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The contribution of bone marrow cells to the formation of bone, fat, and muscle, a commitment disrupted by systemic distress and normalized by mechanical stimulation

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Abstract of the Dissertation

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In excess, adipogenesis and adiposity can compromise the quality of the musculoskeletal system, disrupt progenitor cell populations, and increase susceptibility to diabetes. The extent to which muscle and bone tissue are impaired by obesity-related pathologies is not clear. Additionally, while fat, bone, and muscle are distinct tissue systems, the degree to which bone marrow cells contribute to the fat/muscle phenotype in the pre-obese state is yet to be elucidated.

To combat obesity, exercise is a common treatment modality that is anabolic to bone and muscle and catabolic to fat. However, compliance is low, obese patients may be unable to exercise due to physical limitations, and it can be unsafe for those at risk of fracture. As a surrogate for exercise, our lab has demonstrated the ability of mechanical stimulation in the form of low intensity vibration (LIV) to bias mesenchymal stem cell (MSC) differentiation away from the fat differentiation pathway. Influencing cell differentiation and/or migration patterns may aid in protecting musculoskeletal tissue systems disrupted by obesity-related complications.

To establish a systemic fat insult, eight week C57Bl/6J male mice were subject to either OVX surgery (sham surgery controls) or a 45% high fat diet (regular diet controls) for 10wk. LIV treatment (0.25-3*g*, 90Hz, 15-30min/day, 5day/wk) was administered to OVX animals for 6wk, and high-fat diet or regular diet animals for 8wk. High-fat and regular animals were also subject to bone marrow

transplantations for bone marrow cell tracking purposes. OVX increased adipogenic gene expression in the muscle while high-fat diet increased intermuscular lipid accumulation by 717%, quantified using oil red O staining. Other alterations in the muscular niche include the compromised satellite cell populations in OVX and increased expression of PKCθ in high-fat diet, which influences insulin signaling and demonstrates the negative consequences of an adipose burden on the muscle niche. Bone marrow-derived cells did not contribute to the intermuscular adiposity seen in high-fat diet animals. However, both bone marrow-derived cells and donor MSCs were accelerated to the visceral fat pads of animals on 10wk of high-fat diet, contributing to the expansion of the fat pad. These findings emphasize the notable changes in the musculature and bone marrow populations that precede changes in body habitus leading to obesity, and also the significance of the interactions of bone with muscle and fat tissues. Mechanical stimulation in the form of LIV was effective in mitigating muscular adiposity in both OVX and high-fat animal models and demonstrates the ability to be useful as a therapeutic intervention in preventing the onset of obesity or type II diabetes.

Dedicated to my grandmother, Sylvia Roy, who always believed I would finish, even when I didn't believe it myself.

And to my family, the Frechettes - Dad, Mom, Nicki, Gabbi, and Dog, who have showed me unfailing faith and constant love.


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LIST OF ABBREVIATIONS

AC – age-matched control
ANOVA – analysis of variance
AUC – area under the curve
BMI – body mass index
FoxO1 – forkhead box protein O1
FABP4 – fatty acid-binding protein 4
C/EBPα – CCAAT-enhancer-binding protein α
CD45 – hematopoietic cell marker
CD31 – endothelial cell marker (PECAM-1)
CD34 – reserve satellite cell marker
DAG – diacylglycerol, diglyceride
FAP – fibro/adipogenic progenitor
FoxO1 – forkhead box protein O1
g – earth's gravitational field at 9.81 m/s^2
GLUT4 – glucose transporter protein 4
GTT – glucose tolerance test
Hz – cycle per second
HSC – hematopoietic stem cell
IGF-1 – insulin-like growth factor 1
IRS-1 – insulin-receptor substrate-1
Integrin alpha 7 – satellite cell marker
LIV – low intensity vibration (0.25-0.3 *g*, 90 Hz)
μCT – micro-computed tomography
MSC – mesenchymal stem cell
Myf5 – myogenic factor 5
MyoD – myoblast determination protein
OVX – ovariectomy
ORO – oil red O
Pax7 – paired box 7 protein
PIK3 – phosphatidylinositol 3-kinase
PKCθ – theta isoform of protein kinase C
PPAR – peroxisome proliferator-activated receptor
SAT – subcutaneous adipose tissue
Sca-1 – stem cell marker (non-satellite)
TAG – triglyceride
TAT – total adipose tissue
VAT – visceral adipose tissue

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As I finally make it to the finish line, I cannot help but be proud of the remarkable progress I have made as a research scientist, which I believe is a direct result of the many extraordinary mentors, professors, and peers I have been privileged to learn from and work with during my time at Stony Brook. The most important thing I will take away from this experience is my new and unique way of thinking – how to be critical, persistent, and appropriately inquisitive, which are skills I now have a new found appreciation for and understanding of. In looking back at the past five years, it is without hesitation that I acknowledge how much of a team effort my research was. The work presented here, along with a multitude of other less successful experiments that did not make it into this thesis, was largely a culmination of the efforts and ideas of many and could not have been done alone. I have many people to thank and be grateful for.

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

Health risks in diseases characterized by excess adiposity

Several diseases and conditions are associated with carrying an excess amount of adipose tissue, including aging, obesity, type II diabetes, menopause, and cardiovascular disease. The consequences related to an unhealthy quantity of fat are abundant and range from minor breathing difficulties to decreased life expectancy. The burden excess fat tissue bears on the musculoskeletal system is due to both the heavy physical load bearing on the skeleton and the more detrimental counterpart, accompanying metabolic irregularities. Health complications due to altered endocrine and inflammatory function open the door to a wide variety of chronic diseases that affect both bone and muscle health. The extent to which fat tissue influences skeletal muscle and bone and the interactions between the three tissue systems requires further exploration.

Menopause

Menopause is the progressive decline of ovarian hormone that all women will encounter as they age, typically around the age of 50 (1). The loss of estrogen, progesterone, and other ovarian hormones results in a range of symptoms that can affect up to 85% of women (2) and last for more than ten years (3). Some of the most frequented symptoms include vasomotor symptoms (sweating and hot flashes), sleep loss, mood swings, increased intra-abdominal fat (4, 5), sarcopenia (6), osteoporosis (7), and musculoskeletal pain (8). Menopause increases risk of obesity (9), and, perhaps not unexpectedly, obesity prevalence is currently higher in women (38.3%) than in men (34.3%) (10). Women that are obese are also more susceptible to developing type II diabetes than obese men (11). The physiologic changes that occur during menopause not only lead to psychosocial impairment, social isolation, and ultimately reduced quality of life, but the range of necessary treatments for the physical symptoms are a significant economic burden (3).

The most effective treatments for menopause symptoms are hormone replacement therapies and drugs targeting post-menopausal osteoporosis, such as alendronate and raloxifene (12, 13). While pharmacological treatments have been effective in reducing menopause-related symptoms, these treatments have been associated with additional health risks, including stroke, venous thromboembolism, and cancer (14), demonstrating the need for other alternatives. Non-pharmacological means of reducing symptoms include lifestyle modifications such as exercise (15), which have shown to have positive outcomes through increasing bone mineral density (16) and reducing body fat (17) in postmenopausal women.

Obesity

Obesity is a condition associated with carrying a surplus of fat tissue and is a huge public health concern, as it is both a national and global epidemic affecting people of all ages and races. Over the past decade obesity has maintained its presence as Americans continue to engage in high calorie eating behaviors and a lack of physical activity. Despite significant political and social efforts to reduce or even maintain obesity prevalence, the number of those affected continues to rise. A recent data report from the National Health and Nutrition Examination Survey estimated that as of the years 2011-2014, obesity prevalence was at 36.5% in U.S. adults (20+ years old) and 17% in U.S. adolescents (2-19 years old) (10). Compared with previous years' national health surveys, trends indicate that obesity prevalence has risen since 1999-2000. Accompanying the rise in obesity is significant increases in associated health care costs, which were estimated to be up to $147 billion per year in 2008 (18).

The clinical definition of obesity for adults is having a body mass index (BMI) greater than or equal to 30. BMI is a measure of relative weight (indirect measurement of body fat) and takes into consideration a person's height (m) and mass (kg).

$$BMI = mass/height^2 = kg/m^2$$

While the BMI calculation is simplistic and indirect, and thereby has limitations, it provides a numerical indication that replaces a direct total body fat measurement, which can be difficult or expensive to obtain through methods such as caliper measurements or dual-energy X-ray absorptiometry (DXA) (19).

The negative effects of obesity on human health are rather alarming, as the condition can significantly reduce quality of life (20, 21) and lead to an earlier death (22). Decreased life expectancy is linked to the extensive list of obesity-related chronic medical conditions, including, but not limited to, coronary heart disease, stroke, type II diabetes, and cancer (23). Disruptions in endocrine balance and inflammation due to the large adipose burden, mostly surrounding the abdomen, are largely responsible for the aforementioned secondary diseases. The disease also increases the risk for non-alcoholic fatty liver disease, dyslipidemia, insulin resistance, hypertension, and type II diabetes, in addition to creating a state of chronic inflammation. Obesity can lead to an overall metabolic syndrome, derived from the ectopic fat formation in the pancreas, muscle, and liver tissues, important sites for metabolism. Free-fatty acids also play an important role in the metabolic abnormalities that arise from excess adipose tissue. Increased lipolysis of adipocytes in the visceral compartment releases excess free fatty acids into circulation, which are transported and stored in tissues like the liver and muscle, eventually leading to

insulin resistance (24). Additionally, free fatty acids modulate cytokine production from macrophages, contributing to the overall inflammatory state (25).

Obesity and related complications are some of the leading causes of preventable death (26). Lifestyle intervention through maintaining a healthy diet with limited caloric intake, physical activity, and behavioral therapy are effective in achieving weight loss, which can reduce obesity related complications. A decrease of as little as 5-10% of baseline weight can be associated with health improvements (27, 28). While lifestyle intervention has the potential to be an effective, non-pharmacological treatment modality, many suffering from obesity do not succeed in maintaining clinically meaningful weight loss. Without high-intensity counseling from primary care physicians, compliance is low and patients often do not follow diet and exercise regimens. Alternative expensive and invasive treatments include FDA approved long-term and short-term weight loss drugs (the majority appetite suppressants) (29) and bariatric surgery, the resizing of the stomach to decrease appetite and food consumption.

In modeling obesity for research studies, mouse models are relatively inexpensive and follow the same progression of obesity as observed in the human. There are a range of mutations and dietary models that all impact metabolism differently. Two of the most common models with genetic mutations include mutations in the leptin hormone (*ob*), or the leptin receptor (*db*) genes. These models are robust and obesity and insulin resistance develop early on, however leptin mutations in the human are very rare. A mutation in the *tubby* gene causes a later development of obesity and insulin resistance, along with other complications such as deafness and degeneration of the eye (30). *Carboxypeptidase E* mutation affects the translation of hormones, including proinsulin. This mutation affects the hypothalamus and leads to late-onset obesity (31). Transgenic mouse models consist of a range of gene deletions focused around weight regulation. Knockout models with deletions of the *Mc3r*, *Mc4r*, and *Pomc* genes lead to similar obese phenotypes, although there are many differences between these strains, including susceptibility to obesity and ability to gain weight, timeline for the obese phenotype development, and differences in how the gene deletion affect appetite and metabolism (32). Obesity can also be induced via pharmacological agents, such as in the gold thioglucose mouse model. Many additional mouse models exist, all having varying complications related to the particular gene mutation. Data from transgenic strains or mutation models must be interpreted with care since it may be unclear whether the cause was from the genetic background or obese phenotype.

Diet and levels of physical activity are two primary causes of obesity and manipulating diet in the mouse is a relatively simple way to induce the obese phenotype. A range of diets have been used to develop a similar phenotype and pathogenesis of obesity in the human, however diets drastically range in composition and do not all elicit the same responses in metabolism, which is also true in the human. High-fat diets are the most commonly used diets in murine obesity studies. The fat component can originate from animal lard, beef tallow, plant oils, safflower oil, coconut fat, fish oil or corn oil and the fraction of fat can range from 20%-60% (33). Some diets replace carbohydrate and protein content while others solely add fat content to the diet. In general, high fat diets lead to increased body weight, which is more apparent in younger animals and will lead to hyperglycemia and type II diabetes. Fasting glucose and insulin levels will rise once the obese phenotype is developed. Saturated fat may lead to faster development of insulin resistance while monosaturated and fatty acids have a less severe impact on insulin action (34). High-fat diets also lead to elevated leptin and resistin levels, down regulation of adiponectin, increased triglycerides (although not in fish-oil based diets), impairment of insulin-stimulated glucose uptake in skeletal muscle, and reduced glucose uptake in both white and brown fat (35, 36).

High-carbohydrate diets do not lead to the same robust weight gain and increase in fat depots as seen in high-fat diets. It has even been proposed that diets high in carbohydrates may help prevent obesity (37). Compared to high-carbohydrate diets, high-fat diets have lower satiating effects and the weight gain is due to larger expansion of fat depots needed for energy storage while high-carbohydrate diets have generally lower capacities for glycogen storage. Accumulation of fat in high-carbohydrate diets is likely due to increased glucose oxidation and decline in lipid oxidation, which is why weight gain is more progressive and less severe than diets high in fat (38). Additionally, high-carbohydrate diets do not have as much weight gain in the visceral compartment so complications related to the metabolic syndrome are not as apparent. For these reasons, it is important to bear in mind diet composition when studying the impacts of diet on metabolism. High-fat diets are more robust and better mimic obesity pathogenesis seen in humans.

Type II diabetes

Type II diabetes is characterized by insulin resistance and/or abnormal insulin secretion, and can be a direct consequence of excess fat tissue and metabolic syndrome resulting from obesity. As of 2010, it was estimated that there were 26.8 million adults in the U.S. and 285 million adults worldwide with

diabetes (type I and type II) (39), with type II diabetes accounting for more than 90% of worldwide cases (40). Diabetes prevalence is likely to further increase by 2030 (39). It is estimated that those with diabetes spend 2.3 times more in health care than if they did not have the disease and on average spend approximately $7,900 per year in diabetes-related health care costs. Additionally more than one of every five dollars spent on health care in the U.S. is attributed to diabetes-related treatment (41). Costs include everything from inpatient care to prescription medications and diabetes supplies.

Type II diabetes is the leading cause of kidney failure, causes blindness, increases stroke rate by 1.5 times, and accounts for 60% of all non-traumatic lower-limb amputations. Additionally, in 2010, it was the seventh leading cause of death in the U.S. (42). Intensive lifestyle intervention has been shown to prevent and/or delay diabetes (27) and a variety of pharmacological treatments are available for improving insulin sensitivity. Similar methods for treating obesity are effective in improving the diabetic condition, including weight loss drugs and bariatric surgery.

Role of adipose tissue and its interaction with the musculoskeletal system

Fat tissue is an inhomogeneous energy storage that contains a range of cell types including adipocytes, fibroblasts, endothelial cells, immune cells, and adult stem cells (mesenchymal and hematopoietic) and their progenitors. It is an active endocrine organ that modulates adipokine (*e.g.* leptin, resistin, adiponectin) and inflammatory cytokine (*e.g.* TNFα, IL-6, TGFβ) release (43), impacting both local and systemic environments. Fat tissue plays a role in a range of physiological processes, including immune function (44), inflammation, glucose homeostasis, and energy balance (45) and its signaling directly impacts other tissue systems (46). Conditions in which excess adipose tissue and symptoms of metabolic syndrome are present are believed to be driven by a chronic systemic inflammatory state (47) largely due to adipose endocrine signaling.

Fat tissue can have direct impact on skeletal muscle (48) and excess adiposity can result in ectopic fat formation in the musculature. High levels of intramuscular fat are strong indicators of decreased muscular function and mobility (49). Additionally, the accumulation of fat metabolites within the musculature results in metabolic abnormalities, including interrupted insulin signaling (50) and glucose transport activity (51). These elevated levels of intramyocellular lipids are a direct indication of insulin sensitivity and/or resistance (52), which is commonly seen in obesity and can lead to type II diabetes. Other alterations in the skeletal muscle microenvironment, including local increases in

adipogenesis, may negatively impact resident stem cell populations (satellite cells, MSCs, and fibro/adipogenic progenitors) through altering their function, proliferation or differentiation.

In close proximity to skeletal muscle, bone tissue is an endocrine target in which certain processes and properties (*e.g.* bone remodeling, mass, and structure) are regulated by adipokine release such as leptin from fat tissue (53, 54). Recent clinical evidence suggests that the obese state has a negative impact on bone qualities and increases risk of fracture in lower extremities (55) and the hip (56). In animal models, high-fat diet has been shown to alter bone structure, the bone marrow microniche, and bone marrow cell populations (57, 58). High levels of bone marrow adiposity observed in the aged population (59), obesity (60), and post-menopausal women (61) can potentially alter marrow homeostasis and crowd cell resident marrow populations, including immune and stem cells, and may have negative effects on hematopoiesis resulting from paracrine signaling (62).

Fat, bone, and muscle cells all share a common progenitor, the mesenchymal stem cell (MSC), which has a concentrated population residing in the bone marrow cavity. Here exists a dynamic environment with access to a complex network of vasculature, allowing for exchange of endocrine signals and passage of cells into circulation (63). While fat and muscle tissue both have their own tissue resident populations of MSCs, bone marrow derived-MSCs may contribute to the formation and function of these tissues. Considering the complex signaling cascades initiated by adipose tissue, the obese condition may alter bone marrow stem cell migration and contributions of bone marrow-derived cells to tissues outside of the bone.

Exercise as a treatment modality

Exercise is a desirable treatment modality for obesity-associated metabolic disorders because it's highly effective and a non-pharmacological means of catabolizing fat tissue and building muscle and bone. The public health recommendation of physical activity for adults is to perform a minimum of 150 minutes of moderate-intensity aerobic exercise per week (or other variations) to achieve health benefits (64). Various exercise regimes, from walking to high intensity exercise, have been clinically shown to provide health benefits related to the prevention or treatment obesity/type II diabetes (65-67) and building muscle and bone tissues (68, 69). However, exercise is not always effective as a weight loss intervention. While short-term weight loss may be achieved through exercise, patients often relapse and regain the weight that was lost. Importantly, regular physical activity is critical for maintaining long-term weight loss (70), which will in turn prevent the recurrence of obesity-related health complications.

Compliance with exercise intervention is also a major concern (71), as it is often the case that this particular intervention is considered too strenuous or time consuming for patients to continually incorporate this type of treatment into a permanent lifestyle change. Lastly, successful exercise interventions in the clinic are often coupled with dietary restrictions (27), making it difficult to tease apart the benefits of exercise from diet. While exercise is seemingly effective in catabolizing fat and building muscle and bone, its effectiveness in long-term weight maintenance is likely to require a simultaneous healthy diet and appropriate caloric consumption.

While exercise in many cases may be a desirable method for treating metabolic disorders, certain groups of people may require safer or less strenuous alternatives. The aging or those with osteosarcopenia or osteoporosis are at risk of falls (72) and fracture (73, 74). The recommended moderate-intensity exercise may be undesirable bearing in mind a fracture in the elderly population can significantly reduce quality of life both socially and physically (75). Obese patients may be bedridden or otherwise physically incapable of exercise due to their size. Additionally, those who suffer from motion restricting musculoskeletal disorders or severe musculoskeletal pain may find a less intensive alternative to exercise attractive.

Low intensity vibration

Low intensity vibration (LIV) is a low-magnitude (<1*g* where *g* is earth's gravitational field at 9.81 m/s^2), high frequency (30-90 Hz) mechanical signal typically delivered through the lower extremities and transmitted through the skeleton. LIV biases stem cell differentiation towards osteoblastogenesis and away from adipogenesis (76), making this intervention a non-invasive means of building bone and muscle and preventing the formation of fat - and giving it the potential to be used as surrogate for exercise. Compared to many exercise regimes, LIV can be time consuming and easier to use, and has the potential to increase compliance. LIV is typically delivered in brief (<30 min) bouts five to seven days a week and only requires a person to stand on the plate for the duration of the treatment. Most importantly, the delivery of LIV is safe for up to several hours of use each day as, designated by the International Standards Organization (77, 78). LIV does not put people at risk of fracture in the same way that moderate-intensity exercise may, and, unlike high intensity vibration, LIV is not damaging to the skeleton nor does it cause musculoskeletal pain.

The use of LIV in the clinic has prevented post-menopausal bone loss (79) and improved bone mineral density in children with disabling musculoskeletal conditions (80). Benefits of LIV have also been noted in animal models- LIV has been shown to be anabolic to both bone and skeletal muscle (81), decrease type II diabetes risk factors, and prevent bone marrow MSC differentiation into the adipogenic lineage (82). These studies support the idea that LIV can be utilized as a protection mechanism in both the musculature and bone marrow in the presence of a high-fat environment though promoting bone and muscle anabolism. The frequency and magnitude of whole body vibration are inconsistent across research studies and there is no consensus on the signal parameters that are most effective in preserving bone mass, muscle mass, or preventing adipogenesis. The most effective signal parameters are most likely to be specific to the tissue or cell type being targeted.

Figure 1: LIV platform

LIV is a subtle signal that is not always affective in improving the musculoskeletal system. Whole body vibration treatment given to an elderly population resulted in no preservation of bone mineral density (83) and mechanical intervention in general is thought to be less effective in aged populations due to the decreased responsiveness of stem cells to mechanical signals. Similarly, severe systemic burdens, such as menopause or obesity, may alter cell responsiveness to mechanical signals, and therefore the ability of LIV to bias stem cell differentiation away from adipogenesis. Slatkovska *et al*. also saw no improvement in bone outcomes in postmenopausal women subject to vibration treatment for 12 months (84), however, these subjects did not all have osteoporotic bone at the start of the study. Effectiveness of vibration treatment in improving bone may in part be reliant upon the initial condition of the subjects, and LIV may be more effective in humans and animals that have a pre-existing bone or muscle detriment.

LIV is typically delivered to the skeleton through a vibrating platform (Figure 1). The device is slightly larger than a standard bathroom scale and can be adjusted to the desired frequency and magnitude. When standing on the vibrating plate, the signal is transmitted through the legs and up through the axial skeleton, reaching all the way the skull. For use with small research animals, a partitioned box is set directly on the platform, allowing for simultaneous exposure of up to ten mice. The partitions for the mice are of a size that does not permit animals to move around, allowing the LIV

signal to be delivered through both the hind and forelimbs. An attached accelerometer provides constant monitoring to ensure the steady and consistent delivery of the sinusoidal signal.

Overall objective and hypotheses

The overall goal of this dissertation is to investigate the disruption of a fat insult on the musculoskeletal system and the contributions of bone marrow-derived cells to tissues other than bone, such as visceral fat and skeletal muscle. Additionally, we aim to investigate the use of LIV to protect these tissue systems from obesity-related complications.

Global Hypothesis: Bone marrow cells contribute to the formation of bone, fat, and muscle, a commitment disrupted by systemic distress and normalized by mechanical stimulation.

Sub-hypothesis 1: Bone marrow cells migrate and engraft in tissues outside of the bone, including visceral fat and muscle, a commitment altered by high-fat diet.

