM_07	7.00	24.017	24.032	30.978	31.124	0.04	0.33	24.02	31.05	7.03
P5_CON+FORM_08	8.00	23.486	23.546	31.164	30.434	0.18	1.68	23.52	30.80	7.28
P5_CON+FORM_09	9.00	23.502	23.691	30.219	30.653	0.57	1.01	23.60	30.44	6.84
P5_CON+FORM_10	10.00	23.730	23.458	30.202	30.247	0.81	0.10	23.59	30.22	6.63
P5_CON+FORM_11	11.00	24.360	24.252	31.874	31.648	0.31	0.50	24.31	31.76	7.45
P5_CON+FORM_12	12.00	24.733	25.031	31.110	31.389	0.85	0.63	24.88	31.25	6.37
P18_AGED_13	13.00	24.212	24.096	31.563	32.108	0.34	1.21	24.15	31.84	7.68
P18_AGED_14	14.00	23.813	23.743	31.739	31.073	0.21	1.50	23.78	31.41	7.63
P18_AGED_15	15.00	24.002	24.183	31.714	32.218	0.53	1.12	24.09	31.97	7.87
P18_AGED_16	16.00	24.165	23.900	30.565	30.471	0.78	0.22	24.03	30.52	6.49
P18_AGED_17	17.00	27.419	27.601	29.976	29.956	0.47	0.05	27.51	29.97	2.46
P18_AGED_18	18.00	26.200	25.482	29.187	29.360	1.97	0.42	25.84	29.27	3.43
P18_AGED+FORM_19	19.00	20.979	21.104	29.754	28.919	0.42	2.01	21.04	29.34	8.29
P18_AGED+FORM_20	20.00	21.860	21.942	29.708	30.477	0.27	1.81	21.90	30.09	8.19
P18_AGED+FORM_21	21.00	22.338	21.229	31.072	31.950	3.60	1.97	21.78	31.51	9.73
P18_AGED+FORM_22	22.00	23.893	26.934	31.816	32.433	8.46	1.36	25.41	32.12	6.71
P18_AGED+FORM_23	23.00	26.165	26.385	30.622	31.029	0.59	0.93	26.28	30.83	4.55
P18_AGED+FORM_24	24.00	28.093	26.385	33.191	32.771	4.43	0.90	27.24	32.98	5.74

Table A26

Calculations for NRF1 Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	6.33	0.14	0.91	0.88
	6.48	0.29	0.82	0.80
	6.52	0.33	0.80	0.78
	5.57	-0.63	1.54	1.50
	6.28	0.09	0.94	0.92
	5.98	-0.22	1.16	1.13
AVE	**6.19**		**1.03**	**1.00**
			SEM	***0.11***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	7.03	0.83	0.56	0.55
	7.28	1.09	0.47	0.46
	6.84	0.65	0.64	0.62
	6.63	0.44	0.74	0.72
	7.45	1.26	0.42	0.41
	6.37	0.17	0.89	0.86
			AVE	**0.60**
			SEM	***0.07***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	7.68	1.49	0.36	0.35
	7.63	1.43	0.37	0.36
	7.87	1.68	0.31	0.30
	6.49	0.29	0.82	0.79
	2.46	-3.74	13.35	12.98
	3.43	-2.76	6.78	6.60
			AVE	**3.56**
			SEM	***2.14***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	8.29	2.10	0.23	0.23
	8.19	2.00	0.25	0.24
	9.73	3.53	0.09	0.08
	6.71	0.52	0.70	0.68
	4.55	-1.64	3.13	3.04
	5.74	-0.45	1.37	1.33
			AVE	**0.93**
			SEM	***0.46***

