# Dietary obesity alters muscle stem cell behaviors

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## ABSTRACT

Occurrence of obesity has steadily increased in the human population and, along with it, associated health complications such as systemic insulin resistance, which can lead to the development of type 2 diabetes mellitus. Obesity is a complex metabolic disorder that often leads to chronic inflammation and an overall decline in human and animal health. In mouse skeletal muscle, obesity has been shown to impair muscle regeneration after injury, however, the mechanism underlying these changes in satellite cell (SC) biology have yet to be explored. To test the negative impacts of obesity on SC behaviors, we fed C57BL/6 mice normal chow (NC, control) or high-fat diet (HFD) for 10 wks and performed SC proliferation and differentiation assays *in vitro*. SCs from HFD mice formed colonies with smaller numbers ($P < 0.001$) compared to those isolated from NC mice, and this observation was confirmed ($P < 0.05$) by BrdU incorporation. Moreover, *in vitro* differentiation assays consisting of equally seeded SCs derived from NC and HFD muscles showed that HFD SCs exhibited compromised ($P < 0.001$) differentiation capacity compared to NC SCs. Immunocytochemical staining of cultured SCs demonstrated that the percentage of $Pax7^{+}/MyoD^{-}$ (self-renewed) SC subpopulation decreased ($P < 0.001$) with HFD treatment group compared to the control. In single fiber explants, a higher ratio of SCs experienced apoptotic events as revealed by the expression of cleaved caspase 3 ($P < 0.001$). To investigate further the impact of obesity on SC quiescence and cycling properties *in vivo*, we used an inducible H2B-GFP mouse model to trace the turnover rate of GFP and thus cell division under normal and obese conditions. Flow cytometric analysis revealed that SCs from HFD treatment cycled faster ($P < 0.001$) than their NC counterparts, as reflected by the

quicker loss of the GFP intensity. To test for SC muscle regenerative capacity *in vivo,* we used cardiotoxin (CTX) to induce wide-spread muscle damage in the *tibialis anterior* muscle. After analysis we found that HFD leads to a compromised, though mild, impairment in muscle regeneration. Taken together, these findings suggest that obesity negatively affects SC quiescence, proliferation, differentiation, and self-renewal *in vitro*, *ex vivo* and *in vivo*.

# Dietary obesity alters muscle stem cell behaviors

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## GENERAL AUDIENCE ABSTRACT

The prevalence of obesity in the human population has steadily increased over the past decades and, along with it, associated health complications such as systemic insulin resistance, which can lead to the development of type 2 diabetes mellitus. Obesity is a complex metabolic disorder that often leads to chronic inflammation and an overall decline in human and animal health. Along with the multitude of health disorders associated with obesity, in mouse skeletal muscle, obesity has been shown to impair muscle regeneration after injury. The mechanisms underlying the impairment in muscle regeneration as seen in obesity are unknown. To better understand how obesity affects skeletal muscle, we looked at satellite cells (SC). Satellite cells, or muscle stem cells, are skeletal muscle resident cells that play a vital role in muscle repair after damage. To test the negative impacts of obesity on SC behaviors, we fed mice normal chow (NC, control) or high-fat diet (HFD) for 10 wks to obtain an obesogenic mouse model. Our first experiments involved culturing the SCs derived from the HFD and NC mouse muscles and growing them in an artificial environment. These experiments showed SCs derived from HFD mice had a decreased ability to replicate and divide compared to those isolated from NC mice. Moreover, the SCs from the HFD mice exhibited compromised capacity to form myotubes in culture, an essential part in muscle regeneration after damage. Our next set of experiments conducted looked at individual muscle fibers isolated from mouse muscle. In these experiments the SCs on the HFD muscle fibers had a higher ratio of SCs experiencing cell death in comparison to the control. To test the SC cycling properties in the living mouse we used a mouse model to trace the activity and cell division of SCs under normal and obese conditions.

Using this model revealed that SCs from HFD treatment cycled faster than their control counterparts, even in the absence of notable muscle damage. To test for SC muscle regenerative capacity after muscle damage, we used cardiotoxin (CTX) to induce wide-spread muscle damage in the *tibialis anterior* muscle (leg muscle) of the living mouse. After analysis we found that HFD leads to a compromised, though mild, impairment in muscle regeneration. Taken together, these findings suggest that obesity negatively affects SC behaviors and function.

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# List of Abbreviations

| | |
|---|---|
| AMP | Adenosine monophosphate |
| AMPK | Adenosine monophosphate kinase |
| AP1 | Activator protein 1 |
| bFGF | Basic fibroblast growth factor |
| BMI | Body Mass Index |
| BrdU | Bromodeoxyuridine |
| CSA | Cross sectional area |
| CTX | Cardiotoxin |
| DAPI | 4′,6-diamidine-2′-phenylindole dihydrochloride |
| DMEM | Dulbecco's modified Eagle's medium |
| EDL | Extensor digitorum longus |
| EMCL | Extramyocellular lipids |
| ERK | Extracellular-signal-regulated kinase |
| FACS | Fluorescence activated cell sorter |
| FBS | Fetal Bovine Serum |
| FFA | Free fatty acid |
| GFP | Green fluorescent protein |
| GLUT | Glucose transporter |
| HFD | High-fat diet |
| HS | Horse Serum |
| IFN | Interferon |
| IGF1 | Insulin-like growth factor 1 |
| IκB | Inhibitor of κB |
| IKK | Inhibitor of κB kinase |
| IMAT | Intermuscular adipose tissue |
| IMCL | Intramyocellular lipids |
| IL | Interleukin |
| IRS | Insulin receptor substrate |
| JNK | C-Jun N-terminal kinase |
| MAPK | Ras-mitogen-activated protein kinase |
| MCP | Monocyte chemotactic protein |
| MRF | Myogenic regulatory factor |
| mTOR | Mammalian target of rapamycin |
| Myf5 | Myogenic factor 5 |
| NAD | Nicotinamide adenine dinucleotide |
| NC | Normal Chow |
| NEFA | Non-esterified free fatty acid |
| NFκB | Nuclear factor-κB |
| PBS | Phosphate-buffered saline |
| PI3K | Phosphatidylinositol 3-kinase |
| PKB | Protein kinase B |
| ROS | Reactive oxygen species |
| SC | Satellite cell |
| TA | Tibialis anterior |
| TGFβ | Transforming growth factor β |

| | |
|---|---|
| TNF-α | Tumor necrosis factor - α |
| WGA | Wheat germ agglutinin |

# Chapter 1

# Literature Review

## Satellite Cells and their Function in Muscle Growth and Repair

### Satellite Cells

First discovered by Mauro, SCs are undifferentiated myogenic precursors and are the main source of new myonuclei in postnatal skeletal muscle tissue [1-4]. SCs are located between the sarcolemma and basal lamina and were first coined "satellite cell" due to their anatomical location and intimate association with the plasma membrane of muscle fibers [5, 6]. SCs have unique morphological characteristics by comparison to myofibers and contain reduced organelle content and a small nucleus with an increased amount of heterochromatin [7]. Characteristics of SCs reflect the fact that SCs are quiescent ($G_0$ phase) in nature and are transcriptionally less active than myonuclei; however, on stimulation they become activated and proceed through the cell cycle to proliferate and/or differentiate [8].

### Satellite Cell Cycle and Myogenic Regulatory Factors

As previously stated, SCs typically reside in a state of quiescence and are only activated through regenerative cues such as physical damage or exercise. On exposure to these regenerative signals, SCs exit their quiescent state and start to proliferate, also known as SC activation [9]. These proliferating SCs and their progeny are referred to as adult myoblast or myogenic precursor cells (MPC). Progression of SCs through the myogenic cell cycle is regulated, in part by expression of the paired box transcription factor 7 (Pax7) and the myogenic regulatory factors (MRFs) [10]. When quiescent, SCs express Pax7 and on activation co-express Pax7 with MyoD, a key transcription factor for myogenic differentiation and a member of the

MRF family, which includes MyoD, myogenic factor 5 (Myf5), myogenin, and MRF4 [10-14]. This is illustrated by the fact that MPCs are characterized by their rapid expression of myogenic transcription factors Myf5 and MyoD, which play an essential role in myoblast proliferation and differentiation, respectively [15-21]. After activation, most $Pax7^+/MyoD^+$ SCs proliferate, downregulate Pax7 and proceed to differentiate. In contrast, other proliferating SCs maintain Pax7 but lose MyoD withdraw from the cell cycle and return to a state of quiescence [22, 23]. This ability to exit from the cell cycle is a hallmark of stem cells and is called self-renewal [9].

SCs can divide and self-renew either asymmetrically or symmetrically, both of which allow for maintenance of the SC population [9]. With asymmetrical cell division, one parental SC gives rise to two functionally different daughter cells, one of which self-renews and the other is destined for differentiation [9]. By comparison, symmetrical division gives rise to two daughter cells of equal stemness [9]. The capability of SC self-renewal is essential for the muscle to sustain the capacity to regenerate throughout a human and animal's lifetime.

## The Satellite Cell Niche

Many studies exploring control of SC function by the niche environment revealed that the proliferation and differentiation of SCs during muscle regeneration is influenced by vasculature, hormones, the extent of tissue injury, and nutrition [24-29]. These findings led to the intriguing idea of a SC microenvironment, or SC niche, that affects their cell behaviors [9].

SCs exhibit different intrinsic metabolic properties in states of quiescence, proliferation, and differentiation [30]. When SCs are in the quiescent state, they have low energy demands, low ATP production, as well as low oxygen consumption [31]. In low nutrient conditions, elevated $NAD^+$ levels activate SIRT1, which in turn prevents myogenic differentiation and

promotes myoblast proliferation [32]. This increase in myoblast proliferation through SIRT1 during limited nutrient supply is hypothesized to maintain a pool of muscle SCs; whereas, nutrient overload as seen in obesity could be expected to be detrimental to maintenance of quiescent SCs or SC proliferation during injury [31]. Studies conducted using a short-term caloric restriction in mice found an increase in number and myogenic capacity of SCs and that these SCs had a higher mitochondrial content, enhanced oxidative metabolism, reduced glycolytic capacity, as well as an enhanced regenerative ability [33]. These studies lead to the suggestion that SC metabolism is profoundly influenced by the systemic nutritional environment and that the metabolic or inflammatory state of the organism could also affect the health and activity of the SC pool [31].

Satellite Cells and Skeletal Muscle

SCs have long been a topic of debate concerning their function in muscle growth [34]. During muscle growth in early post-natal development, SCs are necessary for continuous increase in the number of myonuclei in the muscle fibers through SC fusion [3]. Contrary to this supposition, muscle hypertrophy during late post-natal stages and through adulthood takes place without significant contribution of satellite cell fusion, as seen in studies in which muscle hypertrophy was induced [35-39]. One model of induced hypertrophy found to have an effect on SC contribution is compensatory hypertrophy induced by the ablation of synergist muscles [40]. This model causes immediate proliferation and fusion of SCs and a consequent increase in the number of myonuclei [41, 42]. It is thought that SC activate and contribute during muscle hypertrophy primarily when an acute stimulus is involved, specifically under conditions in which some form of muscle damage occurs [43]. To better understand SC function and its role in muscle growth and repair, it is best to first understand how skeletal muscle functions as a tissue.