Sub-hypothesis 2: A system fat insult impairs skeletal muscle satellite cell populations, upregulates indices of fat in skeletal muscle, and impairs insulin signaling.

Sub-hypothesis 3: Mechanical stimulation protects satellite cell proportions, normalizes stem cell migration, and mitigates the accumulation of fat in murine models with a systemic fat insult.

Specific Aim 1*: Investigate the impact of ovarian hormone depletion and mechanical stimulation treatment on skeletal muscle satellite cells and the microenvironment using a murine model of menopause*. C57BL/6J mice were ovariectomized and subjected to LIV or sham operated and handled for six weeks. Adipose and skeletal muscle tissues were evaluated on the organ, tissue, cellular, and molecular levels. Muscle stem cell populations were be quantified via flow cytometry and local adipogenic and myogenic gene expression were evaluated. Abdominal fat and biochemical indices of adiposity were quantified using *in vivo* micro-computed tomography and serum assays at euthanasia.

Hypothesis 1a: Ovariectomy will reduce satellite cell proportions in the muscle, increase systemic adiposity, and increase adipogenic gene expression in the muscle

Hypothesis 1b: Mechanical stimulation will protect satellite cell proportions in the muscle, mitigate systemic fat accumulation, and reduce adipogenic gene expression in the muscle

Specific Aim 2*: Investigate the impact of high-fat diet and mechanical stimulation on satellite cells, skeletal muscle adiposity, and insulin signaling using a murine model of pre-diabetes.* C57Bl/6J mice were fed a high-fat or regular diet for ten weeks and subjected to LIV or sham handling for eight weeks.

Muscle specific satellite cells were quantified via flow cytometry. Intermuscular adiposity and indices of impaired insulin signaling were quantified on the tissue, protein and genetic levels via histological staining, ELISA, and RT-qPCR assays at euthanasia. Systemic indication of pre-diabetes was measured using glucose tolerance testing.

Hypothesis 2a: A systemic high-fat insult will reduce satellite cell proportions, increase intermuscular fat, and upregulate the molecular pathway leading to insulin resistance.

Hypothesis 2b: Mechanical stimulation will protect satellite cell proportions, mitigate intermuscular fat accumulation, and downregulate the molecular pathway leading to insulin resistance.

Specific Aim 3*: Investigate the impact of high-fat diet and mechanical stimulation on bone marrow cell migration and engraftment in a murine model of pre-diabetes*. C57Bl/6J mice were fed a high-fat or regular diet for ten weeks and subjected to LIV or sham handling for eight weeks of treatment. Using GFP cell tracking via whole bone marrow transplantations, bone marrow, visceral fat, muscle, and blood tissues were evaluated for bone marrow cell migration and engraftment using flow cytometry. MSCs were also quantified with flow cytometry in the bone marrow and visceral fat. Bone marrow status was evaluated for the encroachment of adipocytes using histological analysis, and differentiation markers of MSCs were assessed with gene expression assays.

Hypothesis 3a: Bone marrow cells contribute to the formation of fat and muscle tissues through recruitment from the marrow, a process that is accelerated by high-fat diet. Bone marrow-derived MSCs recruitment is accelerated to the visceral fat pads in high-fat diet while the bone marrow niche is encroached by adipocytes.

Hypothesis 3b: Mechanical stimulation normalizes migration of bone marrow-derived cells to tissues outside of the bone and mitigates bone marrow adiposity caused by high-fat diet.

CHAPTER 2: DIMINISHED SATELLITE CELLS AND ELEVATED ADIPOGENIC GENE EXPRESSION IN MUSCLE AS CAUSED BY OVARIECTOMY ARE AVERTED BY LOW-MAGNITUDE MECHANICAL SIGNALS[1]

[1]Originally published as: Frechette, D. M., Krishnamoorthy, D., Adler, B. J., Chan, M. E., and Rubin, C. T. (2015) Diminished satellite cells and elevated adipogenic gene expression in muscle as caused by ovariectomy are averted by low-magnitude mechanical signals. Journal of applied physiology 119, 27-36

INTRODUCTION

Menopause, the age-associated loss of ovarian hormones (estrogen, activin, inhibin, follistatin, etc.), accelerates degeneration of the musculoskeletal system, leading to osteoporosis (7), lower muscle mass (85), reduced strength (86), and altered tissue composition (87). Reduced bone mass increases fracture risk (73) while concurrent weakening of the muscle increases the risk of falls (72), in aggregate increasing the vulnerability of the musculoskeletal system. While sarcopenia (diminishing muscle mass) inherently plays a role in the deterioration of muscle quality in postmenopausal women (6), the progressive decline of estradiol (E2), independent of aging, is believed to have direct consequences on skeletal muscle (88, 89). The absence of this hormone can have detrimental effects on muscle strength, demonstrated by decreased force generating capacity in the muscle of ovariectomized mice (90).
In parallel with a diminished muscle phenotype, menopause is related to an accumulation of abdominal adiposity, particularly in the visceral compartment (5), leading to a higher incidence of obesity in post-menopausal women (9). Increased indices of skeletal muscle fat in post-menopausal women (87) and ovariectomized rats (91) have the potential to negatively affect muscle quality. The changes in muscle composition that occur after menopause can be significant, as women between the ages of 65-80 have approximately twice the amount of non-contractile muscle tissue than women aged 23-57 (92). Significant adipocyte accumulation has also been observed in other conditions where muscle integrity is compromised (93-95). Additionally, ovariectomy has resulted in impaired recovery capabilities of atrophied muscle mass in rats (96), which may be related to fat encroachment and a subsequent disruption of muscle homeostasis and stem cell health.

The ability of skeletal muscle to regenerate following injury is highly dependent on the activation of satellite cells (97) and related gene expression (98) in the muscle tissue. Satellite cells are skeletal muscle stem cells that lie above the sarcolemma and below the basal lamina of muscle fibers (99). As the frontrunners of skeletal muscle repair mechanisms, they have the ability to activate from a quiescent state in healthy tissue (100) and proliferate and/or differentiate into myoblasts during regeneration. Satellite cell activity is affected by circulating estrogen levels. Enns and Tiidus showed increased satellite cell activation and proliferation following downhill running with estrogen supplementation in ovariectomized rats (101). Therefore, it is plausible that the *lack* of estrogen experienced during menopause or following ovariectomy will hamper satellite cell activation and/or proliferation, impairing the overall stem cell pool. Because the source of intramuscular adipocytes is variable (e.g., mesenchymal progenitors (102), fibro/adipogenic progenitors (103), it has been proposed

that satellite cells also possess the ability to commit to an adipogenic lineage (104-106); and thus suggests that the increased adiposity resulting from ovariectomy may in part be a result of biased stem cell differentiation towards an adipogenic fate. Retaining satellite cells may be achieved by suppressing the accumulation of intramuscular fat, ultimately helping to maintain muscle homeostasis and regenerative potential.

As it is well accepted that exercise in general, and mechanical signals in particular, are anabolic to muscle (107) and catabolic to fat (108), it represents a non-drug means to address susceptibility to both obesity and compromised muscle following menopause. As fat encroachment into muscle may arise in part by stem cell populations committing to adipogenesis rather than myogenesis, mechanically biasing stem cell fate towards muscle and away from fat may aid in retaining muscle quality. As a potential adjunct to exercise, mechanical stimulation in the form of low intensity vibration (LIV; <0.4g where g is earth's gravitational field at 9.81m/s2) has been shown to provide a signal that is both anabolic to muscle (81) and biases stem cell differentiation away from adipogenesis (109). As proposed, LIV may be beneficial in protecting muscle homeostasis after menopause by reducing intramuscular adipogenic gene expression and maintaining satellite cell populations that are likely to be negatively affected by the lack of E2 and/or secondary complications of ovariectomy.

This study tested the hypothesis that ovariectomy will compromise skeletal muscle satellite cell populations, increase abdominal adiposity and muscular adipogenic gene expression, whereas daily bouts of LIV would protect the muscle from increased adipogenic gene expression and muscle stem cell impairment. Using an ovariectomized (OVX) C57BL/6 murine model, we identified the effects of ovariectomy on satellite cell populations, adipogenic and myogenic gene expression within the skeletal muscle, and determined if LIV could mitigate this deterioration.

MATERIALS AND METHODS

Experimental design

All experimental procedures were reviewed and approved by the university's Institutional Animal Care and Use Committee. Eight week old, female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) received an ovariectomy (n = 20) or sham surgery (n = 10). Following two weeks of recovery and acclimation, a weight-matched, randomized MATLAB script was used to divide the ovariectomized mice into two groups, ovariectomized (OVX, n = 10) and ovariectomized + vibration treatment (LIV, n =

10). Animals receiving a sham surgery were grouped as age-matched controls (AC, n = 10). Experimental treatment and baseline measurements began two weeks post-surgery at ten weeks of age; this time point is considered the start of the experimental protocol (t = 0 wk). Six weeks following time zero, all 30 mice were anesthetized with isoflurane and euthanized via cervical dislocation (eight weeks post-surgery, 16 wk of age) (Figure 2). End point *in vivo* measurements were taken two days prior to euthanasia. Mice were single housed at 21°C and fed a standard rodent chow diet (LabDiet Prolab RMH 3000, Purina Mills, St. Louis, MO) with *ad libitum* access to food and water.

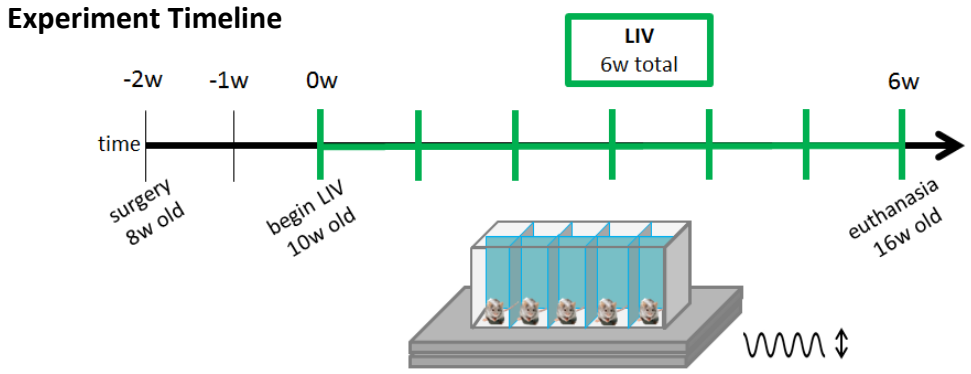

Figure 2: Experimental timeline of mice ovariectomized at 8 wk of age with LIV treatment beginning 2 wk post-surgery, at 10 wk of age, the start of the experimental protocol (t = 0 wk). All mice were euthanized 6 wk following experiment start (8 wk post-surgery).

Mechanical stimulation protocol

Animals receiving mechanical stimulation treatment were subject to low magnitude, high frequency, vertically oscillating vibrations (0.3 *g* @ 90 Hz, where *g* is earth's gravitational force, or 9.81 m/s^2). LIV was administered for 15 min/day, 5 day/week for six weeks. Mice were placed into a partitioned box, which was centered on the vibration plate. AC and OVX were sham handled and placed in the same box, but on an inactive device. The last vibration treatment was administered the day prior to euthanasia.

In vivo micro-computed tomography (µCT) measurements

Total abdominal adiposity was quantified *in vivo* at both baseline (t = 0w) and endpoint (t = 6w) using x-ray µCT (vivaCT 40; Scanco Medical Inc., Brüttisellen, Switzerland). Animals were anesthetized with 2% isoflurane inhalation and stabilized in a custom made, foam scanning bed. Scans of the abdomen (76 µm isotropic resolution, 45kV intensity, 133µA current) were evaluated from the L1-L5

vertebrae (approximately a 20 mm region). Visceral and subcutaneous adipose tissues in this region were segregated using a well-established segmentation script (110). Parameters including visceral adipose tissue (VAT), subcutaneous adipose tissue (SAT), and total adipose tissue (TAT: subcutaneous + visceral,) were reported by volume (mm^3).

Serum biochemical markers

Biochemical markers were quantified in serum (stored at -80°C until analysis) from blood collected at euthanasia. Serum leptin and adiponectin levels were evaluated with commercial ELISA kits (Mouse Leptin ELISA and Mouse Adiponectin ELISA, EMD Millipore, St. Charles, MO) and standard curves were determined according to the manufacturer's protocols.

Quantitative Polymerase Chain Reaction of skeletal muscle gene expression

Following euthanasia, the soleus muscle was excised and stored in RNAlater at 4°C and then moved to -20°C for long-term storage. RNA was isolated using a commercial spin kit for skeletal muscle (RNeasy Fibrous Tissue Mini Kit, Qiagen, Austin, TX). RNA purity was quantified using 1 µl mRNA and a nanodrop spectrophotometer (NanoDrop 1000 Spectrophotometer, Wilmington, DE). cDNA conversion was performed using a high capacity reverse transcription kit (Applied Biosystems, Foster City, CA) and amplification was conducted using TaqMan Gene Expression Assays and TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA). Expression levels of genes critical to adipogenesis, including PPARγ, FABP4, PPARδ, FoxO1, Adipoq, and those critical to myogenesis, including Pax7, MyoD, Myf5, IGF-1, Mstn were analyzed and compared to housekeeping gene β-actin. Relative expression was compared against intact control animals using the delta-delta Ct method of analysis.

Flow cytometric analysis of satellite cells

To evaluate satellite cell status in skeletal muscle, the left gastrocnemius and quadriceps muscles were harvested immediately following euthanasia, minced, pooled for each animal, and stored in Dulbecco's Modified Essential Medium, DMEM, (Gibco, Carlsbad, CA) with 1% penicillin/streptomycin. Satellite cell isolation was adapted from a previous protocol (111) with changes as noted. Tissue fragments were digested with 2 mg/ml collagenase type II, 1.2 units/ml trypsin and 2 mM $CaCl_2$ in PBS. After trituration, samples were neutralized with DMEM supplemented with 1% penicillin/streptomycin and 15% horse serum (Thermo Scientific). Mononuclear cells were filtered with 40 µm nylon cell strainers (BD Biosciences, San Diego, CA). Samples were centrifuged at 2000 RPM for 5 minutes and

resuspended in 1x lysis buffer (Pharm Lyse, BD Biosciences, San Diego, CA) for 5 minutes at room temperature. Cells were again centrifuged, re-suspended in DMEM supplemented with 1% penicillin/streptomycin, and counted. $1x10^6$ cells were removed from each sample for staining. Samples were incubated for 45 minutes in the dark at room temperature with the following antibodies: FITC-conjugated CD34 (eBioscience, Inc., San Diego, CA), APC-conjugated CD45/CD31/Sca-1 (eBioscience, Inc., San Diego, CA), and PE-conjugated Integrin alpha 7 (R&D Systems, Minneapolis, MN). Satellite cells were defined as CD45-/CD31-/Sca-1-/Integrin alpha 7+ with the reserve population as CD34+, and have previously been demonstrated to yield a pure satellite cell population (111). Flow cytometric analysis was conducted using the FACSCalibur system (BD, San Jose, CA) at 200,000 events per sample. Data was analyzed with FlowJo V7.2.5 (TreeStar Inc., San Carlos, CA).

Statistical Analyses

Comparisons between groups were made using a one-way analysis of variance (ANOVA) and Tukey post-hoc test, while comparisons between time points were made with a paired Student's T-test. Correlations were evaluated using two-tailed Spearman rank correlations. All presented data shows the mean ± standard deviation. All data were considered significant when $p \leq 0.05$ and analyses were conducted with SPSS 14.0 software (SPSS Inc., Chicago, IL).

RESULTS

Ovariectomy effects on body mass, tissue mass, and food intake

Ovariectomy induced menopause was confirmed at euthanasia by a significant reduction in uterine mass (data not shown). As expected, ovariectomy resulted in an increase in body weight: Two weeks following surgery and immediately prior to LIV treatment, ovariectomized animals (both OVX and LIV) were 10-12% ($P < 0.05$) heavier than age-matched sham-operated intact controls (AC; Figure 3A). Eight weeks following surgery and after six weeks of LIV, the differences in body weight between AC and OVX remained at 12%, while LIV mice were not significantly different from OVX, indicating these mechanical signals had no impact on body weight (Figure 3A). No differences in food consumption were detected between the three groups, suggesting the weight gain resulting from OVX was driven by metabolic and/or hormonal factors. The soleus muscle and gonadal fat pad extracted and weighed at euthanasia had greater masses in both OVX and LIV groups compared to AC (OVX soleus: +19%, LIV

soleus: +18%, both P < 0.01; OVX gonadal fat pad: +83%, LIV gonadal fat pad: +58%, both P < 0.05) (Figure 3B and C).

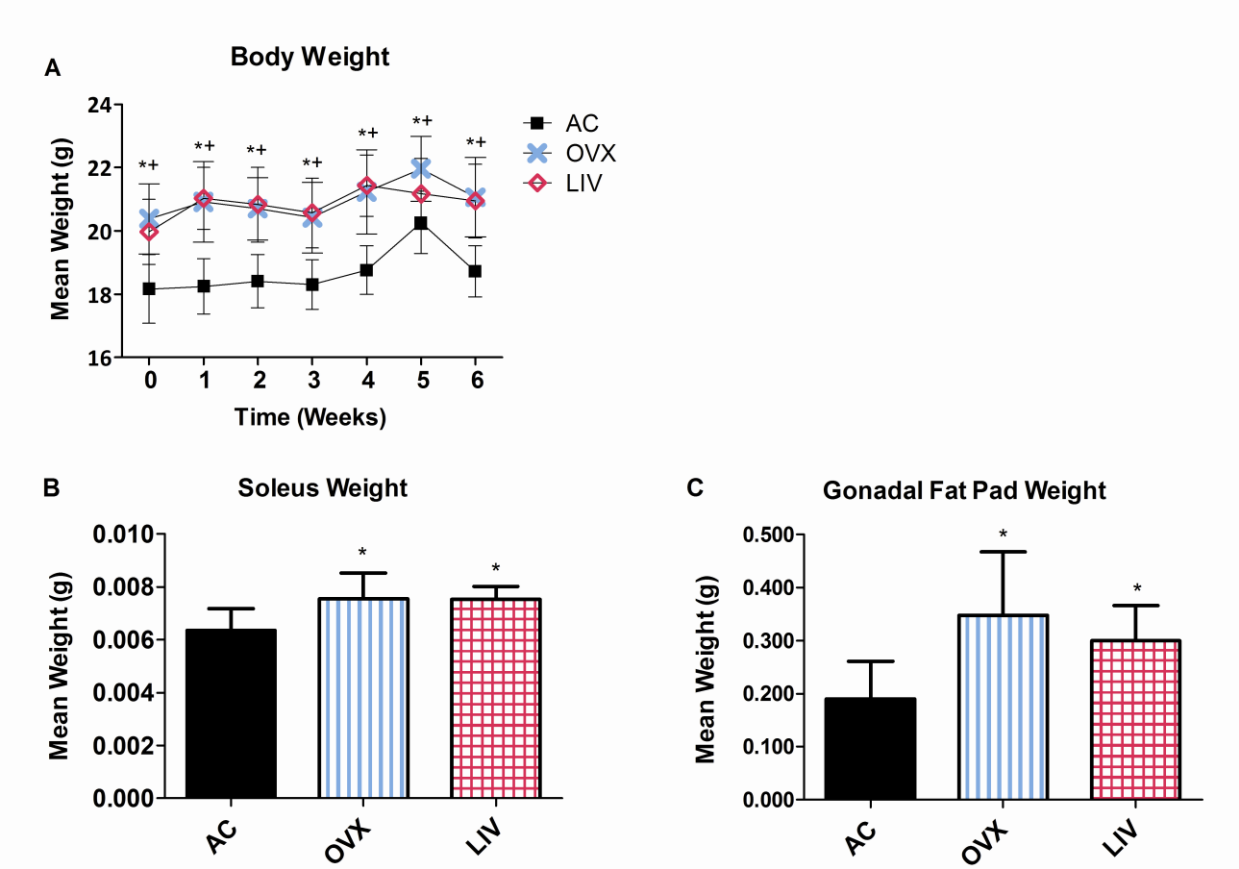

Figure 3: A: Body weights from week 0 (two weeks post-ovariectomy) to week 6. Differences in weight had already occurred due to the ovariectomy surgery two weeks prior to study baseline. *P < 0.05 OVX compared to AC; +P < 0.05 LIV.

Ovariectomy increases serum adipokine concentrations

At sacrifice, adipokine levels were elevated in OVX, with serum leptin up by +220% (P < 0.01) compared to AC, while LIV showed no significant differences from AC (+117%, P > 0.05) (Figure 4A). Leptin levels were positively correlated to TAT volume across all groups at time of euthanasia (P < 0.01, r_s = 0.68, r^2 = 0.46) (Figure 4B). Similarly, serum adiponectin was elevated in OVX animals compared to AC (+79%, P < 0.05), while the increase in LIV mice was not significantly different from AC (+72%, P > 0.05) (Figure 4C). No differences were detected between groups in the leptin/adiponectin ratio (LAR) (P > 0.05) (Figure 4D), but these values were positively correlated to TAT volume (P < 0.05, r_s = 0.40, r^2 = 0.16) (Figure 4E).

