The Relationships of Age, Physical Activity Level, Adiposity, and Diet, with Human Satellite Myogenesis, and Metabolism

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In healthy individuals, satellite cells are partly responsible for muscle repair and preventing atrophy. Previous studies have linked the loss of muscle mass associated with aging to satellite cell dysfunction, postulating that satellite cell function diminishes with age. New evidence suggests that this may not be true as satellite cells collected from healthy aged participants appear indistinguishable from their healthy young counterparts. Satellite cell dysfunction appears to be more mechanistically linked to poor lifestyle factors such as low physical activity, improper diet, and increased adiposity. For this study, satellite cell function was evaluated against the effects of aging, diet, activity level, and adiposity. Satellite cells were collected from the vastus lateralis of sedentary (<2 hours/week activity) male donors categorized into young (18-30 years) and older (60-80 years) groups, as well as a young endurance trained group (18-30 years, 5+ hours/week of running/cycling). Cells were collected in young sedentary males before and after a four-week, high fat (55% of kcal), and hypercaloric (+1000 kcal over DEE) diet (HFHCD). Cells were also subjected to an in-vitro, high substrate media (HSM) challenge, then grown in media with a fivefold increase in glucose (25 mM) and an additional 400 uM of fatty acids (2:1 palmitate:oleate) before seven days of serum starved differentiation. The cells were evaluated for their proliferation rate, ability to differentiate (fusion index), rate of reactive oxygen species (ROS) production, and capacity for substrate oxidation (glucose and fatty acid). The young group exhibited a lower proportion of body fat than the older group (22.4%±8.1 vs. 28.3%±6.3). When compared to the older group, the young group also presented elevated oxidative efficiency (68%, p<0.05) and reduced pyruvate oxidation (~60%, p<0.05) in measures of muscle tissue homogenate. However, isolated satellite cells from the young and older group demonstrated no observable differences in any measures (proliferation rate, fusion index, ROS production, or substrate oxidation), other than increased oxidative efficiency in cells from older vs. younger donors. Cells from young endurance trained donors
demonstrated faster proliferation rates (39%, p<0.05) and elevated early stage fusion (33%, p<0.05) when compared to cells from older individuals. Compared to pre-diet measures, cells collected post HFHCD revealed significantly reduced proliferation rates (-19%, p<0.05). When grown in HSM (as compared to control media), cells from young lean (<25% BF) and trained participants had blunted proliferation rates (-4.8% and -12.6%, p<0.05), fusion index scores (p<0.05), and ROS production rates. Cells collected from participants with higher adiposity (>25% BF) and those collected post HFHCD experienced increased proliferation and fusion when exposed to the HSM. This data suggests that donor activity level, adiposity, and diet but not age are mediating factors for satellite cell function. The cells appear to develop a preference for their in-vivo environment, as cells collected from the leaner and trained participants had their proliferation and fusion rates reduced when exposed to HSM. Conversely, exposure to the HSM accelerated the proliferation and fusion of cells collected from donors with higher body fat and those collected post HFHCD.
General Audience Abstract:

The continually active nature of muscle tissue leaves it vulnerable to physical and chemical damage. Any physical activity especially exercise can cause numerous sites of micro-damage to the muscle tissue. To maintain function, damaged muscle tissue is continually remodeled throughout lifespan. To replace damaged muscle tissue, a special type of muscle specific stem cell, termed a satellite cell, is utilized. Satellite cells lay dormant inside the muscle tissue until their activity is promoted by signals that result from muscle injury. Once activated, satellite cells develop into new muscle cells, a process known as myogenesis. Proper function of satellite cells is required for our muscle tissue to respond to injury. Past studies have demonstrated that aging adversely impacts satellite cell activity, which is thought to contribute to loss of muscle mass and strength typically observed with aging. However, newer evidence suggest that an unhealthy diet and a sedentary lifestyle may contribute to satellite cell dysfunction. This study studied satellite cell function to determine if aging, sedentary lifestyle, and unhealthy diet contributed to satellite cell dysfunction. The results demonstrated that age had no effect on any measures of satellite cell function. Findings suggested that a sedentary lifestyle resulted in diminished satellite cell function regardless of age. This study demonstrated that four weeks on a high-fat/high-calorie “western style” diet decreased satellite cell function. It was also demonstrated that exposing satellite cells to a high-sugar/high-fat treatment altered their cellular function. These data suggest that alterations in macronutrient content of a person’s diet may adversely impact satellite cell function, and decrease the potential for myogenesis. In conclusion aging had no effect satellite cell function. A high-fat/high-calorie diet and sedentary lifestyle did contribute to satellite cell dysfunction. It appears that the loss of muscle mass and strength observed in aged individuals is not associated with satellite cell function.
Dedication

I dedicate this work to my father, Don. He always supported me and I hope this would have made him proud.
Acknowledgements

**Matt:** I immensely appreciate that you decided to give me a chance in your lab and that you took the time to be my mentor. I understand that my initial approach to graduate school was not typical and I now realize the risk you took in bringing in such an unknown factor. There are not enough pages in this document to state how much I appreciate your guidance nor enough to thank you for all the ways you have made life better. When I came to you, I was stagnant with no clue how to proceed, you guided me to the future I have always wanted, and a purpose to keep me grounded.

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Chapter 1: Introduction

Muscle is a highly adaptable and metabolically active tissue that must respond to physiological and environmental demands through constant growth and atrophy. Maintenance of this plasticity is critical for health and survival.\(^1\) Key in muscle’s renewability is the satellite cell, a muscle specific stem cell that exists among working muscle fibers and is the progenitor for new myoblasts and fibers. For much of their lifetime satellite cells remain in a quiescent state awaiting recruitment.

The somewhat fragile nature of muscle fibers and the hazards of repetitive forceful contraction result in a tissue that incurs continual damage.\(^1,2\) Muscle injury activates satellite cells, which are recruited to damaged tissue for maintenance.\(^3\) The activated satellite cells can replace a fiber which is completely lost by fusing together to form a new fiber (hyperplasia) or fuse with preexisting fibers that are still salvageable (hypertrophy). While damaged fibers have the capability to repair on their own, fusion of new cells with existing fibers brings in additional nuclei and organelles, which can improve the capacity of the fiber to heal.\(^1\)

The contribution of satellite cell DNA to an existing multi-nucleated fiber can restore and even increase the transcriptional capacity of the fiber.\(^4\) This not only helps in the immediate repair of the fiber but can also increase the adaptive potential in the future. Increased nuclear content is typically associated with trained muscle tissue and allows that tissue to better respond to the stresses of exercise.\(^4-6\) Lower nuclear content is evident in the muscle tissue of individuals suffering from a range of chronic diseases such as cancer, diabetes and sarcopenia.\(^6\)
Satellite cell involvement in muscle regeneration is partially reliant on the number of quiescent cells available, referred to as the satellite cell pool. The satellite cell pool size varies depending on the muscle, fiber type, and activity level, but it may be affected by aging and disease. With a constant recruitment of cells, the pool size is maintained like that of other tissue specific stem cells. Early in proliferation, before any morphological changes, a proportion of the satellite cells return to the quiescent pool. This asymmetric division protects the cells in the pool from DNA damage due to excessive replication, and can increase pool size (noted in figure 1). Without continual activation, the pool size diminishes.

Satellite cell activation, proliferation, differentiation, and fusion into myofibers is controlled through the coordinated expression of several transcription factors, including DELTA/NOTCH (activation/proliferation), MyoD (differentiation), Myf5 (differentiation), MyoG (differentiation/fusion). Expression of these transcription factors can be altered through hormonal (IGF-1 (insulin-like growth factor-1), myostatin, etc.) and inflammatory (TNF-α (tumor necrosis factor-alpha), IL-6 (interleukin-6), etc.) signaling. This signaling can be

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**Figure 1.1 Satellite Cell Replication (adapted from Yin et al., 2013).**

Transcriptional Regulation:

- Pax-7
- Notch-delta
- MyoD
- MyoD, Myf-5
- Myogenin

Quiescence | Proliferation | Differentiation/Fusion | New/Repaired Myotube
---|---|---|---
![Diagram showing the transition between quiescence, proliferation, differentiation/fusion, and new/repaired myotube stages]

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benificial or detrimental. IGF-1\textsuperscript{16} and IL-6\textsuperscript{15} can increase satellite cell proliferation while excess myostatin\textsuperscript{17} and TNF-\textgreek{a}\textsuperscript{14} can inhibit differentiation (noted in figure 2).

Skeletal muscle repair is auto-regulated by the expression of muscle released cytokines, or myokines, in particular TNF-\textgreek{a}, and IL-6. While the effect of exercise on increasing muscle myokine release has been known for some time, the reasons for this release has still to be understood. Recent studies have shown that IL-6\textsuperscript{18} and TNF-\textgreek{a}\textsuperscript{19} have control over satellite cell maturation. Studies of satellite cells in culture have shown that the addition of IL-6 increases satellite cell proliferation.\textsuperscript{15} Differentiating satellite cells secrete TNF-\textgreek{a} in an autocrine and paracrine fashion.\textsuperscript{19} Treatment with low dose TNF-\textgreek{a} (0.05 ng/ml) can initiate satellite cell differentiation.\textsuperscript{19} This effect is lost when TNF-\textgreek{a} treatments reach higher concentrations at which point differentiation is inhibited.\textsuperscript{20}

Muscle mass correlates with higher metabolic rates and lower rates of obesity.\textsuperscript{21-23} Increased muscle mass is associated with increased quality of life.\textsuperscript{24} This is especially true in older populations, where increased muscle mass and strength increases mobility and decreases risk of injury from falls.\textsuperscript{24,25} Despite the benefits of greater muscle mass, aging is associated with a 1\% decrease in lean mass and a 3-4\% loss in strength per year.\textsuperscript{26,27} The losses from aging can be further exacerbated with the onset of sarcopenia, which increases satellite cell dysfunction.\textsuperscript{25,28} Lower muscle mass is also a common consequence of other chronic diseases including; obesity, cancer, diabetes, COPD, and heart disease.\textsuperscript{29,30}

To explain this phenomenon, it has been reasoned that a loss of muscle mass from aging may be due to cellular level alterations in DNA and protein expression.\textsuperscript{31-34} Due to the
correlation of muscle mass loss with aging, a loss of satellite cell number and functionality with aging has been assumed. This appears to be true in unhealthy aged individuals but is not well studied in healthy aged populations (a schematic of this can be seen in figure 2B). Early experiments in mice and rats have shown that the replicative capacity of satellite cells and their ability to differentiate decreases as the age of the donor increases. These findings were repeated in a few human studies as well. Along with decreases in proliferation there are also studies, which demonstrate a reduction in satellite cell pool size with aging. The limitations of these studies are that they did not account for other confounding factors between the young and aged donors such as activity level, body fat, inflammation, and the existence of chronic disease.

The greatest loss in proliferation potential occurs throughout early human development from fetal cells through adolescence to early adulthood (0 to 20 years of age), reducing nearly 80% during that time. This loss in replicative capacity occurs well before the typical losses in physical function observed in late adulthood (65+ years of age). Despite what is commonly accepted, after physical maturity, chronological aging alone may have very little to do with cellular decline. This decline may be more linked to negative lifestyle factors, which persist as individuals’ age. Both visually and mechanistically, there is high similarity in the proliferation and differentiation of myoblast collected from young and healthy elderly adult human donors. Differences between young and aged population mainly appear when the aged population suffers from underlying conditions, such as obesity, inactivity or even cancer. Research is needed to understand why a loss of physical function is not experienced until late adulthood if the more substantial decline in cellular capabilities occurs in early life.
The relationship between aging and muscle loss may be due to the accumulation of diseases for which the risk increases with age. Decreased physical activity and obesity both contribute to poor satellite cell function in young and old participants, but are more likely in older individuals. Regular physical activity can maintain or increase satellite cell pool size, but a sedentary lifestyle reduces satellite cell numbers and inhibits activation by reducing MyoD expression. Without regular physical activity, older participants are more likely to see a decline in satellite cell function, but this is related to their sedentary lifestyle not their age. Sedentary young participants have satellite cells which function similarly to their older counterparts.

It is not well understood how obesity affects satellite cell function. Obesity is associated with increased blood glucose, free fatty acids, and inflammatory levels all of which could potentially influence myogenesis. In obese Zucker rats, satellite cell myogenesis is inhibited with the suspected cause being elevated blood glucose concentrations. These elevated glucose levels even lead to satellite cell adipogenesis and increased intramuscular fat storage.
effect of blood glucose levels on human satellite cell myogenesis has not been studied. The effects of elevated free fatty acid levels on satellite cell myogenesis have yet to be well studied in any human model.

Obesity is linked with increased expression of IL-6 and TNF-α, there is potential for dramatically reduced myogenesis.\textsuperscript{42} Obese individuals with metabolic syndrome exhibit chronically increased serum levels of IL-6 and TNF-α, both of which impair satellite cell function at different stages.\textsuperscript{15,42} Elevated IL-6 exposure causes cells to over proliferate and increases the likelihood of transdifferentiation and fibrosis.\textsuperscript{43} Elevated TNF-α levels inhibit proliferation and differentiation, and increase apoptosis through NF-kB.\textsuperscript{44} A single exposure to elevated TNF-α can cause epigenetic changes which alter satellite cell metabolism for several generations after the cytokine has been removed.\textsuperscript{14} Older individuals are at an increased risk of both obesity and inflammatory disease and the decreases in their satellite cell function may be more related to their inflammatory state rather than their numerical age.

While these conditions are dangerous, they can be prevented with regular exercise and a healthy diet. Based on the evidence available there is reason to believe that aging alone may not be the root cause of physiological changes, which prevent proper satellite cell function for muscle growth and repair. Exercise in elderly participants can increase satellite cell pool size and myogenesis.\textsuperscript{32} Exercise can even return the satellite cell function of older participants to match that of younger participants.\textsuperscript{45} This is further evidence that lifestyle factors, as opposed to aging, contribute more to diminished satellite cell function.
Chapter 2: Literature Review
Influence of Health, Obesity and Aging on Satellite Cell Function and Metabolism

Introduction

Muscle mass positively correlates to metabolic rate and lower rates of obesity.\textsuperscript{21-23} Increased muscle mass is associated with increased quality of life.\textsuperscript{24} This is especially true in older populations, where increased muscle mass and strength increases mobility and decreases risk of injury from falls.\textsuperscript{24,25} Despite the benefits of greater muscle mass, aging is associated with a 1% decrease in lean mass and a 3-4% loss in strength per year.\textsuperscript{26,27} These losses associated with aging can become pathologic with the diagnosis of sarcopenia (age related degenerative muscle loss), which is linked to greater satellite cell dysfunction.\textsuperscript{25,28} Lower muscle mass is also a common consequence of other chronic diseases including, obesity, cancer, diabetes, COPD, and heart disease.\textsuperscript{29,30}

To explain the loss in muscle mass associated with disease, many assess the mechanisms of tissue repair specifically those mediated by the satellite cell. Due to the correlation of muscle mass loss with aging, it is proposed that there is a loss of satellite cell number and functionality with aging. This concept may be unfounded, as the true effects of aging on satellite cell function have not be fully examined. Early experiments in mice and rats have shown that the replicative capacity of satellite cells decreases as the age of the donor increases.\textsuperscript{35} This is demonstrated in a few human studies as well.\textsuperscript{31,36} The limitations of these previous studies are that they did not account for other confounding factors between the young and old donors such as activity level, diet, adiposity, and chronic disease.