Table A27

Raw Data for TFAM Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**TFAM**	**TFAM**	**RPS13**	**TFAM**	**RPS13**	**TFAM**	**DCt**
P5_CON_01	1.00	21.729	21.559	24.732	24.651	0.56	0.23	21.64	24.69	3.05
P5_CON_02	2.00	21.901	21.898	24.906	24.946	0.01	0.11	21.90	24.93	3.03
P5_CON_03	3.00	21.763	21.963	25.047	24.981	0.65	0.18	21.86	25.01	3.15
P5_CON_04	4.00	21.860	22.092	25.140	25.073	0.75	0.19	21.98	25.11	3.13
P5_CON_05	5.00	21.950	21.716	25.585	25.887	0.76	0.83	21.83	25.74	3.90
P5_CON_06	6.00	22.054	22.011	26.431	27.414	0.14	2.58	22.03	26.92	4.89
P5_CON+FORM_07	7.00	24.017	24.032	25.867	25.842	0.04	0.07	24.02	25.85	1.83
P5_CON+FORM_08	8.00	23.486	23.546	25.739	25.858	0.18	0.32	23.52	25.80	2.28
P5_CON+FORM_09	9.00	23.502	23.691	25.415	25.500	0.57	0.23	23.60	25.46	1.86
P5_CON+FORM_10	10.00	23.730	23.458	25.026	25.042	0.81	0.04	23.59	25.03	1.44
P5_CON+FORM_11	11.00	24.360	24.252	27.211	26.808	0.31	1.05	24.31	27.01	2.70
P5_CON+FORM_12	12.00	24.733	25.031	26.158	26.376	0.85	0.59	24.88	26.27	1.39
P18_AGED_13	13.00	24.212	24.096	26.133	26.207	0.34	0.20	24.15	26.17	2.02
P18_AGED_14	14.00	23.813	23.743	25.293	25.352	0.21	0.17	23.78	25.32	1.54
P18_AGED_15	15.00	24.002	24.183	25.879	25.815	0.53	0.17	24.09	25.85	1.75
P18_AGED_16	16.00	24.165	23.900	25.958	25.615	0.78	0.94	24.03	25.79	1.75
P18_AGED_17	17.00	27.419	27.601	25.595	25.527	0.47	0.19	27.51	25.56	-1.95
P18_AGED_18	18.00	26.200	25.482	24.028	24.221	1.97	0.56	25.84	24.12	-1.72
P18_AGED+FORM_19	19.00	20.979	21.104	26.020	25.723	0.42	0.81	21.04	25.87	4.83
P18_AGED+FORM_20	20.00	21.860	21.942	25.254	25.138	0.27	0.32	21.90	25.20	3.30
P18_AGED+FORM_21	21.00	22.338	21.229	25.967	25.919	3.60	0.13	21.78	25.94	4.16
P18_AGED+FORM_22	22.00	23.893	26.934	26.441	26.378	8.46	0.17	25.41	26.41	1.00
P18_AGED+FORM_23	23.00	26.165	26.385	26.797	26.348	0.59	1.20	26.28	26.57	0.30
P18_AGED+FORM_24	24.00	28.093	26.385	26.633	26.876	4.43	0.64	27.24	26.75	-0.48

Table A28

Calculations for TFAM Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	3.05	-0.48	1.39	1.27
	3.03	-0.50	1.41	1.29
	3.15	-0.37	1.30	1.18
	3.13	-0.39	1.31	1.20
	3.90	0.38	0.77	0.70
	4.89	1.37	0.39	0.35
AVE	**3.52**		**1.10**	**1.00**
			SEM	***0.16***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	1.83	-1.69	3.24	2.95
	2.28	-1.24	2.37	2.16
	1.86	-1.66	3.17	2.89
	1.44	-2.08	4.24	3.87
	2.70	-0.82	1.77	1.61
	1.39	-2.14	4.41	4.02
			AVE	**2.92**
			SEM	***0.38***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	2.02	-1.51	2.85	2.60
	1.54	-1.98	3.95	3.60
	1.75	-1.77	3.41	3.11
	1.75	-1.77	3.41	3.12
	-1.95	-5.47	44.43	40.57
	-1.72	-5.24	37.83	34.54
			AVE	**14.59**
			SEM	***7.30***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	4.83	1.31	0.40	0.37
	3.30	-0.23	1.17	1.07
	4.16	0.63	0.64	0.59
	1.00	-2.53	5.77	5.27
	0.30	-3.23	9.37	8.55
	-0.48	-4.01	16.11	14.70
			AVE	**5.09**
			SEM	***2.33***