Skeletal Muscle Fiber Typing

Skeletal muscle represents between 40-60% of total body weight, is responsible for locomotion, and comprises the most abundant insulin-sensitive tissue, handling around 75-95% of all glucose utilization [50]. As a major metabolic tissue, skeletal muscle plays a key role in the development of metabolic syndrome and obesity [44-46].

Skeletal muscle is a heterogeneous tissue containing fibers which differ in their contractile and metabolic nature and occur in varying proportions in each individual muscle. Muscle fibers are classified into two major types: type I and type II. Type I fibers are characterized as slow-twitch, oxidative fibers and are mitochondria rich [47]. These fibers have a slower time to peak tension and derive ATP mainly from oxidative metabolism, allowing them to be relatively fatigue-resistant [47]. Type IIb fibers, however, are categorized as fast-twitch, glycolytic fibers [47]. Type IIb fibers also have a fast time to peak tension, but a lower mitochondrial density and are overall less dependent on oxidative metabolism than either type I or type IIa fiber. Instead, type IIb fibers primarily utilize glycolysis for ATP production and thus fatigue sooner than other fiber types [47].

Fiber type proportions are not fixed throughout life and exhibit great plasticity and adaptability in response to various signals including exercise and diet [48]. The proportion of muscle fiber types in the body are related to the susceptibility and development of obesity [49]. Obese individuals experience a shift from type I and IIa to an increased proportion of type IIx or IIb fibers, indicating an overall reduction in oxidative capacity [49]. This same trend was shown in the obese, diabetic Zucker rats, where a lower proportion of type IIa fibers were found along with a decrease in expression levels of oxidative metabolism related genes [50]. Other research also suggests that type I fibers may directly or indirectly protect against both dietary induced

insulin resistance and obesity [51, 52]. Further studies suggest that the oxidative capacity of muscle may predict insulin sensitivity more effectively than the concentrations of muscle triglyceride, which is consistent with the observed differences in insulin sensitivity between muscles with a larger proportion of type II insulin resistant fibers versus muscle with a larger proportion of type I insulin sensitive fibers [53, 54].

Skeletal Muscle as a Metabolic Tissue

As the largest insulin-sensitive tissue, any changes in skeletal muscle mass, metabolic rate, hormones and other circulating factors would undoubtedly affect the body's overall metabolism and energy stores [55]. A key characteristic in a healthy individual is the ability to alternate between the use of carbohydrates and lipids in either times of abundance or scarcity respectively [56]. It is this ability that is termed 'metabolic flexibility' [57]. The loss of this, otherwise known as metabolic inflexibility, is an indicator of the beginnings of metabolic syndrome.

Insulin utilizes the IRS-PI3K/Akt pathway in skeletal muscle to translocate GLUT 4 to the sarcolemma to facilitate the entry of glucose into the cell [55]. This intracellular glucose is then quickly phosphorylated by hexokinase and subsequently directed to oxidative or non-oxidative (glycogen synthesis) pathways [55]. Along with the insulin signaling pathway, skeletal muscle can also stimulate glucose uptake and metabolism through contractile activity independent of insulin through $Ca^{2+}$ adenosine monophosphate (AMP)-dependent kinase (AMPK) mediated pathways [58]. This ability allows skeletal muscle to improve insulin sensitivity, glycemic control, and overall metabolic health. To understand better how skeletal muscle responds to external factors, whether nutrient based or damage based, those mechanisms responsible for muscle growth and regeneration need to be understood.

Skeletal Muscle Growth

Muscle growth is characterized by the hypertrophy in muscle mass and fiber size and occurs during both development and in response to mechanical overload [43]. By contrast, a decrease in muscle mass and fiber size, or muscle atrophy, results from varying different factors including aging, starvation, diabetes, and lack of use among others [43]. This regulation of muscle mass and fiber size between hypertrophy and atrophy essentially reflects protein turnover, better summarized as the balance between protein synthesis and degradation within the muscle fibers [59].

Skeletal muscle growth is facilitated primarily through two major signaling pathways: the insulin-like growth factor 1 (IGF1)-PI3K/Akt/PKB-mammalian target of rapamycin (mTOR) pathway, which acts as a positive regulator of muscle growth, and the myostatin-Smad3 pathway, which acts as a negative regulator [43, 59, 60]. The first step in the IGF1-PI3K/Akt/PKB-mTOR pathway and its function to promote muscle growth starts with IGF1. IGF1 is positively correlated with muscle growth and over-expression of muscle specific IGF1 receptor causes muscle hypertrophy and, conversely, inactivation of muscle specific IGF1 receptors results in impaired muscle growth [61, 62]. Although IGF1 activates both the PI3K/Akt and MAPK/ERK pathways, studies have confirmed that only activation of the PI3K/Akt pathway induces muscle hypertrophy [35, 63, 64]. After activation by IGF1, Akt stimulates protein synthesis by activating mTOR, which in turn promotes protein synthesis through the mediation of mTOR complex 1 (mTORC1) containing raptor as opposed to mTOR complex 2 (mTORC2) containing rictor [65, 66]. It is important to note that mTOR is responsible for several cellular processes in addition to protein synthesis, including autophagy, and it responds to various upstream signals besides Akt, including amino acids [43]. The second

major signaling pathway that controls muscle growth is the myostatin-Smad3 pathway. Myostatin is a member of the transforming growth factor β (TGFβ) family and is produced by skeletal muscle to act as a negative regulator of muscle growth [67]. Alternately, in an obese state, these pathways are altered showing a decrease in Akt and mTOR activity, resulting in reduced muscle hypertrophy, as well as an increase in myostatin expression [68-70].

Skeletal Muscle Regeneration

Skeletal muscle has a striking ability to regenerate itself in response to injury [71]. Muscle can experience injury as a result of disease, blunt trauma, exposure to myotoxic agents, or exercise [7]. Muscle regeneration is a highly synchronized and coordinated process that can be broken down into four consecutive phases: 1) necrosis, 2) inflammation, 3) activation and differentiation of SCs, and 4) maturation of newly formed muscle fibers and remodeling of the newly regenerated muscle [72].

Degeneration and necrosis of myofibers is the first step in muscle damage and is characterized by disruption of the myofiber sarcolemma and increased myofiber permeability [7]. The next step in early muscle injury is the infiltration of inflammatory cells into the damaged muscle. These inflammatory cells are not only responsible for mediating damage, they also play a role in the activation of repair processes necessary for muscle recovery [7]. Neutrophils are the first inflammatory cells to invade the injured muscle, increasing in number 1 – 6 h after muscle damage, followed by M1 macrophages which predominate the site of injury around 48 h after damage [73]. The M1 macrophages that infiltrate the damage area develop a pro-inflammatory phenotype and play an important role in promoting migration of SCs to the site of injury, as well as stimulating subsequent SC proliferation through the secretion of IL-6 [74, 75]. Infiltrated neutrophils and macrophages produce TNF- α following muscle injury which

contributes to the pro-inflammatory response and inhibits muscle differentiation through the NF-κB pathway in early muscle damage [76-78]. Although TNF- α plays an inhibitory role in SC differentiation during early stages of damage, it also appears to stimulate muscle differentiation during later stages in the regeneration process and functions independently of the NF-κB pathway [77].

During the regeneration stages of damage, M1 macrophages are converted to an anti-inflammatory M2 phenotype and, whereas the M1 population stimulates muscle proliferation and inhibits differentiation, M2 cells play a key role in the differentiation process [79, 80]. These M2 macrophages stimulate SC differentiation and fusion to existing damaged fibers for repair or SC fusion to one another for new myofiber formation [81]. SCs are the major cell type responsible for skeletal muscle regeneration and the loss of SC function is contributed to diminished muscle regeneration after injury [82-84].

**Obesity and Associated Metabolic Syndrome**

Over the past several decades, the frequency of obesity has drastically increased worldwide and has emerged as a prominent public health concern. Current estimates show that one-third of the United States population is obese and that number is rapidly increasing [85]. According to the World Health Organization, over 500 million people worldwide are overweight or obese and based on the current obesity trends, the United States will have 65 million more obese adults by 2030 [86, 87]. Although genetics plays a role in body size, weight, composition, and the metabolic response to feeding in humans and in animals, genetics cannot solely explain the global increase in obesity occurring in such a short period of time [88, 89]. Studies point to dietary fat intake as the leading factor responsible for the increase in adiposity and human studies

have shown that high-fat diets (HFD), defined as ≥ 30% of energy from fat, can easily induce obesity [90, 91].

Definition of Obesity

Obesity is a chronic disease characterized by the abnormal or excessive accumulation of adipose tissue in the body due to an imbalance between food intake and energy expenditure leading to a multitude of health problems [92]. Body mass index (BMI), which expresses body weight in relation to body height, is the most widely accepted method used to define obesity [93]. The upper limit of normal BMI in adults is 25 $kg/m^2$ and obesity is defined as having a BMI of > 30 $kg/m^2$ with a BMI between these values is designated to be "overweight" [94]. Although using BMI is helpful when estimating body-fat mass at a population level, it is not reliable as a clinical tool for assessing an individual's body fatness. BMI cannot account for the variation in total body mass between individuals with differences in skeletal muscle and other body mass components.

Role of Adipose Tissue

Adipose tissue functions as the major site for storage of excess energy in the body [95]. During positive energy balance, where energy intake exceeds energy output, triglycerides are stored as excess energy in the lipid droplets of adipocytes either through the enlargement in the size of adipocytes (hypertrophy) or the increase in the number of adipocytes (hyperplasia) [96]. Increase in fat mass during adulthood can primarily be attributed to hypertrophy due to the fact that adipocyte number is largely determined during childhood and adolescence and remains relatively constant through adulthood in both lean and obese subjects [97]. On the other hand, when energy is needed, triglycerides stored in adipocytes can be mobilized through lipolysis to

release free fatty acids (FFAs) into circulation, which are then transported and utilized in other tissues as an energy source [95]. These FFAs are generally thought to play a key role in the development of insulin resistance and other obesity-related metabolic disorders [98].