The involvement of satellite cells in muscle function is of importance due to their continual integration into differentiated tissue. Satellite cells are dedicated muscle stem cells that, in fully mature animals, remain in a dormant state until needed. In early growth and development, fetal myoblasts arise from the mesodermal layer and give rise to the skeletal muscle system.\textsuperscript{46} These myoblasts show
high replicative activity and eventually differentiate into myofibers, which lead to the development of most skeletal muscles. A portion of these fetal myoblasts will eventually become classical satellite cells.\textsuperscript{46,47} Once an animal has reached physical maturity and optimal muscling has been achieved, satellite cells enter a state of quiescence. These cells maintain a sentinel like function in that they survey muscle tissue for damage.

Satellite cells are activated by tissue damage accrued either through physical injury (crushing) or exercise (micro-tears).\textsuperscript{48} This damage activates signaling cascades, which release paracrine, and endocrine signaling molecules that begin the satellite cell recruitment process.\textsuperscript{3,19} Once recruited, satellite cells begin to proliferate in great numbers. This proliferation is mitotically asymmetric with a portion of the cells returning to quiescence in order to preserve the satellite cell pool and prevent replicative damage to DNA.\textsuperscript{7} Proliferating cells begin to express typical muscle proteins in a process of differentiation, which may have two fates. In the case of crushing, if the damage is great and a large amount tissue is lost, new cells will fuse together forming completely new myofibers, which replace those that were lost.\textsuperscript{46,48} If the damage is less substantial, as is typically observed in response to exercise, newly differentiated cells will fuse to existing fibers. This fusion will provide new DNA and transcriptional/translational machinery to the damaged myofibers, aiding in repair, and potentially leading to hypertrophy.\textsuperscript{46}

It is not well understood how satellite cell myogenesis is affected by lifestyle factors such as age, diet, and obesity. Past studies have shown that aging is a controlling negative factor for satellite cell myogenesis and with aging comes a decrease in the ability of the satellite cells to proliferate, differentiate, and replace lost muscle tissue. However, new evidence suggests this may not be true. The loss of function that was once attributed to age are now linked to other lifestyle factors such as diet, obesity, and activity level. Such poor lifestyle choices are more apt to be observed in aged populations thus markedly exacerbating diminished satellite cell function in such populations. However, evidence
has shown that older adults with healthier lifestyles have little to no loss in satellite cell function. The purpose of this review is to highlight the losses in satellite cell function associated with aged populations and explain their mechanistic links in the context of diet, obesity, and low levels of physical activity.

**Satellite Cell Myogenesis**

Satellite cells express several markers that designate their myogenic fate. Chief among these is the expression of paired box protein 7 (Pax-7), however Pax-3, myogenic factor 5 (Myf5), and M-cadherin, among others, are also expressed and can be used as satellite cell markers. These markers can vary depending on the origin of the satellite cell and whereas Pax-7 is primarily expressed in human and other mammalian satellite cells, its expression, as well as the expression of the other transcription factors are not always necessary for myogenesis. The expression level of these markers is also variable depending on the function of the satellite cell and the stage of its differentiation. While the roles of Pax-7 are not fully understood, it appears that it is responsible for maintaining satellite cell quiescence and activation while marking its commitment to the myogenic fate. Removal of Pax-7 results in an inability of skeletal muscle to heal and a loss in the satellite cell pool.

The activation and differentiation of satellite cells is crucial for the ability of an individual to recover from exercise and/or injury. The process of differentiation can vary but generally follows a consistent pattern. Activation occurs in response to factors released by cells that are damaged. Notch signaling, like in the majority stem cells, is the key to activating the dormant satellite cells. As stated, Pax-7 is expressed in quiescent satellite cells as the designator of their myogenic fate and is typically ramped up immediately after activation to begin transcription of other myogenic factors. However, this increase may be dependent on the NOTCH signaling pathway and satellite cells must also express NOTCH-1 receptors to be recruited when necessary. At the site of muscle injury, both injured
myotubes and activated satellite cells release DELTA, a NOTCH ligand, which binds to NOTCH-1.\textsuperscript{3,54}

When triggered by DELTA binding, the intracellular section of the NOTCH-1 receptor translocates to the nucleus, where it begins a transcription process that activates the cell from its quiescent state and initiates proliferation.\textsuperscript{3,54}

<table>
<thead>
<tr>
<th>Quiescence</th>
<th>Proliferation</th>
<th>Differentiation/Fusion</th>
<th>New/Repaired Myotube</th>
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**Transcriptional Regulation:**

- Pax-7
- Notch-delta
- MyoD
- MyoD, Myf-5
- Myogenin

Figure 2.1 Satellite cell maturation (taken from Zammit, Partridge et al. 2006).

Shortly after activation, expression of MyoD is upregulated, and initiates differentiation.\textsuperscript{55} Two to three days following the initiation of the differentiation process, Pax-7 levels decrease in those cells committed to differentiation, indicating a cell type switch from satellite cell to functional myocyte. During this same time, MyoD and Myf5 expression begins to increase, peaking after 3-4 days of activation, at which point expression of each begins to recede. MyoD and Myf5 inhibit cell cycle progression, stopping over-proliferation, allowing the cells to transform into their functional phenotype.\textsuperscript{9} The elevation of MyoD and Myf5 corresponds with the initiation of cellular fusion and the consolidation of these now differentiated cells into new or preexisting myotubes.\textsuperscript{49}

Myogenin (MyoG) levels rise, simultaneously coordinated with receding MyoD and Myf5 expression. This acts to complete the differentiation process. MyoG increases the expression of functional muscle proteins such as myosin heavy chains and acetylcholine receptors.\textsuperscript{49,56} The completion of this process results in the addition of new nuclei to existing myofibers or the creation of
new multi-nucleated myofibers. The addition of these new nuclei, in conjunction with added machinery for transcription and translation, increases the rate of hypertrophy to promote faster muscle growth.\textsuperscript{39} Elevated myofiber nuclei count may expedite recovery after an event of muscle atrophy.\textsuperscript{57} This leads to a positive adaptation that allows trained individuals to recover faster from bouts of exercise.

The pattern of satellite cell activation and differentiation may be constant across study models, but it must still be tested to see if this pattern holds true across the human species. Different population subsets (aged, trained, obese, etc.) may have more or less advantaged patterns of proliferation and differentiation. A miscue in any of these activation or differentiation signaling pathways due to certain lifestyle factors could inhibit muscle cell repair and contribute to sarcopenia. Improper differentiation can have fatal consequences in diseases of muscular dystrophy and myopathy.\textsuperscript{58}

**Pluripotent Satellite Cells**

Satellite cells are pluripotent cells that have the potential to differentiate into a variety of cell types to aid in muscle function. Satellite cells have shown, both \textit{in-vitro} and \textit{in-vivo}, a potential to differentiate into fibroblast, angiogenic precursor cells, osteocytes, and adipocytes.\textsuperscript{59,60} This pluripotency can be beneficial, as demonstrated by increased vascularity and fat storage in the muscle of trained endurance athletes.\textsuperscript{61} However, unintended activation of these pluripotent pathways in satellite cells leads to transdifferentiation and is a potential cause of disease, especially heart disease and cancer. Age related sarcopenia is associated with an increase in satellite cell fibrogenesis, leading to a loss of muscle function and fibrosis. Cancers related to muscle and other soft tissues such as sarcomas are associated with increased satellite cell angiogenesis. Obesity may cause satellite cell adipogenesis and increased lipid accumulation.\textsuperscript{41} Repetitive crushing contact leads to muscle ossification and calcification which can lead to muscle immobilization and potentially loss of limb.\textsuperscript{62}
It is important to note that though Pax-7 expressing satellite cells should undergo myogenesis when activated, they still have a pluripotent potential. If not differentiated into muscle tissue, satellite cells can form fibroblasts. Like all organ tissues, muscle is susceptible to fibrosis, which is mostly attributed to miscues in the immune response. In a proper function, fibrosis is a quick action to heal a large wound and quickly restore partial function to a torn muscle. Muscle tissue works closely with cells of the immune system to orchestrate this type of repair. However, this fibrotic pathway may also be unintentionally activated by smaller injuries (micro-tears) due to diseases associated with chronic inflammation and/or an altered immune responses. This causes normal muscle tissue to be replaced by fibrotic tissue and results in a loss of muscle function.

Macrophages control the secretion of transformative growth factor-beta (TGF-β). TGF-β is released by injured tissue and initiates fibrosis by activating the SMAD pathway, which inhibits myogenin expression and myogenesis. Inhibiting the SMAD pathway restores myogenesis and reduces fibrosis. There does appear to be some need for TGF-β in muscle repair as complete inhibition of the TGF-β cytokine reduces fibrosis, but also impedes satellite cell activation and differentiation. For proper function, it appears that TGF-β must be released at the site of injury to activate the repair process, but recruited macrophages must intervene and reduce the TGF-β signals in order to prevent it from causing altered differentiation of satellite cells into fibroblasts. If macrophages do not function properly or are inhibited from accessing the site of injury, satellite cell activation and recruitment is slowed. This could result in the damaged muscle tissue being replaced with fibrotic tissue.

Myostatin and Wnt signaling also contribute to fibrosis. Myostatin is primarily thought of as a regulator of muscle breakdown, but has recently demonstrated the ability to increase proliferation of fibroblasts in muscle. Myostatin activates the SMAD pathway, like TGF-β, but also activates MAPK and Akt. Myostatin may have an autocrine function as it binds to the fibroblasts from which it is released, increasing fibroblast function. Fibrosis related to myostatin is enhanced by Wnt signaling.
Growing mouse satellite cells in sera from aged mice induces Wnt signaling through activation of the frizzled receptor.\textsuperscript{78} Wnt signaling alters the myogenic fate of satellite cells causing them to differentiate into fibroblasts.\textsuperscript{78} Inhibitors of myostatin and Wnt signaling have both been used to successfully reduce fibrosis.\textsuperscript{77,78}

Satellite cells have demonstrated adipogenic potential. High glucose and high fat treatments in culture cause muscle derived satellite cells to differentiate in adipocytes.\textsuperscript{79,80} Inducing adipogenesis is also possible in culture by growing cells in a highly oxygenated environment.\textsuperscript{81} Adipogenesis may be controlled through myostatin as well, through inhibiting the expression of MyoD and myogenin.\textsuperscript{17} In-vivo, obese Zucker rats show increased conversion of satellite cells to adipocytes, which can be mechanistically linked to increased blood glucose levels.\textsuperscript{41} The satellite cells from these obese rats have similar MyoD and MyoG expression levels compared to their lean counterparts, but they show blunted insulin sensitivity, increased leptin secretion, and increased PPARγ expression. All of these may contribute to increased adipogenesis.\textsuperscript{41}

This adipogenic conversion appears to be specific to certain satellite cells as those from type I fibers show greater adipogenic potential.\textsuperscript{82} This is demonstrated by comparing satellite cells collected from muscles having higher proportions of type I fibers and satellite cells collected from muscles having higher proportions of type II fibers, where higher adipogenic rates are exhibited in cells from type-I fibers and to lower adipogenic rates are associated with cells from type-II fibers.\textsuperscript{83} Even in satellite cells that maintain their myogenic fate and express muscle proteins, low MyoD expression can result in mass accumulation of lipids in a similar process observed in adipocytes.\textsuperscript{84}

**Inflammation and Myogenesis**

Satellite cell myogenesis is partially controlled through cytokine signaling. Acute and chronic inflammation can both be hindrances to proper myogenesis. Muscle released cytokines or myokines
have autocrine and paracrine functions in the regulation of myogenesis.\textsuperscript{19} Of the cytokines affected by muscle contraction, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-\(\alpha\)) appear to have the highest myogenic regulatory influence. IL-6 concentrations typically increase several fold immediately following exercise while TNF-\(\alpha\) secretion is suppressed for several hours before increasing later.\textsuperscript{85}

Myokines can be stimulated by immune cells. Monocytes attracted to the site of damage release a range of soluble factors that contribute to the makeup of the muscle sera, and promote the release of IL-6.\textsuperscript{15} Whether or not immune cells adopt a pro- or anti-inflammatory phenotype has a dramatic influence on their effect on myogenesis. Pro-inflammatory macrophages oppose myogenesis by inhibiting satellite cell fusion, both \textit{in-vitro} and \textit{in-vivo}.\textsuperscript{86} Anti-inflammatory macrophages promote satellite cell differentiation and fusion.\textsuperscript{86} Macrophage polarization may regulate muscle repair by first taking a pro-inflammatory role to limit over production of satellite cells, then switching to an anti-inflammatory role to ensure their addition to damaged fibers.\textsuperscript{86}

The myokine IL-6 has apparent regulatory control over myogenesis and satellite cell proliferation. Released by contracting muscle tissue, IL-6 has a paracrine function to stimulate proliferation in pax-7 positive satellite cells.\textsuperscript{87} This is done through downstream activation of STAT3, a transcription factor that promotes c-myc, MyoD, and cyclin D1, which are conserved regulators of cellular proliferation.\textsuperscript{87-90} STAT3 activation is a target of many pharmacological treatments that aim to promote satellite cell myogenesis and muscle regeneration.\textsuperscript{91} IL-6\textsuperscript{-/-} mice do not respond to exercise training and exhibit minimal muscle hypertrophy, which is in part due to a lack in proliferation of satellite cells.\textsuperscript{87} Showing a need for precise regulation, IL-6 overload prematurely activates differentiation in C2C12 cells. IL-6 treatment of C2C12 cells over-stimulated STAT3, which inhibited proliferation and activated differentiation, however these cells were unable differentiate properly or fuse into functional myotubes.\textsuperscript{90} Muscle derived IL-6 is revealing itself to be a potent regulator of muscle function, however it appears to reason that chronic inflammation will alter myogenesis.
Reducing basal TNF-α levels may be essential for proper myogenesis. Low level TNF-α expression is essential for satellite cell differentiation but this effect will be overridden if TNF-α levels are chronically elevated. Elevated TNF-α appears to inhibit satellite cell expression of Pax-7 through activation of the p38/polycomb repressive complex 2 (PRC2). Tissue from regenerating healthy and MDX mice treated with Infliximab (a TNF-α antibody) showed increased myogenesis and enhanced proliferation of Pax-7 expressing satellite cells.