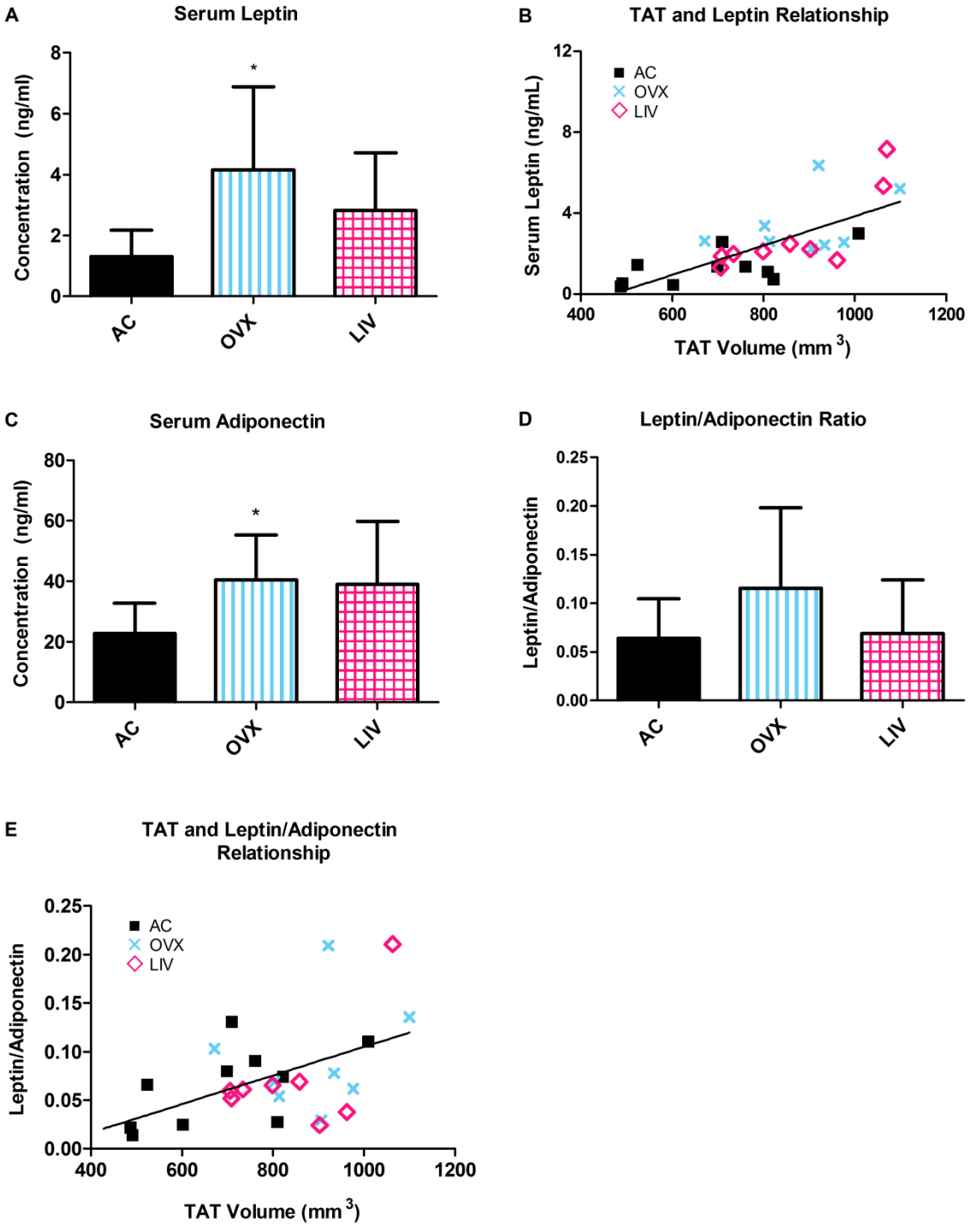

Figure 4: Serum adipokine levels measured at euthanasia. A: Leptin levels measured with ELISA. B: Leptin levels correlated to TAT volume. Linear regression shown. C: Adiponectin levels measured with

ELISA. D: Leptin/adiponectin ratio (LAR). E: LAR correlated to TAT volume. Linear regression shown. *P < 0.05 compared to AC.

Abdominal adiposity is increased by OVX while LIV trends towards mitigated adipose accretion

At the study baseline, two weeks post-ovariectomy and immediately before LIV, adipose distributions in both OVX and LIV groups were significantly different from AC, while there was no difference in TAT at this point in time (Figure 5B). VAT represented 19% of TAT in both OVX and LIV groups at baseline compared to 26% in AC ($P \leq 0.05$), while SAT represented 81% of TAT in both OVX and LIV, as compared to 74% in AC ($P \leq 0.05$).

Over the course of the study, OVX animals gained 43% in TAT ($P < 0.001$) while LIV gained 21% ($P = 0.01$) and AC, +7% (Figure 5B). The TAT percent change from baseline for each individual animal in OVX mice was 44% greater than AC ($P < 0.05$), while the 23% increase in mice subject to LIV was not significantly different than AC (Figure 5C). After 6 weeks, both OVX and LIV animals had greater subcutaneous fat volumes (658 ± 124 mm^3 and 621 ± 58 mm^3, respectively) compared to AC (507 ± 93 mm^3; $P < 0.01$, $P < 0.05$, respectively). OVX animals indicated a trend towards greater VAT volume compared to AC, with a 70% increase by the study conclusion ($P = 0.06$), while LIV animals only had a 33% increase (Figure 5D). Compared to baseline, VAT accumulation increased in ovariectomized animals 125% ($P < 0.001$), as compared to 70% ($P < 0.01$) with LIV, while control animals gained 1.4% VAT over this same period of time (Figure 5D).

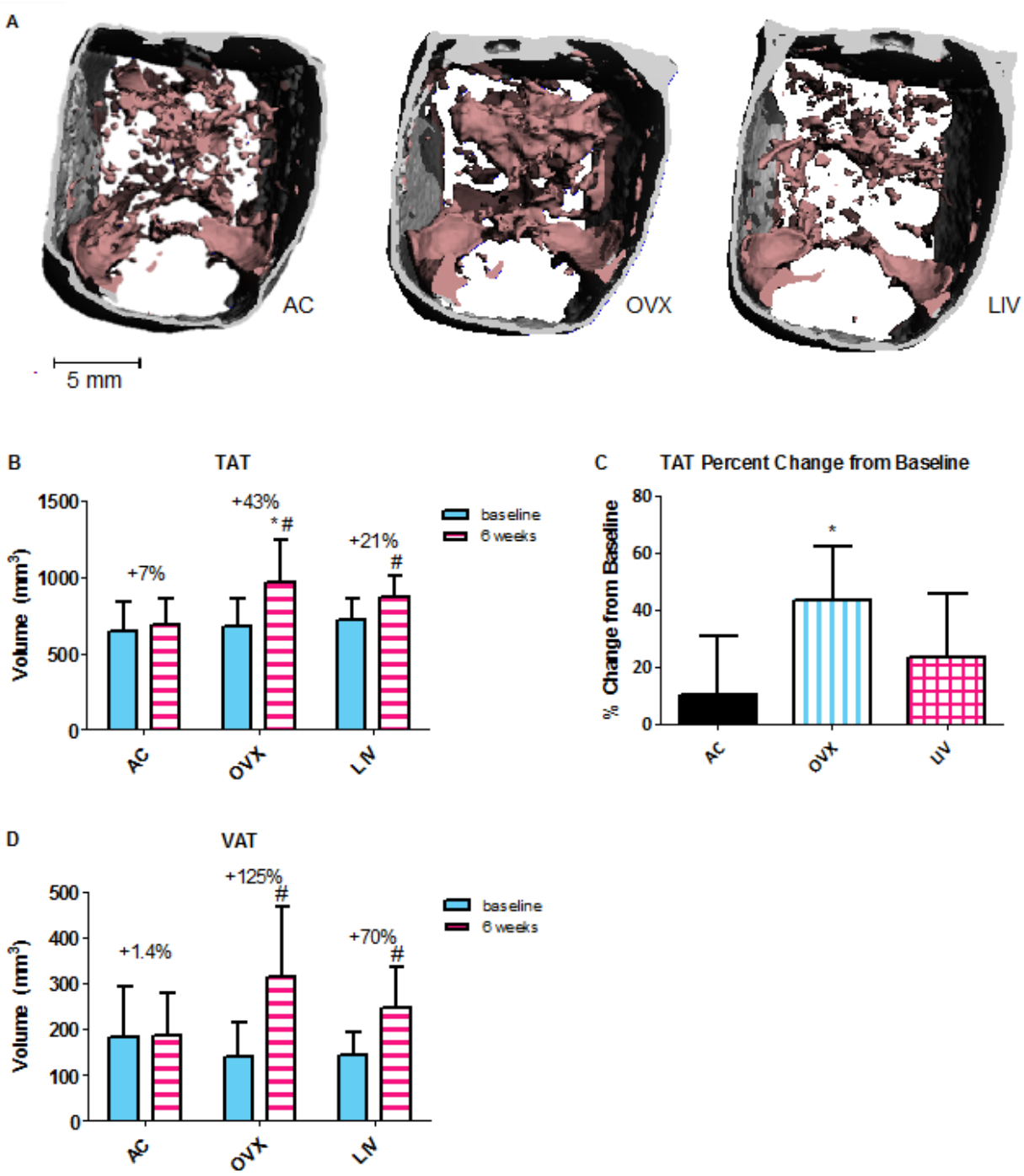

Figure 5: In vivo µCT three dimensional reconstructions (76 µm resolution) of transverse abdominal sections depicting endpoint (after 6 wk of LIV treatment) TAT. B: TAT volume. Percent increases shown are the differences between the group's average TAT volume at baseline versus the group's endpoint volume. C: TAT volume percent change was calculated for each individual animal from its own baseline value, which were all used to obtain a group average. D: VAT volume. *P < 0.05 compared to AC at particular time point. #P < 0.05 compared to baseline measurement.

OVX increases adipogenic gene activity in muscle

PPARδ and PPARγ increased 120% and 78%, respectively, in the soleus muscle of OVX as compared to AC ($P \leq 0.001$, Figure 6). In contrast, expression levels of PPARδ increased by 27% and PPARγ decreased by 15% in LIV, as compared to AC ($P > 0.05$), representing a differential expression of LIV relative to OVX of -42% and -52%, respectively ($P \leq 0.001$). Compared to AC, FABP4 was upregulated in OVX by 93% ($P < 0.001$), and downregulated in LIV by 46% as compared to OVX ($P < 0.001$). Similarly, FoxO1 was upregulated by 113% in OVX compared to AC ($P < 0.001$), while LIV reduced this expression by 59% compared to OVX ($P < 0.001$). No differences in Adipoq expression levels were detected.

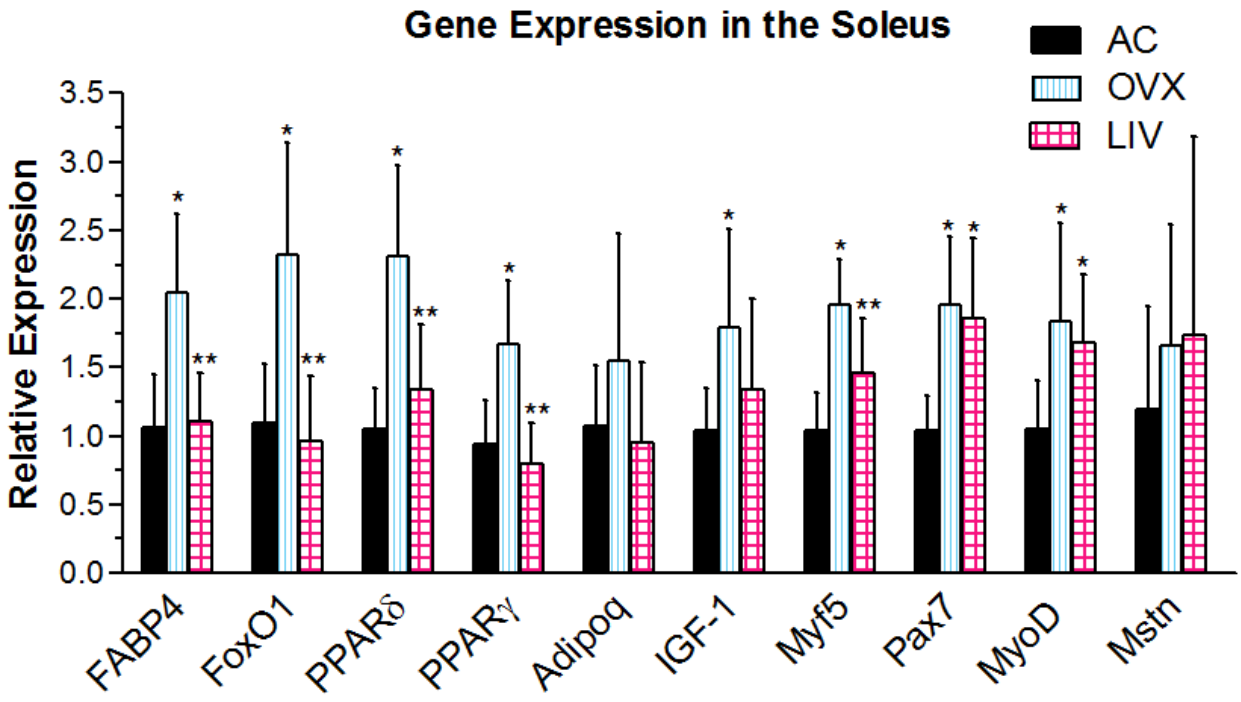

Figure 6: Gene expression analysis in the soleus muscle. Adipogenic (FABP4, FoxO1, PPARδ, PPARγ, Adipoq) and myogenic (IGF-1, Myf5, Pax7, MyoD, Mstn) genes were measured. Analyses were conducted using Real-time PCR and the delta-delta Ct method of analysis. *$P < 0.05$ compared to AC, **$P < 0.05$ compared to OVX.

OVX modulates myogenic gene expression in the soleus

Pax7 and MyoD were upregulated in both the OVX and LIV mice compared to AC, with Pax7 up 89% ($P < 0.001$) and 70% ($P < 0.01$), and MyoD elevated by 74% ($P < 0.01$) and 60% ($P < 0.05$), respectively (Figure 6). Expression levels of Myf5 were elevated in OVX compared to AC by 89% ($P < 0.001$), while LIV was 26% lower than OVX ($P < 0.05$). IGF-1 was upregulated in OVX by 72% ($P < 0.05$)

compared to AC, while changes in LIV (+28%, $P > 0.05$) were not significantly different from AC. Lastly, no differences were detected in Mstn expression levels.

OVX compromises satellite cells populations

Satellite cell populations measured in the pooled gastrocnemius and quadriceps muscles (Figure 7A-B), positive for Integrin alpha 7 and negative for markers CD31, CD45 and Sca-1 (111), were 21% lower in OVX compared to AC ($P < 0.01$; Figure 7C). In contrast, there were no significant differences between AC and LIV (-11%; $P > 0.05$). This relationship is consistent in the proportion of reserve satellite cells in the muscle tissue, as indicated by the positive marker CD34 (111). OVX mice had a 28% reduction ($P < 0.01$) in percent CD34+ satellite cells as compared to AC, while LIV mice had proportions reduced by 17% ($P > 0.05$; Figure 7D). LIV was not different than OVX in either cell population. No differences were measured in the number of CD34- cells (data not shown). Additionally, total cell number was not different between any of the three groups, indicating true changes in satellite cell populations (data not shown).

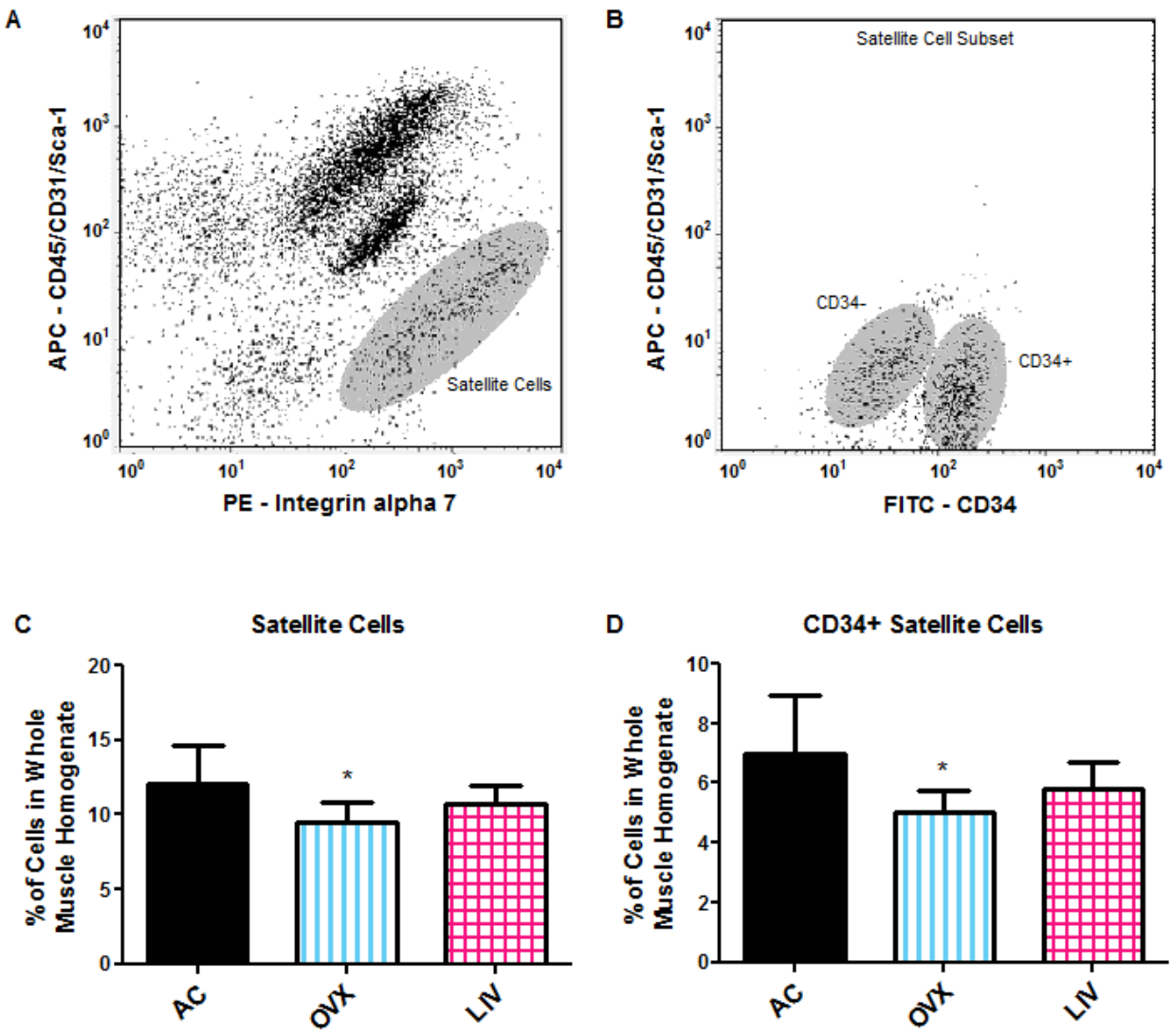

Figure 7: Satellite cell quantification in the gastrocnemius and quadriceps muscles quantified via flow cytometry. A. Flow cytogram representing the isolated satellite cell population negative for APC (CD45/CD31/Sca-1) and positive for PE (Integrin alpha 7). B. Flow cytogram showing a satellite cell subset depicting both FITC (CD34) positive and negative populations. C: The percentage of satellite cells (CD45-/CD31-/Sca-1-/Integrin alpha 7+) in the hind limbs. No differences were measured in total cell number. *P < 0.01 compared to AC. D: The proportion of reserve satellite cells in the hind limbs, positive for CD34. *P < 0.01 compared to AC.

DISCUSSION

Ovariectomy in the mouse was used to model phenotypic changes that follow menopause in the human, with a specific focus on the rapid escalation of systemic adiposity and changes in muscular composition that parallel estrogen deficiency. It is well established that OVX in mice increases

susceptibility to obesity (112, 113), and the data reported here indicate that the transformation in body habitus occurs within two weeks following ovariectomy. While estrogen depletion resulted in a higher average body mass of 11% compared to controls only two weeks post-surgery, the following six weeks resulted only in an additional 1.5% increase relative to controls. Certainly, when considering the consequences of OVX on the mouse, the reality that this response happens so rapidly must be recognized. In retrospect, the introduction of the LIV signal – or any intervention - to this model beginning two weeks post-surgery, rather than immediately after surgery, would appear to constitute more of a test of 'reversal of' rather than 'protection from' the impact of OVX.

LIV has previously been shown to suppress adipogenesis in murine models of diet induced obesity (82, 109), and the OVX data presented here indicate trends toward this mitigation. However, when comparing gonadal fat pad weights and abdominal adiposity as quantified by μCT, the data suggest LIV continues to suppress adiposity, despite no effects on total body mass. While both OVX and LIV mice had significant increases in adiposity from baseline (2w following OVX), TAT and VAT volume increases in LIV mice at the conclusion of six weeks were approximately half of that measured in the OVX group. And certainly, the challenges of catabolizing extant adipose tissue that existed after two weeks are different from that of suppressing the formation of fat.

The idea that adipogenesis is being mitigated by these low level mechanical signals is further supported by differences in serum adipokine levels measured at the end of protocol. Circulating leptin levels were 220% higher in OVX relative to age matched controls, similar to trends previously published (114), and may reflect metabolic abnormalities related to excessive adipose tissue. In some contrast, no significant differences in leptin levels were measured in LIV mice when compared to controls. Circulating adiponectin concentrations showed similar trends. Expressing leptin and adiponectin as a ratio can be indicative of insulin resistance and may be a better indication of adipocyte or metabolic health than solely leptin or adiponectin (115-118). However, the data reported here show no differences in LAR despite a relatively strong correlation to TAT. These data indicate that mechanical signals represent a reasonable means to control adipokine levels, even in the face of systemic pressures to become imbalanced.

The systemic increase in adipose burden which followed ovariectomy, measured as increased fat depots in the subcutaneous and visceral compartments, was also realized locally in the musculature, as reflected by the elevation of adipogenic gene expression measured in the soleus. Regulators of adipogenesis, including PPARγ, PPARδ, FABP4, and FoxO1, were significantly upregulated in muscle from

OVX mice, suggesting that despite a conservation of mass, the quality of the muscle was deteriorating. Increases in the PPAR genes indicate alterations in fatty acid metabolism (119), an outcome supported by the upregulation of FABP4. The measured increases in PPARγ expression, a major regulator of adipocyte differentiation, are likely linked to increased fatty acid transport and binding in the muscle tissue. Fatty acids bind with high affinity to fatty acid binding proteins, such as FABP4, which are upregulated as a result of both fatty acid transport and elevated PPARγ expression. PPARδ is also activated by fatty acids and plays a role in preadipocyte proliferation (120), which activates PPARγ and may ultimately contribute to an overall increase in adipocyte encroachment into the muscle. Transcription factor FoxO1 is downstream of PPARδ and plays a role in the mediation of oxidative metabolism and preadipocyte differentiation. Paired regulation of PPARδ and FoxO1 expression levels have been reported in the OVX model, although both genes were downregulated (121). Differences in expression trends are possibly due to specific muscle selection (soleus versus quadriceps) and differing fiber compositions. No differences were measured in Adipoq (adiponectin), an anti-adipogenic gene that is associated with decreased skeletal muscle triglyceride content in mice (122) and increases fatty acid oxidation in skeletal muscle cells (123).

That the marked elevations in circulating adipokine concentrations following ovariectomy were mirrored by elevated adipogenic gene expression in the muscle tissue implicates the depletion of ovarian hormones in the decline of multiple tissue systems. Whether the noted increase in intramuscular adiposity is a result of adipocyte encroachment or adipogenic differentiation of pre-existing stem cells within the muscle tissue requires further investigation. Furthermore, while these genes are known to participate in adipogenesis, all are involved in other skeletal muscle metabolic processes, such as muscular mitochondrial biogenesis and glucose metabolism (124); changes in expression may have additional influences on the muscle tissue.

High levels of intramuscular adiposity are associated with both aging (125) and obesity (126), however, an “athlete’s paradox” exists that demonstrates similarly elevated skeletal muscle adiposity in well-trained endurance athletes (127). Fat is a key regulator of many physiological processes (128-130) and increased intramuscular fat may not translate to decreased function. In this particular case, a buildup of fat in the muscle due to ovarian hormone depletion is likely to put muscle function at risk (131), including a reduction in muscular strength (132), which intrinsically plays a role in functional performance and contractile properties (133). In some contrast to the adipogenic bias measured in the muscle of OVX, there was a significant suppression (> 40%) of those parameters in mice subject to LIV,

suggesting that these mechanical signals provided some form of a protective mechanism against adipogenesis that may ultimately lead to a reduction in the number of intramuscular adipocytes and the protection of muscle quality. As exercise is known to reduce adiposity and is often prescribed to post-menopausal women to help maintain muscular integrity, it is possible that the mechanical signals derived from LIV serve as a form of exercise surrogate to provide similar salutary benefit. Considering that LIV has been shown, in humans, to protect postural stability (134) and reduce falls in the elderly (135), it is possible that these clinical outcomes are achieved both by retaining quality and neuromuscular control of the muscle (136).