A major role of adipose tissue is as an endocrine organ and adipocytes secrete multiple adipokines, including chemokines, cytokines, and hormones, many of which are involved in energy homeostasis and inflammation [95]. Of these adipokines, the two most well-known are leptin and adiponectin [99]. The production of leptin is positively correlated with the accumulation of triglycerides and is therefore generally used an indicator of body adipose mass [100]. Leptin is utilized to negatively regulate appetite, drive physical activity, and promote insulin sensitivity in an effort to maintain metabolic homeostasis [101]. With obesity, however, leptin levels are chronically elevated and result in a state of leptin resistance in the body [101]. Adiponectin, on the other hand, functions to promote insulin sensitivity and in a lean state produces insulin-sensitizing effects [95]. In obesity, adiponectin is inversely related to leptin; its production diminishes as adiposity increases in the body, contributing to insulin resistance due to a loss in insulin-sensitizing effects [102].

As well as secreting proteins, adipose tissue releases lipids that act locally and systemically. In a lean state, insulin signaling is responsible for inhibiting lipolysis and non-esterified free fatty acid (NEFA) release from adipocytes [100]. Whereas, in an obese state, this function is hindered. As a result, limited insulin response causes an increase in lipolysis as well as an associated increase in elevated levels of circulating NEFAs thought to play a factor in obesity-related metabolic dysfunctions, particularly insulin resistance [95, 100].

Obesity and Chronic Inflammation

Adipocytes play an integral role in the development of obesity-induced inflammation by increasing the secretion of various pro-inflammatory cytokines and chemokines [103, 104]. Many of these pro-inflammatory adipokines, notably monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-1β, and IL-8, promote insulin resistance [105-108]. The macrophage content of adipose tissue increases with body mass and adipocyte size [109]. The increase in adipose tissue not only increases the macrophage content in the body, but it also causes a phenotypic shift in these cells from a primarily anti-inflammatory M2 polarized state to a primarily pro-inflammatory M1 polarized state [110]. This switch in macrophage content and type causes an increase in the secretion of a variety of pro-inflammatory cytokines and chemokines and this increase is believed to contribute to obesity-related insulin resistance [111, 112]. The decrease of M2 macrophage accumulation leads to the decrease of adipose tissue remodeling, including the clearance of dead or dying adipocytes [113]. Furthermore, obesity increases the levels of pro-inflammatory immune cells, such as interferon (IFN)-$\gamma^+$ T helper type 1 cells and $CD8^+$ T cells, as well as decreases the secretion of insulin-sensitizing adiponectin as discussed previously [102, 114]. All of these together leads to an increase in insulin resistance.

Obesity as a Metabolic Disease and Insulin Resistance

Obesity, especially abdominal obesity, is one of the predominant factors linked to an increased risk of developing metabolic syndrome [115]. Metabolic syndrome is prominently defined in humans as the development of insulin resistance, as well as, hyperinsulinemia, hypertension, impaired glucose tolerance, chronic inflammation, high blood pressure, and dyslipidemia [100]. Insulin resistance is characterized as the decreased ability of tissues to respond to insulin action. For adipose and skeletal muscle, this translates to decreased insulin-

stimulated blood glucose uptake; for the rest of the body, insulin resistance means a failure to inhibit glucose production by the liver, as well as a failure to inhibit triacylglyceride lipolysis and NEFA release from adipose storage [116].

In comparison, the MAPK pathways are involved in stimulating mitogenic and growth effects of insulin [95]. The MAPK family includes extracellular-signal-regulated kinase (ERK) 1/2, C-Jun N-terminal kinases (JNKs), ERK5, and p38 MAPK [117]. Of these, p38 MAPK is a stress-activated serine/threonine protein kinase with major functions in stress responses, inflammation, and apoptosis [118]. Research shows that obesity increases p38 MAPK signaling in adipose tissue and along with this it has been suggested that pro-inflammatory TNF-α induces insulin resistance via the p38 MAPK pathway in skeletal muscle [119, 120]. Further, p38 MAPK is not the only MAPK pathway thought to play a role in inflammation and insulin resistance. JNK and inhibitor of κB (IκB) kinase (IKK) are linked to inhibition of insulin signaling through the phosphorylation and subsequent inhibition of IRS-1, thus blunting the insulin signaling cascade [121]. JNK and IKK signaling also play a role in inflammation through the activation of transcription factors important to inflammation, such as activator protein 1 (AP1), c-Jun/Fos, and nuclear factor-κB (NFκB).

Previous studies found through the use of genetic knockout models and pharmacologic treatments that, although the actions of inflammatory kinases such as JNK and IKK play a central role in the development in obesity and insulin resistance, decreasing these kinase activities *in vivo* does not necessarily correlate to decreased obesity [122-126]. Animals with absent or diminished stress kinase activation were found to remain insulin sensitive despite lipid accumulation [124]. Although adiposity and lipid accumulation are linked to inflammation and insulin resistance, the accumulation of lipids by itself is not always a determinant in reduced

insulin sensitivity; instead, the activation of the inflammatory response is crucial to the development of insulin resistance [100].

Obesity is a multifactorial metabolic disease and a variety of these factors can be linked to the overall impaired insulin signaling that occurs with obesity. These factors include an accumulation of intramuscular lipids and lipid by-products, decreased mitochondrial oxidative capacity, a difference in muscle fiber-type composition, and reduced muscle contractile activity [55, 127-129]. The exact methods as to how the accumulation of intramuscular lipids affects SC function is still largely unknown.

**Effects of Obesity on Satellite Cells and Muscle Physiology**

A continuous positive energy balance leads to a homeostatic metabolism shift, often inducing systemic insulin resistance. This change in metabolic nature affects the insulin-sensitive skeletal muscle tissue and causes a shift in muscle fiber typing, as well as a shift in whole body fatty acid oxidation. The implications associated with chronic-inflammation induced by obesity can be detrimental. While many intrinsic and extrinsic factors affect insulin resistance, understanding how skeletal muscle adapts and changes in an environment of excess energy is important to further understanding how these changes can affect SC population and function during obesity.

<u>Skeletal Muscle Lipid Accumulation</u>

Obesity causes an elevation in adipose storage in not only subcutaneous and visceral adipose depots, but also in non-adipose organs, which has been termed ectopic lipid accumulation [130]. High ectopic lipid deposition occurs in skeletal muscle during obesity and can partially be from the increase in fatty acid uptake from circulation, as obese individuals also

have elevated levels of FFA in circulation [131-135]. Lipid accumulation found in skeletal muscle is made up of two separate types: extramyocellular lipids (EMCL), which are localized in adipose cells found between myofibers, and intramyocellular lipids (IMCL), which are located within the myofibers [132, 136]. A proportion of EMCL closely associated with the muscle, referred to as intermuscular adipose tissue (IMAT), is positively correlated with insulin resistance and reduced muscle in obese patients, but does not seem to affect muscle mass [137-139]. The accumulation of ectopic lipids found in obese people leads to the increased deposition of long-chain acyl CoAs, diacyglycerols, and ceramides in the skeletal muscle causing a negative effect on cell signaling and metabolism; collectively these defects are called lipotoxicity [140-146]. Lipotoxicity and its associated metabolism effects could influence the way SCs function and respond during times of quiescence and activation.

Chronic Inflammation and Satellite Cells

As previously discussed, obesity is recognized as a state of chronic inflammation with increased levels of circulating pro-inflammatory cytokines, namely TNF-α, IL-1β, and IL-6 [105-108]. The effects of chronically elevated cytokines on SC function and maintenance are not well understood, but studies have shown a distinct effect of cytokines on myoblast proliferation and differentiation during acute exposure. In one such study, a mouse model in which TNF-α is constitutively expressed in lung and becomes chronically elevated in circulation, myoblast proliferation and differentiation were reduced and skeletal muscle was atrophied in response to mechanical loading [147]. Similar defects in muscle growth are also seen in chronic, local delivery of IL-6 in muscle of young rats, contributing to the idea that during chronic inflammation the normal coordination between SCs and macrophages is impaired and may lead impairment in SC function [31, 148].

Along with increased levels of pro-inflammatory cytokines, myostatin, whose function is known to prevent muscle regeneration and growth, are also increased during obesity [68]. In a study utilizing C2C12 myoblasts, overexpression of myostatin led to a decrease in proliferation and in a different study using myostatin-null mice, SC proliferation markedly increased [149, 150]. Myostatin also represses expression of MyoD and myogenin through direct activation of Smad2/3 proteins, leading to an approximately two-fold decrease in MyoD and myogenin in obese people [151-153]. Therefore, increased myostatin may contribute to SC maintenance and regeneration of muscle.

**Summary and Implications**

Obesity is a growing epidemic and its known association with metabolic diseases and other health disorders continue to be explored. During chronic inflammation, the increase of circulating FFAs, and ectopic lipid accumulation in the skeletal muscle due to obesity, overall muscle metabolism is negatively affected and this often leads to systemic insulin resistance. The effect obesity has on muscle metabolism and regeneration has been documented, but the effects and mechanisms in which obesity affects muscle SC niche and function remain elusive. Obesity is a multifactorial disease and many of its effects on the body could also influence SC maintenance; although, which factor of obesity plays the largest role in affecting SCs is also unknown.

With the trend in obesity seemingly increasing each year, future researcher is needed to better understand the interaction between SCs and obesity to find ways to manage and prevent this metabolic disease. The aim of the following work was to look specifically at the SC population in a HFD mouse model using *in vitro, ex vivo,* and *in vivo* methods.

# Chapter 2

## Impacts of dietary obesity on muscle stem cell behaviors

### Introduction

Obesity is a metabolic disease characterized by an accumulation of adipose tissue in the body due to a positive energy balance in which energy intake is greater than energy expenditure [92]. Prevalence of obesity in the population has dramatically increased over the past several decades worldwide and with it a greater occurrence of cardiovascular disease, Type 2 diabetes mellitus, renal dysfunction, asthma, sleep disorders, infertility and others [154, 155]. Along with these pathologies, obesity is linked to an increased risk of Metabolic Syndrome, which is prominently defined in humans as the development of insulin resistance [100]. Physiologically, insulin resistance is characterized as decreased insulin-stimulated blood glucose uptake by skeletal muscle and adipose tissue as well as a failure to inhibit lipolysis and glucose production in the liver [95, 100]. Another major characteristic of obesity is the development of a state of low-grade inflammation in the body, known to influence the risk for insulin resistance and subsequent progression to Type 2 diabetes [156, 157]. Adipose inflammation, particularly macrophage-mediated inflammation, is linked to the development of systemic insulin resistance in both obese animals and humans; some studies show as much as a 4-5 fold increase in macrophage content in the adipose tissue from a lean to an obese state [109, 112].

Along with adipose tissue, skeletal muscle is metabolically active tissue in the body that is affected by obesity. Skeletal muscle is the most abundant insulin-sensitive tissue and handles 75-95% of all mediated glucose utilization [55]. It is unsurprising then that a dysregulation in body metabolism would affect muscle metabolism as well. Excess lipid accumulation in adipose

tissues and ectopic lipid accumulation in skeletal muscle affects not only muscle insulin signaling, but also muscle maintenance and regeneration [31]. Although the underlying mechanisms causing an impairment in muscle repair are not fully understood, it has been suggested that muscle satellite cells could be negatively affected by obesity.