In healthy conditions, low levels of TNF-α are released by differentiating satellite cells and stimulate MAPK and myogenesis. Treatment with low-level (0.05 ng/ml) TNF-α is enough to stimulate differentiation. TNF-α at low levels appears to be essential for satellite cell differentiation as complete inhibition of endogenous TNF-α release blocks differentiation. This effect on differentiation is lost when TNF-α levels are elevated. High-level TNF-α treatments (0.5 and 5 ng/ml) effectively reduce human satellite cell mRNA expression of MyoD and MyoG delaying differentiation and satellite cell fusion into myotubes. Elevated TNF-α inhibits myogenesis through the suppression of myocyte enhancer factor 2C (MEF-2C), which can result in abnormal motor function. Higher levels of TNF-α can also activate catabolism of previously differentiated muscle fibers, destroying existing muscle tissue.

Elevated TNF-α appears to stimulate the activation of the nuclear factor-kappa B (NF-kB), which is disruptive to the differentiation process. TNF-α induction of NF-kB suppresses MyoD transcription, inhibiting differentiation. This pathway is similar for muscle decay associated with cancer and cachexia, which are also regulated through NF-kB activation. Treatment with interferon gamma (IFN-γ) has similar effects on differentiation as TNF-α due to its activation of NF-kB. The genetic deletion of NF-kB in satellite cells increases their viability when re-injected into damaged muscle tissue.

Elevated pro-inflammatory conditions may have a lasting epigenetic effect on the DNA of satellite cells. TNF-α treatment contributes to DNA methylation, causing a ‘memory’ of diminished
satellite cell function, which can persist for several population doublings after the removal of the cytokine. In a study on C2C12 cells, it was found that acute exposure to TNF-α caused losses in function as was indicated by slower proliferation, decreased myogenesis, and decreased metabolic efficacy. TNF-α increased methylation of MyoD CpG sites, silencing the gene. Thirty population doublings after the TNF-α treatment had been removed, MyoD CpG site methylation persisted and, compared to immediately post exposure, cell function had worsened.

**Satellite Cells and Oxidative Damage**

Increased oxidative damage is displayed in satellite cells experiencing dysregulated myogenesis. Satellite cells cultured from elderly individuals exhibit decreased mitochondrial efficiency and lower expression of antioxidant enzymes, which can cause elevated ROS production. This increased ROS production results in a decreased ability to differentiate.

Increased satellite cell ROS production may be a component of aging. Lipid oxidation increases in an age dependent manner, and mirrors the increased oxidation in sarcopenic tissue. Differentiated aged satellite cells had a fivefold increase in levels of the lipid oxidation marker malondialdehyde (MDA), compared to young samples. This increase in oxidation was also associated with a 30% reduction in myogenity and 50% reduction in fusion. Along with increases in ROS production there are also age dependent decreases in antioxidant defense systems. Catalase and enzyme components of the glutathione system both show decreased expression in satellite cells cultured from older donors compared to young. Cell oxidation is also associated with diminished cellular function as demonstrated by the existence of defective excitation-contraction coupling apparatus in cells with high oxidative damage.

Increased oxidation is confounded by the fact that these cells also exhibit faults in their machinery to remove damaged proteins, which disrupts proper enzyme turnover. Aged cells have
reduced expression of enzymes needed for autophagy and ubiquitination, limiting proper protein removal. Endoplasmic reticulum stress in aged cells further contributes to this problem by increasing the amount of misfolded proteins in the cell, which cannot be properly removed. This leads to a situation in aged cells where misfolded proteins and proteins damaged by oxidative stress build up in the cell.

Non-cancerous satellite cells cultured from elderly participants with a variety of other types of cancers such as lymphomas and melanomas have dramatically impaired myogenesis partially due to increased oxidation. While the cultured cells themselves did not exhibit the typical hallmarks of cancer, they did display diminished function. These satellite cells show significantly decreased oxidative defense, with lower levels of SOD2, GCLM, and Nrf2. In these satellite cells collected from patients with cancer, Pax-7, Pax-3 and MyoD levels were increased, allowing the satellite cells to proliferate, but MyoG levels were decreased inhibiting fusion and muscle repair. Cancer may create an environment that promotes oxidative damage and deregulated cellular proliferation, or the development of cancer in these participants was more likely due to genetic alterations that increased global cellular oxidative damage. However, the existence of both ailments creates a compounding effect with one of the consequences being excessive satellite cell proliferation but inhibited differentiation.

The increased ROS production and oxidative damage in both aged and cancer patient satellite cells may be due to increased Wnt signaling. Increased activation of the Wnt pathway is thought of as a hallmark of cancer. Wnt activation may be a promoter of cancer growth or a physiological defense against its progression. What has been shown is that Wnt signaling leads to increased ROS production by reducing expression of SOD enzymes, possibly to limit cell growth. Inhibition of Wnt signaling causes deregulation of numerous types of stem cells, including satellite cells, and leads to carcinogenesis. Wnt signaling has been identified as a main cause of increased ROS production due to
The Wnt/β-catenin pathway is increased in stem cells collected from aged donors and thus these cells show an increase in their rates of ROS production and a diminished ability to proliferate and form functional tissue. Treatment with pharmacological anti-oxidant agents in aged stem cells reduced activation of the Wnt/β-catenin pathways, decreased observed DNA damage, and restored proper stem cell function.

Oxidative damage alone may not be detrimental enough to cause problems with differentiation. Human satellite cells treated with hydrogen peroxide (H$_2$O$_2$) exhibited slower proliferation rates but this treatment did not alter their differentiation nor their ability to fuse into myotubes. Undifferentiated satellite cells appear to be protected from oxidation and treatments with H$_2$O$_2$ and are able to increase their expression of antioxidant enzymes (SOD1, SOD2, and GPX). Proliferating satellite cells can be negatively affected by H$_2$O$_2$ treatments but differentiated myofibers appear to incur the greatest damage. These effects are relative to the level of treatment as higher concentrations of H$_2$O$_2$ inhibited expression of anti-oxidative enzymes in a dose dependent manner.

**Hormonal/Growth Factor Regulation of Satellite Cell Myogenesis**

Perhaps the most influential regulation over stem cell activation and growth occurs through hormonal signaling. As a component of muscle hypertrophy, satellite cells are sensitive to a wide variety of anabolic and catabolic hormones and growth factors. Growth hormone (GH) and insulin like growth factor 1 (IGF-1) promote satellite cell activation and myogenesis, while myostatin and cortisol inhibit cell activation. As stated earlier, myostatin and TGF-β disrupt myogenesis by diverting differentiation into alternate cell types.

IGF-1 is a potent stimulator of muscle hypertrophy. IGF-1 activates a strong signaling pathway that leads to the downstream activation of Akt and eventually mTOR. In IGF-1 activated satellite cells, mTOR promotes G$_1$/S cell cycle progression, leading to increased proliferation.
treatment in-vitro also promotes differentiation and fusion. Myotubes formed by satellite cells activated by IGF-1 show increased accretion of the protein MHC, indicating a more robust fiber. Muscle tissue injury results in the release of IGF-1 for repair. In-vivo, IGF-1 treatment can restore mass and function of muscles that have atrophied, while decreased IGF-1 expression is enough to cause atrophy. Notably, the effects of IGF-1 are overridden in the presence of TGF-β.

Several growth factors have the ability to regulate satellite cell activity. Hepatocyte growth factor (HGF) is known to bring satellite cells out of quiescence. This is done through interaction with the c-met receptor which activates the cell cycle and begins proliferation. HGF is proposed to improve muscle regeneration after injury by increasing the available myoblast population. However, these effects may come at a cost, while HGF promotes proliferation, it also inhibits satellite cell differentiation. Expression of MyoD, myogenin, and MHC is significantly decreased in proliferating satellite cells exposed to HGF. A controlled exposure of HGF improves muscle regeneration, but overexposure to the growth factor has the potential to cause overgrowth and the development of transdifferentiated fibrous masses.

**Influence of miRNA on Satellite Cell Differentiation**

Emerging in the field of satellite cell research are the controlling effects of micro RNAs (miRNAs). These miRNAs appear to be dramatically altered by disease and aging and have vast regulatory control over myogenic transcription factors. MiR-431 counteracts satellite cell transdifferentiation by inhibiting the SMAD pathway activated by TGF-β and myostatin. Inhibition of miR-431 results in reduced myogenesis while miR-431 treatment restores differentiation potential in satellite cells from diseased models.

Micro RNAs have even shown regulatory control over muscle fiber type expression. MiR-494 expressed in differentiating satellite cells inhibits the transcription of proteins needed for type IIa fibers
without affecting type I or type IIx related proteins.\textsuperscript{129} miR-494 also affects mitochondrial biogenesis by inhibiting mitochondrial transcription factor A (mtTFA) and Forkhead box j3 (Foxj3), reducing synthesis of new mitochondria.\textsuperscript{130} Due to these effects, miR-494 may be key in inhibiting fiber type switching from fast twitch to slower more oxidative fibers.

**Exercise and Satellite Cell Function**

There are several mechanistic pathways to target in order to improve satellite cell function. The concentration of inducible quiescent satellite cells in the muscle tissue sarcolemma, known as the satellite cell pool, can be amplified. The proliferation potential of a given set of satellite cells can be increased to allow more available cells to be added to the site of injury when needed. The differentiation and fusion potential of the activated cells can be enhanced to ensure their incorporation into damaged tissue. Also, the functional capacity of the cell, once fully differentiated into working tissue, can be amended for greater expression of efficient muscle proteins, increased oxidative defense enzymes, and a better metabolic profile. Exercise training has the potential to increase these parameters.

A 12-week resistance training program effectively increased the number of satellite cell pool populations in the sarcolemma of trained muscle fibers in both males and females.\textsuperscript{131} Resistance training also restores NOTCH receptor expression, which improves the likelihood that satellite cells will respond to muscle injury and may increase their proliferative capacity.\textsuperscript{3,32,132} Expression of cyclin D1 is increased within one week of resistance training and is associated with increased cellular proliferation.\textsuperscript{133} MyoD, linked to improvements in cellular differentiation, also increases along with cyclin D1 within one week of exercise training.\textsuperscript{133}

A single bout of exercise is enough to recruit the addition of satellite cells into muscle fibers.\textsuperscript{133,135} As soon as 6 hours post exercise, levels of cyclin D1 and MyoD begin to increase, and there is a
noticeable increase in muscle fiber DNA indicating cellular fusion has begun.\textsuperscript{133,134} The time course for satellite cell recruitment may vary depending upon the method of exercise. Although a combined endurance/resistance exercise bout led to no changes in satellite cell activation within 9 hours of exercise, significant increases in satellite cell pools resulted overnight.\textsuperscript{135}

Adaptations to exercise appear to be fiber type specific. Intense eccentric loading leads to increased DNA and satellite cell content in type II fibers but had no effect on type I.\textsuperscript{136} Endurance training through cycling increases DNA and MHC-1 content in type I fibers.\textsuperscript{136} It is important to note that these adaptations to endurance activity occurred without significant changes in muscle fiber size, indicating that satellite cells additions to muscle fibers can occur in the absence of hypertrophy.\textsuperscript{136}

Exercise is associated with increased expression of myosin heavy chain,\textsuperscript{137} observed both \textit{in-vivo} and \textit{in-vitro}. Satellite cells cultured post exercise express more contractile protein \textit{in-vitro} indicating this change is more permanent in the satellite cell and is not just an adaption in functional tissue.\textsuperscript{133}

**Obesity and Satellite Cell Function**

Alterations in the extracellular environment can have lasting effects on stem cells of many lineages including satellite cells. Obesity may have lasting or even permanent negative effects on satellite cell function. Individual satellite cell genetics may prevent or exacerbate the development of obesity. If left untreated, obesity can lead to further complications such as the development of insulin resistance and metabolic syndrome. These negative effects from these diseases can evidenced in both muscle tissue and cultured satellite cells.

Metabolic syndrome is associated with chronic increases of low-level inflammation. Increased inflammatory markers include IL-6 and TNF-\(\alpha\) which have been shown to disrupt satellite cell proliferation and differentiation in culture. This increase in inflammation may be associated with endotoxemia due to systemic low-levels of lipopolysaccharides (LPS). LPS have a direct effect on
satellite cell function by decreasing myogenesis.\textsuperscript{138} LPS treatment decreases satellite cell viability causing the death of a significant portion of proliferating satellite cells.\textsuperscript{139} Treatment of skeletal muscle with LPS decreases the ability of the cells to proliferate.\textsuperscript{139}

Although not thoroughly studied, the idea that obesity could influence the satellite cell population of an individual is not new. In 1984, a study on the muscle from \textit{ob-/ob-} mice found that obesity resulted in small and less nucleated fibers in the soleus and gastrocnemius muscles.\textsuperscript{140} These obese mice had fewer nuclei per length of fiber when compared to control mice. This study also indicated that these effects were seen regardless of age noting no change in nuclei count from 3 to 15 weeks in either the controls or \textit{ob-/ob-} mice.\textsuperscript{140}

Aside from inflammation, obesity can affect other cellular pathways. C57BL/7J mice with diet induced obesity (DIO) have significantly reduced circulating levels of hepatocyte growth factor (HGF), which was associated with slower satellite cell proliferation in-\textit{vivo}.\textsuperscript{141} HGF is a common ingredient used in skeletal muscle cell growth media to activate proliferation and myogenesis by inducing expression of the transcription factors MyoD and myogenein.\textsuperscript{141,142}

Studies have shown that AMPK has an important role in repairing muscle damage by increasing metabolic efficiency to allow for rapid proliferation. This action is reduced in obese participants, as they show decreased AMPK expression and activation.\textsuperscript{143,144} Knocking out \textit{AMPKα1} in mice results in a significantly reduced ability of satellite cells to activate, proliferate, and differentiate.\textsuperscript{143} Induced obesity in mice results in a similar reduction in AMPK levels which also lead to reduced satellite cell function and an inability to recover from muscle damage.\textsuperscript{143} Notably, the treatment of obese mice with AICAR (an AMPK activator) restored muscle recovery.\textsuperscript{143}

Satellite cells are not the only stem cell type that can be functionally altered by obesity. Adipogenic precursor cells biopsied from obese human donors exhibited insulin resistance when
differentiated into white adipocytes.\textsuperscript{145} This shows that phenotypic changes to an individual reach further than the functioning tissue and can cause epigenetic changes to the core set of stems cells.

**Aging and Satellite Cell Function**

Several previous studies have found links between satellite cell function and aging, however, these studies may not have considered moderating factors. Perhaps the largest investigation into the link between aging and satellite cell function was a 165-participant study on males, 0 to 86 years of age, performed by Verdijk et. al.\textsuperscript{146} Significant findings from this work showed that declines in satellite cells appear to be fiber type specific, with aging causing significant losses in muscle cross sectional area and satellite cell pool size in type II fibers, but only minimal losses seen in type I fibers.\textsuperscript{146} The inclusion criteria for this study did exclude participants with skeletal and cardiovascular abnormalities as well as those with type II diabetes. The study also required that its participants be sedentary, defined as not participating in any structured exercise for at least 3 years prior to the study, leading to the possibility of the older participants being sedentary for much longer than their younger counterparts. In other words, the researchers had a 3-year minimum requirement with no maximum limit for a sedentary lifestyle. This study, like many others, did not take into account their participants’ daily unstructured physical activity nor their level of obesity.