Increases in intramuscular adiposity will invariably disrupt the muscle microniche, ultimately encroaching upon satellite cell populations and thus impacting the ability for repair and regeneration of muscle tissue. Indeed, in parallel with the increased adipogenic activity of muscle in OVX, there was a significant decrease in the proportions of satellite cells in comparison to total cells in the muscle. It has been noted that CD34 expression on satellite cells is a reversible state of activation that influences stem cell quiescence. CD34+ satellite cells have been identified as a reserve population of satellite cells that divide early on in response to injury. These cells will eventually become CD34- for later proliferation involved in the reparative process (111). The proportion of CD34+ cells in OVX was reduced compared to controls, which may suggest the immediate ability of the muscle to respond to injury or demands for myogenesis is limited. The simultaneous increase in local adipogenic gene expression may be what is driving the reduction of satellite cells and/or CD34+ satellite cells, through biased stem cell differentiation or disrupted muscle homeostasis.

While LIV did not have higher satellite cell proportions compared to OVX, no differences in overall satellite cell proportion or CD34+ satellite cells were observed in LIV as compared to age-matched controls, suggesting that these mechanical signals served to protect these progenitors. Preserving the CD34+ reserve population of satellite cells may be critical in maintaining muscle quality and quantity during a progressive loss of ovarian hormone. Mechanical signals delivered in the form of exercise - or when that is not possible, perhaps as LIV, may play a critical role in the regeneration, rehabilitation, and restoration of function of muscle tissue.

Myogenic gene expression influences the proliferation and differentiation of satellite cells. Proliferating satellite cells simultaneously express Pax7 and MyoD (137), both of which were upregulated in OVX. While satellite cell proliferation and differentiation genetic markers were increased, the number of satellite cells in the OVX group was lower than that of controls. Even though the actual

proportion of satellite cells in the muscle is compromised, it appears that OVX is responding to injury by upregulating myogenic gene expression to repair overall muscle homeostasis. MyoD, a gene also expressed by myocytes, was also upregulated, possibly due to the initiation of proliferating muscle cells. Myf5 promotes satellite cell renewal and myoblast differentiation and was again upregulated in OVX animals, perhaps as another effort to repair and regenerate damaged muscle. IGF-1 was also upregulated in the OVX group. Overexpression of IGF-1 stimulates muscle hypertrophy (138) through satellite cell activation and the upregulation of protein synthesis (139) during active postnatal muscle development or adult regeneration (140). Both OVX and LIV groups had heavier soleus weights at euthanasia, consistent with heavier muscle weights found in other OVX murine models (141-143). There were no changes in the expression of Mstn (myostatin), which is responsible for preventing muscle growth (144).

LIV mice also had elevated levels of Pax7 and MyoD, an indication that despite the exercise surrogate, the muscle tissue was still signaling the process of repair. While mechanical stimulation is generally found to increase the expression of myogenic factors (145, 146), there was a significant decrease in Myf5 expression in LIV relative to OVX. Myf5 is a one of the earliest markers of active satellite cell commitment to the myogenic lineage. The noted changes of Myf5 in the LIV group may be a result of the transition of stem cells into a myoblast differentiation phase where Myf5 is consequently downregulated (147). Overall, LIV resulted in less of an impact on myogenic markers than those related to adipogenesis, which suggests the primary influence of LIV is associated with suppressing adiposity rather than influencing myogenesis.

Our gene expression profiles consisted of chiefly anabolic genes, which we saw respond towards an adipogenic profile. Certainly, a concurrent assessment of catabolic genes would give a more complete analysis of the effects of LIV on OVX skeletal muscle tissue. While two catabolic genes in this study were assessed (adiponectin and myostatin), no differences in their expression profile were detected between any groups. Clearly, the systemic insult of OVX is significant, and the mechanisms involved in the consequences – and the mechanical protection – are certain to be complex. Ultimately, a more comprehensive transcriptional profile must be performed before a more complete picture is possible.

The reported changes in gene expression due to OVX and vibration treatment cannot be attributed to solely one cell population and rather represent variations across the muscular environment. Changes in the stem cell niche may be due to a number of cell types involved in skeletal

muscle regeneration and maintenance, e.g., fibroblasts, endothelial cells, fibro/adipogenic progenitors, telocytes, motor neurons, mesenchymal and hematopoietic stem cells and precursors, and are likely to influence satellite cell activity. Additionally, while all muscles used for this study were extracted from the hind limbs, muscle groups differed among assays due to assay cell number and tissue mass requirements. Variations in gene expression and satellite cell numbers may occur depending on anatomic location. Further investigation into the mechanism behind OVX's impact, and LIV's salutary influence on adipogenesis and mygoenesis, in addition to local variations in the skeletal muscle, is necessary. It is also important to note that future studies must include the assessment of muscular composition on the phenotypic level (lipid content, adipocyte infiltration, etc.) and functional testing to demonstrate changes in muscular strength and quality.

The work presented here provides evidence for the rapid and marked consequences of ovariectomy on a number of physiologic systems. In particular, this work shows that the removal of ovarian hormones increases adipogenic gene expression in muscle, reducing its quality, and simultaneously suppresses the number of satellite cells available for muscle regeneration and repair. These data also indicate that extremely low level mechanical signals, introduced for brief periods each day using LIV, reduce levels of total adiposity and suppress muscular adipogenic gene expression, while protecting the number of satellite cells.

Exercise, representing the primal mechanical stimulus, is a critical modality to slow the range of complications which arise from menopause, including the deterioration of muscle quality and strength. Ironically, however, the menopause and its sequelae, such as the rapid decline of the musculoskeletal system, can erode the safety, ease and compliance required of exercise. While there is no true substitute for exercise, the data presented here suggest that LIV may have the potential to serve as a surrogate to exercise for the injured or impaired. Ultimately, alternatives to exercise, either chemical or physical, that help retain the progenitor population and its microniche sooner rather than later, may help preserve the integrity of a range of physiologic systems.

CHAPTER 3: MECHANICAL STIMULATION MITIGATES INTERMUSCULAR LIPID ACCUMULATION CAUSED BY HIGH-FAT DIET WHILE DIET HAS NO EFFECT ON SATELLITE CELLS

INTRODUCTION

Ovariectomy compromised the musculature by increasing local adipogenic gene expression and suppressing satellite cell populations. While it is plausible that the noted changes in adipogenic gene expression had a direct effect on satellite cell populations in the OVX model, the abrupt and severe decrease in ovarian hormone production is a confounding variable that cannot be ignored as a primary contributor in the study. A model that eliminates the systemic endocrine insult is more effective in determining the direct effects of adiposity on the skeletal muscle microenvironment and local stem cell populations.

Using the insult of high-fat diet, which eventually leads to excessive fat gain (diet-induced obesity, DIO) and glucose intolerance, we sought to determine whether this particular fat insult, or the process of gaining excess adiposity from high-fat diet, suppresses satellite cells and if LIV can protect this stem cell population in the same manner as it did in OVX. We also investigated the skeletal muscle phenotype to determine if changes in stem cell populations were accompanied by local increases in fat. While the observed changes in adipogenic gene expression in OVX were robust, it was necessary to further explore the influence of fat on muscle tissue and the extent to which LIV is able to protect the muscular environment, *i.e.* whether or not the benefits of LIV extend to improving the muscular phenotype. Lastly, we looked into the molecular pathway influenced by fat metabolites in the muscle that is believed to lead to impaired insulin signaling.

The detrimental impact of high-fat diet affects a range of tissue systems, including bone (57, 148) and skeletal muscle (149, 150), while adipose depots increase in mass and inflammatory state (151-153). Besides an energy store, adipocytes are active endocrine signalers involved in a range of different physiological roles. Different types of fat, such as brown, white, or beige, differ in metabolic function (154), and, location of the fat depot (*i.e.* visceral, subcutaneous, or ectopic) also influences the role of the adipocyte. Brown and beige adipocytes are sites of glucose breakdown and thermogenesis, and contain high numbers of mitochondria (154). The primary function of white adipose tissue is the storage of triglycerides and release of fatty acids upon adipocyte lipolysis. White adipocytes are also active in regulating metabolism through adipokine release, such as leptin, adiponectin, and resistin (155).

Fat pad expansion can be achieved through adipocyte hyperplasia and hypertrophy and is initiated through the adipogenic differentiation program (*i.e.* PPARγ, C/EBPα, etc.) of cells from the mesenchymal lineage (156). During obesity and excess caloric consumption, fat masses become larger particularly in the visceral compartment, due to the need for space for excess energy storage. VAT is

associated with insulin resistance and impaired glucose tolerance and precedes the complications related to the metabolic syndrome (157) caused by obesity. With expanding masses of VAT in obesity, adipocyte turnover increases due to remodeling of the tissue, resulting in macrophage infiltration and overall inflammation (158). Eventually, ectopic adipocytes and enlarged lipid storage begin to form in tissues such as such as liver (159) and skeletal muscle (94), which can lead to abnormalities (*i.e.* insulin resistance) and disruption of tissue homeostasis.

In addition to DIO altering levels of fat in both the visceral compartment and in non-traditional tissues, previous research supports that models of DIO alter cell populations within the bone marrow cavity (57, 62, 148). Considering the dynamic state of the marrow, high-fat diet may alter migration patterns and contributions of bone marrow cells to the formation and/or function of muscle and fat. To minimize our animal use, we used one model of high-fat diet combined with GFP+ bone marrow cell tracking to investigate: 1. the muscular environment and insulin resistance; and 2. bone marrow cell migration and contributions of bone marrow-derived cells to abdominal adipose depots and muscle tissue. The skeletal muscle phenotype and insulin resistance are addressed in Chapter 3 while bone marrow cell tracking is discussed in detail in Chapter 4.

It should be noted that there is a distinct difference between the influence of obesity and the influence of diet on tissue systems. The metabolic abnormalities that arise from excess amounts of fat tissue or ectopic adipocytes differ from the changes that occur when there is a modification in diet, preceding the obese phenotype. This has been demonstrated in the bone marrow cavity, where Adler *et al.* showed that high-fat diet induced alterations to leukocyte populations in the bone marrow of mice are progressive and transform as the obese phenotype develops. For example, a reduction in the B-cell population was evident at 1 wk, but not 2 days, while increases in the myeloid population were not apparent at 1 wk, but were at 6 wk (148). These data demonstrate both the immediate (<1 wk) changes caused by diet and long-term (6 wk) complications that are likely to be caused by a combination of diet and excess amounts of adiposity. High-fat diet animals at the 6 wk time point had over a 400% increase in abdominal adiposity compared to their regular-diet controls, suggesting alterations in bone marrow phenotype were influenced by existing adipocytes. The work presented here investigated the impact of diet on the musculature rather than the impact of obesity on tissue systems, allowing for identification of changes in skeletal muscle that occur in the development of obesity and prediabetes.

Obesity is associated with decreased muscle functional capabilities in the elderly (160, 161), but it is unclear if these changes are due to alterations in the satellite cell pool or the extent to which age is

a confounding factor. As demonstrated in OVX, satellite cells can be affected by systemic insult, and a similar depletion in this cell population has been seen in the aged population (>70 years of age) (162). Fu *et al.* recently demonstrated that impaired muscular regeneration in obese mice is due to low AMPKα1 activity in satellite cells, likely affecting satellite cell proliferative and myogenic potential (163). Another group also showed DIO impairs satellite cell activation, but does not impact overall number of stem cells (164). Further evidence is needed to determine if a systemic fat insult impairs the satellite cell population. Additionally, while it has been demonstrated on numerous occasions that exercise replenishes the satellite cell pool and increases stem cell proportions (165, 166), we investigated whether mechanical stimulation can increase satellite cell populations in the presence of high-fat diet.

A recent hypothesis suggests that muscular insulin resistance results from the accumulation of lipids within the muscle cells and activation of the theta isoform of protein kinase C (PKCθ) (153, 167), inhibiting insulin signaling and glycogen synthesis. Upon activation of this pathway, insulin-receptor substrate-1 (IRS-1) serine phosphorylation is activated while insulin-induced tyrosine phosphorylation of the substrate is blocked, leading to the deactivation of phosphatidylinositol 3-kinase (PIK3). This signaling cascade decreases glucose transporter activity (glucose transporter protein 4, GLUT4) and consequently glucose oxidation and glycogen uptake. The metabolite responsible for the activation of the PKCθ pathway is believed to be diacylglycerol (diglyceride, DAG), a fat intermediate that is a precursor to the most common form of fat storage in muscle, triglycerides. The ectopic accumulation of fat within the musculature is solely responsible for the interrupted insulin signaling that leads to the diabetic condition. However, clinical studies have demonstrated that a reduction of ectopic lipid is associated with an improvement in insulin resistance (168, 169).

The reduction of intramuscular fat can be achieved through weight loss, which invariably improves glucose sensitivity. Weight loss through bariatric surgery is effective, but invasive and costly (170). Other non-pharmacological interventions include dietary restrictions and increased physical activity, however compliance is low and long-term weight loss is only achieved by a few (170). Additionally, those who are obese may have difficulties exercising due to their physical size and movement restrictions. As a surrogate for- or adjunct treatment to exercise, the use of mechanical stimulation in the form of low intensity vibration (LIV, <0.4 *g* where *g* is earth's gravitational field) may provide a signal that is successful in protecting the muscular microenvironment. Studies using LIV have demonstrated increased cross-sectional area of both fiber types in murine hindlimb muscle (81), improved murine neuromuscular activity and function as indicated with behavioral testing (136), and

reaped other muscular benefits in clinical investigations (171-173). Considering LIV can be anabolic to muscle (81), suppress adipogenesis (82, 109, 174), and influence bone marrow stem cell fate selection (109), this treatment modality has the potential to mitigate intermuscular fat formation and muscular insulin resistance.

This study tested the hypothesis that a systemic high-fat insult will compromise muscle stem cell populations, increase intermuscular fat, and predispose the musculature to insulin resistance, while LIV will protect satellite cell proportions and mitigate intermuscular fat accumulation and interrupted insulin signaling. Using a murine model of pre-diabetes, we identified the effects of high-fat diet on satellite cell proportions, the muscular phenotype, and molecular pathway of insulin resistance, and determined if LIV could protect these impairments.

MATERIALS AND METHODS

Experimental design

All experimental procedures were approved by the Stony Brook University Institutional Animal Care and Use Committee. Forty 8 week old C57BL/6 male recipient mice (The Jackson Laboratory, Bar Harbor, ME), were lethally irradiated and given bone marrow transplantations of GFP+ whole bone marrow from 5-8 week old GFP+ hemizygous transgenic, male mice (C57BL/6-Tg(CAG-EGFP)1Osb/J, Stock #003291, The Jackson Laboratory, Bar Harbor, ME). The day following transplantation, recipients were randomly divided into two groups using a weight matched MATLAB script and started on diet regimens, either regular diet (Energy [kcal/g]: 10.2% fat, 18% protein, 71.8% carbohydrates; vanHeek Series 58Y2, TestDiet, Richmond, IN, USA) or 45% high-fat diet (Energy [kcal/g]: 46.1% fat, 18.1% protein, 35.8% carbohydrates; vanHeek Series 58V8, TestDiet, Richmond, IN, USA). After two weeks of diet induction, diet groups were divided into two additional groups, with half were subjected to LIV for 8 weeks, yielding the following four groups: regular diet (RD, n=9), regular diet with vibration treatment (RDV, n=10), high-fat diet (HD, n=10), and high-fat diet with vibration treatment (HFV, n=10). Respective diets were maintained throughout all ten weeks of the experiment. An additional group of age-matched controls were sham irradiated and handled, received a saline injection for the bone marrow transplantation, and were fed a regular diet (AC, n=10). All animals had *ad libitum* access to food and water and were singly housed in a 12 hr/12 hr light/dark cycle at 21°C. At the completion of the experiment (2 weeks diet induction + 8 weeks LIV and diet; Figure 8) mice were anesthetized with isoflurane and euthanized via cervical dislocation. Glucose tolerance testing was performed two days prior to euthanasia and all tissue weights were obtained immediately following extraction.

As an add-on to this study, two additional high-fat diet murine models were used to further investigate impact of high-fat diet on satellite cells. The first animal model (referred to in the satellite cell results as “non-irradiated 45% kcal diet”) received the same diets and diet induction period of two weeks followed by six weeks of vibration treatment. Seventeen week old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were divided into groups based on a weight-matched program and were subjected to either a regular diet (RD-45, n=8) or 45% high-fat diet for two weeks. After the two week diet induction, high-fat diet animals were either sham vibrated (HD-45, n=8) or subjected to low intensity vibration treatment (90 Hz, 0.2*g*) for five days a week and six weeks total. Two vibration regimens were used: one bout of 30 min/day (HDV-45, n=9) or two bouts of 15 min/day with a five hour

refractory period (HDVR-45, n=9). All animals had *ad libitum* access to food and water and were singly housed in a 12 hr/12 hr light/dark cycle at 21°C. Mice were anesthetized with isoflurane and euthanized via cervical dislocation.

The second subsequent analysis (referred to in the satellite cell results as "non-irradiated 60% kcal diet") was done with a more robust diet regimen. Seven week old C57BL/6J male mice (The Jackson Laboratory, Bar Harbor, ME) were randomly weight-matched into either a regular diet (RD-60, n=9) or 60% high-fat diet (HD-60, n=10) (Energy [kcal/g]: 61.6% fat, 18.1% protein, 20.3% carbohydrates; vanHeek Series 58Y1, TestDiet, Richmond, IN, USA) group and were subjected to the assigned diets for a total of six weeks. All animals had *ad libitum* access to food and water and were singly housed in a 12 hr/12 hr light/dark cycle at 21°C. Mice were anesthetized with isoflurane and euthanized via cervical dislocation.

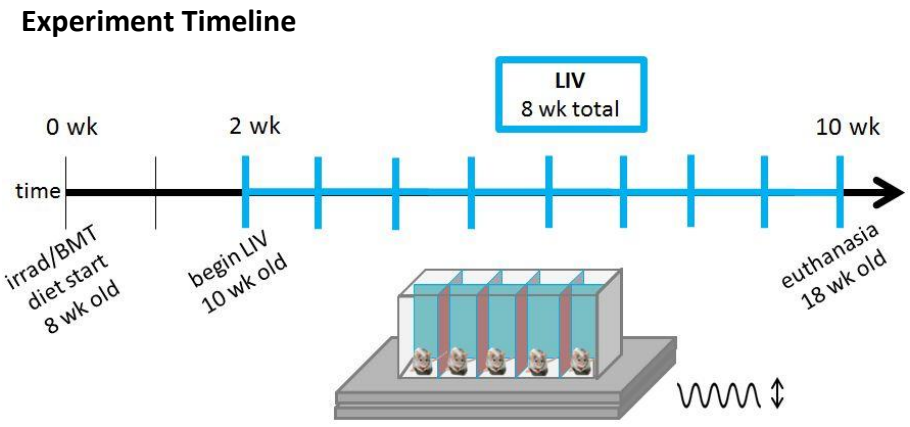

Figure 8: Experiment timeline. Lethally irradiated animals were rescued within 24 hrs with a bone marrow transplant (t = 0w). Diet regimens began the day following bone marrow transplantation at t = 1 d. At t = 2 wk, appropriate groups began LIV treatment (8 wk duration).

Bone marrow transplantation

To eliminate host bone marrow, recipient mice were exposed to a lethal dose of fractionated total body ^{137}Cs gamma irradiation at (6+6) Gy with a four hour interval at a dose rate of 0.607 Gy/min. Twenty-four hours following irradiation, recipients were rescued with $10x10^6$ whole bone marrow cells from GFP+ transgenic donors through an intravenous tail injection, using a 27G needle and 300 µL injection volume. Transgenic donor mice express enhanced GFP through a chicken beta-actin promotor and cytomegalovirus enhancer, causing cells to fluoresce green when excited. Body weight was monitored every other day for the first two weeks following irradiation and every week thereafter.

Mechanical stimulation protocol

Low magnitude, high frequency vibration treatment (0.2-0.25*g* and 90 Hz, where g is earth's gravitational force at 9.81 m/s^2) was administered five days a week for either 30 min/day or two bouts of 15min with a five hour refractory period. Mice were placed in a partitioned box set directly on the vertically oscillating vibration plate. All non-vibrated groups were sham handled and placed on the inactive device for the same duration of time.

Flow cytometric analysis of satellite cells

Cells from the hind limb muscles were prepared as previously described in Aim 1. Briefly, hind limb muscles were harvested at euthanasia, minced, pooled, and stored in supplemented DMEM (Gibco, Carlsbad, CA, USA). Tissue was digested, triturated, and neutralized with supplemented DMEM containing 15% horse serum (Thermo Scientific, USA). Mononuclear cells were filtered and red blood cells were lysed from samples. Samples were then centrifuged, resuspended in supplemented media, and counted to remove 1x10^6 cells from each sample. Samples were incubated with PE-conjugated Integrin alpha 7 (R&D Systems, Minneapolis, MN), APC-conjugated CD45, CD31, and Sca-1 (eBioscience, Inc., San Diego, CA), and either PerCP/Cy5.5-conjugated CD34 (BioLegend, San Diego, CA) or FITC-conjugated CD34 (BD, San Jose, CA). Satellite cells were gated as CD45-/CD31-/Sca-1- (APC-) and Integrin alpha 7+ (PE+) with CD34 (PerCP/Cy5.5 or FITC) determining the quiescent satellite cell pool. Flow cytometric analysis was conducted using the FACSCalibur system (BD, San Jose, CA, USA) at 200,000 events per sample. Data was analyzed with FlowJo V7.2.5 (TreeStar Inc., San Carlos, CA).

Neutral lipid quantification using oil red O

Upon animal euthanasia, soleus muscles were excised, stretched to *in situ* length, and pinned to a small board. Once covered in Tissue-Tek® OCT compound (Sakura Finetek USA Inc, Torrance, CA, USA), muscles were rapidly frozen in chilled isopentane and then immediately submerged in liquid nitrogen. Muscles were kept at -80°C for long-term storage. For sectioning, muscles were first cut in half perpendicular to the long axis of the muscle and then sectioned on a cryostat at 5 µm to obtain transverse cross-sections of the fibers. All slides were stored at -80°C until staining. The staining protocol for neutral lipids by oil red O (ORO) was adapted from Mehlem and colleagues (175). Briefly, slides came to room temperature and were incubated in ORO (Sigma-Aldrich, St. Louis, MO) for 10 minutes. Slides were then rinsed in running tap water for 30 minutes and mounted with aqueous mounting medium

(EcoMount, Biocare Medical, Concord, CA). Slides were sealed with a coverslip and imaged with bright field microscopy within 8 hours. A minimum of 8 fields of view per slide were used for analysis. Neutral lipids were stained bright red and were quantified in terms of pixel area using a dedicated threshold applied to all images with ImageJ software (ImageJ, NIH).