Satellite cells (SCs), also known as muscle stem cells, are myogenic precursors that reside on the outside of the muscle fiber between the sarcolemma and basal lamina [1, 5, 6]. SCs are responsible for the maintenance and repair of adult skeletal muscle and the loss or impairment of SCs is contributed to blunted muscle regeneration after damage [82-84]. SCs are normally quiescent and are only activated in response to external stimuli such as growth factors, hormones, or muscle injury [9]. When activated, SCs proceed through the myogenic lineage pathway to increase numbers, or proliferate, then subsequently exit the cell cycle to either differentiate into new myofibers, fuse to existing myofibers, or self-renew back into a quiescent state to maintain the SC population [158]. SC progression through the myogenic cell cycle is governed through the regulation of paired box transcription factor 7 (Pax7) and myogenic regulatory factor (MRF) expression [10]. Pax7 is expressed in the majority of quiescent SCs and is widely used as a marker of SC nuclei [10]. In contrast, cells expressing MRFs, such as MyoD, Myf5, MRF4, and myogenin, are no longer quiescent and are ready to continue with the myogenic cycle [65]. The ability of SCs to activate, proliferate, and differentiate in response to stimuli is essential in skeletal muscle repair and regeneration post injury. Likewise, SCs capability to self-renew after activation and revert to a quiescence state is necessary for long-term maintenance of muscle SC pool as well as continued skeletal muscle regeneration during an animal or human lifetime.

Though various studies indicate a difference in satellite cell behavior during obesity, these mechanisms are unresolved. Therefore, understanding how obesity alters satellite cell behaviors is essential. Hence, the aim of this research was to determine changes in SC function in response to an obesigenic, high-fat diet, in mice using *in vitro*, *ex vivo*, and *in vivo* protocols.

## Materials and Methods

### Mouse Model

C57BL/6J mice were fed either a normal chow (NC) or high-fat diet (HFD) starting at either 4 or 6 wks of age [159]. The HFD formula derives 60% of energy from fat (Teklad, East Millstone, NJ) and were fed ad libitum for at least 10 weeks. Mice were weighed each week until euthanasia. All animal procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee.

### Tissue Sample Collection

Mice were euthanized by carbon dioxide ($CO_2$) administration followed by cervical dislocation. *Tibialis anterior* (TA) and *gastrocnemius* (GA) muscles were collected, placed in freezing compound (O.C.T. Thermo Fisher Scientific, Fisher Healthcare, Houston, TX), and immediately frozen in isopentane pre-cooled in liquid nitrogen. Samples were stored in -80$^{o}$C until analyses. Ten micron thick sections were made using a Microm HM550 cyrostat (Thermo Fisher Scientific, Waltham, MA) and mounted on 3-aminoproprytriethoxysilanesy (silane, Sigma-Aldrich, St. Louis, MO) coated microscope slides for immunocytochemistry or traditional histology.

### Satellite Cell Isolation and Culture

Immediately after euthanasia, mice were rinsed briefly in 70% ethanol and muscles from the hind limbs, lower back, and diaphragm were exposed, incised whole and transferred to sterile phosphate-buffered saline (PBS). Muscles were washed and excess connective tissue, adipose tissue, blood, and hair were removed. Pooled muscles were then dissected and minced with sterile scissors to yield a fragmented muscle suspension. Muscle suspensions were digested in Ham's F10 medium (Fisher Scientific, Hampton, NH) containing 10% horse serum ((HS) (Invitrogen, Carlsbad, CA)), and collagenase II (500 units per mL; Invitrogen) in a 15 mL centrifuge tube for 90 min at 37$^{o}$C under agitation. After a 90 min digestion, digests were triturated 20 times to separate the single fibers using a 10 mL serological pipette. Digestions were then centrifuged at 500 X g for 1 min to pellet down the myofibers. Supernatants were discarded and pellets were suspended in 10 mL washing buffer (Ham's F10 medium containing 10% HS and1% penicillin-streptomycin (pen/strep, Sigma-Aldrich, St. Louis, MO)). Pellets were triturated 10 times and allowed to incubate for 1 min to allow the clusters of non-digested fibers containing fibroblasts to fall to the bottom of the tube. Supernatants containing single fiber fragments were then transferred into a new 15 mL tube and centrifuged. After centrifugation, supernatants were discarded and 10 mL of washing buffer was added and the pellet was triturated again 10 times and centrifuged. This step was repeated for a total of 3 washes. Fragmented myofibers were then digested in 3 mL of pre-warmed Ham's F-10 containing 10% HS, 0.5 U/mL dispase (Invitrogen), and 38 U/mL collagenase type II (US Biological, Salem, MA) in a 15 mL centrifuge tube for 30 min at 37$^{o}$C with agitation. After digestion 10 mL of wash buffer was added to the digest and satellite cells were liberated from the myofibers by trituration 10 times with a 20-gauge syringe and centrifuged. Supernatants were filtered through 40-µm sterile filters. The elute flow-through was centrifuged at 1000 X g for 5 min to pellet

satellite cells. Supernatants were discarded and cells were suspended in 1 mL of Ham's F-10 containing 20% fetal bovine serum ((FBS) Genesee Scientific, San Diego, CA), 1% pen/strep, and 5ng/mL basic fibroblast growth factor ((bFGF) Thermo Fisher Scientific, Gibco, Gaithersburg, MD). Cells were triturated 10 times to disperse and were quantified using a hemocytometer. Cells were seeded on collagen-coated 12-well plates at 0.1 x $10^6$ cells/well for proliferation assays and on matrigel-coated 6-well plates at 0.1 x $10^6$ cells/well for differentiation studies. Plates were incubated at 5% $CO_2$ at 37ºC.

BrdU Incorporation

Either 3 or 7 d after isolation, bromodeoxyuridine (BrdU) labeling reagent (Invitrogen, Carlsbad, CA) was added to each well at a 1:100 concentration. Cultures were incubated at 37ºC for 1 hr, after which media were discarded and cell monolayers were washed once with ice-cold PBS, fixed in 1 mL of ice-cold 70% ethanol for 5 min at room temperature, and washed with PBS. After removal of PBS, plates were treated with 0.5 mL of 1.5M hydrochloric acid and allowed to sit at room temperature for 30 min. Plates were washed twice with PBS and blocked in PBS with 5% goat serum (Thermo Fisher Scientific) for 1 h. Plates were then incubated with an anti-BrdU antibody (clone G3G4, DSHB, Iowa City, IA), diluted 1:100 in PBS containing 5% goat serum. Plates were incubated overnight at 4ºC. The following day, plates were washed three times with PBS, and a secondary antibody, Alexa Fluor 555 goat anti-mouse IgG (Life Technologies, Eugene, OR) diluted 1:1000 in PBS containing 5% goat serum, was applied. Cultures were incubated in the dark at room temperature for 2 hr. Plates were washed in PBS, and fluorescent mounting medium was added to each well. 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) staining was used to identify nuclei. Images were collected using a Nikon ECLIPSE Ti-E fluorescent microscope (Nikon Instruments Inc., Melville, NY). Number

of nuclei positive for BrdU was quantified as a percent of total number of nuclei, and the percentage was used as an indicator for cell proliferation rate.

Myogenic Differentiation

Cells plated for differentiation were cultured in growth medium for 7 days or until reaching a density of 95% confluence. Once cells reached confluence the cells were washed 2X in PBS and released by putting 1mL of 1:5 trypsin (Thermo Fisher Scientific) diluted in PBS in each well. Plates were incubated at 37$^{o}$C until the cells detached from the bottom. Growth medium was added to neutralize the trypsin and cells for each mouse were combined and centrifuged at 300 X g for 5 min to pellet cells. Supernatants were discarded and 2 mL of growth medium was added to each tube. Cells were pre-plated on non-coated 35 mm plates at 37$^{o}$C for 20 min. Media containing the SCs was transferred to a new tube and enumerated. Cells were plated at confluence on matrigel-coated 24-well plates at 0.2 x $10^{6}$ cells/mL/well. To induce differentiation, growth medium was switched to differentiation medium the following day (high-glucose DMEM containing 3% horse serum and 1% pen/strep), and allowed to incubate at 37$^{o}$C for 3 days. Plates were washed once with ice-cold PBS and fixed with 1 mL of ice-cold 100% methanol at room temperature for 10 min followed by a PBS wash, blocking, and staining using Pax7 (1:50 dilution, DSHB, Iowa City, IA), MyoD (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies, and/or an antibody against myosin (clone MF20, DSHB, Iowa City, IA) and secondary antibodies (Alexa Fluor 555 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG), including DAPI, at a dilution of 1:1000 and 1:500 respectively. Plates were incubated at room temperature in the dark for 2 hr then washed with PBS. Fluorescent mounting medium was added to each well. Ten images per well were taken using a Nikon ECLIPSE Ti-E fluorescent microscope (Nikon Instruments Inc., Melville, NY)

Muscle Oil Red O Staining

Cyrosectioned muscle samples on silane-coated slides were allowed to dry at room temperature for 30 min prior to staining. Sections were placed in propylene glycol for 2 min and then incubated in concentrated Oil Red O solution for 6 min. Sections were then placed in 85% propylene glycol for 1 min. Slides were rinsed in distilled water and stained with hematoxylin for 2 min, rinsed with running tap water for 5 min, and then rinsed for 2 min in distilled water. Slides were cover-slipped and mounted in Permount mounting medium (Thermo Fisher Scientific, Waltham, MA). Ten images per sample were taken using a Nikon ECLIPSE 80i light microscope (Nikon Instruments Inc).

Muscle Immunohistochemistry

Frozen muscle cyrosections were dried on silane-coated slides for 30 min at room temperature prior to staining. Slides were washed once in PBS, then fixed in 4% paraformaldehyde for 10 min at room temperature followed by three washes with PBS and permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 15 min then washed 2 more times with PBS. Sections were incubated with wheat germ agglutinin ((WGA, Thermo FIsher) diluted 1:500 and DAPI diluted 1:500 in PBS at room temperature in the dark for 1 hr. Sections were washed 3 times in PBS, then mounted with fluorescent mounting medium. Ten images per sample were taken using a Nikon ECLIPSE Ti-E fluorescent microscope (Nikon Instruments Inc).