It is possible that the loss of CSA and satellite cell pool size attributed to aging is partly the effect of a prolonged sedentary lifestyle. The fiber type specific losses are similar to patterns seen in atrophy related to immobilization and hind limb unloading regardless of age.\textsuperscript{147,148} A 12-week resistance-training program performed by elderly participants in this study was able to nearly abolish the losses in type II fibers. This is further evidence that lack of activity, and not aging, contributes most to muscle loss and satellite cell dysfunction.
Obesity is controllable throughout one’s lifespan through diet and exercise. Therefore, body-fat levels should be evaluated as an individual variable and not considered as a side effect of aging. However, in studies comparing young and aged participants, aged participants routinely have higher body fat levels. In a study designed to elicit the mechanistic difference between young and old satellite cells, Cyclin D1 and MyoD were determined to have different patterns. In response to exercise, MyoD increased in the younger group as expected but there was no increase in the older group.\textsuperscript{133} The older participants had significantly higher levels of Cyclin D1 compared to the younger group in which cells are trapped in the growth phase preventing them from properly differentiating.\textsuperscript{133} While both of these findings were contributed to the effects of aging it is important to note that the body-fat levels between the young and old group were significantly different with the 10 young participants at 15.4\% (+/-2.9\%) and the 10 elderly participants at 27.4\% (+/-1.9\%). These numbers put the young participants in the healthy range for body-fat and the elderly in the overweight/obese ranges. With both factors being significantly different between the study groups, it should be stated that age and/or body-fat percentage contribute to disrupted MyoD and cyclin D1 levels.\textsuperscript{149}

While a correlation may exist between age and satellite cell function there is not enough evidence to suggest causality. The most common method of determining cellular age is to measure the length of the telomere, the end cap of the DNA strand. With each cellular replication, the length of the telomere shortens. Eventually a telomere will become too short for the cell to replicate and the cell will enter senescence. The only way to extend telomere length is through telomerase activity.\textsuperscript{150} Aged satellite cells do not exhibit shortened telomeres.\textsuperscript{151,152} While satellite cells have not been shown to have telomerase activity, telomere length does not appear to be different in satellite cells from young and elderly donors.\textsuperscript{34} New evidence suggests that activity level, regardless of age, determines telomere length and can be increased by physical training.\textsuperscript{152} In fact, there is very little discernable difference in
satellite cells between healthy young and healthy elderly donors. A small study looking at satellite cells from two young and two elderly donors found no difference in the proliferation and differentiation between the groups.

Several negative differences exist between the *in-vivo* environment of young and old muscle. Aged cells show a decline in NOTCH receptor expression. This decline decreases satellite cell activation and proliferation; however, an absence of NOTCH does not stop differentiation. Differentiation of satellite cells without proliferation leads to the disappearance of the satellite cell pool in aged muscle. Along with decreased NOTCH receptor expression in aged cells, there is also an increase in the antagonist receptor NUMB, which acts to further inhibit cell activation. Deficiencies in aged satellite cells can be restored by treatments to increase expression of the NOTCH-1 receptor or its ligand, DELTA. Exercise training can eliminate the differences in NOTCH receptor signaling between young and elderly human participants.

Increased myostatin contributes to the decreased muscle mass seen in old tissue compared to young. While other hormonal factors, such as IGF-1 and GH, improve in aged participants with exercise, training does not decrease elevated myostatin levels. Myostatin may contribute to the fiber type specific losses seen in aging, as myostatin levels in aged muscle were 67% higher in type II fibers compared to type I. These effects have led many investigators to design strategies to inhibit myostatin in order to restore muscle mass in aged participants, with some success in mice and rats.

Perhaps the most detrimental factor affecting muscle mass is the transdifferentiation caused by aging. Compared to young, aged satellite cells are more likely to differentiate into fibroblasts or adipocytes. This is mostly due to increases in TGF-β, Wnt signaling, and myostatin. These factors all act through downstream activation of the SMAD pathway described earlier to inhibit MyoD
and myogenin.\textsuperscript{154} Increased TGF-\(\beta\) may be the activator of the mechanistic pathway by which sarcopenia causes pathological fibrosis.\textsuperscript{158}

The effects of aging on muscle loss may be explained by the increased adiposity and lower physical activity seen in older individuals. As shown earlier, regular physical activity and exercise are key in maintain proper functioning satellite cell pools. Several studies have shown that with aging there is a dramatic decrease in physical activity combined with increases in body-fat percentage.\textsuperscript{159-161} The positive side of this is several studies of exercise training programs with older participants show a complete restoration of satellite cell function.\textsuperscript{162} Both endurance and resistance training are capable of restoring the muscle tissue microenvironment of older participants.\textsuperscript{163,164} Training programs can restore satellite cell pool size and activation levels to that of their young counterparts.

\textbf{Conclusion}

Aging is associated with a decline in satellite cell phenotype. However, it must be examined if aging alone is the cause of this decline or if aging is simply allowing more time for the accumulation of detriments that negatively affect satellite cell phenotype. Several of the factors that negatively affect satellite cell function are associated with aging including; decreased physical activity, chronic inflammation, obesity, oxidative damage, cancer, hormonal changes, and other diseases. The easiest of these factors to amend in physical activity. Several studies have shown that even modest exercise training programs can amend the satellite cell function of older participants to match that of their younger counterparts.

Most factors that occur with aging and disrupt satellite cell function occur in-vivo. In-vitro, when satellite cells from young and old donors are grown and differentiated in identical conditions the differences tend to disappear. This would suggest that there are no changes to the cells themselves but they are reacting to the environment for which they are exposed. The clearest demonstration of this
concept is satellite cells from old donors grown in sera collected from young participants have their proliferation rates restored.\textsuperscript{165} Conversely, young cells grown in sera collected from unhealthy aged participants experience the same faults in myogenesis as aged cells. If the tissue microenvironment is the main contributing factor for the loss of muscle mass during aging, then future research must be aimed at restoring this environment.
Chapter 3: Study Goal

The first purpose of this study is to develop measures to evaluate satellite cell myogenesis and metabolism by evaluating proliferation, differentiation, and substrate metabolism. These measures will define the *in-vitro* satellite cell phenotype. The second purpose is to determine if donor age, activity level, adiposity, or diet are associated with variations in measured phenotype. This study will use a high substrate (high fat and high glucose) treatment as an in-vitro mimic of a high-fat diet to determine the effects of substrate availability on satellite cell phenotype, and if these effects can be influenced by donor age, activity level, adiposity, or diet.
Specific Aims

Aim 1: Evaluate how the age and physical activity level of healthy human donors influences the *in-vitro* myogenesis and metabolism of their satellite cells.

**Hypothesis:** There will be no significant difference in proliferation rate or differentiation potential between satellite cells from young and older healthy donors. We also hypothesize that glucose and fatty acid metabolism and ROS production will be similar in differentiated satellite cells from young and older healthy donors.


**Hypothesis:** Higher donor body fat levels will negatively correlate with measures of satellite cell function. Including decreases in proliferation and differentiation, blunted metabolism and elevated ROS production. The high-fat/hypercaloric diet is predicted to reduce proliferation, differentiation, and oxidative efficiency while increasing ROS production. Cells treated with elevated substrate media will exhibit reduced initial proliferation and differentiation, as well as, increased glucose oxidation, increased fatty acid oxidation with reduced efficiency, and increased ROS production.
Chapter 4: Approach

Satellite Cell Culture

To complete these aims, several in-vitro experiments were conducted on satellite cells in culture. Satellite cells were collected during two prior studies and during this study from tissue biopsies of the vastus lateralis muscle of male participants. Muscle samples weighing ~50 mg were collected from donors ranging in age (18-80 years) and body-fat % (7-40%). The tissue was minced and non-muscle tissue types were removed. The tissue samples were then placed in a trypsin cocktail (2.5% trypsin, 2% EDTA, 1% collagenase type IV, and 1% BSA) for 40 min to release the cells. The digested tissue samples were then placed in non-collagen coated flasks with DMEM media and incubated for 3 hours, this incubation allowed non-satellite cell types to adhere to the uncoated flask. The tissue samples were then placed in collagen coated flasks with DMEM and incubated overnight. When satellite cell colonies began to adhere to the flask the tissue debris was removed and the media was replaced. When the cells reached 70% confluence they were frozen down in DMEM media with DMSO for future use. Satellite cells were cultured and differentiated in multinucleated myotubes as previously described (Muoio et al., 2002b)\textsuperscript{166}. Satellite cell isolation was approved by the Virginia Tech institutional Review Board (IRB# 14-1234)

Aim 1 Experiments

Develop measures to properly evaluate satellite cell function
The satellite cell phenotype was evaluated by measures meant to predict the functional capacity of donor satellite cell in-vitro. These measures evaluated the ability of the satellite cell to restore functional muscle tissue, which has been lost due to exercise damage, injury, or atrophy. The phenotype was measured as the satellite’s cell’s ability to proliferate, differentiate into myofibers, and properly metabolize substrates. Proliferation was determined by tracing the rate of cellular doublings through a 6-day period. Differentiation was evaluated by exposing confluent satellite cells to 7 days of differentiation media (low serum). After 7 days, the myogenic index was calculated as percent multi-nucleated (the number of nuclei contained within fibers with 2 or more nuclei compared to the total nuclei count) and percent highly-nucleated (the number of nuclei contained within fibers with 10 or more nuclei compared to the total nuclei count). Differentiation was evaluated as the total protein concentration expressed over total DNA expression (mg/nuclei), as an attempt to develop a simpler measure of differentiation that is less subjective. Metabolism measures were performed in cells that have undergone 7 days of differentiation, complete fatty acid oxidation (FAO) (fully oxidized palmitate), incomplete FAO (acid soluble metabolites remaining in media), Total FAO (Complete + Incomplete), and oxidative efficiency (ratio of Complete FAO to Incomplete FAO), as well as glucose oxidation were measured. The rate of cellular ROS production was included as a metabolic measure in 7 day differentiated cells.

**Evaluate the relationship of age on the satellite cell phenotype**

Collected satellite cells from young (18-35 years) and older (50-80 years) donors were evaluated. For each group (young/old) 10-15 participants were phenotyped. Including the 6-day proliferation rate, myogenic index, fatty acid and glucose oxidation as well as measures of
cellular ROS production (experimental methods for these procedures are explained in full detail in the Methods section). Findings from each group were compared to determine if age influences satellite cell phenotype.

**Determine the relationship between training status and the satellite cell phenotype**

Satellite cells were collected from sedentary and endurance trained young (18-35) donors (n=3-5 per group). The satellite cells of each donor were evaluated to determine if cells form regular endurance training displayed a more beneficial phenotype. Endurance training in young is expected to improve phenotype, through increases in proliferation and myogenesis as well as substrate oxidation.

**Aim 2 Experiments**

**Relationship of donor body fat and satellite cell function**

Satellite cells were collected from donor with body fat % ranging from 8-38%. These measures were recorded using DEXA scan. Satellite cell phenotypes were evaluated to determine if donor body fat % correlated with any facet of the satellite cell phenotype. Higher body-fat levels are expected to correlate with decreased proliferation and differentiation.

**Effect of high substrate media on satellite cell function in-vitro**

To mimic obesity cells were treated with high substrate media (HSM), a high fat/high glucose growth media with additional supplementation of 400uM 2:1 oleate/palmitate, and 20 mM glucose, as noted in Hulver et. al.\textsuperscript{167} This media is an approximate of levels observed in the skeletal muscle environment of individuals on a high-fat diet.\textsuperscript{168-170} Collected satellite cells from
young, older, and trained donors were grown in control and HSM, then differentiated in control differentiation media (low serum). This was meant to determine if exposure to HSM during growth could alter satellite cell phenotype during the growth phase and if this exposure would last throughout differentiation. Exposure to the HSM is expected to inhibit the differentiation process.

![Figure 4.1 High Substrate Media treatment schedule. HSM and control medias applied at day one and replaced at day five with identical serum starve differentiation media (replaced every 2 days).](image)

**Effect of a high-fat/hypercaloric diet on satellite cell phenotype**

Satellite cells were collected from human donors before and after a four-week, 55% fat, +1000 kcal diet. Satellite cells were evaluated to determine if the effects of a high-fat/hypercaloric diet (HFHCD) would alter the satellite cell phenotype once the cells were isolated. It was expected that the diet will decrease satellite cell proliferation and differentiation.

![Figure 4.2 High-fat/Hypercaloric diet and biopsy schedule](image)

**Methods**

**Cell Medias**
**Growth Media:** DMEM with 5mM glucose, 10% FBS, fetuin and 0.05% BSA. Supplemented with growth factors EGF (10 ng/ml) and dexamethasone (0.4 ug/ml). Amphotericin-B (0.5 ug/ml), antimitotic (for penicillin 100U/ml and streptomycin 100 ug/ml), and gentamycin (0.01 mg/ml) will be added for protection against bacterial and fungal infections. Growth media is changed every 5 days.

**High Substrate Media (HSM):** DMEM with 25mM glucose, 400uM 2:1 oleate:palmitate, 10% FBS, fetuin and 0.05% BSA. Supplemented with growth factors EGF (10 ng/ml) and dexamethasone (0.4 ug/ml). Amphotericin-B (0.5 ug/ml), antimitotic (for penicillin 100U/ml and streptomycin 100 ug/ml), and gentamycin (0.01 mg/ml) will be added for protection against bacterial and fungal infections. The oleate:palmitate mixture is complexed with 0.5% BSA for 30 min at 37°C before added to the media.

**Differentiation Media:** DMEM with 5mM glucose, 10% FBS, fetuin and BSA. Amphotericin-B (0.5 ug/ml), antimitotic (for penicillin 100U/ml and streptomycin 100 ug/ml), and gentamycin (0.01 mg/ml) will be added for protection against bacterial and fungal infections. Differentiation media is changed every 2 days.

**Serum Free Media (starve):** DMEM with 5mM glucose, fetuin and BSA Amphotericin-B (0.5 ug/ml), antimitotic (for penicillin 100U/ml and streptomycin 100 ug/ml), and gentamycin (0.01 mg/ml) will be added for protection against bacterial and fungal infections.

**Cell Proliferation**

Satellite cells from 6-10 donors were seeded in separate T-25 flasks with growth media. After 2-3 days, cells from the T-25 were lifted with trypsin (0.25%) and transferred to a T-75
flask. Once cells reached 50% confluency in the T-75 flask they were again lifted and counted with a hemocytometer. Cells were seeded at 2,500 cells/well in six separate 96-well plates with six replicates per cell line. On days 1-6 plates were collected and nuclear material was counted using thermofisher’s CyQUANT® NF Cell Proliferation Assay Kit. Values were imputed into PRISM and doubling times will be calculate using the PRISM exponential growth calculator equation. Proliferation are reported as doubling time in hours (hrs/DT) and doublings per day (dbs/day)

**Cell Differentiation/Myogenic index**

Satellite cells in were differentiated through exposure to low serum differentiation media once they reach 70-80% confluency. Differentiation proceeded for 7 days with media being changed every 2 days as previously described by Frisard et al.\textsuperscript{171} After day seven nuclear content will be analyzed with CyQUANT Dye. Cells were fixed in 4% formaldehyde for 15 min at room temperature then stained with hematoxylin and eosin. Cells were imaged at 10x magnification. Nuclei number in multi-nucleated and highly-nucleated fibers as well as total nuclei number were counted. Myogenic index was calculated as the percentage of nuclei within multi-nucleated (2+ nuclei per fiber) and highly nucleated (10+ nuclei per fiber) fibers compared to total number of nuclei as described by Kamli, M., et al.\textsuperscript{172} Myogenic index is reported as a percent ± SEM. Total protein as evaluated through BCA and nuclei content was measure through CyQUANT assay. Protein/nuclei ratio is expressed as a measure of differentiation in ug/nuclei ± SEM.