Table A29

Raw Data for PGC-1β Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**PGC1B**	**PGC1B**	**RPS13**	**PGC1B**	**RPS13**	**PGC1B**	**DCt**
P5_CON_01	1.00	22.064	22.089	31.267	31.345	0.08	0.18	22.08	31.31	9.23
P5_CON_02	2.00	22.431	22.266	31.072	31.376	0.52	0.69	22.35	31.22	8.88
P5_CON_03	3.00	22.064	21.960	30.978	30.863	0.33	0.26	22.01	30.92	8.91
P5_CON_04	4.00	21.855	21.614	32.357	31.911	0.78	0.98	21.73	32.13	10.40
P5_CON_05	5.00	21.950	21.716	30.978	31.782	0.76	1.81	21.83	31.38	9.55
P5_CON_06	6.00	22.054	22.011	31.929	31.255	0.14	1.51	22.03	31.59	9.56
P5_CON+FORM_07	7.00	24.697	24.920	31.890	32.494	0.64	1.33	24.81	32.19	7.38
P5_CON+FORM_08	8.00	24.117	24.092	31.758	31.817	0.07	0.13	24.10	31.79	7.68
P5_CON+FORM_09	9.00	23.828	23.819	31.512	31.861	0.03	0.78	23.82	31.69	7.86
P5_CON+FORM_10	10.00	22.914	22.570	31.598	31.287	1.07	0.70	22.74	31.44	8.70
P5_CON+FORM_11	11.00	24.360	24.252	31.831	31.111	0.31	1.62	24.31	31.47	7.17
P5_CON+FORM_12	12.00	23.536	23.573	31.322	32.059	0.11	1.64	23.55	31.69	8.14
P18_AGED_13	13.00	24.127	24.141	32.809	32.161	0.04	1.41	24.13	32.49	8.35
P18_AGED_14	14.00	23.505	23.373	32.039	32.269	0.40	0.51	23.44	32.15	8.71
P18_AGED_15	15.00	23.654	23.668	33.197	33.537	0.04	0.72	23.66	33.37	9.71
P18_AGED_16	16.00	23.265	23.309	31.945	31.732	0.13	0.47	23.29	31.84	8.55
P18_AGED_17	17.00	23.041	23.022	31.726	32.013	0.06	0.64	23.03	31.87	8.84
P18_AGED_18	18.00	23.455	23.299	29.757	29.362	0.47	0.94	23.38	29.56	6.18
P18_AGED+FORM_19	19.00	23.877	24.743	32.035	31.386	2.52	1.45	24.31	31.71	7.40
P18_AGED+FORM_20	20.00	23.612	23.755	30.253	31.342	0.43	2.50	23.68	30.80	7.11
P18_AGED+FORM_21	21.00	23.860	24.095	32.000	31.420	0.69	1.29	23.98	31.71	7.73
P18_AGED+FORM_22	22.00	25.484	25.574	32.232	32.286	0.25	0.12	25.53	32.26	6.73
P18_AGED+FORM_23	23.00	25.306	25.220	31.981	33.117	0.24	2.47	25.26	32.55	7.29
P18_AGED+FORM_24	24.00	26.355	26.328	31.794	32.502	0.07	1.56	26.34	32.15	5.81

Table A30

Calculations for PGC-1β Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	9.23	-0.19	1.14	1.08
	8.88	-0.54	1.46	1.38
	8.91	-0.51	1.43	1.35
	10.40	0.98	0.51	0.48
	9.55	0.13	0.92	0.86
	9.56	0.14	0.91	0.86
AVE	**9.42**		**1.06**	**1.00**
			SEM	***0.14***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	7.38	-2.04	4.10	3.87
	7.68	-1.74	3.33	3.15
	7.86	-1.56	2.94	2.78
	8.70	-0.72	1.65	1.55
	7.17	-2.25	4.77	4.51
	8.14	-1.28	2.44	2.30
			AVE	**3.03**
			SEM	***0.43***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	8.35	-1.07	2.10	1.98
	8.71	-0.71	1.63	1.54
	9.71	0.29	0.82	0.77
	8.55	-0.87	1.83	1.72
	8.84	-0.58	1.50	1.41
	6.18	-3.24	9.43	
			AVE	**1.49**
			SEM	***0.20***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	7.40	-2.02	4.05	3.83
	7.11	-2.31	4.94	4.67
	7.73	-1.69	3.22	3.04
	6.73	-2.69	6.45	6.09
	7.29	-2.13	4.39	4.14
	5.81	-3.61	12.23	
			AVE	**4.35**
			SEM	***0.51***