Muscle Single Fiber Isolation

*Gastrocnemius* muscles were isolated and digested in Dulbecco's modified Eagle's medium ((DMEM), Thermo Fisher Scientific) high glucose, L-glutamine with 110 mg/mL

sodium pyruvate with 110 mg/mL sodium pyruvate containing 0.2% collagenase type I at 37°C for 1 hr. Digested muscles were then switched to DMEM containing 1% pen/strep and a large bore pipette was used to triturate the muscle and release myofibers from the muscle. A small bore pipette was used to transfer single fibers to wash media. Once a desired number of myofibers were collected, fibers were fixed in pre-warmed 4% paraformaldehyde for 5 min, washed with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min. After incubation, two additional washes were performed with PBS and followed by a second incubation in 5% goat serum in PBS for 1 hr. Fibers were incubated in primary Pax7 and cleaved caspase 3 antibodies diluted 1:50 and 1:200 in blocking buffer, respectively at 4°C overnight. The next day fibers were washed and incubated in secondary antibodies (Alexa Fluor 555 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG), including DAPI, diluted to 1:1000 and 1:500 respectively for 1 hr at room temperature in the dark followed by three PBS washes and transferred to microscope slides with fluorescent mounting medium. Images of all $Pax7^+$ nuclei per fiber were taken using a Nikon ECLIPSE Ti-E fluorescent microscope (Nikon Instruments Inc).

Flow Cytometry

One *gastrocnemius* muscle was damaged by intramuscular injection of 300 µL cardiotoxin (CTX) dissolved in PBS 3 d prior to harvest and both muscles were collected for SC and immune cell analysis. GA muscles were minced and digested in collagenase B/dispase II for 1 hr with trituration every 15 min. Digestions were neutralized with FBS and pelleted at 350 X g. Samples used to analyze for SCs were stained with CD31-APC, CD45-APC, Sca1-APC, and Vcam-1-biotin. After a brief wash Sav-PE-Cy7 conjugated secondary antibody was applied and PI and Calcein violet stains were added prior to analysis. For immune cell analysis, samples

were stained with CD45-APC, Ly6G/C-PE-Cy7, CD206-PE, and F4/80-Alexa 488 and PI and Calcein violet stains were added prior to analysis. Samples were recorded using a flow cytometer and analyzed using FlowJo software.

Muscle Injury

The *tibialis anterior* and *gastrocnemius* muscles of mice were damaged by intramuscular injection of 50 µL and 300 µL cardiotoxin (CTX) dissolved in PBS respectively. TA muscles were damaged either 3 or 10 days prior to harvest and GA muscles were damaged 3 days prior. Samples were collected and processed for histochemistry as outlined previously.

Statistical Analysis

Data are presented as means ± standard error of the mean (S.E.M.), with significance set as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

**Results**

To evaluate SC function during an obese state, mice were subjected to a HFD. Diets for the HFD mice consisted of 60% of total energy derived from fat. Mice were fed either NC or HFD starting at 4 weeks of age and were continuously fed this diet ad libitum for at least 10 weeks when an obese phenotype was observed. Mice fed the high fat diet had a greater ($P < 0.001$) body weights after 4 wks of dietary intervention and this continued throughout the 10 wks study (Figure 1A). Consistently, NMR scans of the mice indicated an increase in body fat mass in the HFD compared to the NC, but no differences were noted in skeletal muscle mass (Figure 1B). Oil Red O staining for lipid accumulation also an increase in ectopic lipid in the skeletal muscle of HFD mice in comparison to NC (Figure 1C). These results confirm that our HFD mouse model induces obesity and excess adipose tissue in the body.

To determine the effect of obesity on SC behaviors, we isolated and cultured SCs *in vitro* from muscle of mice exposed to different dietary treatments. Although no noticeable differences in SC numbers were evident between NC and HFD mice, SCs of HFD fed mice muscle had a reduced capacity to proliferate compared to that of NC mouse muscle (Figure 2D). This is consistent with clonal assay quantification, although no difference in the number of cells per clone were evident at D3, but by D7, fewer cells per clone ($P < 0.001$) were evident in SC cultures from HFD mice muscle (Figure 2E).

To examine the possible differences in SC cycling and differentiation capacities caused by diet, SCs were isolated and allowed to reach confluence in culture for 7 d. Once cells reached confluence, an equal number of SCs from NC and HFD were re-plated and induced to differentiate for 3 d. After 3 d of differentiation, cells were either stained with Pax7 and MyoD antibodies, or with a myosin antibody to identify myotubes (Figure 3A and E). SCs derived from muscle of HFD mice possessed diminished ability to differentiate *in vitro* as evidenced by decrease in myotube diameter, smaller nuclear domain, and a trend for less nuclei per fiber ($P < 0.001$) than NC (Figure 3 B-D). $Pax7^{-}$;$MyoD^{+}$ cells are committed myogenic SCs, while $Pax7^{+}$;$MyoD^{+}$ cells indicate SCs in the process of returning to quiescence, and $Pax7^{+}$;$Myf5^{-}$ cells represent quiescent SCs that have already gone through the lineage progression and have self-renewed [65, 160]. After 3 d of differentiation SCs derived from HFD have a smaller pool of $Pa7^{+}$;$MyoD^{-}$ cells ($P < 0.001$) and a greater percentage of $Pax7^{-}$;$MyoD^{+}$ cells ($P < 0.001$) than muscle from NC mice suggesting muscle of mice fed a high fat diet have SCs with less self-renewal capabilities and may cycle slower (Figure 3F).

To study the properties of SCs in *ex vivo*, single muscle fibers were isolated from the GA of both NC and HFD mice. After isolation these fibers were immediately fixed and stained with

Pax7 and cleaved caspase 3 to identify possible SC apoptosis (Figure 4A). SC apoptosis was quantified as the ratio of cleaved caspase $3^+$;$Pax7^+$ cells over the total number of $Pax7^+$ cells per fiber. At D0 of single fiber isolation fiber from muscle of HFD mice experienced greater occurrence of SC apoptosis ($P < 0.001$) than those from muscle of NC mice (Figure 4B).

To test the SC cycling properties *in vivo*, a H2B-GFP mouse model was used in conjunction with our obesogenic model. In H2B-GFP mice, administration of tetracycline, often given in the form of doxycycline (dox), conditionally activates a TetOn system which causes incorporation of green fluorescent protein (GFP) into histone H2B causing cells to fluoresce green [161, 162]. Once tetracycline is omitted from the diet, cells slowly lose GFP as they divide and GFP is diluted with each division (Figure 5A). This is a useful method for studying traditional quiescent or less active cells in the body, such as SCs. To allow for maximal incorporation of GFP, mice were fed dox food for a period of 6 wks. After 6 wks, mice were chased with either NC or HFD for 10 wks to induce obesity (Figure 5A and B). At the end of 10 wks, SCs were isolated from muscle and subjected to fluorescence activated cell sorting (FACS). Using positive and negative SC markers to identify the SC population, SCs number was reduced in muscle of HFD mice ($P < 0.001$) compared to controls (Figure 5C). The GFP intensity of the gated SC pool was then measured and SCs from muscle of HFD mice had the vast majority of GFP expression lost, whereas, those of NC mice appeared to maintain a greater population of $GFP^+$ SCs (Figure 5D). This loss in GFP in SCs purified from muscle of HFD mice suggests fewer quiescent SCs exist in muscle of mice fed high fat diets.

To assess the role of diet on the ability of adult SCs to facilitate regenerative myogenesis, the left TA was injected with cardiotoxin (CTX) to induce muscle damage in both NC and HFD mouse models, while the right TA was left non-damaged. Ten days after damage, TA muscles

were collected, sectioned, and stained for muscle fiber characteristics that could be used to determine fiber cross sectional area (CSA) and nuclear domain (Figure 6A). Once quantified, data suggested a trend for smaller CSA in muscle of HFD mice at D10 and non-damaged (Figure 6B). After D10 post injury, muscle fibers from HFD mice exhibited a decreased nuclear domain ($P < 0.05$) compared to controls (Figure 6C).

To identify immune cell populations in non-damaged and damaged muscle, the GA was damaged intramuscularly with CTX and harvested 3 days post injury. The SCs were isolated from GA muscles and analyzed using FACS. Positive and negative cell markers were used to identify neutrophil and macrophage populations in both damaged and non-damaged muscle. HFD exhibited a decreased neutrophil population ($P < 0.001$), as well as an increased macrophage population ($P < 0.05$) at D3 (Figure 7B and C).

**Discussion**

Obesity and a high adipose tissue accumulation in the body without doubt causes major shifts and alterations in body composition and metabolism [43]. Mice fed a HFD show considerable changes in fat mass, body weight, as well as a noticeable ectopic lipid residence in muscle fibers. Previous work has discovered a decrease in skeletal muscle metabolic flexibility, as well as a decreased ability to regenerate after damage. Our work contributes to the extant literature by exploring how HFD impacts SC function and muscle regeneration, and our *in vitro*, *ex vivo*, and *in vivo* data show that HFD impairs SC proliferation and differentiation in culture, SC viability in isolated muscle fibers, and muscle regeneration.

When removed from their niche and cultured, SCs derived from muscle of HFD mice have a marked decrease in proliferation in culture, while closer to initial culturing, no marked

differences were noted between treatments. This observation suggests that the niche cultivated by diet negatively impacts SC behaviors such that SCs have impaired proliferative capacity *in vitro*. The fact that SCs from muscle of HFD mice exhibit no signs of slowdown in growth in the first 3 days of culture may be related to the fact that SCs take some 48 hrs to activate and begin to proliferate after receiving the stimulus [163]. To that end, any inherent differences, if any, may not be detected at such an early stage. Although the exact mechanisms of the delayed proliferation is unknown, these data suggest that SCs are impacted by *in vivo* cues, most likely related to their local niche.