Glucose tolerance testing

Mice were fasted for 18 hours prior to beginning the glucose tolerance test (GTT). Fasting blood glucose levels were measured by a tail snip using a glucometer (Precision Xtra, Abbott Diabetes Care Inc., Almeda, CA USA). Animals received an intraperitoneal injection of 0.75 mg/g body weight of 20% dextrose solution under light isoflurane anesthesia. Blood glucose levels were taken at the following time points after baseline measurement: 15, 30, 45, 60, 90, and 120 min. The area under the curve (AUC) was calculated using the trapezoidal rule.

Diacylglycerol quantification in skeletal muscle

The gastrocnemius muscle was excised after euthanasia and ½ of the tissue sample was immediately fast frozen in liquid nitrogen and kept at -80°C for long-term storage. Muscle tissue was weighed and mechanically homogenized in NP-40 (Invitrogen, Camarillo, CA) using stainless beads (Next Advance, Averill Park, NY) in a tissue homogenizer (Bullet Blender, Next Advance, Averill Park, NY). Diacylglycerol was quantified using a commercial colorimetric ELISA kit (Mouse diacylglycerol (DAG/DG) ELISA kit, Bioassay Technology Laboratory, Shanghai, China) and the standard curve was calculated according to the manufacturer's protocol.

Quantitative Polymerase Chain Reaction of skeletal muscle gene expression

Immediately following euthanasia, the gastrocnemius muscle was excised, cut, and one half of the tissue was stored in RNAlater at -20°C for short-term storage and then moved to -80°C until use. Samples were homogenized in TRIzol (Thermo Scientific, USA) using a tissue homogenizer (Bullet Blender, Next Advance, Averill Park, NY). RNA was extracted from the samples using a commercial spin column kit (PureLink RNA Mini Kit, Life Technologies, Carlsbad, CA). RNA purity was tested using 1 µL of mRNA sample on a nanodrop spectrophotometer (NanoDrop 1000 Spectrophotometer, Wilmington, DE) and was then converted to cDNA using a high capacity reverse transcription kit (Applied Biosystems, Foster City, CA). PCR amplification was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) using TaqMan Gene Expression Assays and TaqMan Gene Expression Master

Mix (Applied Biosystems, Foster City, CA). Genes involved in insulin resistance, including PKCθ, IRS-1, GLUT4, IGF-1, and genes involved in adipogenesis, including PPARδ, FoxO1, PPARγ, FABP4, were compared to housekeeping gene β-actin. Relative expression was compared against intact, age-matched controls using the delta-delta Ct method of analysis.

Statistical Analyses

Comparisons between three or more groups were made using a one-way ANOVA and Tukey post-hoc test. An unpaired, two-tailed T-test was used for data with a singular comparison. All data shown is mean ± standard deviation. All data were considered significant when $P \leq 0.05$ and all analyses were done using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Bone marrow transplant survivability and diet effects on body weight

At the completion of the ten week experiment, 39 of the 40 animals that received lethal irradiation and rescue bone marrow transplants survived, demonstrating successful reconstitution of the bone marrow cavity (Figure 9A). RD had 90% survival while all other groups maintained 100%. All irradiated groups lost approximately 11% of their body weight within the first three days, and steadily gained weight thereafter, although never reaching that of controls (Figure 9B). AC had greater body weights than all irradiated groups beginning 3 days after irradiation ($P \leq 0.005$) and maintained this weight difference for the duration of the study. High-fat diet groups never achieved significantly higher body weights than regular diet groups during the ten weeks of diet, however, after 49 days, high-fat diet groups started to trend toward gaining weight. Vibration had no effect on body weight.

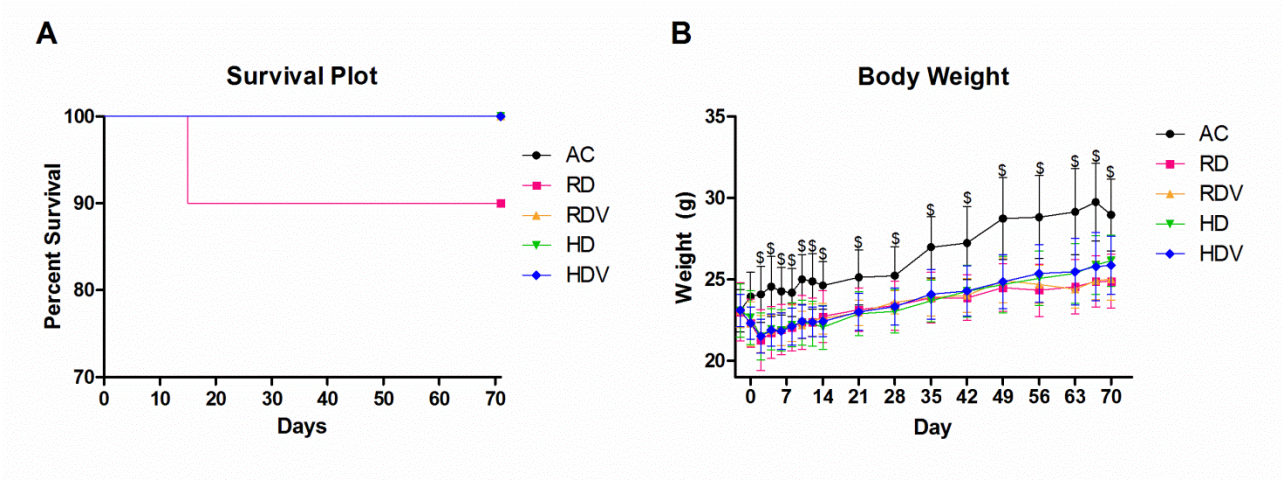

Figure 9: A: Successful reconstitution of the bone marrow was demonstrated by 90-100% survivability at the study conclusion. B: Age-matched controls (sham irradiated animals) maintained higher body weight compared to all irradiated groups beginning 3 days after irradiation and from that point forward. $P < 0.05 compared to all irradiated groups.

Impact of high-fat diet on muscle hypertrophy

At the completion of the ten week experiment, there were no differences in soleus and quadriceps weights between any groups (Table 1). However, the gastrocnemius weight of the HD group was 17% higher than RD ($P < 0.05$), while there were no other differences in muscle weight.

Table 1: Body weight and hind limb muscle wet weights at euthanasia.

Group	Body (g)	Soleus (mg)	Gastrocnemius (mg)	Quadriceps (mg)
AC	28.96 ± 2.21$^{\$}$	8.36 ± 2.63	124.14 ± 17.04	106.25 ± 30.25
RD	24.89 ± 1.65	7.32 ± 2.81	118.14 ± 15.49	119.10 ± 13.68
RDV	25.02 ± 1.28	8.4 ± 0.95	125.44 ± 19.07	126.68 ± 15.25
HD	26.15 ± 1.58	7.55 ± 2.60	144.79 ± 27.45$^{\#}$	118.47 ± 15.64
HDV	25.86 ± 1.78	6.37 ± 2.08	123.38 ± 11.25	119.48 ± 10.99

Weights are ± standard deviation. #P < 0.05 compared to RD; $P < 0.05 compared to all irradiated groups.

High-fat diet has no influence on satellite cell proportion

At the completion of the study, proportions of satellite cells in the quadriceps muscles, positive for Integrin alpha 7 and negative for CD45, CD31, and Sca-1 (Figure 10A), were lower in all irradiated groups compared to non-irradiated controls (RD: -28%, RDV: -46%, HD: – 40%, and HDV: -36%; all P <

0.05) (Figure 10B). No differences in satellite cell proportions were detected between diets or any irradiated groups. Additionally, no differences in number of total muscle cells (Figure 10C) or satellite cell absolute numbers (Figure 10D) in the quadriceps muscles were found (P > 0.05).

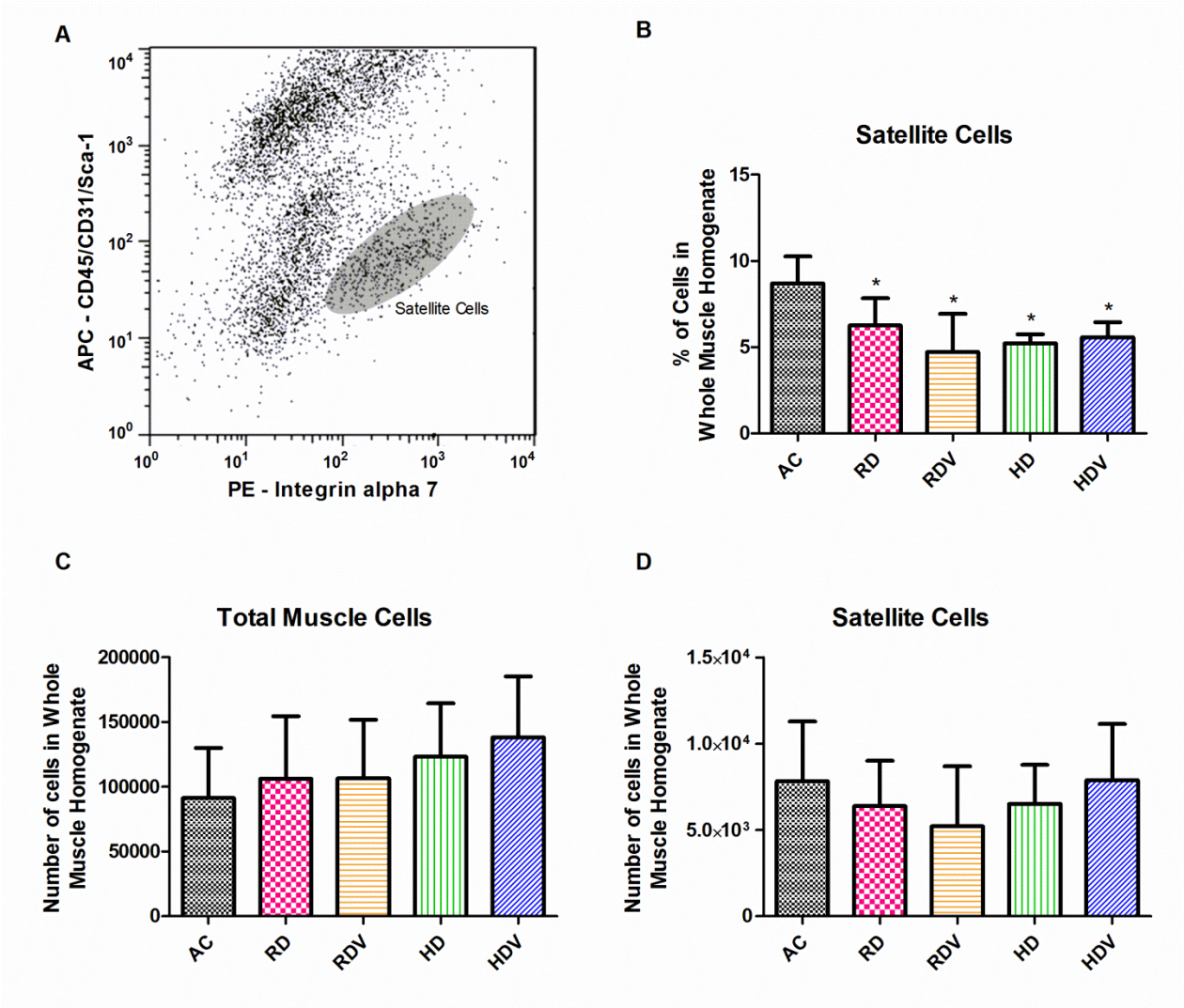

Figure 10: Satellite cell quantification in the quadriceps muscles quantified via flow cytometry. A: Flow cytogram representing the isolated satellite cell population negative for APC (CD45/CD31/Sca-1) and positive for PE (Integrin alpha 7). B: The percentage of satellite cells (CD45-/CD31-/Sca-1-/Integrin alpha 7+) out of the total number of muscle cells in the sample. C: The total number of muscle cells in the sample. D: Satellite cells represented in absolute numbers. *P < 0.01 compared to AC.

High-fat diet has no influence on satellite cell proportions in non-irradiated animals

Our findings indicated that high-fat diet does not impact satellite cell proportions, but it is plausible that the influence of radiation masked any detriment to the cell population caused by diet. To confirm the findings found in the original study, we performed the same analyses in two additional animal models subjected to high-fat diets that were not irradiated. In the animals on the same 45% kcal from fat as the original study, all three high-fat diet groups developed the obese phenotype and were heavier than RD-45 (average of +25% body weight) at the conclusion of the study (RD-45: 27.8 ± 2.5g; HD-45: 35.4 ± 3.7g; HDV-45: 35.1 ± 2.9g; HDVR-45: 33.7 ± 3.4g) (all $P < 0.05$) (data not shown). There were no differences in weights between any groups on the high-fat diet. No differences were detected between RD-45 and HD-45 animals in proportion of satellite cells ($P > 0.05$) (Figure 11A), absolute number of satellite cells ($P > 0.05$) (Figure 11B), total cell number, or muscle weights (data not shown).

Similarly, in the more robust 60% kcal from fat diet, HD-60 animals were 23% heavier than RD-60 (RD-60: 24.1 ± 1.7g; HD-60: 29.6 ± 2.8g) ($P < 0.001$) (data not shown) at the time of euthanasia. No differences were detected in either satellite cell proportion (Figure 11C) or absolute number of satellite cells (Figure 11D) after six weeks of high-fat diet. There were also no differences in total cell number or muscle weights between groups ($P > 0.05$) (data not shown).

LIV increases satellite cell proportions in non-irradiated animals

While there were no differences in satellite cell number between diets, LIV treatment enhanced satellite cell populations in the high-fat diet groups. With LIV treatment there was a 77% increase in satellite cell proportion in HDV-45 ($P < 0.01$) and a 61% increase in HDVR-45 ($P \leq 0.05$) relative to RD-45. Similarly, there was an 89% increase in absolute number of satellite cells in HDVR-45 compared to RD-45 ($P < 0.01$) and a 44% increase in HDVR-45 compared to HD-45 ($P = 0.06$). Since there were no differences detected in total number of cells or muscle weights between any groups ($P > 0.05$) (data not shown), differences in satellite cell proportion are due to true increases in satellite cells.

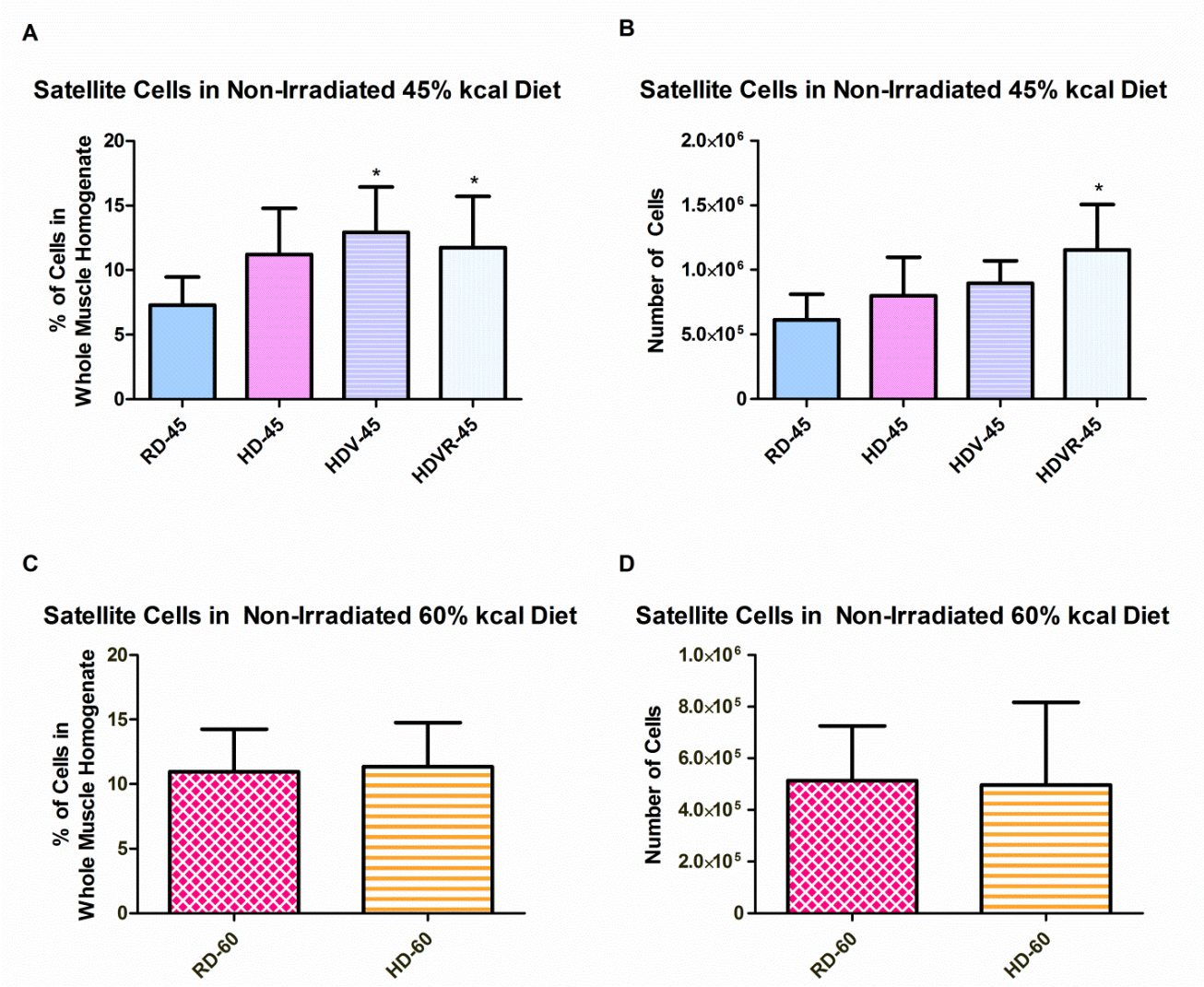

Figure 11: Satellite cell quantification in the hindlimb muscles of animals subjected to high-fat diet and vibration regimens without radiation exposure. A: Proportion of satellite cells in animals subjected to a 45% high-fat diet and 30 minutes of LIV with and without a refractory period. B: Absolute number of satellite cells in animals subjected to a 45% high fat diet and 30 minutes of LIV with and without a refractory period. C: Proportion of satellite cells in animals subjected to a 60% high-fat diet. D: Absolute number of satellite cells in animals subjected to a 60% high-fat diet. *P < 0.05 compared to the regular diet group of the same study.

Effects of high-fat diet on CD34 expression

A subset of satellite cells characterized by CD34 expression (Figure 12A) represents a reversible state of quiescence. Satellite cells expressing CD34 are part of the quiescent portion of the satellite cell pool while those without the protein are more primed for proliferation and differentiation in response

to injury or contribution to muscle hypertrophy. CD34+ satellite cells made up between 3.4-5.3% of all muscle cells while CD34- satellite cells comprised of 1.8-3.8% (Figure 12B). RD, HD, and HDV all had lower percentages of CD34- cells relative to CD34+ cells (all $P \leq 0.001$). Interestingly, the ratio of CD34+:CD34- cells was greater than 1 in all groups (AC: 1.6, RD: 2.1, HD: 1.8, HDV: 2.7) except RDV, where the population had a shift to more CD34- cells than CD34+ and a ratio of 0.9 (Figure 12C). The CD34+:CD34- ratio for RDV was lower than RD ($P < 0.05$) while HDV was greater compared to RDV ($P \leq 0.001$).

In the non-irradiated 45% kcal from fat study, no differences were found in the ratios of CD34+:CD34- cells ($P > 0.05$), indicating that LIV may only affect satellite cell CD34 expression in animals on a regular diet. Elevated satellite cell populations with LIV in this study were due to an increase in CD34+ satellite cells; proportions of CD34+ satellite cells were higher in HDV-45 compared to RD-45 (+84%, $P < 0.05$) while there were no differences in CD34- cells ($P > 0.05$) (data not shown).

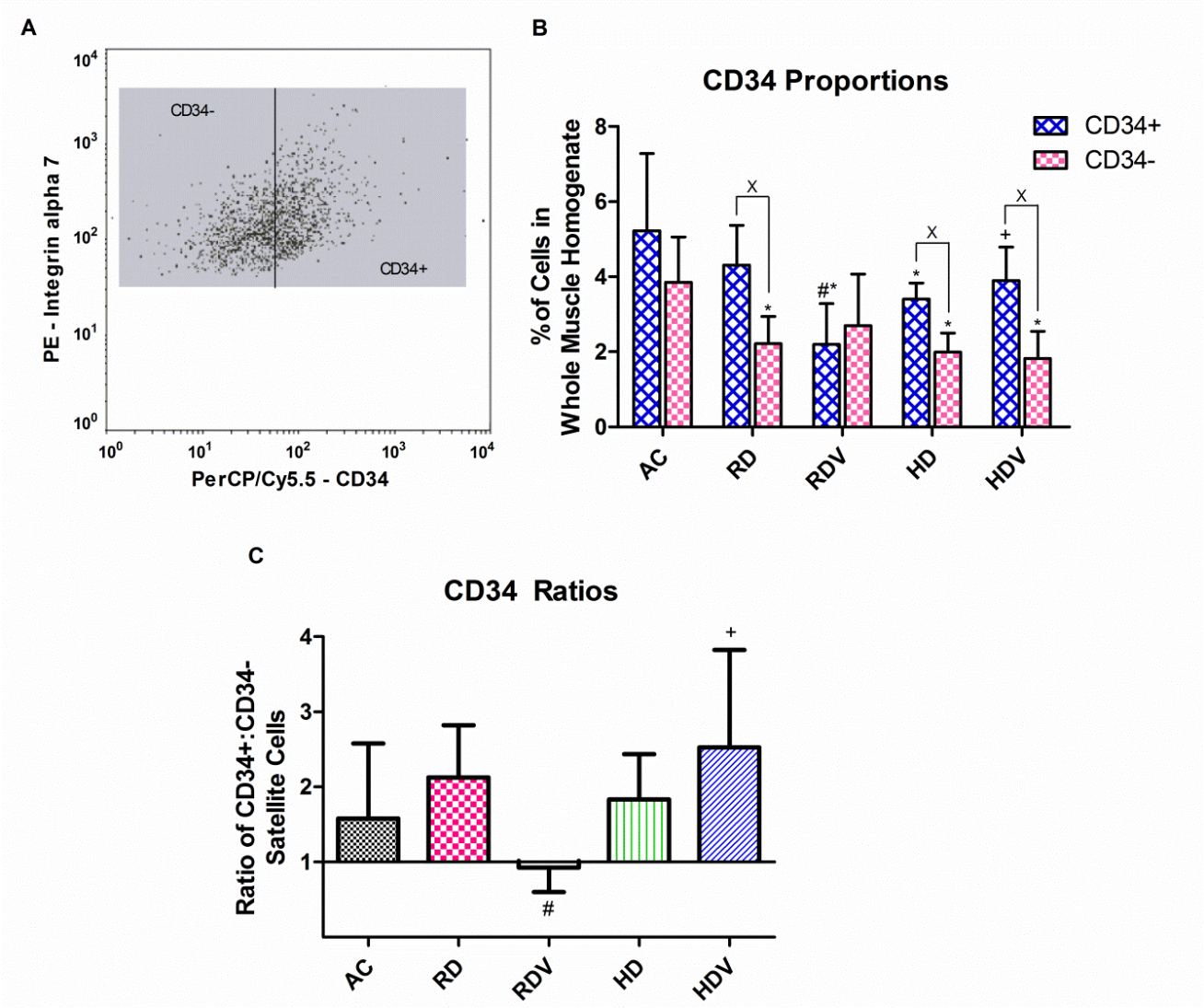

Figure 12: CD34 quantification in the quadriceps muscles quantified via flow cytometry. A: Flow cytogram representing the satellite cell population subset PerCP/Cy5.5 positive (CD34+) and negative (CD34-). B: Percentages of CD34+ and CD34- satellite cells. C: The ratio of CD34+:CD34- satellite cells. *P < 0.05 compared to AC, #P < 0.05 compared to RD, +P < 0.05 compared to RDV, XP < 0.05 compared to CD34+ population of the same group.