**Glucose and Fatty Acid Oxidation**
Satellite cells were grown to 50% confluency in a T-75 flask. Cells were lifted from the flask and seeded in 12-well plates then grown to 70-80% confluency. Cells were then differentiated for 7-days. On day seven cells were serum-deprived overnight with serum-free media. Fatty acid oxidation was determined by measuring and summing $^{14}$CO$_2$ production and $^{14}$C-labeled acid soluble metabolites from the oxidation of [1-$^{14}$C] palmitic acid (American Radiolabeled Chemicals, St. Louis, MO). Briefly, cells were incubated in media containing radiolabeled substrate for 3 hours at 37C, 5% CO2. Following incubation, media was removed and acidified with 70% perchloric acid to elute gaseous $^{14}$CO$_2$. $^{14}$CO$_2$ will be trapped in 1M NaOH over the course of 1 hour. The NaOH was then placed in a liquid scintillation counter and counted. Data is expressed as means ± SEM and is normalized to total protein content. Glucose oxidation was assessed by measuring $^{14}$CO$_2$ production from the oxidation of [U-$^{14}$C] glucose (American Radiolabeled Chemicals, St. Louis, MO) in a manner similar to fatty acid oxidation except for the substitution of glucose in place of palmitic acid.

**Cell ROS production**

Satellite cells were seeded in 96-well plates at 5,000 cells per well. Cells were grown to 70-80% confluency and differentiated for 7 days. The CM-H2DCFDA (General Oxidative Stress Indicator) dye kit was used to detect ROS production. ROS production was detected at day 0 and day 7 of differentiation. The maximal rate of ROS production is expressed per nuclei content as int/min/nuclei ± SEM.

**RNA extraction and qRT-PCR**
Cells were grown in either control or HSM for 4 days prior to differentiation. RNA was collected at select time points of the differentiation process on days 0, 1, 2, and 7. RNA was extracted using an RNeasy Mini Kit (Qiagen) and DNase I treatment (Qiagen, Valencia, CA), according to the manufacturer's instructions. qRT-PCR was performed using an ABI PRISM 7900 Sequence Detection System instrument and TaqMan Universal PCR Master Mix used according to the manufacturer's specifications (Applied Biosystems, Foster City, CA). Target gene expression in rodent skeletal muscle and cell culture was normalized to β-actin RNA levels. Target gene expression in human skeletal muscle primary cell culture was normalized to cyclophilin B RNA levels. Primers and 5# FAM-labeled TaqMan probes were purchased as prevalidated assays (ABI). Relative quantification of target genes was calculated using the 2−ΔCT method. Derivation of the 2−ΔCT equation has been described in Applied Biosystems User Bulletin no. 2 (P/N 4303859). As previously described by Frisard et al.173

Statistical Analyses

All calculations were performed in PRISM or SPSS statistical software. The alpha value for statistical significance was set at 0.05 for all tests and a p-value of ≤ 0.05 was considered significant. Proliferation rates, myogenic index, and protein/DNA ratio, were correlated to age, body-fat percentage, and physical activity scores. PRISM linear regression was used to determine whether slope has any significant deviation from zero. Age was screened as a covariate against body-fat and physical activity to determine if a correlation still exists once the deviation in age has been accounted for.

A paired t-test was used to determine changes in satellite cell function for tissue samples taken pre-and post- HFD. One-way ANOVA was used to determine differences between participants from trained and untrained groups
One-way ANOVA was used to determine the effect of HSM on satellite cell function between different treatment groups. One-way ANOVA was used to determine if exposure to HSM affected proliferation rate, myogenic index or metabolism. The effect of the media was expressed as percent difference between the HSM and control (\(\frac{\text{HSM} - \text{control}}{\text{control}}\times 100\)). Two-way ANOVA was used to determine if the media effect was influenced by diet (pre- vs. post-HFD) or activity level (sedentary vs. active). Media effect was correlated to age and body fat through linear regression.
Chapter 5: Donor Age does not Affect Human Satellite Cell Myogenesis or Metabolism in-vitro

Abstract:
Aging is associated with a rapid decline in muscle mass of about 1% per year. This can result in a sometimes-debilitating loss of strength, increasing a person's risk for fall injuries and chronic disease. Satellite cells are skeletal muscle progenitor cells that aid in the repair and replacement of damaged muscle fibers. Previous evidence has suggested that progenitor cells may lose potency due to the aging process. Satellite cells must be studied to determine if it is a loss in their cellular function that leads to overall decreased skeletal muscle mass and strength during aging. Satellite cells were collected from the vastus lateralis of young sedentary, young trained, and older sedentary males. These cells were evaluated for their ability to proliferate, differentiate, and oxidize lipids and glucose. There were no differences between sedentary young and older cells in their proliferation rates, fusion index scores, or measures of substrate oxidation, other than increased oxidative efficiency in the cells from older donors. Trained cells did show an increased capacity to proliferate (39%, p<0.05) and spontaneously differentiate (33%, p<0.05) when compared to both sedentary young and older cells. These findings suggest the common lack of activity exhibited by the elderly population may be the cause of their persistent losses in muscle mass, which may be mediated through satellite cell dysfunction. A chronically sedentary lifestyle has the potential to cause debilitating losses in muscle mass among younger individuals similar to what is currently associated with older populations, as it was not possible to distinguish a difference between cells from sedentary young or older individuals.

Introduction:
Muscle mass correlates with increased metabolic rate and lower rates of obesity, as well an improved overall quality of life. Increased muscle mass and strength is more vital to overall health in elderly populations, as it increases mobility and decreases risk of injury from falls. Aging is associated
with a 1% decrease in lean mass and 3-4% decrease in muscular strength per year.\textsuperscript{6,7} In extreme cases of sarcopenia, accelerated muscle loss associated with aging and frailty syndrome, there is documented dysfunction in muscle progenitor cells which inhibits regrowth.\textsuperscript{5,8} Lower muscle mass is also a common consequence of other age related chronic diseases including; obesity, cancer, diabetes, COPD, and heart disease.\textsuperscript{9,10}

To explain the loss in muscle mass associated with disease, many researchers have assessed mechanisms of tissue repair, specifically those mediated by the satellite cells. The satellite cell is a muscle progenitor cell responsible for replacing atrophied or damaged fibers.\textsuperscript{11,12} These cells replicate and form new fibers or fuse to existing fibers to donate new DNA helping increase transcription and protein expression. This process is done through a myriad of transcription factors,\textsuperscript{13} which must be expressed in a timed and coordinated effort to allow for proper proliferation, differentiation and fusion of satellite cells.\textsuperscript{11,13}

Satellite cell activation, proliferation, differentiation, and fusion into myofibers is controlled through the coordinated expression of several transcription factors, including DELTA/NOTCH (activation/proliferation)\textsuperscript{14-16}, MyoD (differentiation)\textsuperscript{16,17}, Myf5 (differentiation)\textsuperscript{16}, and MyoG (differentiation/fusion)\textsuperscript{18}. Expression of these transcription factors can be altered through hormonal [IGF-1 (insulin-like growth factor-1), myostatin, etc.]\textsuperscript{19,20} and inflammatory [TNF-\alpha (tumor necrosis factor-alpha, IL-6 (interlukin-6), etc.]\textsuperscript{21-23} signaling. This signaling can be beneficial or detrimental. IGF-1\textsuperscript{24} and IL-6\textsuperscript{22} can increase satellite cell proliferation while excess myostatin\textsuperscript{25} and TNF-\alpha\textsuperscript{23} can inhibit differentiation.

Myokine (muscle cytokine) autocrine secretion may affect expression of the transcription factors necessary for satellite cell activation, proliferation, and differentiation. Chiefly IL-6 promotes satellite cell proliferation while TNF-\alpha is secreted in higher levels during differentiation.\textsuperscript{22,26} Both
cytokines have a bell shaped dose response where just the right levels are required. Over exposure to IL-6 or TNF-α leads to blunted differentiation, while excessive IL-6 alone results in over proliferation.\textsuperscript{22,26} With aging there is a systemic increase in the secretion of both IL-6\textsuperscript{27,28} and TNF-α.\textsuperscript{28} Increased plasma levels of these cytokines are correlated with a decrease in muscle mass in older men and women.\textsuperscript{28} However, it remains to be identified which organ system is the source of these elevated plasma levels. While muscle can cause spikes in plasma cytokine levels in response to exercise\textsuperscript{29}, chronic elevation is typically linked to adipose tissue secretions.\textsuperscript{30,31} Whether or not aging alters myokine secretion from muscle tissue is still not known.

There are clear losses in muscle tissue and strength in elderly individuals that lower quality of life and contribute to frailty syndrome. On a whole body and tissue level, aging leads to decay and fibrosis. There is a decreased ability for tissues to regenerate from their tissue specific stem cells. These decreases are facilitated in part by disruptions in stem cell growth, differentiation, metabolism, oxidative stress and cytokine secretion. In the case of satellite cells, what needs to be determined is when and how these disruptions arise. The question we must ask is, do quiescent cells incur damage simple due to the length of their existence or is it the detrimental environment associated with aging individuals that causes these disruptions once the cell is activated?

This study was designed to discover any innate losses in satellite cell function due to chronological aging alone. To do this the cells of a sedentary young population were compared to the cells from a sedentary but health aged population healthy. To test this, cells from sedentary young and sedentary older donors as well as cells from endurance trained donors were compared in an in-vitro environment. Cells were evaluated on measures to evaluate myogenic ability. Such as the ability to proliferate, differentiate, and become metabolically functional.
Methods:

Participant Selection: Skeletal muscle biopsies previously were obtained under three IRB-approved human clinical studies, and satellite cells were subsequently isolated. Participants were categorized based on activity level as either sedentary or endurance trained. Sedentary participants engaged in less than ≤2 days, 20 min per day of low-intensity physical activity. Endurance-trained individuals ran or cycled for a self-reported 5+ hours per week and had to of engaged in at least one organized running race event of a distance of 10+ kilometers within the past year. All participants were male and grouped as, young (18-30 years, sedentary), older (60-80 years, sedentary), and trained (18-30 years, endurance trained). Young sedentary samples were collected from a controlled feeding study performed in 2013. Samples of older individuals were collected during a resistance training study aimed at improving the mitochondrial function of older males participants through a resistance training program performed from 2013-2014. Samples from trained participants were acquired through a study designed to determine the effects of a western style diet on highly active endurance athletes. All participants were considered healthy and free of any chronic disease assessed through a medical history questionnaire. Body composition was assessed through DEXA (dual-energy x-ray absorptiometry) scan.

Satellite Cell Isolation/Growth/Differentiation: Satellite cells were previously collected from tissue biopsies of the vastus lateralis muscle of male participants. Muscle samples weighing ~ 50 mg were collected from donors ranging in age (18-80 years) and body-fat percentage (7-40%). Satellite cells were cultured and differentiated in multinucleated myotubes as previously described (Muoio et al., 2002). Cells were seeded and allowed to proliferate in growth (DMEM with 5mM glucose, 5% fetal bovine serum, rhEGF (10n/ml) and dexamethasone (0.4 ug/ml)) media for 5 days, until they reached 60-70% confluency. After proliferation, cells were differentiated in low serum (DMEM with 5mM glucose and 2% horse serum) media to activate differentiation. Cells were differentiated for seven days with media changed every 2 days. Amphotericin-B (0.5 ug/ml), antimitotic (for penicillin 100U/ml and
streptomycin 100 ug/ml), and gentamycin (0.01 mg/ml) were added for protection against bacterial and fungal infections. Satellite cell isolation was approved by the Virginia Tech Institutional Review Board (IRB# 14-1234)

**Proliferation Assay:** Cells were counted with a hemocytometer. Cells were seeded as 2,500 cell/well in separate 96-well plates. Plates were collected on days 1 through 6 plates and nuclear material was counted using thermofisher’s CyQUANT® NF Cell Proliferation Assay Kit. Doubling times were calculated using the PRISM exponential growth equation. Proliferation is reported as doubling time in hours (hrs/Db ± SEM) and doublings per day (dbs/day ± SEM).

**Myogenic Index:** Myogenic index methods were adapted from Kamli et. al. Cells were fixed in 4% formaldehyde for 15 min at room temperature. Cells protein was stained red with eosin, while nuclei were stained green with CyQUANT. Cells were imaged at 10 times magnification. Nuclei number within multi-nucleated (two or more nuclei per fiber), and super-nucleated (5 or more nuclei per fiber) fibers as well as total nuclei number was counted. Myogenic index was calculated as the percentage of nuclei within multi-nucleated fibers compared to total observed nuclei, as described by Kamli, M., et al. Myogenic index is reported as both multi-nucleation (proportion of nuclei in fibers with 2 or more nuclei per fiber divided by total nuclei) and Robust-nucleation (proportion of nuclei in fibers with 10 or more nuclei per fiber divided by total nuclei). Myogenic index is reported as a percent ± SEM. Total protein was evaluated through BCA and nuclei content was measure through CyQUANT assay. Protein/nuclei ratio is expressed as a measure of differentiation as µg/nuclei ± SEM

**Substrate Oxidation:** Substrate metabolism measures were performed in muscle tissue homogenate removed from the vastus lateralis and differentiated cells as previously described in Frisard et. al. Complete fatty acid oxidation (FAO) (fully oxidized palmitate), incomplete FAO (acid soluble
metabolites remaining in media), Total FAO (Complete + Incomplete), and oxidative efficiency (ratio of Complete FAO to Incomplete FAO), as well as glucose oxidation were measured in cells after seven days of differentiation (in homogenate tissue pyruvate oxidation was measured in place of glucose).

**Reactive Oxygen Species Production:** The CM-H2DCFDA (General Oxidative Stress Indicator) dye kit was used to detect ROS production. ROS production was measured at day zero (last/fifth day of proliferation) and day seven of differentiation. The maximal rate of ROS production is expressed per nuclei content as int/min/nuclei ± SEM.

**Cytokine Secretion:** Cytokine levels were measured on the last/fifth day of proliferation and the seventh day of differentiation. Cayman Chemical ELISA kits were used to measure media secretions of IL-6 and TNF-α (Cayman Chemical, Ann Arbor, MI). Cytokine levels are expressed as pM/ug ± SEM.