In parallel to the aforementioned observations, SCs, derived from muscle of mice fed an obesogenic diet, have diminished capacities differentiate as noted by decreases in myotube diameter, myonuclear domain, and distribution of number of nuclei per fiber. This could be due to a decreased expression of MyoD, myogenin, and myosin heavy chain as reported previously [164]. A decrease in the ability of muscle cells to differentiate had been observed *in vitro* and could be linked to the chronic inflammation associated with obesity, where the M1 macrophage population is systemically elevated causing a failure to convert to M2 macrophages and an inhibition in differentiation [79, 80]. Regardless, these results further support the notion that high fat diets impact SC function *in vivo* as SCs derived from NC and HFD muscles were ultimately cultured in the same media *in vitro.*

In addition to a decreased capacity to proliferate and differentiate *in vitro,* we also observed diet-induced decreases in SC content *in vivo* using FACS analysis. After feeding the mice with HFD for 10 wks, we isolated myofibers and found that HFD fibers contained fewer SCs, and a proportion of SCs were positive for cleaved caspase 3, an apoptotic marker. This result may help explain why SCs *in vitro* have impaired proliferation, as indicated by clonal

assay and BrdU incorporation assay. Although it is unclear what causes an increase in SC apoptosis on muscle fibers, various factors associated with obesity could be involved. With obesity there is a noted elevation in reactive oxygen species (ROS), oxidative stress, and mitochondrial dysfunction which could factor into the increase in apoptotic SCs in HFD mice. [165]

Moreover, our *in vivo* GFP tracking experiment may explain why SCs have reduced number *in vivo* and limited proliferative or cycling, capacity *in vitro*. The enhanced SC cycling rate, as indicated by a more rapid loss of GFP, *in vivo*, suggests that the adverse HFD niche may force SCs awake from their quiescent status. After awakening, certain SCs may undergo apoptosis, whereas others may repeat the more frequent cycling period as compared to the NC SCs. As such, aberrant cycling would have two negative impacts on SC number. First the number of resident SCs would be reduced due to apoptosis. Alternatively, the capacity of SCs to cycle may be exhausted, further reducing SC number *in vivo*. Satellite cells have inherently limited cycles of replication to repair the damaged tissue. This is best illustrated by the etiology of human Duchenne muscular dystrophy and its associated mouse models [166]. In our case, repeated wakening of SCs by high fat diet-induced niche effects may mimic the dystrophin-deficiency-induced Duchenne model in which SCs are repeatedly activated and expand to regenerate, or repair damaged muscle fibers. Although the exact mechanism for the effects of HFD on SCs remains elusive at this stage, it is clear that HFD creates an adverse niche for SCs to survive. There is an increase in M1 macrophages associated with obesity, which may be responsible for the recruitment of pro-inflammatory cytokines as well as the migration and proliferation of SCs during injury through secretion of IL-6 [74, 75]. While M1 macrophages only infiltrate tissue during time of damage, M1 macrophages will reside in the tissue longer,

especially during obesity. This constant presence in the tissue could partially be responsible for continual activation and proliferation of SCs as seen with the H2B-GFP. Thus, it may be interesting to investigate the molecular mechanism of how SCs interact with its inflammatory niche, and how interference of such an interaction may lead to therapeutic interventions for obese patients.

Long-term high fat feeding (8 months) results in a marked decrease in TA muscle regeneration as demonstrated by a reduction in muscle mass, smaller myofibers, increased collagen deposition, and larger interstitial spaces in comparison to NC mice [167]. Another study using a shorter high caloric feeding paradigm (3 wks) showed similar results in young mice aged 3-6 weeks old [168]. However, these findings are not repeatable in HFD models fed for an intermediate amount of time. For example, a study feeding high fat diets to mice for 12 wks failed to observe a marked decrease in the size of regenerating fibers after inducing injury of the extensor digitorum longus (EDL) with cardiotoxin [169]. Our findings are consistent with this study, showing only a mild impairment in muscle regeneration after an intermediate feeding time period. The only difference in the two studies was our study exploited the TA instead of the EDL. While both the TA and EDL are made up of primarily fast-twitch IIB fiber types, the TA consists of a larger proportion of IIA fibers and thus could have an impact on muscle regeneration after injury [170]. Regardless of the muscle type differences, our results and that from Nguyen, et al. indicate that a mild impairment in HFD-feeding may suggest a compensatory mechanism exist to recover muscle after insult. Since muscle regeneration is a complex process which involves a hierarchy of cellular events, including but not limited to SCs, it is reasonable to speculate that the negative impacts of HFD on SCs may be somehow buffered *in vivo* as compared to *in vitro.* For example, HFD mice exhibit hyperinsulinemia, and a high level of

circulating insulin, a well-known myoblast proliferation and differentiation enhancer, may boost SC function and thus compensate HFD-caused SC harm *in vivo*, at least in a temporary manner. Thus, a long-term, multiple-round muscle injury model could be exploited to study muscle regeneration in general, and SC behaviors in particular, will help elucidate the impacts of HFD microenvironment on muscle physiology.

Obesity remains a widely researched area and its effects on the whole body metabolism are substantial. Although the mechanisms by which obesity affects SCs remains largely uncertain, our study has added more insights into SC niche interaction. Future studies focusing on the signaling pathways emanating from the HFD niche will help expound the molecular mechanism responsible for SC homeostasis in a given pathological setting, which in our case, obesity.

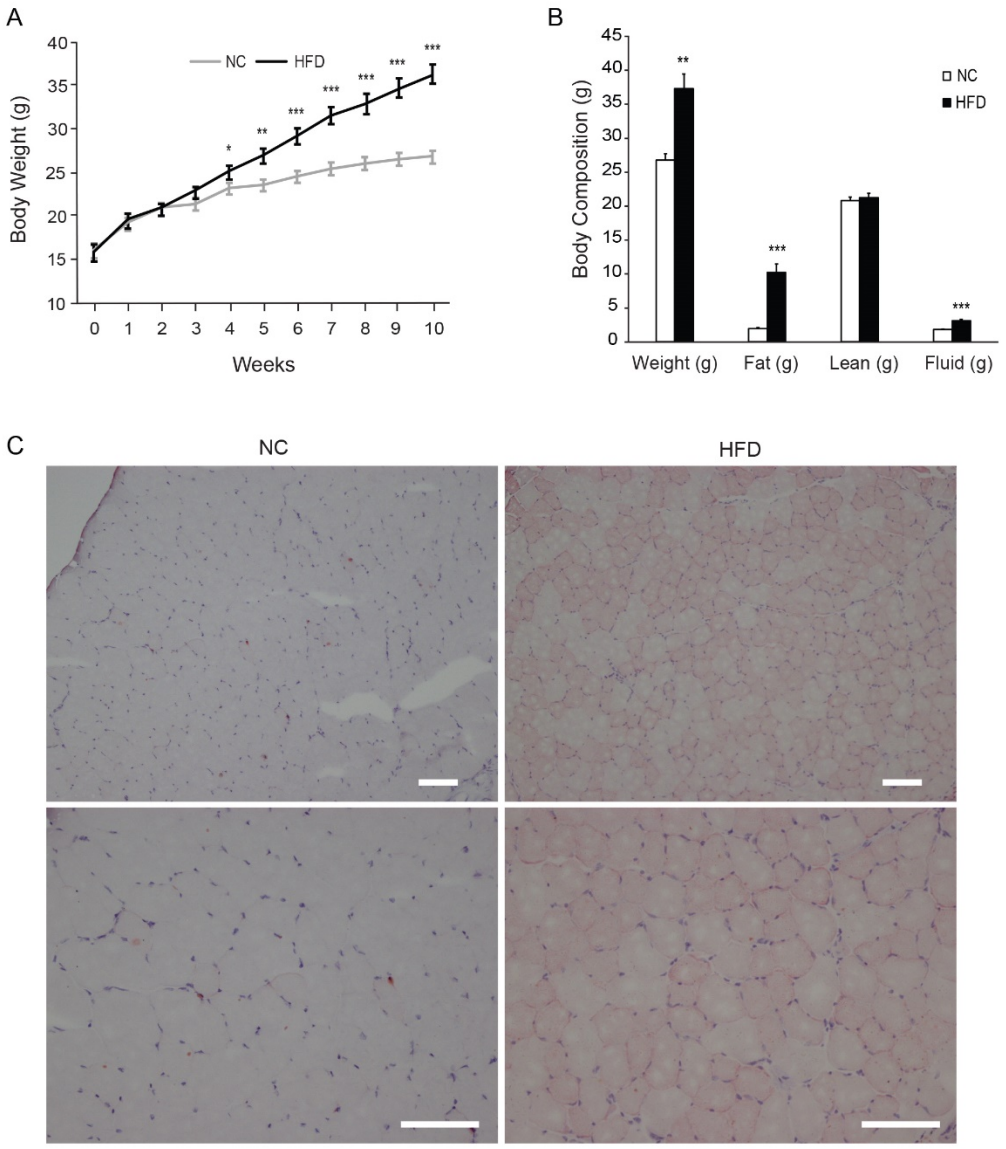


Figure 1. HFD mouse model show increase in weight and fat accumulation. C57BL/6J mice fed either NC or HFD at 4-6 weeks for at least 10 weeks. (A) NC vs HFD body weight over 10 weeks. (B) NC vs HFD body composition after 10 weeks. (C) Oil Red O stain on TA muscle after 10 weeks. Scale bars, 100 μm. Red arrows indicate intramyocellular lipid accumulation. N=5 from each phenotype. Data represent means ± SEM. * *P < 0.05*, ** *P < 0.01*, *** *P < 0.001* compared to NC.