LIV mitigates intermuscular fat accumulation caused by high-fat diet

To determine if high-fat diet influenced the phenotype of the muscular niche, we evaluated the musculature using oil red O staining to quantify neutral lipids in frozen sections of the soleus. High-fat diet caused a marked increase in skeletal muscle lipid content (Figure 13). Lipid levels in HD animals were 717% higher than RD ($P < 0.01$) and 260% higher than RDV ($P < 0.01$). Interestingly, LIV treatment

was able to mitigate part of this fat accumulation and no differences were detected between RD and HDV groups. With the application of LIV, lipid content in HDV was 54% percent lower than HD animals, which did not receive treatment (P = 0.06).

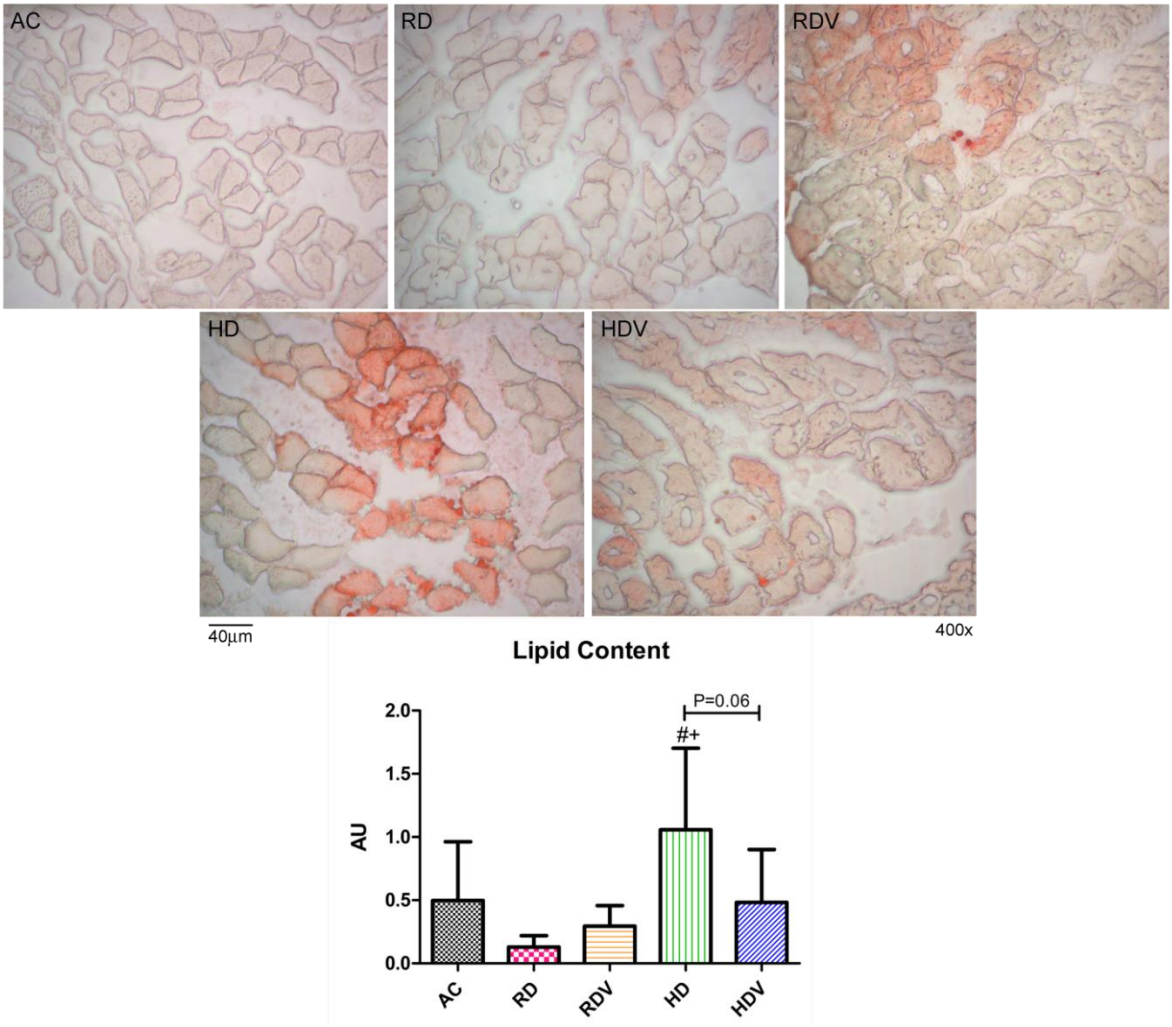

Figure 13: Neutral lipids in the soleus muscle. Representative images of frozen soleus cross sections and neutral lipids stained with oil red O. Quantification of neutral lipids in the soleus is represented as arbitrary units: densiometric count of neutral lipids/area of image (pixels^2/µm^2). #P < 0.05 compared to RD; +P < 0.05 compared to RDV.

Diet and LIV effects on glucose tolerance

To test the systemic impact of high-fat diet on glucose metabolism, we performed the GTT test with peripheral blood prior to animal euthanasia. RDV had significantly lower plasma glucose than controls at 60 (-19%, P < 0.05), 90 (-18%, P < 0.01), and 120 min (-19%, P ≤ 0.001) time points, while RD had significantly lower levels at 60 min compared to controls (-19%, P < 0.05) (Figure 14A). At the final

time point (120 min), RDV had lower plasma glucose levels than HD (-19%, $P < 0.05$). RDV was the only group to have a lower AUC compared to controls (-14%, $P < 0.05$), indicating greater glucose tolerance (Figure 14B). HD animals had a higher AUC than RDV (+16%, $P < 0.05$) while there was no difference between HDV and RDV (Figure 14B). No differences were detected in fasting glucose levels (data not shown).

Skeletal muscle diacylglycerol content

Skeletal muscle DAG content was evaluated in the gastrocnemius muscle using ELISA to determine if this particular fat metabolite may be related to the changes seen in glucose tolerance in the GTT assay (Figure 14C-D). No differences were seen between diets in either DAG concentration or total DAG content, however, high-fat diet animals undergoing LIV treatment had a 23% decrease in DAG concentration compared to RD animals ($P < 0.05$).

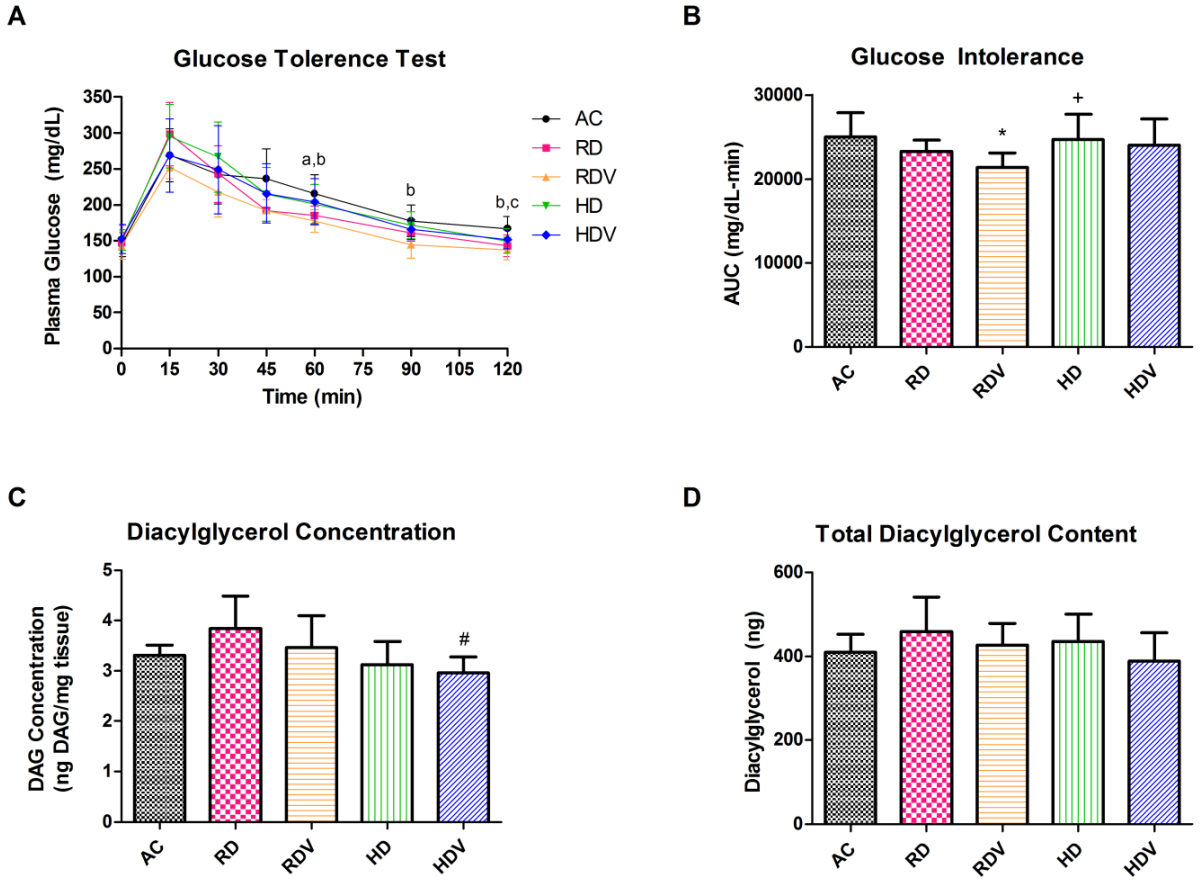

Figure 14: Indications of the development of glucose intolerance and impaired insulin signaling. A: Plasma glucose levels during glucose tolerance testing using an intraperitoneal dose of 0.75mg 20% dextrose solution/g body weight. B: Glucose tolerance testing "area under the curve" (AUC) calculated using the trapezoidal rule. C: Diacylglycerol concentration in the gastrocnemius muscle using a commercial ELISA kit and measured in ng diacylglycerol/mg tissue. D. Total diacylglycerol content in the gastrocnemius muscle.*$P < 0.05$ compared to AC, #$P < 0.05$ compared to RD, +$P < 0.05$ compared to RDV, $^{a}P < 0.05$ for AC compared to RD, $^{b}P < 0.05$ for AC compared to RDV; $^{c}P < 0.05$ for RDV compared to HD.

High-fat diet alters genes involved in the insulin resistance pathway

Differences in systemic glucose tolerance with LIV and diet indicated that molecular pathways involved in glucose metabolism may be affected in skeletal muscle. Skeletal muscle mRNA was assessed in the gastrocnemius muscle of the hindlimb to identify genes related to insulin resistance that are affected by high-fat diet and vibration treatment (Figure 15). Expression of PKCθ was upregulated 56% in high-fat animals compared to RDV ($P < 0.05$) while no difference in expression was detected between RD and HD or any other groups. Expression levels of IRS-1 were down regulated in HDV compared to

RDV by 55% (P < 0.05). Adipogenic genes were also evaluated to determine if high-fat diet upregulates adipogenic differentiation in skeletal muscle and if LIV could attenuate fat accumulation on the genetic level (Figure 15), which may influence muscular insulin resistance. No significant differences were found in PPARγ, a major regulator of adipocyte differentiation. LIV showed trends in the downregulation of FABP4 by 35% (P = 0.099) in HDV animals compared to HD.

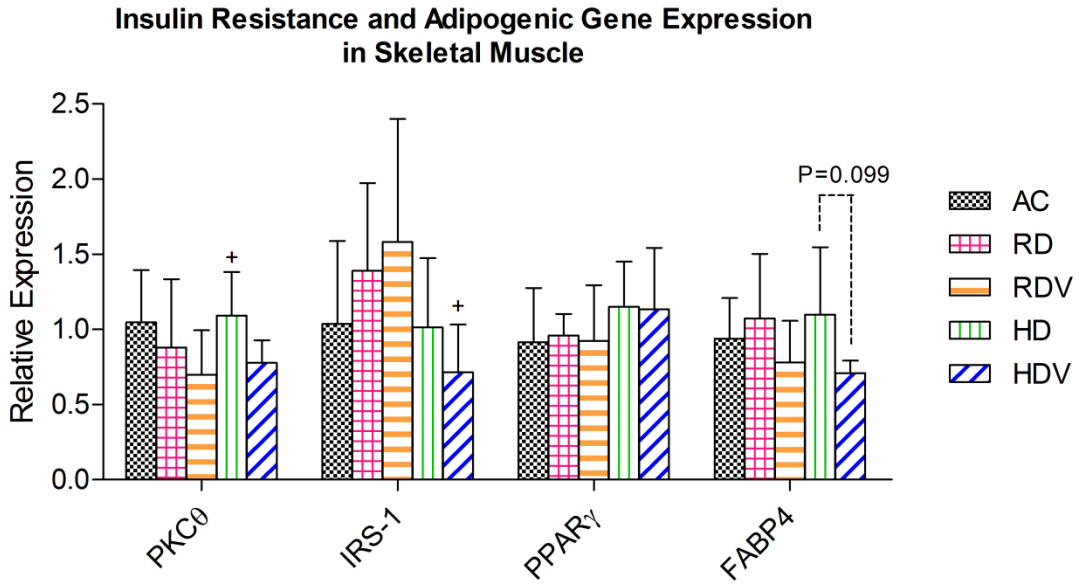

Figure 15: Genes involved in skeletal muscle insulin resistance (PKCθ, IRS-1) and adipogenesis (PPARγ, FABP4) evaluated in the gastrocnemius muscle. Analyses were conducted using Real-time PCR and the delta-delta Ct method of analysis. +P < 0.05 compared to RDV.

DISCUSSION

In this study, a high-fat diet and bone marrow transplantation protocol were used in mice to track the migration of bone marrow cells to tissues outside of the bone (discussed in Aim 3) and to investigate the progression of pre-diabetes in the muscle. We aimed to determine the impact of high-fat diet on the muscular niche, including resident satellite cells and indications of insulin resistance. Modeled after the development of type 2 diabetes in the obese human, we used a moderate high-fat diet that would allow us to study the progression of type 2 diabetes before a severe adipose burden was established. While there is no definitive measure for obesity in the mouse, the animals in this study did not develop a phenotype characteristic of DIO, evidenced by no differences in body weight at the

conclusion of the study. The 45% kcal from fat diet chosen for this study is mild relative to the 60% kcal from fat diet known to rapidly induce type 2 diabetes (176), allowing for slower weight gain and more progressive impact on tissue systems. In addition to the modest diet used, body habitus was also affected by the systemic insult of total body radiation. Irradiated animals gained less of their original body weight compared to non-irradiated controls over the course of the study. Despite these conditions, differences between diets began to occur after two weeks of BMT recovery and immediately prior to LIV treatment. Age-matched, healthy controls had grown an additional 6.7% of their original body weight while pooled regular diet animals had lost 1.6% body weight and pooled high-fat animals 3.6%. At the conclusion of the study and after ten weeks of diet, both RD and RDV had gained less weight than age-matched controls while there were no differences in HD or HDV relative to sham-irradiated animals. These data demonstrate that the high-fat diet protocol influenced body development despite there being no differences in body weight at endpoint. Most importantly, it should be noted that this model was able to induce physiological changes on the cellular and molecular levels caused by high-fat diet, which allows for evaluation of the mechanism of progression of pre-diabetes.

Skeletal muscle wet weights extracted from the hind limbs did not differ from one another in either soleus or quadriceps muscles, demonstrating no phenotypic influences from irradiation or high-fat diet. However, gastrocnemius wet weight was higher in the high-fat group relative to regular diet group. Previous work has shown that irradiation prevents muscular hypertrophy (177, 178) so it is plausible that the increase in weight is not due to an increase in myofibers (via satellite cell proliferation, differentiation, and fusion), but perhaps increased adiposity due to the diet regimen. Elevated levels of intramyocellular lipid have been found in the muscles of obese subjects with larger cross-sectional areas (179), and the gastrocnemius muscle accumulates more fat over time than other muscle groups in the lower limb (131). Inconsistent trends in wet weights may be due to differences in muscle anatomic location, fiber composition, or oxidative capacity. For example, clinical work by Bachmann *et al.* showed that accumulation of intramuscular fat due to a high-fat diet was greater in oxidative muscle (180). A similar increase in gastrocnemius weight was not seen in high-fat animals exposed to vibration treatment compared to animals on a regular diet. Evidence showing that mechanical stimulation in the form of exercise reduces muscular fat content in humans (169) and that LIV prevents adipogenesis in mice (109) could explain this difference. Comparative histological measurements of intermuscular content and fiber size between the three hindlimb muscles would help provide an explanation for differing phenotypes due to diet and mechanical signal exposure.

In the bone marrow cell tracking study, evaluation of satellite cells in the quadriceps muscles showed there was no impact of high-fat diet or LIV treatment on satellite cell proportions at the conclusion of ten weeks. Despite ten weeks of recovery, our data did indicate radiation exposure at a lethal dose of (6+6) Gy reduced satellite cell proportions - all irradiated groups had significantly lower proportions of satellite cells compared to non-irradiated controls. It is hypothesized that irradiation impairs satellite cell activity, and that changes in this stem cell population are responsible for stunted muscle regeneration following exposure (181). Similarly, Boldrin *et al.* show exposure of muscle to irradiation in *mdx* mice results in a significant decrease in percentage of satellite cells per fiber four weeks following exposure (182). It should be noted that in this study there were no differences in either absolute number of satellite cells or total number of muscle cells between any groups. Insignificant differences in absolute cell numbers, both total cell count and satellite cell count, resulted in the presented proportion changes. Since there were no changes in absolute cell numbers, the reduction in proportion of satellite cells alone is insufficient to conclude that radiation exposure has a detrimental effect on satellite cell proportions. With that being said, the importance of the satellite cell pool for muscle homeostasis and anabolism cannot be overlooked, and reductions in satellite cell proportions may translate to significant complications in the stem cell response to injury or mechanical loading.

Irradiation is severe insult to tissue systems that may have masked the impact of high-fat diet on satellite cell proportions. To address this, we performed the same flow cytometric analysis on satellite cells in hindlimb skeletal muscle in two different high-fat diet animals, one with the same 45% kcal from fat diet for eight weeks and the other with a more robust 60% kcal from fat diet for six weeks. High-fat diet animals in these studies developed the obese phenotype and were significantly heavier than regular diet animals. Both analyses demonstrated no differences in either satellite cell proportion or absolute number of satellite cells, confirming our original findings. Similarly, work by D'Souza and colleagues showed no changes in overall satellite cell number in mice following eight weeks of a 60% kcal from fat diet (164). We hypothesized the reduced satellite cell proportion we saw in the OVX animal model was due to a local increase in fat. Differences in our satellite cell data may be because stem cell reductions in OVX were influenced by a confounding variable (*i.e.* estrogen depletion) or because we used two different animal models and high-fat diet does not elicit the same type of systemic fat insult (*e.g.* different mechanism of action).

Despite there being no changes in satellite cell proportions between diets, it is possible high-fat diet altered the proliferative and functional capabilities of the stem cells. D'Souza and colleagues' work

also demonstrated that even though there were no changes in satellite cell number, DIO caused impairment in satellite cell activation and *ex vivo* proliferation (164). As with OVX, we investigated the reversible state of activation of satellite cells using CD34, following ten weeks of diet and eight weeks of LIV treatment. Ieronimakis *et. al* showed that regardless of anatomic location, there were consistently more CD34+ cells than CD34- across skeletal muscle groups, while the ratio of CD34+:CD34- in C57BL/6 male mice is relatively similar (111). The results in the presented study are consistent with this finding, apart from the regular diet animals exposed to LIV for eight weeks. In the same study, Ieronimakis *et al.* demonstrated a shift in proportions towards CD34- following cardiotoxin induced muscle injury, which was due to the initiated myogenic repair response. This same shift of satellite cells from CD34+ to CD34- was observed in our study in RDV, suggesting that mechanical stimulation is priming the satellite cell pool for myogenesis. To support this conjecture, it is well-known that exercise is anabolic to muscle and that mechanical stimulation in the form of LIV has also been shown to increase the cross-sectional area of muscle fibers in murine hindlimbs (81) as well as influence satellite cell populations in mice (174). Our data also indicate that the satellite cell response to mechanical stimulation is influenced by diet. In the non-irradiated 45% high-fat diet study, there was no shift in CD34+:CD34- satellite cells with either high-fat diet or vibration. Similar to our original tracking study, the shift towards the CD34- population only occurred in animals on regular diet, not high-fat.

Changes in muscular composition significantly alter the overall health of the muscle tissue and are likely to occur in high-fat diet regimens. Increases in intermyocellular lipid and ectopic adipocytes in the musculature are found in a range of conditions with decreased muscle mass and strength, including aged muscle (183), disuse (184), and muscular dystrophies, such as Duchene muscular dystrophy (DMD) (185). Fatty muscle has also been identified in the pathogenesis of obesity (126) and linked to poor muscular mechanical properties (179) and insulin resistance (186). We examined skeletal muscle tissue on the histological level to determine if the muscular composition of the soleus was altered in the presence of high-fat diet. Our data show that high-fat diet animals fed a 45% kcal from fat high-fat diet for only ten weeks had a 717% increase in lipid content compared to regular-diet animals on a 10% kcal from fat diet and a 260% increase relative to regular diet animals subject to vibration. Importantly, these data demonstrate that intermuscular fat accumulation precedes the formation of large visceral fat depots, or a body habitus in the mouse that would parallel the obese condition in the human. Clinical work using nuclear magnetic resonance spectroscopy has associated increased skeletal muscle fat with insulin resistance (187, 188), which may suggest that the muscle phenotype seen in the high-fat animals

of this study will lead to impaired insulin signaling or insulin resistance. Additionally, the observed levels of lipid may indicate compromised mechanical properties of the muscle. Indeed, accumulation of fat in skeletal muscle is not homogenous and our findings in the soleus may not be paralleled in other muscle groups. High-fat diet induced accumulation of fat tends to be greater in oxidative muscle, such as the soleus (180), and all muscle groups must be analyzed for a comprehensive evaluation of skeletal muscle tissue.