Table A31

Raw Data for ATG1 Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**ATG1**	**ATG1**	**RPS13**	**ATG1**	**RPS13**	**ATG1**	**DCt**
P5_CON_01	1.00	21.564	21.516	28.647	28.585	0.16	0.15	21.54	28.62	7.08
P5_CON_02	2.00	21.833	21.813	30.409	29.281	0.07	2.67	21.82	29.84	8.02
P5_CON_03	3.00	21.492	21.383	28.272	28.554	0.36	0.70	21.44	28.41	6.98
P5_CON_04	4.00	21.290	21.161	28.922	29.211	0.43	0.70	21.23	29.07	7.84
P5_CON_05	5.00	21.596	21.475	29.047	29.208	0.40	0.39	21.54	29.13	7.59
P5_CON_06	6.00	21.660	21.642	29.141	28.776	0.06	0.89	21.65	28.96	7.31
P5_CON+FORM_07	7.00	24.390	24.325	30.228	30.216	0.19	0.03	24.36	30.22	5.86
P5_CON+FORM_08	8.00	23.719	23.570	30.160	30.152	0.45	0.02	23.64	30.16	6.51
P5_CON+FORM_09	9.00	23.758	25.718	Undetermined	Undetermined	5.60	#DIV/0!	24.74	#DIV/0!	#DIV/0!
P5_CON+FORM_10	10.00	23.720	24.211	30.202	30.868	1.45	1.54	23.97	30.53	6.57
P5_CON+FORM_11	11.00	23.788	23.790	29.899	29.686	0.01	0.51	23.79	29.79	6.00
P5_CON+FORM_12	12.00	24.831	24.793	31.575	31.127	0.11	1.01	24.81	31.35	6.54
P18_AGED_13	13.00	24.215	24.200	30.272	29.825	0.05	1.05	24.21	30.05	5.84
P18_AGED_14	14.00	23.765	23.771	30.070	29.717	0.02	0.84	23.77	29.89	6.13
P18_AGED_15	15.00	24.590	24.849	30.820	30.470	0.74	0.81	24.72	30.64	5.93
P18_AGED_16	16.00	23.310	23.250	29.572	29.485	0.18	0.21	23.28	29.53	6.25
P18_AGED_17	17.00	23.006	23.063	29.580	29.094	0.18	1.17	23.03	29.34	6.30
P18_AGED_18	18.00	23.455	23.299	28.431	28.224	0.47	0.52	23.38	28.33	4.95
P18_AGED+FORM_19	19.00	23.308	23.277	28.825	28.770	0.09	0.14	23.29	28.80	5.51
P18_AGED+FORM_20	20.00	24.770	24.695	30.102	30.120	0.21	0.04	24.73	30.11	5.38
P18_AGED+FORM_21	21.00	25.110	24.951	30.735	31.461	0.45	1.65	25.03	31.10	6.07
P18_AGED+FORM_22	22.00	26.654	26.458	31.469	32.000	0.52	1.18	26.56	31.73	5.18
P18_AGED+FORM_23	23.00	25.306	25.220	31.182	31.501	0.24	0.72	25.26	31.34	6.08
P18_AGED+FORM_24	24.00	27.450	27.456	31.789	31.770	0.02	0.04	27.45	31.78	4.33

Table A32

Calculations for ATG1 Gene Expression.

ALL expressed relative to D4 CON				
P5_CON_1-6	**dct**	**ddct**	**fold**	**norm**
	7.08	-0.39	1.31	1.27
	8.02	0.55	0.68	0.66
	6.98	-0.49	1.41	1.36
	7.84	0.37	0.77	0.75
	7.59	0.12	0.92	0.89
	7.31	-0.16	1.12	1.08
AVE	**7.47**		**1.04**	**1.00**
			SEM	***0.12***
P5_CON+FORM_7-12	**dct**	**ddct**	**fold**	**norm**
	5.86	-1.60	3.04	2.94
	6.51	-0.96	1.94	1.88
	#DIV/0!	#DIV/0!	#DIV/0!	
	6.57	-0.90	1.87	1.80
	6.00	-1.47	2.76	2.67
	6.54	-0.93	1.90	1.84
			AVE	**2.22**
			SEM	***0.24***
P18_AGED_13-18	**dct**	**ddct**	**fold**	**norm**
	5.84	-1.63	3.09	2.99
	6.13	-1.34	2.54	2.45
	5.93	-1.54	2.91	2.82
	6.25	-1.22	2.33	2.25
	6.30	-1.17	2.24	2.17
	4.95	-2.52	5.73	
			AVE	**2.53**
			SEM	***0.16***
P18_AGED+FORM_19-24	**dct**	**ddct**	**fold**	**norm**
	5.51	-1.96	3.90	3.77
	5.38	-2.09	4.26	4.11
	6.07	-1.40	2.64	2.55
	5.18	-2.29	4.89	4.72
	6.08	-1.39	2.62	2.53
	4.33	-3.14	8.83	8.53
			AVE	**4.37**
			SEM	***0.91***

Table A33

Raw Data for MuRF1 Gene Expression.