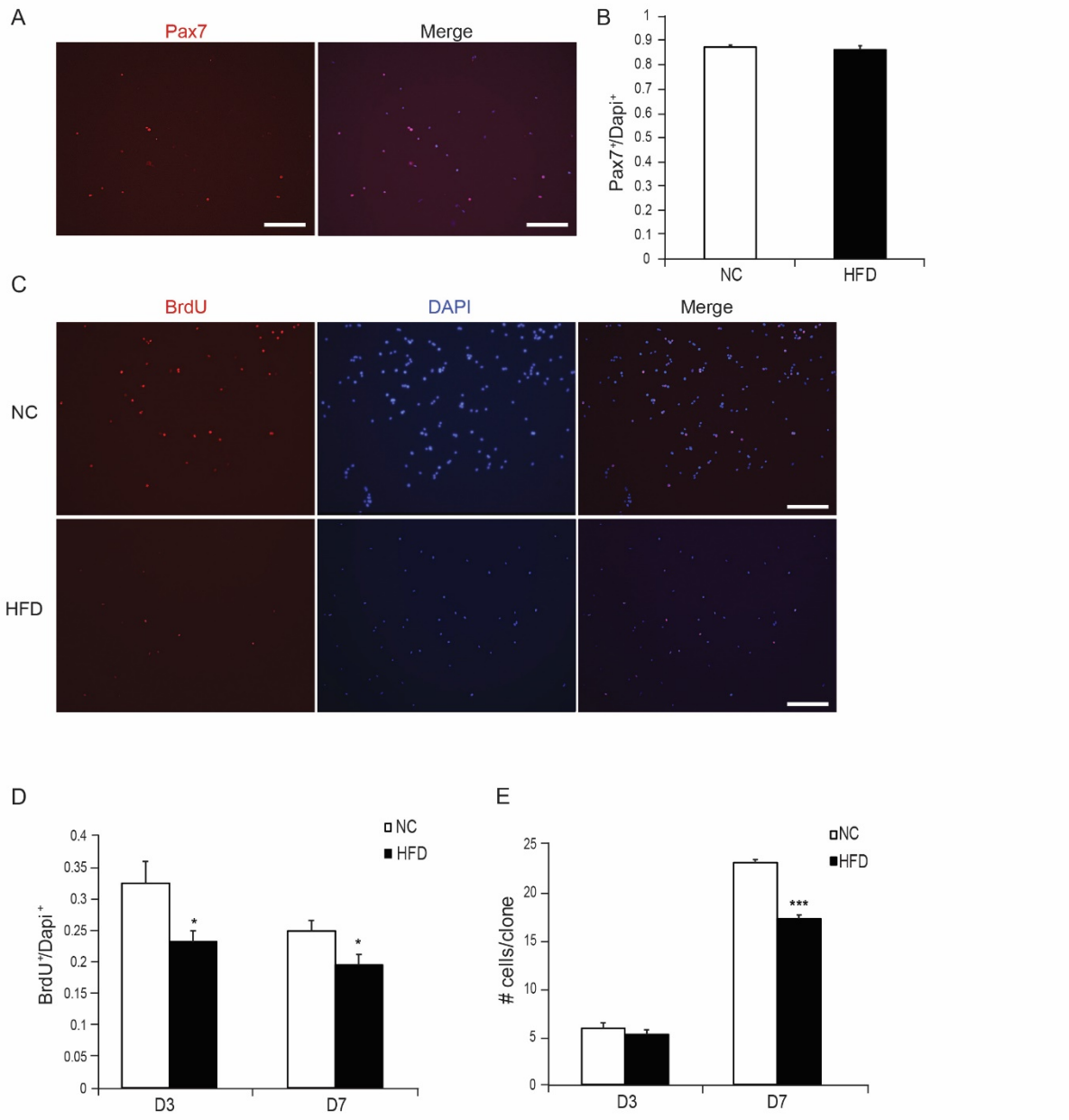


Figure 2. Satellite cells derived from HFD have impaired proliferative capacity *in vitro*. (A) Identification of SCs. SCs stained with Pax7 (red) and nuclei were counterstained with DAPI (blue). Scale bars, 50 µm. (B) Quantification of SCs 24 hrs. after isolation. (C) SCs were isolated from mice and cultured in growth medium for 3d or 7d. 10 µM of BrdU was added to the growth medium for 1h, cells were fixed and stained with BrdU antibody. Nuclei was stained with DAPI (blue). Scale bars, 50 µm. (D) Quantification of number of cells per clone 3d or 7d culture. (E) Quantification of BrdU+ cells expressed as percent of total number of nuclei. N=7 from each phenotype. Data represent mean ± SEM. ** $P < 0.05$*, **** $P < 0.001$* compared to NC.

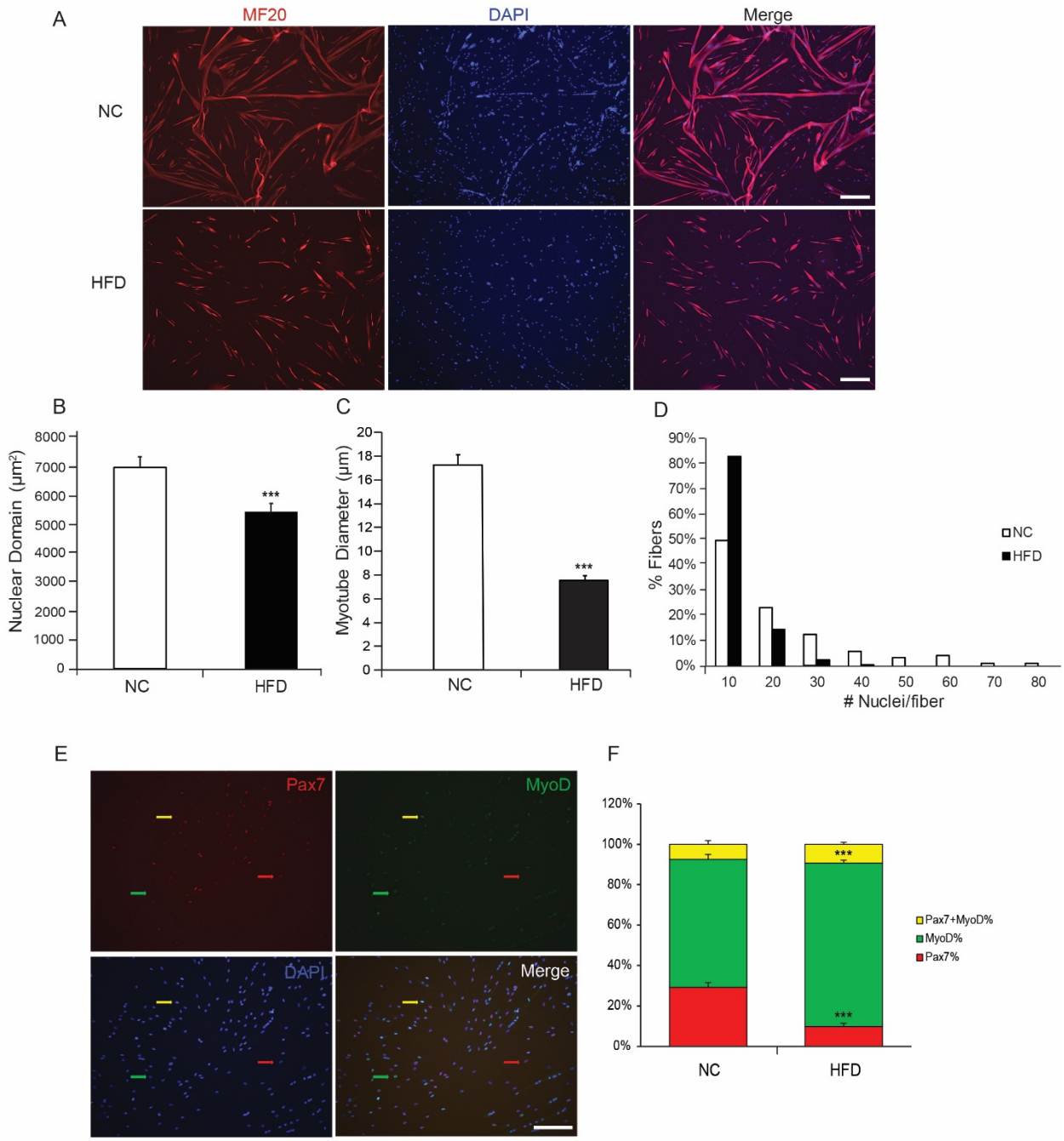


Figure 3. Satellite cells derived from HFD have impaired differentiative capacities and self-renewal. (A) SCs were isolated from NC and HFD mice and cultured for 7d to cofluence. Equal number of SCs were plated and induced to differentiate for 3d. Myotubes were stained with DAPI for nuclei and MF20 for myosin. Scale bar, 200 μm. (B) Nuclear Domain (C) Myotube diameter (D) Histograms showing the distribution of number of nuclei per fiber. (E) Representative images showing the staining of Pax7 (red), MyoD (green), and nuclei (DAPI, blue). Red arrow, $Pax7^+$ only; green arrow, $MyoD^+$only; yellow arrow, $Pax7^+MyoD^+$. Scale bar, 50 μm. (B) SC subpopulation in NC and HFD after 3d differentiation. $Pax7^+$, $MyoD^+$, and $Pax7^+MyoD^+$ cell subpopulations were calculated as percent of total. N=5 from each phenotype. Data represent mean ± SEM. *** $P < 0.001$ compared to NC.

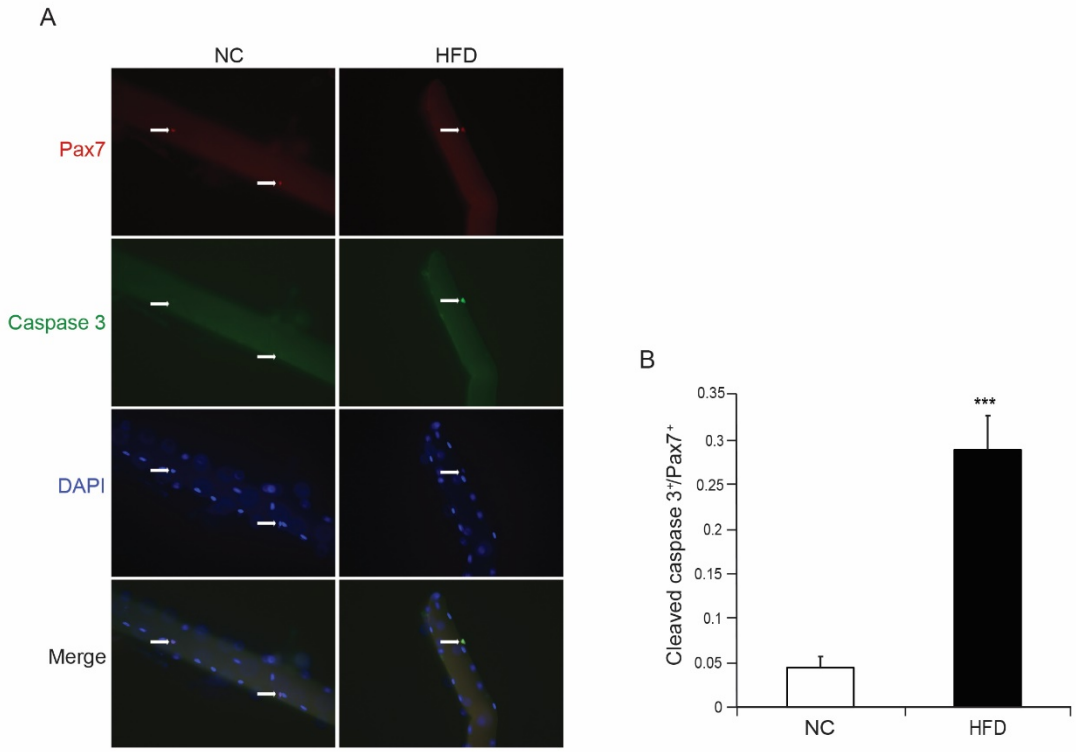


Figure 4. HFD induces SC apoptosis *ex vivo*. (A) Single fibers were isolated and fixed immediately. Representative images showing the staining of Pax7 (red), cleaved caspase 3 (green), and nuclei (DAPI, blue). (B) Bar graph of number of cleaved caspase $3^+/Pax7^+$ cells over total $Pax7^+$ cells. N=5 from each phenotype. Data representmeans ± SEM. *** $P < 0.001$ compared to NC.