**RNA extraction and qRT-PCR:** RNA was collected at select time points of the differentiation process on days 0, 1, 2, and 7. RNA was extracted using an RNeasy Mini Kit (Qiagen) and DNase I treatment (Qiagen, Valencia, CA), according to the manufacturer’s instructions. qRT-PCR was performed using an ABI PRISM 7900 Sequence Detection System instrument and TaqMan Universal PCR Master Mix used according to the manufacturer's specifications (Applied Biosystems, Foster City, CA). Target gene expression in rodent skeletal muscle and cell culture was normalized to β-actin RNA levels. Target gene expression in human skeletal muscle primary cell culture was normalized to cyclophilin B RNA levels. Primers and 5# FAM-labeled TaqMan probes were purchased as prevalidated assays (ABI). Relative quantification of target genes was calculated using the 2−ΔCT method. Derivation of the 2−ΔCT equation has been described in Applied Biosystems User Bulletin no. 2 (P/N 4303859). As previously described by Frisard et al.173

**Statistical Analysis:** One way-ANOVA was used to compare proliferation rates between the young sedentary, older sedentary, and trained groups, with a Tukey post hoc test to evaluated
difference between individual groups. Pre- and post- HFHCD samples and control vs. HSM samples were compare using paired t-test. Differentiation and the myogenic index was analyzed using repeated measures ANOVA and a Tukey post-hoc analysis. Age, body-fat %, and BMI correlations were analyzed using Pearsons’ correlation, with $r^2$-values reported. The HSM effect is reported as percent change from control values. Data is presented as mean ± SEM, Statistical significance was set at a $p$-value of $\leq 0.05$.

**Results:**

<table>
<thead>
<tr>
<th></th>
<th>Sed. Young</th>
<th>Sed. Older</th>
<th>Trained</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Participants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>23</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>23.8 ± 4.1</td>
<td>66.6 ± 4.5*</td>
<td>22.8 ± 2.2^</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>22.4 ± 8.1</td>
<td>28.3 ± 6.3*</td>
<td>16.5 ± 4.9^</td>
</tr>
<tr>
<td>Proliferation Rate (dlbs/day)</td>
<td>0.547 ± 0.124</td>
<td>0.487 ± 0.110</td>
<td>0.678 ± 0.126^</td>
</tr>
<tr>
<td>Multi-Nucleated (%)</td>
<td>82.6 ± 19.8</td>
<td>79.9 ± 12.5</td>
<td>82.6 ± 22.4</td>
</tr>
<tr>
<td>Highly-Nucleated (%)</td>
<td>22.1 ± 14.9</td>
<td>47.7 ± 14.1*</td>
<td>28.6 ± 0.6</td>
</tr>
<tr>
<td>PRO/DNA (mg/nuclei)</td>
<td>9.8E-05 ± 1.1E-05</td>
<td>7.6E-05 ± 1.3E-05</td>
<td>8.1E-05 ± 1.2E-05</td>
</tr>
<tr>
<td><strong>Metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle Tissue Homogenate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete FAO</td>
<td>0.78 ± 0.23</td>
<td>0.91 ± 0.66</td>
<td></td>
</tr>
<tr>
<td>Incomplete FAO</td>
<td>7.28 ± 1.79</td>
<td>13.26 ± 4.11*</td>
<td></td>
</tr>
<tr>
<td>Efficiency</td>
<td>0.11 ± 0.02</td>
<td>0.07 ± 0.03*</td>
<td></td>
</tr>
<tr>
<td>Total FAO</td>
<td>8.05 ± 1.92</td>
<td>14.17 ± 4.60*</td>
<td></td>
</tr>
<tr>
<td>Pyruvate Oxidation</td>
<td>146.14 ± 76.99</td>
<td>366.82 ± 131.8*</td>
<td></td>
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<tr>
<td>In-Vitro Cellular Metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete FAO</td>
<td>0.26 ± 0.14</td>
<td>0.32 ± 0.19</td>
<td>0.32 ± 0.16</td>
</tr>
<tr>
<td>Incomplete FAO</td>
<td>2.23 ± 1.11</td>
<td>1.65 ± 0.53</td>
<td>1.54 ± 0.39</td>
</tr>
<tr>
<td>Efficiency</td>
<td>0.12 ± 0.05</td>
<td>0.19 ± 0.10*</td>
<td>0.21 ± 0.05*</td>
</tr>
<tr>
<td>Total FAO</td>
<td>2.49 ± 1.22</td>
<td>1.97 ± 0.65</td>
<td>1.87 ± 0.55</td>
</tr>
<tr>
<td>Glucose Oxidation</td>
<td>4.00 ± 1.93</td>
<td>2.78 ± 1.40</td>
<td>4.65 ± 0.82</td>
</tr>
</tbody>
</table>

Table 5.1. Values for young (18-30 years) older (50-80 years) and trained (>5 hours/week running/cycling) ± standard deviation. (* indicates difference from young, ^ indicates difference between older and trained).

Participant data: Satellite cell cultures of 51 participants were used in this analysis. Of those, 23 were young, 23 were older, and 5 were categorized as endurance trained participants. Between the
young and trained groups, there were no differences in age (23.8 ± 4.1 vs 22.8 ± 2.2 years) or body fat % (22.4% ± 8.1 vs 16.5% ± 4.9). The older group had significantly higher body fat levels compared to the young (22.4 ± 8.1 vs 28.3±6.3, p = 0.011, table 5.1).

**Proliferation:** There was no significant difference in proliferation rate between the young and older groups (p = 0.31, figure 5.1-A, and B). Cells from the trained group exhibited higher proliferation rates compared to older group (p = 0.02), but not young group (p = 0.13). In sedentary participants, age did not correlate with proliferation rate (p = 0.23). There was no significant correlation between proliferation rate and body fat % (p= 0.39). When the proliferation rates of the young sedentary and older cells were matched based on body fat percentage the mean difference between the groups decreased from -0.060 ± 0.040 to -0.052 ± 0.049 and the difference remained non-significant (Figure 1-C).

**Differentiation:** Adiposity did not significantly affected measures of in-vitro myogenesis (figure 5.2). The young, older and trained groups achieved similar fusion rates after seven-days of differentiation, and while the trained group appeared higher it was not significant (p = 0.74) (figure 5.2-A). There were differences between the groups throughout the process of differentiation particularly in the initial stages. Before the end of the proliferation stage fusion of cells from trained participants had increased from 15.9% to 79.1% (p = 0.01) (figure 5.2-C and D). This spontaneous fusion was not observed in the young and older groups, as they did not experience significant fusion until incubation in differentiation media for at least two days. Neither BMI nor body fat percentage had any correlation with myogenicity. The cells from the older participants expressed a greater proportion of highly-nucleated fibers (47.7 ± 14.1) compared to the young group (22.1±14.9) (p = 0.01).

**Substrate Metabolism:** Skeletal muscle homogenate from older participants exhibited higher rates of incomplete fatty acid oxidation, as well as higher rates of pyruvate oxidation when compare to
younger participants (figure 5.3-D, and J). Older participants also displayed lower levels of oxidative efficiency (Figure 5.3-F). In tissue homogenate, complete and total fatty acid oxidation positively correlated with participant BMI (p = 0.016, 0.036) (figure 4-A, and C) while incomplete oxidation and BMI trended towards a significant correlation (p = 0.065) (figure 5.4-C). Though there were significant metabolic differences between groups in muscle tissue homogenate (Figure 5.3-B, D, F, H, and J), there were no apparent metabolic differences in differentiated satellite cells due to donor age, adiposity, or training status other than the higher oxidative efficiency displayed by older and trained cells when compared to young (figure 5.3-E).

**ROS Production:** Cellular ROS production did not differ between young, older or trained groups during proliferation or after differentiation (figure 5.5). ROS production did not appear to be affected by donor weight status or adiposity.

**Cytokine Secretion:** Young and older cells showed no differences in IL-6 secretion during proliferation (Figure 5.6-A). However, older cells displayed significantly lower IL-6 secretory levels after differentiation when compared to the young group (p = 0.047) (Figure 5.6-B). No differences were detected in TNF-α secretion between young and older participants during either proliferation or differentiation (figure 5.6-C and D). Compared to those from young donors, cells from trained participants had significantly higher secretory levels of TNF-α during proliferation and after differentiation (p = 0.048 and p <0.01, respectively) (Figure 5.6-C and D). IL-6 secretion was markedly higher in fully differentiated cells compared to cells still in the proliferation stages (p < 0.01). IL-6 secretion inversely correlated to donor body fat, with higher rates of secretion in leaner participants (p = 0.035, r² = 0.6226) (Figure 5.6-G).

**Transcription Factor Expression:** No significant differences were observed in the RNA expression of Pax-7, MyoD, or MyoG between the sedentary young, older, and trained groups (Figure 5.7).
Figure 5.1. Proliferation rates of satellite cells from older, young, and young trained donors (A), Correlation between proliferation rates and age of sedentary participants (B). Proliferation correlated to body-fat percentage (C). Body fat matched proliferation rates of sedentary young and older cells (D). (*) indicates statistical difference between groups (p <0.05)
Figure 5.2. Myogenic index (A) and Super-Myogenic index (B) values after seven day of differentiation. Time course of MGI (C) and S-MGI (D) from growth through differentiation. (*) indicates statistical difference between groups (p < 0.05)
Figure 5.3. Satellite cells from young sedentary (18-30 yrs, n = 9), older sedentary (50-80 yrs, n = 9), and trained (18-40 yrs, n = 2) human donors (n= 4) differentiated for seven days in serum starve media and then measured for; (A) complete FAO, (C) incomplete FAO, (E) total FAO, (G) oxidative efficiency, and (I) glucose oxidation. Tissue homogenate taken from the vastus lateralis of sedentary young (n = 11) and sedentary older (n = 14) participants measured for; (B) complete FAO, (D) incomplete FAO, (F) total FAO, (H) oxidative efficiency, and (J) PDH activity. (*) indicates statistical difference between groups (p <0.05)
Figure 5.4. Correlations of participant BMI and muscle homogenate measures of; complete FAO (A), incomplete FAO (B), total FAO (C), oxidative efficiency (D), and glucose oxidation (E).
Figure 5.5. ROS production of satellite cells from older, young, and trained donors during proliferation (A) and after differentiation. (*) indicates statistical difference between groups (p<0.05)
Figure 5.6. IL-6 (A, B) and TNF-α (C, D) secretion during the fourth day of proliferation and seventh day differentiation grouped as young sedentary (18-30 yrs, n = 9), older sedentary (50-80 yrs, n = 9), and trained (18-40 yrs, n = 2). Daily media secretion of IL-6 (E) and TNF-α (F) throughout proliferation and differentiation grouped as young sedentary (18-30 yrs, n = 9), older sedentary (50-80 yrs, n = 9), and trained (18-40 yrs, n = 2). Correlation of IL-6 expression during day four of proliferation to participant body-fat % (G). (*) indicates statistical difference between groups (p <0.05).
Figure 5.7. RNA expression (Delta:Delta CT) of Pax-7 (A), MyoD (B), and MyoG (C) throughout differentiation grouped as young sedentary (18-30 yrs, n = 2), older sedentary (50-80 yrs, n = 2), and trained (18-40 yrs, n = 2). Average relative RNA expression (% of maximum) of Pax-7, MyoD, and MyoG (D).

Discussion

This study was designed to access the variability in human satellite cell function. With satellite cell function defined as the ability of the cells to proliferate, differentiation, and become metabolically functional in culture. A secondary goal was to determine if the anthropometric characteristics of a human donor correlate to the functionality of their satellite cells in-vitro. Compared to previous studies, a relatively large sample size ranging in age, body-fat, and training status was used to illustrate how wide an array there is in satellite cell function. Chiefly, this study was an attempt to understand if human satellite cells are affected by their donor’s age independent of activity level, weight status, or level of adiposity.

It has been proposed that age related muscle loss could be at least partly caused by damaged and/or dysfunctional satellite cells. More research should be done on this topic, as the functionality of the satellite cells though to the aging process has not been completely examined. Pioneering research
in rodent animal models demonstrated a decreased ability of satellite cells to proliferate at increased ages.\textsuperscript{38} These results were replicated in a few human studies as well.\textsuperscript{39,40} However, these studies did not account for the lower activity levels and increased body fat of the older participants.\textsuperscript{41,42} Though body composition typically shifts towards higher fat mass and lower lean body mass with age, with proper diet and exercise higher lean body mass is maintainable throughout the lifespan\textsuperscript{43}. Elevated body-fat should not be treated as a consequence of aging, but instead be evaluated as an individual variable. There is an inherent difficulty in this regard as in studies comparing young and older participants, older participants routinely have higher body-fat levels.

This current study found that age alone accounted for no significant differences in satellite cell function. Cells from older humans showed no differences in their ability to proliferate, fuse, and differentiate. It is important to note that while sedentary both groups were considered healthy and free from any chronic disease. The only difference detected between cells from sedentary young and older donors was an increase in oxidative efficiency in the older cells. No differences were observed between the young and older groups in markers of cellular stress such as ROS production or cytokine secretion (IL-6 or TNF-\(\alpha\)). These results are contrary to current opinions that aging alters the function of stem cells at their core. Aging has been linked to disrupted muscle tissue metabolism which is associated with decreased lean mass and changes in body composition.\textsuperscript{44} This is demonstrated in this study by the reduced oxidative efficiency, elevated incomplete FAO, and elevated pyruvate oxidation in the older muscle tissue homogenate compared to the young. However, these disruptions are most likely attributed to the environmental factors the tissue is grown in, for when the young and older cells are isolated from their donors and grown in equal conditions the age related dysfunction disappears.

The effects of aging on muscle loss may be more associated with the increased body-fat and lower physical activity observed in older individuals. Regular physical activity and exercise are key in
maintaining proper functioning satellite cell pools, groups of cells that are called upon during muscle regeneration. However, several studies have shown that with aging there is a dramatic decrease in physical activity combined with increases in body-fat percentage. These lower activity levels are associated with decreases in satellite cell availability and function in humans and mice. The results of this study highlight how cells from a sedentary young population are equal in function to those from a sedentary older population.

Recent studies have demonstrated that IL-6 and TNF-α, as a myokines, regulate myogenesis. These cytokines are also elevated in the serum of older individuals. IL-6 activates STAT3 and the downstream expression of MyoD, which is potentially how IL-6 mediates myogenesis in response to exercise. The results of this study revealed that neither IL-6 nor TNF-α secretion appeared to be affected by age. However, cellular IL-6 secretion was inversely correlated to donor body-fat percentage. Chronic exposure to elevated inflammatory cytokines may epigenetically blunt muscle release of IL-6 through a negative feedback loop. Obesity is associated with chronic adipose derived inflammation, exposure to which may blunt necessary autocrine myokine secretions needed for myogenesis. Exercise may alleviate these effects as elevated lean mass is correlated with decreased inflammation in obese participants.