		Duplicates				CV %		Means		
ID	**Plate ID**	**RPS13**	**RPS13**	**MuRF1**	**MuRF1**	**RPS13**	**MuRF1**	**RPS13**	**MuRF1**	**DCt**
P5_CON_01	1.00	21.564	21.516	Undetermined	34.541	0.16	#DIV/0!	21.54	34.54	13.00
P5_CON_02	2.00	21.833	21.813	Undetermined	Undetermined	0.07	#DIV/0!	21.82	#DIV/0!	#DIV/0!
P5_CON_03	3.00	21.492	21.383	Undetermined	Undetermined	0.36	#DIV/0!	21.44	#DIV/0!	#DIV/0!
P5_CON_04	4.00	21.290	21.161	33.687	Undetermined	0.43	#DIV/0!	21.23	33.69	12.46
P5_CON_05	5.00	21.596	21.475	Undetermined	Undetermined	0.40	#DIV/0!	21.54	#DIV/0!	#DIV/0!
P5_CON_06	6.00	21.660	21.642	Undetermined	33.528	0.06	#DIV/0!	21.65	33.53	11.88
P5_CON+FORM_07	7.00	24.390	24.325	Undetermined	Undetermined	0.19	#DIV/0!	24.36	#DIV/0!	#DIV/0!
P5_CON+FORM_08	8.00	23.719	23.570	Undetermined	Undetermined	0.45	#DIV/0!	23.64	#DIV/0!	#DIV/0!
P5_CON+FORM_09	9.00	23.758	25.718	Undetermined	Undetermined	5.60	#DIV/0!	24.74	#DIV/0!	#DIV/0!
P5_CON+FORM_10	10.00	23.720	24.211	Undetermined	Undetermined	1.45	#DIV/0!	23.97	#DIV/0!	#DIV/0!
P5_CON+FORM_11	11.00	23.788	23.790	Undetermined	Undetermined	0.01	#DIV/0!	23.79	#DIV/0!	#DIV/0!
P5_CON+FORM_12	12.00	24.831	24.793	Undetermined	Undetermined	0.11	#DIV/0!	24.81	#DIV/0!	#DIV/0!
P18_AGED_13	13.00	24.215	24.200	Undetermined	Undetermined	0.05	#DIV/0!	24.21	#DIV/0!	#DIV/0!
P18_AGED_14	14.00	23.765	23.771	Undetermined	Undetermined	0.02	#DIV/0!	23.77	#DIV/0!	#DIV/0!
P18_AGED_15	15.00	24.590	24.849	Undetermined	33.675	0.74	#DIV/0!	24.72	33.68	8.96
P18_AGED_16	16.00	23.310	23.250	Undetermined	Undetermined	0.18	#DIV/0!	23.28	#DIV/0!	#DIV/0!
P18_AGED_17	17.00	23.006	23.063	Undetermined	Undetermined	0.18	#DIV/0!	23.03	#DIV/0!	#DIV/0!
P18_AGED_18	18.00	23.455	23.299	Undetermined	Undetermined	0.47	#DIV/0!	23.38	#DIV/0!	#DIV/0!
P18_AGED+FORM_19	19.00	23.308	23.277	Undetermined	Undetermined	0.09	#DIV/0!	23.29	#DIV/0!	#DIV/0!
P18_AGED+FORM_20	20.00	24.770	24.695	Undetermined	Undetermined	0.21	#DIV/0!	24.73	#DIV/0!	#DIV/0!
P18_AGED+FORM_21	21.00	25.110	24.951	Undetermined	Undetermined	0.45	#DIV/0!	25.03	#DIV/0!	#DIV/0!
P18_AGED+FORM_22	22.00	26.654	26.458	Undetermined	Undetermined	0.52	#DIV/0!	26.56	#DIV/0!	#DIV/0!
P18_AGED+FORM_23	23.00	25.306	25.220	Undetermined	Undetermined	0.24	#DIV/0!	25.26	#DIV/0!	#DIV/0!
P18_AGED+FORM_24	24.00	27.450	27.456	Undetermined	Undetermined	0.02	#DIV/0!	27.45	#DIV/0!	#DIV/0!