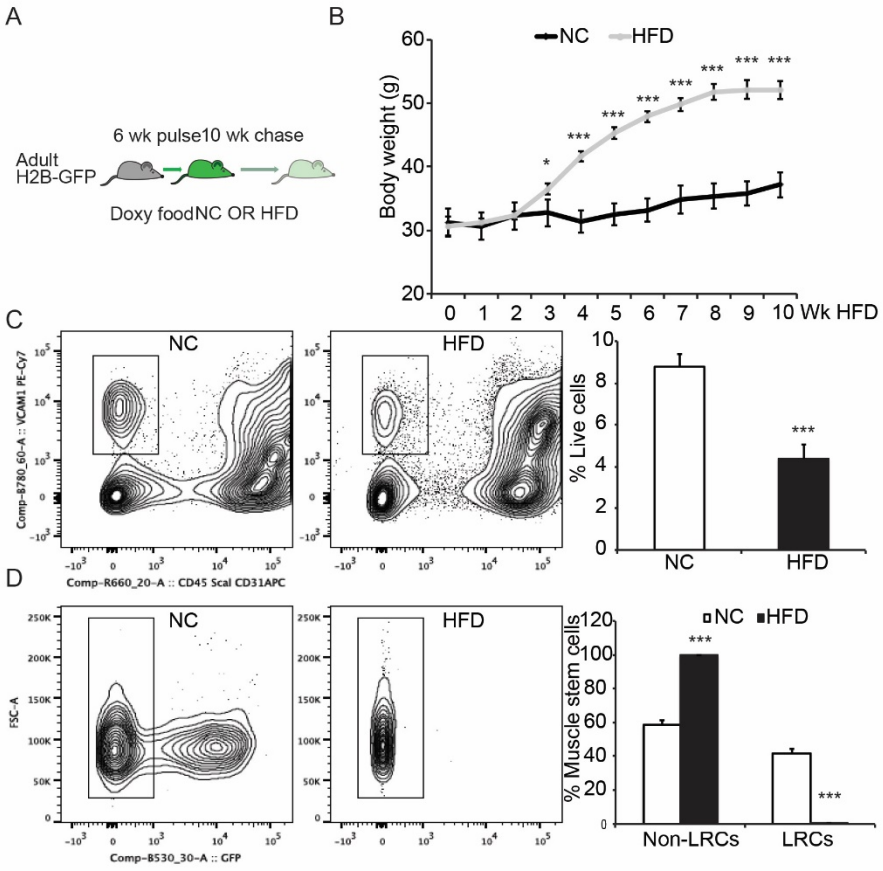


Figure 5. Dietary obesity reduces MuSC content and enhances its cycling rate *in vivo*. (A) Experimental scheme for *in vivo* lineage tracing. Adult mice, 7-week-old. NC, normal chow; HFD, high fat diet. (B) Body weight following HFD feeding. (C) FACS analysis of muscle stem cell content based on surface marker VCam1$^{+}$CD45$^{-}$CD31$^{-}$Sca1$^{-}$. (D) Non-label retension cells (Non-LRCs) and LRCs of the muscle stem cell population in NC and HFD mice. Data represent means SEM from n=5 in (B), n=4 in (C-D) mice. ** P < 0.05*; **** P < 0.001* compared to the NC treatment.

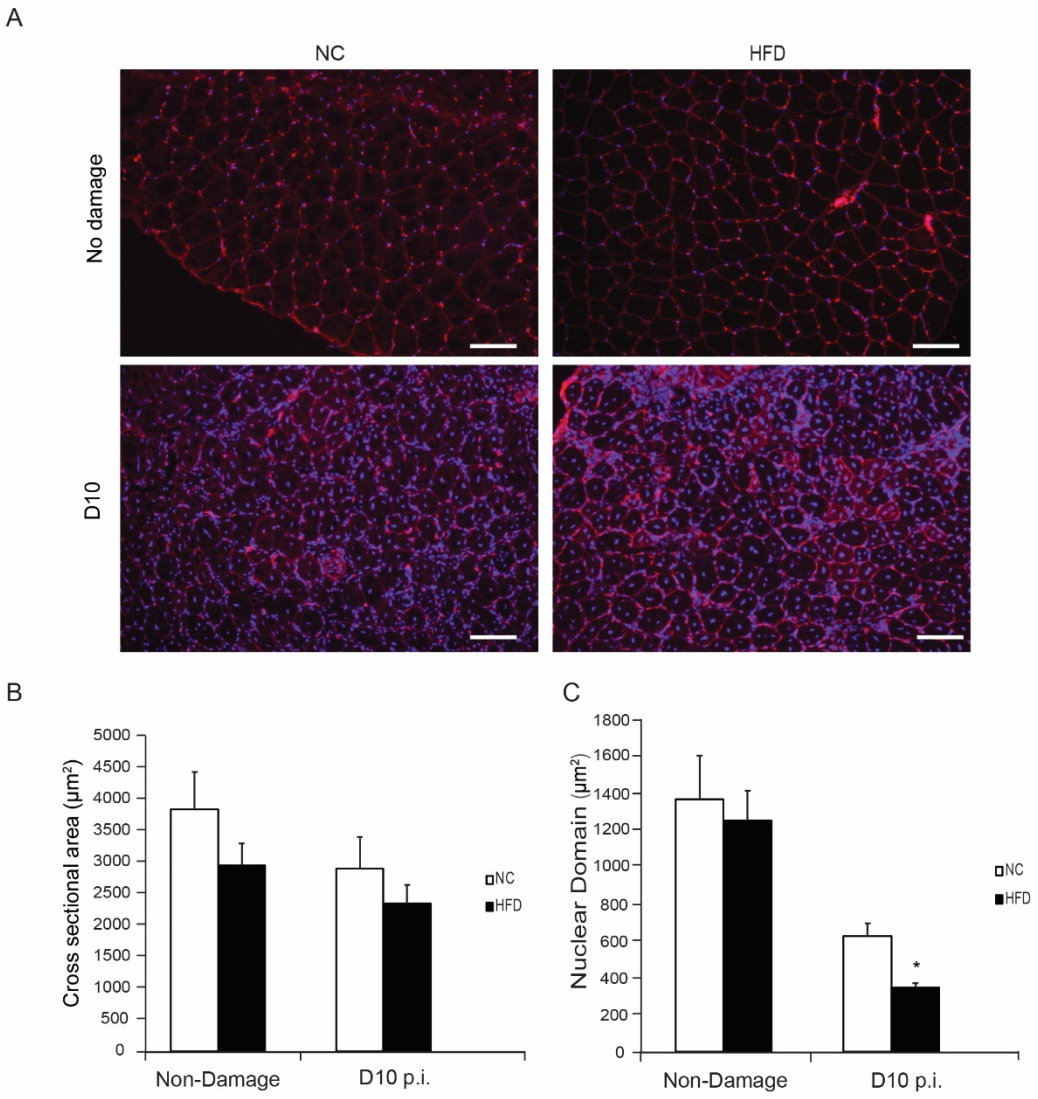


Figure 6. HFD exhibit slight impairement in muscle regeneration. TA muscles from mice were injected with 50 μL caridotoxin to induce injury. Muscle samples were harvested 10d post injury. (A) WGA staining. Scale bars, 100 μm. (B) Muscle fiber cross sectional area. (C) Nuclear Domain. N=4 from each phenotype. Data represent mean ± SEM. * $P < 0.05$ compared to NC.

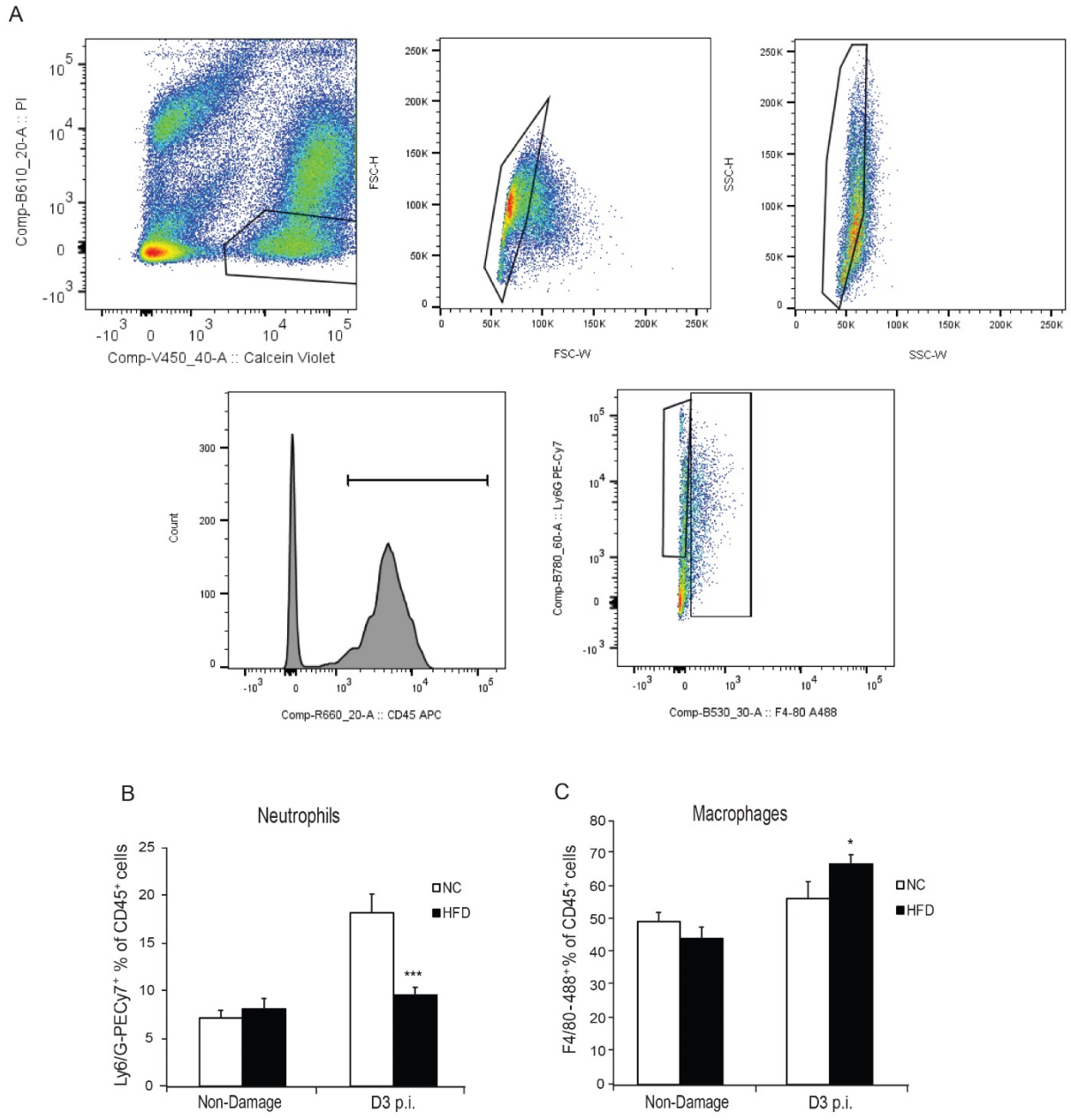


Figure 7. HFD exhibit a shift in immune cell population after damage. (A) GA muscles in mice were injected with 300 µL cardiotoxin to induce injury. 3d post injury muscles were harvested for FACS analysis. (B) FACS analysis of neutrophil content based on surface marker Ly6/G-PECy7$^{+}$CD45$^{+}$. (C) FACS analysis of macrophage content based on surface marker F4/80-488$^{+}$CD45$^{+}$. N=8 per phenotype. Data represent mean ± SEM. * $P < 0.05$ and *** $P < 0.001$ compared to NC.

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