Donor adiposity did not correlate with any of the functional measures in this current work other than IL-6 secretion. In mice, diet-induced obesity is associated with decreased myogenesis through inhibition of satellite cell recruitment. This is thought to be mechanistically linked to the decreases in the response and secretion of growth factors in the muscles of obese mice. The effects of obesity on satellite cell function may exist more dominantly in-vivo, as once the satellite cells are removed from the obese environment and grown in-vitro they are no longer participantd to disrupted adipokine signaling. A link between body sizes as reported by BMI did correlate with satellite cell oxidative
capacity. With greater rates of incomplete and total FAO observed in the cells of donors with higher BMIs. This finding is similar to those of Chen et al, which demonstrated disrupted substrate oxidation and AMPK activation in cells isolated from obese type II diabetics.60

In direct opposition to the effects of a sedentary lifestyle, older sedentary participants who participate in exercise training programs show a near complete restoration of satellite cell function.61 Both endurance and resistance training are capable of restoring the muscle tissue microenvironment of older participants.45,62,63 Training programs can restore satellite cell pool size and activation potential to that of their young counterparts, suggesting that it is the sedentary lifestyle is more detrimental to maintenance of lean muscle mass than aging. It is possible that the loss of CSA and satellite cell pool size attributed to aging is partly the effect of a prolonged sedentary lifestyle.

In previous work done by Roberts et. al. eliciting the mechanistic difference between young and old satellite cells, Cyclin D1 and MyoD were determined to have different patterns. In response to exercise, MyoD increased in the young group as expected but there was no increase in the old group.64 While this finding was contributed to the effects of aging, it is important to note that the body-fat levels between the young and old group were significantly different with the 10 young participants at 15.4% (+/-2.9%) and the 10 older participants at 27.4% (+/-1.9%). These numbers put the young participants in the healthy range for body-fat and the old in the overweight/obese ranges. This is a common complication observed in aging studies, as it is difficult to recruit a lean older population because such a population seldom exists. Even in the participant demographics for this current study there was a significant difference between the adiposity of the young and older group. With body fat differences being so prevalent in aging studies, we must ask if the differences currently attributed to age are in fact caused by adiposity.
Most factors that arise with aging and disrupt satellite cell function occur in-vivo. The findings from this study have shown that when satellite cells from young and old donors are grown and differentiated in identical conditions differences in function was not discernable. Even though, in-vivo, there were clearly differences between the age groups. This concept has been demonstrated before as satellite cells with poor function collected from unhealthy older donors had their function restored when grown in sera collected from young participants. Conversely, young cells grown in sera collected from unhealthy older participants experience the same faults in myogenesis as unhealthy older cells. If the tissue microenvironment is the main contributing factor for the loss of muscle mass during aging, then future research must be aimed at restoring this environment knowing that even in older participants their cells will be able to respond.

**Conclusion**

The findings of this study demonstrate that age alone does not negatively affect satellite cell function. This finding is mostly due to the large ranges observed among human samples collected at any age. While the young participants did have slightly higher proliferation and fusion rates compared to the old, the variability in the groups made them indistinguishable. There is a well-studied link between age and loss of muscle function. There have also been findings that suggest satellite cells do not function properly in older individuals, in-vivo. However, this study’s results suggest these cells function normally, in-vitro, in a healthy environment. This supports the notion that there are no innate problems with the satellite cells of older individuals and that dysfunction occurs when the cells are exposed to a suboptimal extra-cellular environment. Future studies must be aimed at adjusting the extra-cellular environment of older individuals. This would allow healthy stem cells to restore tissue function without being compromised once they are activated.
References


Chapter 6: A High-Fat/Hyper Caloric Diet (in-vivo), and High Substrate Media Treatment (in-vitro) Alter Human Satellite Cell Myogenesis and Metabolism

Abstract:
Satellite cells are responsible for skeletal muscle growth and regeneration. Dysfunction in satellite cell mediated skeletal muscle maintenance has the potential to cause or at least exacerbate several diseases including obesity, diabetes, and sarcopenia. Dysfunction could also reduce the capacity for physical activity, resulting in a loss in overall quality of life. While there is a correlation between obesity and poor musculoskeletal function, the effects of obesogenic lifestyle factors on satellite cell function are not understood. For this study, satellite cells were isolated from the vastus lateralis of young (18-30 years) sedentary and endurance trained men. Satellite cells we also collected in the sedentary participants after a four-week, high-fat (55%) hypercaloric (+1000 kcal) (HFHCD). In-vitro, cells were exposed to a high substrate growth media (+20mM glucose and +400 uM 2:1 olate:palmitate). Cells were assessed on their ability to proliferate, differentiate, and their ability to oxidize lipids and glucose once differentiated. Satellite cells collected from participants post-HFHCD had significantly reduce proliferation rates (-19%, p<0.05). Changes in cellular proliferation in response to the high substrate media was correlated to the participant’s body-fat percentage ($r^2=0.612$, p<0.01). Cells from young lean (<25% BF) and trained participants had blunted proliferation rates (-4.8% and -12.6%, p<0.05) and, fusion index scores (p<0.05) when exposed to HSM. Cells from higher percent body fat donors (>25%BF) experienced enhanced growth when exposed to the high substrate media while cells from trained and leaner participants exhibited diminished proliferation when exposed to elevated substrate availability. These results demonstrate that both diet and participant adiposity have the potential to alter satellite cell function. Satellite cells appear to develop a preference for their in-vivo environment and substrate availability. This may possibly reduce the ability of obese individuals or individuals on a high fat diet to respond properly to exercise.
Introduction:

Alterations in the extracellular environment can have lasting effects on stem cells of many lineages, including satellite cells. Given the effects of muscle mass and exercise on daily energy expenditure/weight loss, any scenario where myogenesis is inhibited may contribute to obesity. Obesity may have a permanently negative effect on satellite cell function and myogenesis. An Individual’s satellite cell genetics may prevent or exacerbate the development of obesity by up or down regulating the myogenic response to diet and exercise. If left untreated, obesity can lead to further complications such as the development of insulin resistance and metabolic syndrome, both of which have compounding effects on muscle loss. These diseases can have permanent epigenetic effects on muscle tissue and satellite cells, which can be retained in culture.

Other stem cell types have already demonstrated an ability to be functionally altered by obesity. Adipogenic precursor cells biopsied from obese human donors exhibit insulin resistance when differentiated into white adipocytes. This shows that phenotypic changes to an individual reach further than the functioning tissue and can cause epigenetic changes to the core set of stems cells. Obesity may also cause stem cells to differentiate improperly and acquire the phenotype of other tissues. Differentiation into another cell type is a primary cause of organ fibrosis, as tissue specific stem cells lose their desired function and take the role of fibroblasts.

Obesity and a fatty diet have previously demonstrated negative effects on many tissue types including muscle. inflammatory adipokine signaling from the visceral adipose tissue of obese individuals has the ability to decrease myogenesis and muscle contractility. A high-fat diet also induces an inflammatory state, which impairs myogenesis and can cause muscle atrophy. What is not yet known is if these effects are exerted only on expressed muscle tissue or also on quiescent satellite cells. When activated satellite cells fuse with existing fibers bringing new DNA into the fiber. If the satellite
cells are damaged due to obesity or diet and the new DNA they supply is negatively altered, then exercise may not be enough to restore muscle function in these situations.

Altered inflammatory signaling may be the root cause of obesity and diet induced dysfunction. Metabolic syndrome is characterized with chronic increases of low-level inflammatory markers. Both Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) are increased by metabolic syndrome and have been shown to disrupt stem cell proliferation and differentiation in culture. Although not thoroughly studied, the idea that obesity could influence the satellite cell population of a participant is not new. In 1984, Campion et al demonstrated in ob/ob mice that obesity was associated with smaller and less nucleated fibers in the soleus and gastrocnemius. These obese mice had fewer nuclei per length of fiber when compared to control mice.

Inflammatory signaling may not be the only mechanism for obesity induced stem cell dysfunction. There may be dysregulation due to substrate availability. AMPK has an important role in repairing muscle damage by increasing metabolic efficiency to allow for rapid proliferation. This action is reduced in obese participants, as they show decreased AMPK expression and activation. Knocking out AMPKα1 in mice resulted in a significantly reduced ability of the satellite cells to activate, proliferate, and differentiate. Diet-induced obesity in mice results in a similar reduction in AMPK expression, also leading to reduced satellite cell function and an inability to recover from muscle damage. Treatment of obese mice with AICAR (an AMPK activator) restored muscle recovery.

This study was designed to measure the effects of elevated lipid and carbohydrate supply to satellite cells. The effects of a high-fat/hyper-caloric diet (in-vivo), as well as a high-lipid/high-carbohydrate treatment (in-vitro) were tested. The purpose of this study was to assess the effects of a variety of in vivo physiological stimuli (high fat diet or endurance trained) on skeletal muscle satellite cell function. Satellite cell function was also assessed in response to growth conditions of excess substrate
(glucose and fatty acids) availability. With satellite cell function defined as the ability of the cells to proliferate, differentiation, and become metabolically functional in culture.

**Methods:**

**Participant Selection:** Skeletal muscle biopsies previously were obtained under three IRB-approved human clinical studies, and satellite cells were subsequently isolated (described below). Participants were categorized based on activity level as either sedentary or endurance trained. Sedentary participants engaged in less than ≤2 days, 20 min per day of low-intensity physical activity. Endurance-trained individuals ran or cycled for a self-reported 5+ hours per week and had to be engaged in at least one organized running race event of a distance of 10+ kilometers within the past year. All participants were male and grouped as, sedentary (18-30 years, sedentary), and trained (18-30 years, endurance trained). All participants were assessed through a medical history questionnaire and free of any chronic diseases or medication use. Body composition was assessed through DEXA (dual-energy x-ray absorptiometry) scan. Satellite cell isolation was approved by the Virginia Tech Institutional Review Board (IRB# 14-1234)

**Dietary Intervention:** participants were exposed to a 2-week lead-in diet immediately followed by a high-fat hypercaloric diet (HFHCD) for four weeks as described by Osterberg, et al. The participant’s daily energy expenditure (DEE) was assessed as described. The lead in diet was isocaloric as determined by DEE and consisted of 30% fat, 50% carbohydrate and 20% protein. The HFHCD was set to DEE plus an additional 1000 kcal with a macronutrient break down of 55% fat, 20% carbohydrate and 15% protein. Biopsies were collect on three occasions; before the lead-in diet, at the completion of the lead-in diet/start of the High fat, and following the 4-week high-fat challenge diet.

**Satellite Cell Isolation/Growth/Differentiation:** Satellite cells were previously collected from tissue biopsies of the vastus lateralis muscle of male participants. Muscle samples weighing ~ 50 mg
were collected from donors ranging in age (18-80 years) and body-fat percentage (7-40%). Satellite cells were cultured and differentiated in multinucleated myotubes as previously described (Muoio et al., 2002). Cells were seeded and allowed to proliferate in growth (DMEM with 5mM glucose, 5% fetal bovine serum, rhEGF (10n/ml) and dexamethasone (0.4 ug/ml)) media for 5 days, until they reached 60-70% confluency. After proliferation, cells were differentiated in low serum (DMEM with 5mM glucose and 2% horse serum) media to activate differentiation. Cells were differentiated for seven days with media changed every 2 days. Amphotericin-B (0.5 ug/ml), antimitotic (for penicillin 100U/ml and streptomycin 100 ug/ml), and gentamycin (0.01 mg/ml) were added for protection against bacterial and fungal infections.

**High Substrate Media Challenge:** High substrate media (HSM) was applied during the proliferation stage. HSM contained all the same ingredients as the control growth media with additional supplementation of 400mM, 2:1, oleate:palmitate and the glucose concentration increased from 5mM to 25mM. Control growth media and high substrate media were always replaced at the same time when required. For the proliferation assay high substrate media was applied one day after the cells were plated and replaced again after 4 days. For all differentiation protocols, high substrate media was applied one day after the cells were plated. Once the proliferation process was complete both the cells exposed to control media and HSM were differentiated identically in the low serum differentiation media. Cells were allowed to grow in the high substrate media for four days at which point the differentiation process was initiated by the removal of the high substrate media and exposure to the differentiation media. All high substrate challenged cells and matching control cells were grown on the same plate and differentiated at the same time.

**Proliferation Assay:** Cells were counted with a hemocytometer. Cells were seeded as 2,500 cell/well in separate 96-well plates. Plates were collected on days 1 through 6 plates and nuclear material was counted using thermofisher’s CyQUANT® NF Cell Proliferation Assay Kit. Doubling times
were calculated using the PRISM exponential growth equation. Proliferation is reported as doubling time in hours (hrs/Db ± SEM) and doublings per day (dbs/day ± SEM).

**Myogenic Index:** Myogenic index methods were adapted from Kamli et. al.\(^{18}\) Cells were fixed in 4% formaldehyde for 15 min at room temperature. Cells protein was stained red with eosin, while nuclei were stained green with CyQUANT. Dye (Thermo Fisher). Cells were imaged at 10 times magnification. Nuclei number within multi-nucleated (two or more nuclei per fiber), and super-nucleated (5 or more nuclei per fiber) fibers as well as total nuclei number was counted. Myogenic index was calculated as the percentage of nuclei within multi-nucleated fibers compared to total observed nuclei, as described by Kamli, M., et al.\(^{19}\) Myogenic index is reported as both multi-nucleation (proportion of nuclei in fibers with 2 or more nuclei per fiber divided by total nuclei) and Robust-nucleation (proportion of nuclei in fibers with 10 or more nuclei per fiber divided by total nuclei). Myogenic index is reported as a percent ± SEM. Total protein was evaluated through BCA and nuclei content was measure through CyQUANT assay. Protein/nuclei ratio is expressed as a measure of differentiation as µg/nuclei ± SEM

**Substrate Oxidation:** Substrate metabolism and tissue homogenate measures were performed on differentiated cells as previously described in Frisard et. al.\(^{20}\) Complete fatty acid oxidation (FAO) (fully oxidized palmitate), incomplete FAO (acid soluble metabolites remaining in media), Total FAO (Complete + Incomplete), and oxidative efficiency (ratio of Complete FAO to Incomplete FAO), as well as glucose oxidation were measured in cells after seven days of differentiation.

**Reactive Oxygen Species Production:** The CM-H2DCFDA (General Oxidative Stress Indicator) dye kit was used to detect ROS production. ROS production was measured at day zero (last/fifth day of proliferation) and day seven of differentiation. The maximal rate of ROS production is expressed per nuclei content as int/min/nuclei ± SEM.
**Cytokine Secretion:** Cytokine levels were measured on the last/fifth day of proliferation and the seventh day of differentiation. Cayman Chemical ELISA kits were used to measure media secretions of IL-6 and TNF-α (Cayman Chemical, Ann Arbor, MI). Cytokine levels are expressed as pM/μg ± SEM.