Exercise is anabolic to muscle and catabolic to fat, and exercise interventions in the clinic have proved to positively impact metabolism and a multitude of physiological systems. Exercise reduces muscular fat content in humans (126, 169) and muscle triglycerides in animal models of high-fat diet (189). This reduction of ectopic lipid is associated with improved insulin resistance (168, 169). Additionally, a *lack* of physical activity is associated with increased intermuscular adipose tissue in people with type II diabetes (190). It has been proposed that decreases in fat and improved metabolism, as achieved through exercise, can also be attained via mechanical signals of much lesser magnitude (<1*g*, where *g* is earth's gravitational acceleration at 9.81m/s^2) than traditional exercise regimes. This allows for the incorporation of a safe musculoskeletal therapy for patients who are in need of reaping the benefits of exercise, but are unable to exercise due to mobility limitations or frailty. Previous work using LIV treatment has demonstrated the ability of this mechanical signal to be anabolic to muscle (81), prevent adipogenesis in a murine model of diet-induced obesity (109), and reduce adipogenic gene expression in murine skeletal muscle (174). Using histological evaluation of oil red O staining, our work exhibited a trend towards a diet-specific reduction in adipogenesis in skeletal muscle on the cellular level. In line with our hypothesis, high-fat diet animals that received LIV treatment accumulated 54% less lipid than non-treated animals. While the difference between HD and HDV is only a trend (P = 0.06), a decrease of fat by half is encouraging, considering the brief treatment period of eight weeks for only 30 minutes/day. Similar reductions in lipid were not seen in regular diet animals that received LIV treatment, which may be a result of these animals lacking excess depots of fat. The use of LIV to mitigate the accumulation of intermuscular fat may prove to be useful as an adjunct therapy for patients suffering from obesity, type II diabetes, or other debilitating musculoskeletal disorders, helping to maintain healthy muscle tissue.

The development of type II diabetes is gradual and is often initiated by excess caloric consumption, lack of physical activity, or some combination of the two. Insulin resistance and subsequent β–cell compensation will lead to β–cell dysfunction and eventually hyperglycemia and type

II diabetes (191). While it is well-established that obesity causes impaired glucose sensitivity or glucose intolerance in both humans (192) and animal models (193), the molecular mechanisms behind the development of insulin resistance are still debated. Here, we investigated indices of impaired insulin signaling in skeletal muscle using a murine model that follows the pathogenesis of type II diabetes in the human (176), allowing for study of early state insulin resistance development.

First, we investigated the influence of high-fat diet and LIV treatment on systemic glucose tolerance in mice as measured by the GTT assay. As expected, animals in this study were not diabetic and there were no differences in fasting glucose levels at the completion of the study. Similarly, animals fed a high-fat diet did not have lower glucose tolerance than regular diet animals nor non-irradiated controls. Interestingly, LIV did have an effect on the regular diet vibration group, improving glucose tolerance compared to age-matched controls in both AUC and in plasma glucose levels at multiple time points in duration of the GTT assay. Additionally, high-fat diet animals had a 16% higher AUC (lower glucose tolerance) than RDV and higher plasma glucose levels at the final GTT time point, demonstrating LIV was able to improve glucose tolerance in regular diet animals. Moreover, a similar increase in glucose intolerance relative to RDV was not seen in the HDV group, suggesting LIV may also be effective in improving glucose tolerance in animals fed a high-fat diet. A reduction of total fat improves glucose tolerance and LIV has been shown to inhibit adipogenesis in a mouse model (82). While LIV had no effect on body mass, reductions in fat on the cellular level, as seen in oil red O staining, may contribute to the improvements in glucose tolerance seen with LIV treatment. Duncan *et al.* also demonstrated that improved insulin sensitivity in humans can be achieved through exercise without there being any weight loss (194). Further investigation into the mechanism of how LIV influences glucose tolerance and insulin sensitivity is needed.

Despite no clear differences between diets as measured with the GTT in the blood, increased intermuscular adipose tissue may have had effects on muscular insulin resistance, insulin signaling, and ectopic fat formation. Muscular insulin resistance can develop independent of systemic adiposity (195), emphasizing the importance of local signaling. Contrary to the Randal hypothesis that attributes insulin resistance to increased fatty acid infiltration and the subsequent inhibition of pyruvate dehydrogenase activity and lower glucose oxidation (196), strong evidence suggests the mechanism of development is actually mediated by the intermediate fat metabolite diacylglycerol and subsequent activation of the theta isoform of protein kinase C (PKCθ), ultimately inhibiting insulin signaling and glycogen synthesis (197). The ectopic accumulation of fat within the musculature is solely responsible for the interrupted

insulin signaling that leads to the diabetic condition. Despite seeing markedly increased lipid content in the soleus of high-fat animals, we did not see any differences in DAG content in the gastrocnemius muscle of high-fat diet animals compared to either regular diet or age-matched controls. Curiously, the only difference found was a reduction in HDV DAG concentration relative to RD, perhaps through the same mechanism that LIV was able to reduce adipogenic gene expression in muscle in OVX (174).

Previous work has shown transient increases in DAG due to lipid infusion in the skeletal muscle of both rats (198) and humans (199). Additionally, elevated DAG concentrations have been seen in humans that are obese and have type II diabetes (199). While data suggests DAG leads to insulin resistance, it should be noted that DAG plays a complex role in the muscle and higher concentrations of this metabolite do not necessarily translate to impaired insulin signaling. For example, total myocellular DAG content was found to be greater in well-trained athletes who also had higher insulin sensitivity compared to obese, sedentary adults (200). This echoes the "athlete's paradox", which demonstrates well-trained endurance athletes often have higher levels of skeletal muscle adiposity in the form of triglycerides (127). Despite these considerations, our data are likely to not have demonstrated increased DAG in high-fat diet animals because of the time point of sampling (missing the transient increase of DAG) or nature of the assay; DAG concentrations are typically measured *in vivo* using magnetic resonance spectroscopy.

Although DAG concentrations were not elevated in high-fat diet animals after ten weeks of diet, we observed changes on the genetic level in the insulin signaling pathway of high-fat diet animals. DAG initiates the upregulation of PKCθ, which in turn upregulates serine phosphorylation of IRS-1 (site Ser 1101) and prevents the tyrosine phosphorylation of IRS-1; this is followed by a series of downstream events leading to reduced translocation of GLUT4 and reduced glucose uptake (197). PKCθ expression was upregulated in HD relative to RDV and IRS-1 expression levels were influenced by vibration treatment only in the high-fat diet group. This suggests DAG content may have been elevated at an earlier point in time and disrupted this signaling cascade.

PPARγ is a key transcription factor for adipocyte differentiation and no differences were seen in expression levels in the muscle. However, as demonstrated with histology, high-fat diet animals had significant lipid accumulation in the soleus. Notably, even with excessive lipid accumulation, ectopic adipocytes were not observed in histological sections, conceivably explaining the lack of PPARγ expression in the muscle. The observed lipid accumulation following ten weeks of high-fat diet, likely occurring in FAPs, occurred prior to the differentiation of these cells into adipocytes, but after - or in

conjunction with - alterations in the insulin signaling pathway. The trend of a reduction of FABP4 expression in HDV compared to HD parallels the trend seen in the soleus with vibration reducing lipid content in HDV. This indicates vibration is able to mitigate accumulation of fat in the muscle on both the phenotypic and genetic levels.

The status of the satellite cell pool and muscular insulin signaling are two characteristics of the muscle microenvironment important in maintaining overall homeostasis and health of the tissue. In this model of pre-diabetes, we established that high-fat diet increases intermuscular fat and alters the insulin signaling pathway, while LIV is able to reduce fat in the muscle tissue, which may invariably translate to improved insulin resistance in the long-term. Importantly, formation of intermuscular fat precedes systemic weight gain. We also determined that high-fat diet does not alter stem cell proportions in this particular model, but may influence how satellite cells respond to mechanical stimulation. These findings indicate that LIV may be an effective alternate or adjunct therapy to exercise that improves the condition of the muscle and may prevent the onset of insulin resistance in the pre-obese population. A closer investigation into the signaling pathways involved in insulin resistance and mechanism of ectopic fat formation in the muscle is necessary to get a clearer picture of how high-fat diet impacts the muscle microenvironment and the mechanism in which LIV may protect the tissue.

CHAPTER 4: HIGH-FAT DIET ACCELERATES BONE MARROW CELL MIGRATION TO VISCERAL ADIPOSE DEPOTS

INTRODUCTION

Using high-fat diet to model pre-diabetes in the mouse, we demonstrated ten weeks of high-fat diet increases intermuscular fat, preceding changes in body habitus. The shift in phenotype seen in skeletal muscle is driven by cellular and/or molecular changes and requires further exploration. In the development of obesity, the expansion of fat depots is mediated through fat pad remodeling and adipocyte lineage commitment (201). Adipocytes are derived from the MSC lineage and differentiation towards the adipogenic fate is primarily driven by the expression PPAR genes (202). MSCs are residents to multiple tissue sources (203), including adipose tissue and bone marrow, although the marrow cavity is a primary environment for the stem cell population. While there is evidence that the functional capabilities of MSCs are not equal due to differences in tissue origin (102, 204), both bone marrow-derived and adipose tissue-derived MSCs are capable of differentiating into adipocytes and may play a role in the upregulation of fat seen in animals on a high-fat diet.

The significant adipose accumulation associated with DIO fat pad expansion may in part depend on the recruitment of bone marrow-derived MSCs to visceral fat depots rather than solely abdominal fat stem cell populations. In a model where the expansion of fat is rapidly occurring, it is plausible that migration and recruitment of stem cells are accelerated or altered due to the changes in cytokine and adipokine release into the circulation. Moreover, the infiltration of macrophage and leukocyte populations that occurs during an obese state (204, 205) may result from either resident fat tissue origin or recruitment from the bone marrow niche, which is also a primary site of haematopoiesis (62). The dynamic state of the bone marrow (63) and identification of signaling molecules that play a role in regulating bone marrow cell migration (206-208) suggest bone marrow cells are able to leave the marrow niche and engraft in peripheral tissues, including fat, muscle, and blood. Evidence in both humans (209) and mice (82, 210, 211) suggests the expansion of fat depots is not solely a result of utilizing permanent resident adipogenic progenitors; however, the extent to which bone marrow cells participate in the formation or function of abdominal fat is inconclusive.

To reduce abdominal fat caused by excess caloric consumption or a fatty diet, exercise is a commonly prescribed methodology that is well known to be catabolic to adipose tissue. As an adjunct to - or surrogate for exercise, the use of mechanical stimulation in the form of LIV (<0.4*g* where g is earth's gravitational field at 9.81m/s^2) has been shown to alter stem cell differentiation in favor of osteoblastogenesis (109), consequentially preventing the formation of fat (82, 109). Considering the ability of LIV treatment to bias stem cell differentiation, LIV may aid in preventing the differentiation of

MSCs into adipocytes in visceral fat pads or skeletal muscle tissues in the presence of high-fat diet. Additionally, mechanical stimulation may also normalize bone marrow cell migration patterns altered in the high-fat diet model.

Tracking the migration and engraftment of bone marrow cells in the presence of an adipose burden allows for a better understanding of the influence of a fatty diet. Understanding the dynamic roles bone marrow-derived cells play in the maintenance of tissue regeneration and repair can be useful for both the basic understanding of obesity and therapeutic applications of cellular transplants in obese patients. Using bone marrow transplantation methodologies, animals in this aim were subjected to a lethal dose of radiation to first eliminate host bone marrow cells and were then rescued with a bone marrow transplant that would aid in the reconstitution of the marrow cavity. GFP+ whole bone marrow was used to track the migration, engraftment, and/or differentiation of bone marrow cells to other tissue sites, including visceral fat and skeletal muscle.

This study tested the hypothesis that bone marrow cells support visceral fat formation through the recruitment of bone marrow cells to abdominal fat tissue, a process that is accelerated by high-fat diet and mitigated by mechanical stimulation. Using a bone marrow transplantation model, we tracked GFP+ bone marrow cells from transgenic donors to peripheral tissues in mice and identified the impact of high-fat diet on bone marrow-derived cellular migration and the ability of LIV to reverse alterations in migration caused by high-fat diet.

MATERIALS AND METHODS

Experimental design and animal procedures

All experimental procedures were approved by the Stony Brook University Institutional Animal Care and Use Committee. Experimental timeline and all animal procedures, including irradiation, bone marrow transplantation, diet regimes, and vibration treatment can be found in detail in Specific Aim 2. Briefly, forty 8 week old C57BL/6 male mice were exposed to (6+6) Gy of whole body gamma irradiation and rescued within 24 hrs with whole bone marrow transplants ($10x10^6$ cells from GFP+ transgenic donors). The day following transplantation, animals were put on a regular (10% kcal from fat) or high-fat (45% kcal from fat) diet. After two weeks of recovery on assigned diets, half the animals from each diet group were exposed to LIV treatment (0.25*g*/ 90 Hz, 30 min/day, 5 day/wk for 8 wk), yielding the following four groups: regular diet (RD, n=10), regular diet with LIV (RDV, n=9), high-fat diet (HD, n=10),

and high-fat diet with LIV (HDV, n=10). Age-matched control animals were sham irradiated, injected, and vibrated (AC, n=10). All animals had *ad libitum* access to food and water and body weight was monitored every other day for two weeks following irradiation and every week thereafter. Animals were euthanized via cervical dislocation ten weeks following experiment start and at 18 wk of age.

Quantitative Polymerase Chain Reaction of gene expression in the fat and bone marrow

Following euthanasia, the mesenteric fat pad was excised, cut, and stored in RNAlater at -20°C. Bone marrow was flushed from the femur directly into RNA later and also stored at -20°C. RNA from the fat pad was isolated using a commercial spin kit for fat tissue (RNeasy Lipid Tissue Mini Kit, Qiagen, Austin, TX) and the RNeasy Mini Kit (Qiagen, Valencia, CA) for bone marrow. RNA purity was quantified using 1 µl mRNA and a nanodrop spectrophotometer (NanoDrop 1000 Spectrophotometer, Wilmington, DE). cDNA conversion was performed using a high capacity reverse transcription kit (Applied Biosystems, Foster City, CA) and amplification was conducted using TaqMan Gene Expression Assays and TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA). Expression levels of genes critical to adipogenesis, PPARγ, FABP4, and PPARδ, were analyzed in the fat pad and those critical to MSC differentiation, PPARγ and RUNX2, were analyzed in the bone marrow (TaqMan gene expression assays, Applied Biosystems, Foster City, CA). All genes were compared to housekeeping gene GAPD and relative expression was compared against intact control animals using the delta-delta Ct method of analysis.

Flow cytometric analysis of cell populations

To evaluate GFP in skeletal muscle, cells from the quadriceps muscles were prepared as previously described in Aim 1. Briefly, muscles were harvested at euthanasia, minced, pooled, and stored in supplemented DMEM (Gibco, Carlsbad, CA, USA). Tissue was digested, triturated, and neutralized with supplemented DMEM containing 15% horse serum (Thermo Scientific, USA). Fat tissue was similarly minced upon euthanasia, stored in supplemented DMEM, and digested using collegenase. For GFP analysis in all tissues (skeletal muscle, bone marrow, blood, and fat) and MSC quantification (Sca-1+, C-Kit+, CD44+, and CD90.2+) in fat and bone marrow, mononuclear cells were filtered with 40 µm nylon cell strainers (BD Biosciences, San Diego, CA). Samples were centrifuged at 2000 RPM for 5 minutes and resuspended in 1x lysis buffer (Pharm Lyse, BD Biosciences, San Diego, CA) for 5 minutes at room temperature. Cells were again centrifuged, re-suspended in DMEM supplemented with 1% penicillin/streptomycin, and counted. $1x10^6$ cells were removed from each sample for staining. Flow

cytometric analysis was conducted using the FACSCalibur system (BD, San Jose, CA, USA) at 200,000 events per sample. Data was analyzed with FlowJo V7.2.5 (TreeStar Inc., San Carlos, CA).

Immunohistochemistry staining of GFP in the gonadal fat pad

At euthanasia, gonadal fat pads were extracted, minced, and fixed in 4% paraformaldehyde for 24 hours and then moved to PBS for long-term storage. Fad pads were embedded in paraffin and sectioned at 8 μm on a sledge microtome (Leica, Bannockburn, IL). Paraffin sections were deparaffinized and rehydrated with a series of ethanol gradients. Antigen retrieval was achieved using a commercial kit (Retrievagen A, BD Biosciences, San Diego, CA). Sections were then blocked with 3% hydrogen peroxide for 10 min and 2% non-fat milk for 1 hr at room temperature. Sections were stained with primary antibody Goat anti-GFP (ABCAM) at 1:200 overnight and at 4°C. Secondary antibody Rabbit anti-goat IgG, Alexa Fluor 488 (ABCAM) was diluted at 1:200 and incubated for 45 min at room temperature.

Bone marrow adipocyte encroachment

Upon animal euthanasia, tibias were extracted and fixed in 4% paraformaldehyde for 24 hours and stored long-term in PBS at 4°C. Bones were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) adjusted to a pH of 7.4 for two weeks. Following decalcification, bones were dehydrated using a series of ethanol solutions and embedded in paraffin wax (Leica, Richmond, IL). Specimens were sectioned on a sledge microtome (Leica, Bannockburn, IL) at 8 μm thickness approximately 1200 μm into the lateral side of the metaphysis. Sections were subsequently deparaffinized, rehydrated, and stained using wright-giemsa stain (Sigma-Aldrich, St. Louis, MO). Slides were imaged at 2.5x in bright-field on a Zeiss microscope. Processing of the bone tissue removed all adipocytes from the marrow space, leaving white ghosts easily distinguishable from the surrounding blue tissue. Sections were analyzed below the growth plate and small tears were subtracted from the total area. All analyses were done blinded using ImageJ software (ImageJ, NIH).

Statistical Analyses

Comparisons between groups were made using a one-way ANOVA and Tukey post-hoc test. All data is mean ± standard deviation. All data were considered significant when $P < 0.05$ and all analyses were done using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Diet induced weight gain

Raw body weight data can be found in Chapter 3, Figure 9. In short, age-matched controls maintained significantly higher body weight than all four irradiated groups and diet did not have an effect on raw body weight. Age-matched controls also maintained a greater percent increase in body weight (relative to weight pre-irradiation) compared to all irradiated groups beginning Day 0 (one day following irradiation) and maintained this difference for all time points ($P < 0.001$) (Figure 16A). Immediately prior to the start of LIV treatment (Day 14), pooled RD and HD groups had lower body weight changes compared to the 6.7% weight gain in AC (-1.6% in RD and –3.6% in HD, $P < 0.001$) while HD groups trended toward having lost more weight than RD groups ($P = 0.068$) (Figure 16B). Total weight gain from diet start shows both regular diet groups gained less weight throughout the course of the study compared to AC (average of -39%, $P \leq 0.01$) while there was no difference in either high-fat diet group compared to AC ($P > 0.05$) (Figure 16C). Vibration had no effect on body weight.

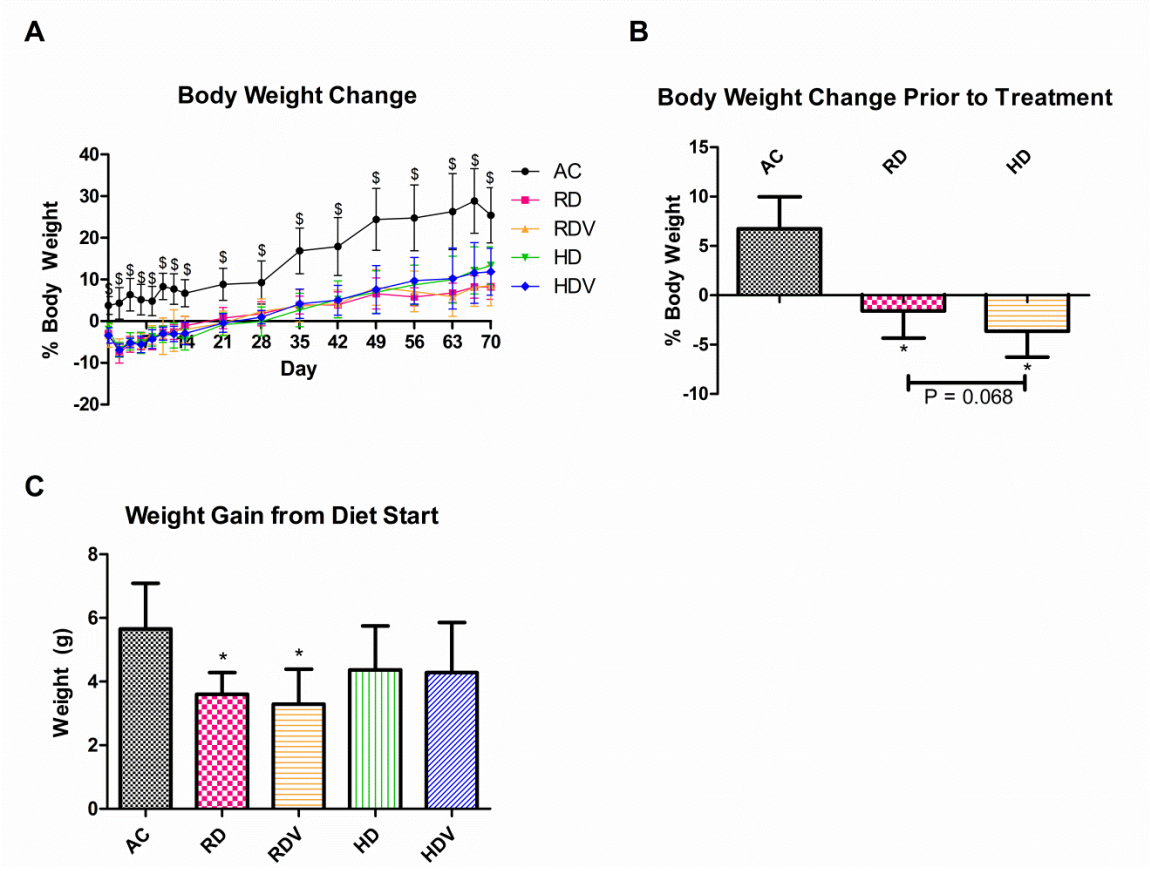

Figure 16: A: Percentage of body weight increase throughout the duration of the study. B: Percentage of body weight increase immediately prior to LIV treatment (day 14 of study) with diet groups pooled. C: Total weight gain over the 10 wk period. $P < 0.005 compared to all four irradiated groups, *P ≤ 0.01 compared to AC.