**RNA extraction and qRT-PCR:** Cells were grown in either control or HSM for 4 days prior to differentiation. RNA was collected at select time points of the differentiation process on days 0, 1, 2, and 7. RNA was extracted using an RNeasy Mini Kit (Qiagen) and DNase I treatment (Qiagen, Valencia, CA), according to the manufacturer's instructions. qRT-PCR was performed using an ABI PRISM 7900 Sequence Detection System instrument and TaqMan Universal PCR Master Mix used according to the manufacturer’s specifications (Applied Biosystems, Foster City, CA). Target gene expression in rodent skeletal muscle and cell culture was normalized to β-actin RNA levels. Target gene expression in human skeletal muscle primary cell culture was normalized to cyclophilin B RNA levels. Primers and 5# FAM-labeled TaqMan probes were purchased as prevalidated assays (ABI). Relative quantification of target genes was calculated using the 2−ΔCT method. Derivation of the 2−ΔCT equation has been described in Applied Biosystems User Bulletin no. 2 (P/N 4303859). As previously described by Frisard et al.173

**Statistical Analysis:** One way-ANOVA was used to compare proliferation rates between the sedentary, high-adiposity, and trained groups, with a Tukey post hoc test to evaluated difference between individual groups. Pre- and post- HFHCD samples and control vs. HSM samples were compare using paired t-test. Differentiation and the myogenic index was analyzed using repeated measures ANOVA and a Tukey post-hoc analysis. Age, body-fat %, and BMI correlations were analyzed using Pearsons’ correlation, with r²-values reported. The HSM effect is reported as percent change from control values. Data is presented as mean ± SEM, Statistical significance was set at a p-value of ≤ 0.05.
Results:

<table>
<thead>
<tr>
<th></th>
<th>Sed. Young</th>
<th>Post-HFHCD</th>
<th>Trained</th>
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<tbody>
<tr>
<td>n</td>
<td>23</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>23.8 ± 4.1</td>
<td>22.7 ± 4.0</td>
<td>22.8 ± 2.2^</td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>22.4 ± 8.1</td>
<td>25.4 ± 10.2</td>
<td>16.5 ± 4.9^</td>
</tr>
<tr>
<td>Proliferation Rate (dbls/day)</td>
<td>0.547 ± 0.124</td>
<td>0.446 ± 0.114*</td>
<td>0.678 ± 0.126^</td>
</tr>
<tr>
<td>Multi-Nucleated (%)</td>
<td>82.6 ± 19.8</td>
<td>92.4 ± 4.7</td>
<td>82.6 ± 22.4</td>
</tr>
<tr>
<td>Highly-Nucleated (%)</td>
<td>22.1 ± 14.9</td>
<td>19.2 ± 13.5</td>
<td>28.6 ± 0.6</td>
</tr>
</tbody>
</table>

Table 6.1. Values for young (18-30 years) post-High-fat (55%)/Hypercaloric (+1000kcal) diet (50-80 years) and trained (>5 hours/week running/cycling) ± standard deviation. (* indicates statistical difference from young, ^ indicates statistical difference between older and trained).

Effects of High-Fat/Hyper-Caloric Diet and Obesity: Exposure to the 4-week high-fat hypercaloric diet decreased in-vitro satellite cell proliferation rate an average of 19.4%, from 0.55 ± 0.03 to 0.45 ± 0.03 doubling per day (p < 0.01)(figure 6.1A). The HFHCD did not appear to affect satellite cell differentiation when comparing the MGI scores of cells collected pre- and post- intervention. There were significant differences observed between pre and post diet conditions in tissue homogenate metabolism (Figure 6.3). These differences were not detected in-vitro after the satellite cells were isolated from each condition. Inflammatory secretions of TNF-α and IL-6 during proliferation of differentiation did not differ between cells collected pre and post diet.

Effect of High Substrate Media: Treatment with HSM appeared to alter several aspects of satellite cell myogenesis, and the affect, either positive or negative, was dependent on the condition of the donor. The proliferation rates of satellite cells collected from trained participants and leaner sedentary participants (BF < 25%) were blunted when exposed to HSM (figure 6.1B). However, cells collected from more participants with higher adiposity (BF > 25%) displayed accelerated growth when exposed to HSM (Figure 6.1B+D). In sedentary participants (18-30 years), the effect of HSM on satellite cell proliferation rate was significantly correlated (r=0.612, p<0.01) to donor adiposity (Figure 6.1C).
Cell differentiation was blunted by HSM across all ranges of donor adiposity. HSM decreased multi-nucleation by an average of 6.6%, and robust fusion by 11.9% (Figure 6.2A+B). HSM treatment resulted in an initial delay in cellular fusion but the differences between the treatment and control groups lessened as differentiation progressed, and the cells were further removed from the HSM exposure (Figure 6.2A). Cells from trained participants exhibited spontaneous differentiation during the growth phase (45% fusion in control on the 4th day of proliferation vs. 10% in HSM) before exposure to differentiation media (Figure 6.2C+D). This spontaneous differentiation did not occur in trained cells when exposed to the HSM during the growth phase. HSM appeared to have no effect on the differentiation of trained cells after they were exposed to differentiation media.

Exposure to HSM during the growth phase increased fatty acid oxidation in differentiated cells (Figure 6.3). Complete, incomplete, and total fatty acid oxidation measures as well as the ratio of complete to incomplete oxidation were all significantly increased in cells exposed to HSM after differentiation (p<0.05). The HSM treatment had no effect on cellular glucose oxidation. HSM exposure led to reduced cellular ROS production during both the growth and proliferation phases (Figure 6.5).

Effect of HSM on Post-HFHCD Cells: Pre and post HFHCD cells responded in opposite fashion when exposed to HSM. Cells collected pre dietary intervention responded to HSM treatment with decreased proliferation rates and fusion index scores (Figure 6.1B+2E). However, post diet cells reacted to HSM with increased proliferation and fusion rates. These differences are highlighted in images in figure 4 where fusion is blunted in pre-diet cells exposed HSM but large robust fibers developed in the post-diet cells exposed to HSM. HSM did not affect RNA expression of Pax-7, MyoD, or MyoG (Figure 6.6).
Figure 6.1. Changes in proliferation rate after completion of a four-week, high-fat (55%), hypercaloric (+1000 kcal) diet (HFHCD) (n = 4) (A). Effect of high substrate media (HSM) on proliferation rates in sedentary lean (BF<25%, n = 5), sedentary high adiposity (BF>25%, n = 6), trained (n = 4), and post-HFHCD (n = 2) reposted as percent change from control conditions (B). Effect of HSM on proliferation rate (% change) correlated with participant body-fat % (n = 11) (C). Effect of HSM on proliferation rate pre and post HFHCD (n = 2) (D). (*) indicates statistical difference between groups (p <0.05)
Figure 6.2. Myogenic index of sedentary (n=10) (A) and trained (n=2) cells (C) and super-myogenic index of sedentary (B) and trained cells (D) grown in control growth media and high substrate media. Effect of HSM on myogenicity of cells collected pre and post HFHCD (n=6) (E). (*) indicates statistical difference between groups (p <0.05)
Figure 6.3. Satellite cells from human donors (n= 4) grown in control or high substrate media (HSM) for four days prior to seven days of serum starve differentiation and then measured for; (A) complete FAO, (C) incomplete FAO, (E) total FAO, (G) oxidative efficiency, and (I) glucose oxidation. Tissue homogenate taken from the vastus lateralis of sedentary lean male participants pre and post four-week, high-fat (55%), hypercaloric (+1000 kcal) diet (n = 20) measured for; (B) complete FAO, (D) incomplete FAO, (F) total FAO, (H) oxidative efficiency, and (J) PDH activity. (*) indicates statistical difference between groups (p <0.05)
Figure 6.4. Differentiated Cells isolated pre and post four-week, high-fat/hypercaloric diet. Grown in control or high substrate media (HSM) for four days prior to seven days of serum starve differentiation. (protein stained red with eosin and nuclei stained green with CyQuant dye)

Figure 6.5. ROS production during the fourth day of proliferation and seventh day of differentiation. Grown in control or high substrate media (HSM) for four days prior to seven days of serum starve differentiation (n = 8).
Discussion:

The HFHCD did cause a reduction in satellite cell proliferation and altered substrate preference towards the HSM. This is an indication that these cells can be significantly impacted by diet. In healthy lean control cells, exposure to the HSM decreased measures of both proliferation and differentiation. However, in cells collected from participants with higher adiposity (>25% BF%) and participants post HFHCD, exposure to the HSM resulted in an increase in both functions. These results highlight the capacity of satellite cells to be influenced by and adapt to their in-vivo environment.

These data suggest that satellite cells are susceptible to changes in substrate availability both in-vivo and in-vitro. Exposure to elevated fats and carbohydrates may result in epigenetic changes that permanently effect how satellite cells proliferate and differentiate. These results are similar to a previous study by Sharples et al, which showed that exposure to LPS could increase the DNA methylation of genes necessary for myogenesis. This prior study demonstrated that after exposure to LPS, DNA methylation increased with each new generation as the cells proliferated. This may explain
how exposure to the HSM during growth continues to affect the cells once they have been fully differentiated. This is an indication that obesity or improper diet could have lasting or worsening effects even after an individual has improved their diet or lost weight.

Exposure to HSM during growth resulted in decreased cellular ROS production after differentiation, indicating a potentially positive adaptation to the treatment. A similar response was observed in lipid metabolism as exposure to HSM also increased cellular oxidative capacity and efficiency. In-vivo, high-fat diets (5+ days) have shown to increase lipid oxidation in the muscle tissue of sedentary\textsuperscript{22} and trained\textsuperscript{23} participants. These previous works concluded that increased lipid oxidation was a beneficial response the change in diet. In this study, the cells respond to the elevated substrate with an increase in the capacity to oxidize these substrates which could also be perceived as a protective response. However, it cannot be said with certainty as to how long this response would last. This improved capacity for lipid oxidation is a plausible mechanism for the observed decreases in ROS production observed in cells post exposure to HSM.

There may be acclimation to lifestyle factors as cells post-HFHCD, and cells from participants with higher adiposity began to function better in the HSM treatment over the control. In most cases, HSM decreased the function of cells collected from healthy donors. Pre-HFHCD cells displayed decreases in their proliferation rates and their ability to differentiate when treated with HSM. However, cells collected from participants with higher adiposity and post-HFHCD cells seemed to prefer the HSM treatment as it increased their proliferation rates and did not affect their ability to differentiate. A cellular preference for an obesogenic environment may be an indication of why long term weight loss is so difficult.\textsuperscript{24} More research must be done to determine if the substrate preference of an individual with higher adiposity could return to that of a lean individual after a weight loss intervention.
Increased inflammatory signaling is a hallmark of obesity and a western style diet. Obesity causes chronically elevated levels of IL-6 and TNF-α, which are mainly secreted from dysfunctional adipose tissue. This study found that neither the HFHCD nor the HSM treatment had a profound effect on satellite cells cytokine secretion during proliferation or after differentiation. Elevated IL-6$^{2,25}$ and TNF-α$^{26}$ decrease myogenesis both in-vitro and in-vivo. However, this study gives evidence that elevated inflammatory cytokines may not come from the muscle tissue. Disruptive elevations in cytokine production are the result of inflamed adipose tissue shown to result from obesity.$^{11}$

Reducing the substrate availability to cells collected post-HFHCD and cells collected from more participants with higher adiposity decreased myogenesis. These cells preferred the HSM with elevated lipids and glucose. Changing the diet of individuals with higher adiposity and individuals on a high-fat diet may result in reduced myogenesis. The time required for satellite cells to acclimate to an environmental change in substrate availability is unknown. Exercise training has shown in this study and several previous works to improve satellite cell myogenesis but a dietary change may interfere with the positive effects of training. Weight loss program that combines both a dietary change and exercise intervention are common.$^{27}$ While these combined programs are perceived as effective, evidence from this study outline a mechanistic reasoning for staggering the start of the dietary change until sometime after the training program start to allow the cells time to adapt to one intervention then the other.

**Conclusion**

This study demonstrates that satellite cell function is susceptible to diet and the availability of metabolic substrates. High levels of lipids and carbohydrates can inhibit satellite cell proliferation and myogenesis. This presents a potential problem to individuals with higher adiposity, as their ability to respond to exercise may be inhibited until their blood substrate levels are normalized. The effects of the HFHCD were retained in culture highlighting the potential for cell culture models to be an effective means of studying dietary effects. Satellite cell fatty acid metabolism and ROS production did improve
after exposure to high substrate media, suggesting the cells do have some ability to protect themselves in a metabolically challenging environment.  

References


Chapter 7: Conclusion/Future Directions

Proper skeletal muscle function is a necessity of life that must be maintained through all ages. Aside from providing locomotion, skeletal muscle is a highly metabolically active tissue that can affect the fate of many chronic diseases. Loss of muscle mass is often associated with aging and/or a sedentary lifestyle. These losses in muscle mass may be mediated by satellite cell dysfunction. This work has demonstrated that at its core chronological aging alone does not dictate improper function to the satellite cell. As long as the satellite cells themselves remain functional, there is a potential for muscle tissue to maintain proper if not superior function throughout the majority of a person’s lifetime.

Many factors can affect satellite cell function but the two most controllable are diet and activity level. While the beneficial effects of activity on skeletal muscle have been well studied for many years the means by which diet affects muscle tissue and myogenesis is still somewhat unknown. This work has found that a high fat, hypercaloric diet can significantly negatively affect satellite cell myogenesis in as few as four weeks. The negative impacts of such a diet are retained in cultured cells and can be mimicked through high substrate media treatments.

There is an initial protective response to elevated substrate availability in satellite cells. This protective response is even retained throughout myogenesis. Cells exposed to elevated lipids and glucose during their proliferative stage displayed elevated capacity for fatty acid oxidation and decreased ROS production once differentiated. How long this metabolic elevation can be maintained is still unknown. This effect on fatty acid oxidation was elicited after a short exposure to the elevated substrates. Long-term exposure to such a treatment could prove to be damaging and eventually result in decreased oxidative capacity.
Perhaps most interesting from these findings is the fact that satellite cells develop a preference for their in-vivo environment. Cells from obese donors or donors on a high fat diet performed better in in-vitro conditions of elevated substrate meant to mimic their in-vivo environment, while cells from lean and trained donors were impaired by such an environment. What is not clear is whether these effects are reversible. There is most likely some sort of epigenetic changes that occur in a satellite cell due to poor diet or obesity. These changes alter satellite cell myogenesis and substrate preference. If these effects are permanent, it could prove detrimental to individuals trying to switch from a poor to healthier diet. Due to the potential negative effects of a dietary change on myogenesis, a staggered approach may be effective when initiating an intervention involving diet and exercise.

These results focus mainly on the detrimental effects of a high fat, hypercaloric diet. More work must be done to determine if a health conscious diet could improve satellite cells function or at least halt the negative effects associated with sedentary and older populations. Increased muscle mass improves disease risk and raises metabolic rate, making weight loss more possible. Exercise is a common means of improving muscle mass and overall health but there is difficulty initiating such a program for frail or highly obese individual. If a dietary intervention could improve muscle mass in such populations it would create a stepping stone towards participation in more proven treatment methods.
**IRB Approval**

Virginia Tech: Institutional Review Board

- The Effects of Resistance Training on Aged Skeletal Muscle
  IRB# 12-652

- Effect of High Fat Diet on Muscle Metabolism
  IRB# 06-367

- Human Satellite Cell Isolation
  IRB# 14-1234
References