High-fat diet increases adipogenic gene expression in visceral fat pad

No differences in wet weight were detected between any irradiated groups in the visceral fat pads (Figure 17). Compared to non-irradiated controls (AC), RD had lower wet weights in the gonadal fat pad (-38%, P < 0.01) and mesenteric fat pad (-33%, P < 0.05) due to irradiation exposure, as did RDV (-37%, P < 0.01) and HD (-28%, P < 0.05) in the gonadal fat pad (Figure 17A-B). While there were no apparent differences in fat pad weight due to diet regimen, high-fat diet did increase genetic expression of key genes involved in adipogenesis (Figure 17C). Relative to RD, both HD and HDV had greater expression of PPARγ (+82% and +112%, respectively, P < 0.01) and PPARδ (+93% and 104%, respectively, P < 0.05) at the end point of the experiment. HDV expression of PPARγ was also 39% higher than RDV (P < 0.01). No differences in expression were detected in FABP4.

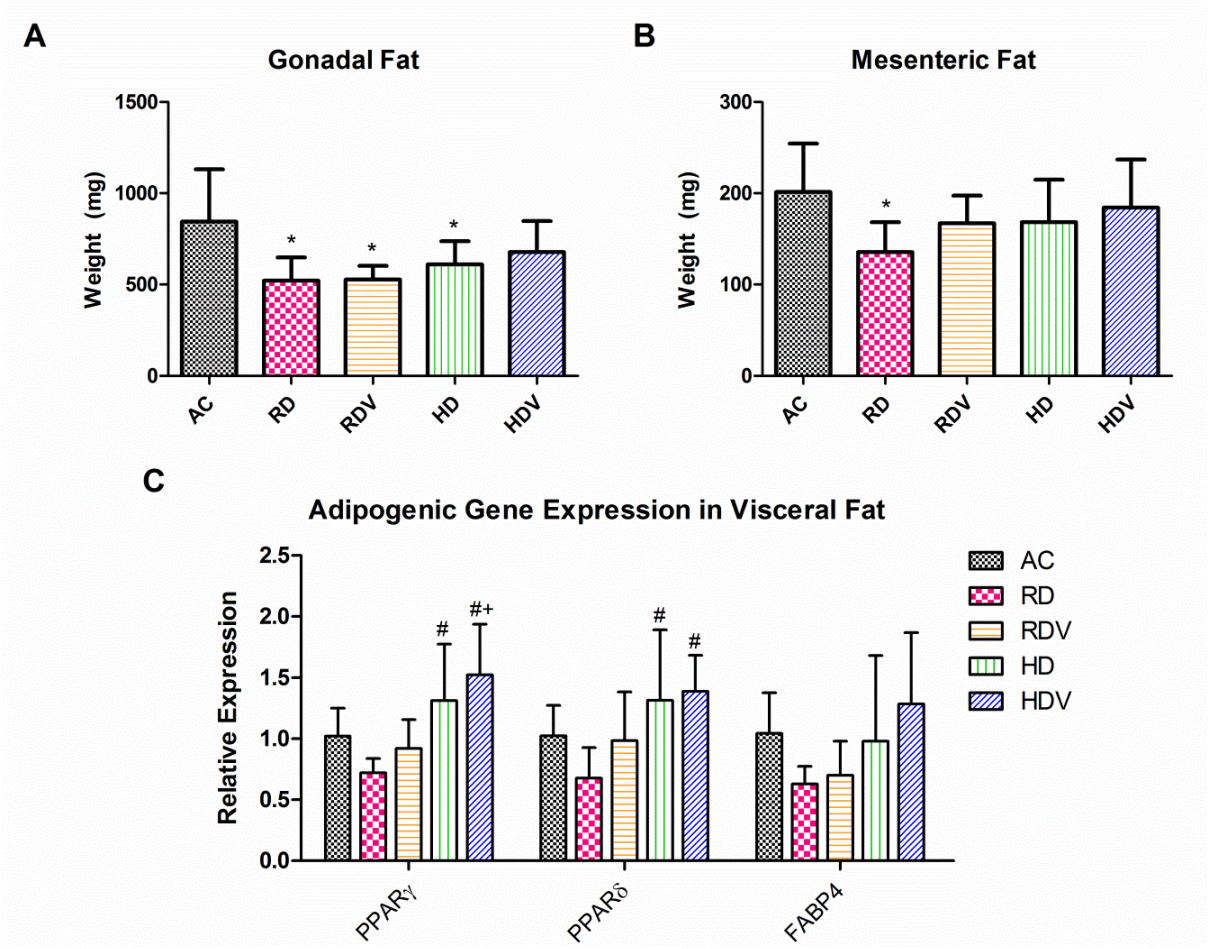

Figure 17: A: Gonadal fat pad wet weight at euthanasia. B: Mesenteric fat pad wet weight at euthanasia. C: Adipogenic gene expression analysis in the mesenteric fat pad (PPARγ, PPARδ, FABP4). Analyses were conducted using Real-time PCR and the delta-delta Ct method of analysis. *P < 0.05 compared to AC, #P < 0.05 compared to RD, +P < 0.05 compared to RDV.

High-fat diet influences migration of bone marrow cells

After 10 wk, transplanted bone marrow cells (GFP+) were detected in the fat pads of all BMT groups (between 21-30% of total fat cell population excluding adipocytes), suggesting that bone marrow cells contribute to the formation and/or function of visceral fat depots (Figure 18A). Interestingly, the proportion of transplanted bone marrow cells in the gonadal fat pad was higher in both HD and HDV groups compared RD (+38% and +43%, respectively; P < 0.05) despite no differences in visceral fat pad weights (Figure 18A), demonstrating that high-fat diet accelerates bone marrow cell migration to visceral fat. While there was substantial GFP engraftment in the bone marrow (40-44%) and blood (27-40%) of all irradiated groups, no differences were detected between diets in these tissues (Figure 18B-

C). Skeletal muscle engraftment of transplanted bone marrow cells was detected at levels about 1000-fold lower than in the fat, bone marrow, and blood, at 0.01-0.03% (Figure 18D). Compared to RD, RDV had a 95% increase in engraftment ($P < 0.05$). Both HD and HDV groups had lower engraftment than RDV, with 50% and 66% reductions, respectively (both $P < 0.05$).

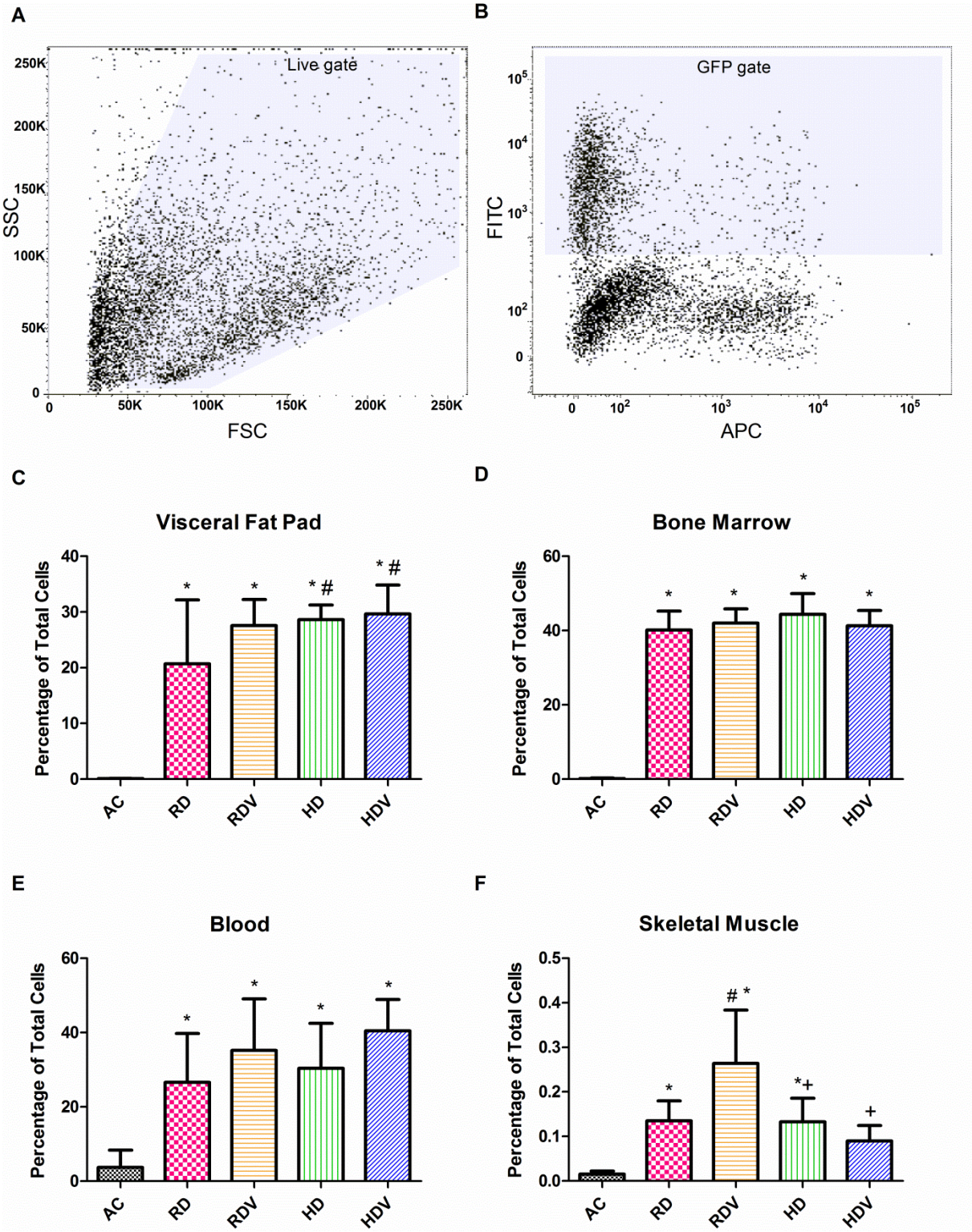

Figure 18: Migration of transplanted GFP+ bone marrow cells quantified via flow cytometric analysis and represented as the percentage of total cell count, which excludes red blood cells for all tissues. A:

Visceral fat pad (gonadal); total cell count includes the stromal vascular fraction and excludes adipocytes. B: Bone marrow. C: Peripheral blood. D: Skeletal muscle (quadriceps); total cell count is of whole muscle homogenate. *P < 0.05 compared to AC, #P < 0.05 compared to RD, +P < 0.05 compared to RDV.

High-fat diet accelerates MSC migration

Bone marrow-derived MSCs (Sca-1+, C-Kit+, CD44+, CD90.2+, GFP+) were quantified in the gonadal fat pad using flow cytometric analysis and endogenous GFP signal. No differences were detected between any groups except for increased MSC proportions in HD compared AC (P < 0.05), suggesting that not only does high-fat diet accelerate total bone marrow cells to visceral fat, but specifically recruits MSCs (Figure 19A). Total MSC populations quantified in the bone marrow show HDV having lower proportions than healthy controls (-39%, P < 0.05) and RDV similarly having a trend toward a lower MSC proportions (-32%, P = 0.095) (Figure 19B). Immunohistochemical analysis of the gonadal fad pad embedded in paraffin wax shows the localization and engraftment of bone marrow derived cells (Figure 19C). GFP+ cells in the tissue section depict both adipocytes and cells in the stromal vascular fraction, showing that injected bone marrow cells, which excluded adipocytes, migrated and differentiated into adipocytes in visceral fat tissue.

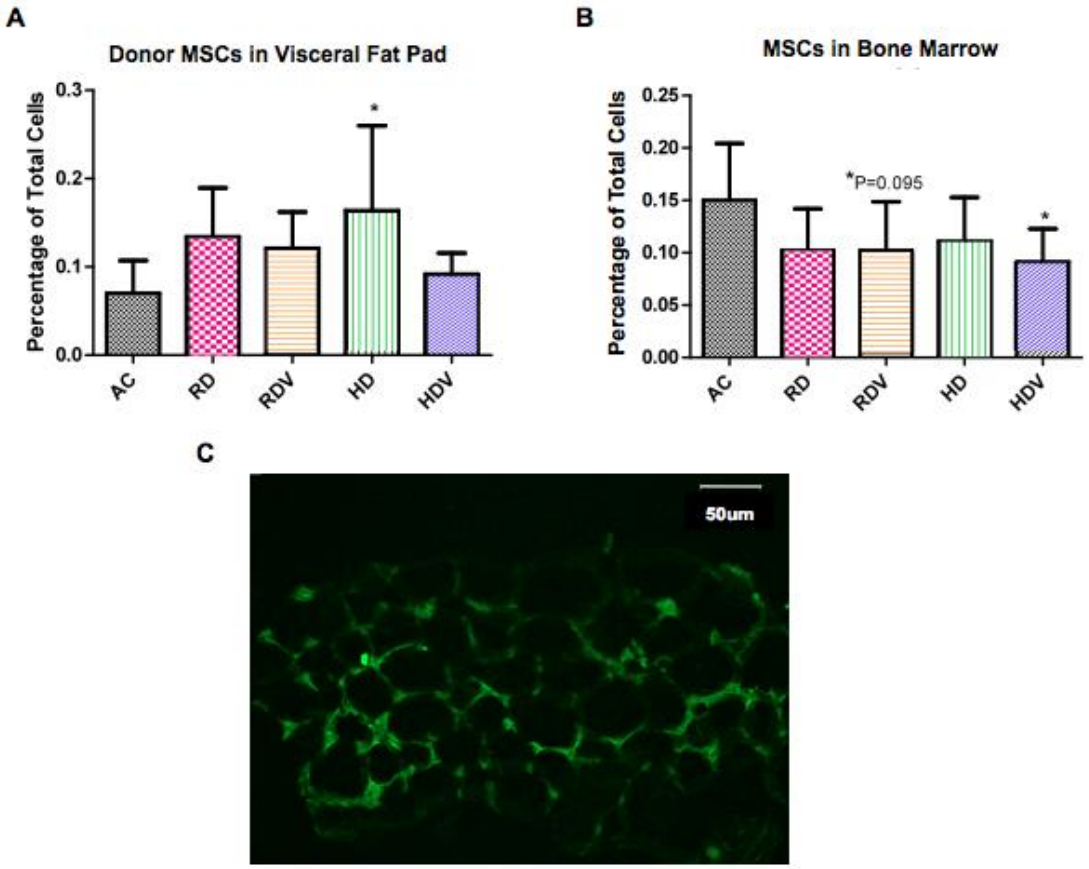

Figure 19: Donor bone marrow cells and MSCs (Sca-1+, C-Kit+, CD44+, and CD90.2+) found in the visceral fat pad and bone marrow cavity. Cells were quantified via flow cytometric analysis and are represented as the percentage of total cell count, which excludes red blood cells for both tissues. A: Visceral fat pad (gonadal); total cell count includes the stromal vascular fraction and excludes adipocytes. B: Bone marrow. C: Representative image of donor cell engraftment in the gonadal fat pad of the RD group using immunohistochemical staining of GFP. *P < 0.05 compared to AC.

Bone marrow adiposity

To investigate the condition of the bone marrow and cellular changes in the bone marrow niche, perhaps in part what is influencing bone marrow cell migration, we evaluated the influence of diet and LIV on fat accumulation by quantifying the number of adipocytes, average adipocyte cell size, and apparent adiposity in the proximal tibial metaphysis (Figure 20A). The number of adipocytes was greater in HDV relative to both RD and RDV, by 120% and 167%, respectively ($P < 0.05$) (Figure 20B). High-fat diet increased the average size of adipocytes in the marrow in both HD and HDV groups by 41% compared to RDV (both $P \leq 0.05$) (Figure 20C). Lastly, apparent adiposity was calculated to see the overall impact of both adipocyte size and number. HDV had an increase of 273% total adiposity

compared to RDV ($P < 0.05$) and had a trend towards a difference relative to RD, with a 192% increase ($P = 0.06$) (Figure 20D).

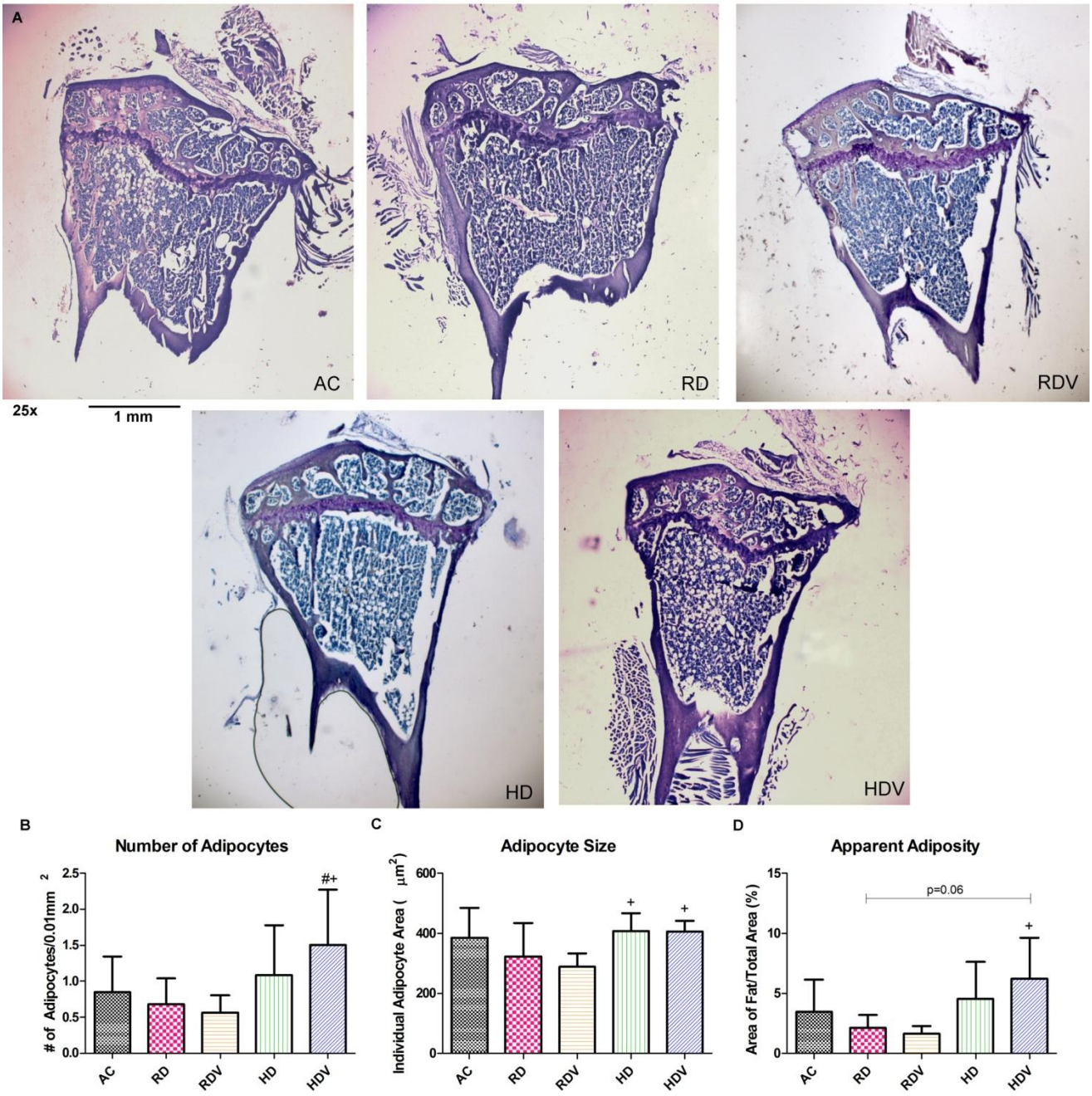

Figure 20: Bone marrow histomorphometry using wright-giemsa stain in paraffin sections to visualize and quantify bone marrow adiposity. A: Representative images of the proximal tibial metaphysis. B: Average number of adipocytes per area. C: Average size of individual adipocyte cells. D: Apparent

adiposity represented as area occupied by fat/total area below the growth plate. #P < 0.05 compared to RD; +P < 0.05 compared to RDV.

Bone marrow gene expression

Changes in the bone marrow phenotype due to high-fat diet in the proximal tibia may indicate changes in MSC differentiation on the genetic level. We investigated two major regulators of MSC differentiation, PPARγ and RUNX2, to see if MSCs were being biased towards the adipogenic or osteoblastic lineage in the bone marrow of the femur (Figure 21). Largely due to variability in the data, no differences were detected in either PPARγ or RUNX2 expression between any groups.

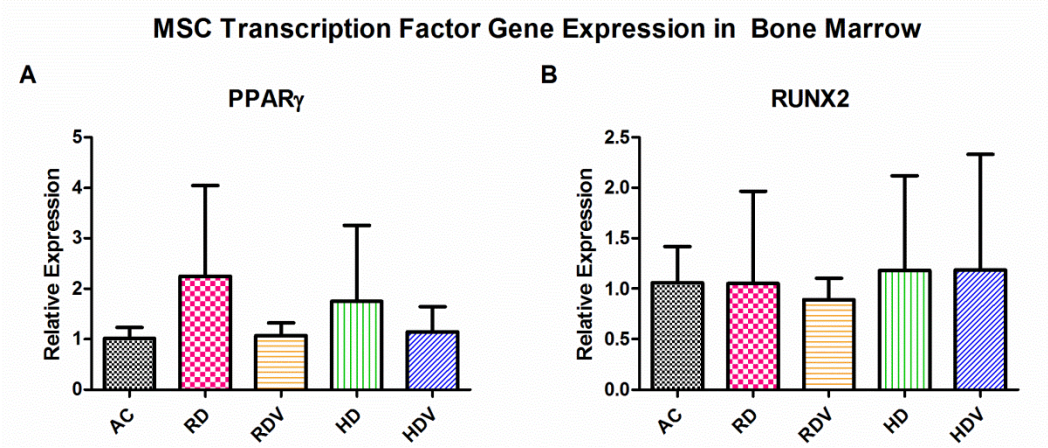

Figure 21: Adipogenic (PPARγ) and osteoblastic (RUNX2) gene expression analysis in the bone marrow of the femur. Analyses were conducted using Real-time PCR and the delta-delta Ct method of analysis.

DISCUSSION

In this study, a bone marrow transplantation protocol was used to track the migration of GFP+ bone marrow cells to tissues outside of the bone in the presence of high-fat diet in mice. The bone marrow niche is a dynamic environment (63) and identification of signaling molecules that play a role in regulating bone marrow cell migration (206-208) suggest bone marrow cells are able to leave the marrow niche and engraft in peripheral tissues. Following the delivery of whole bone marrow into the circulation of mice exposed to whole body irradiation, we analyzed migrated and engrafted bone marrow cells via flow cytometric analysis in the visceral fat pads, bone marrow, blood, and skeletal muscle tissues using the endogenous GFP signal of bone marrow derived from GFP+ transgenic mice. Successful rescue of recipient mice using bone marrow transplantation (10,000 GFP+ whole bone

marrow cells excluding adipocytes) was confirmed via survivability 8 weeks following injection (total survivability of 98%; data presented in Chapter 3, Figure 9). The chosen total body fractionated dose (6+6 Gy) and interval (4 hr) has been demonstrated to yield high survival and low graft failure rates in allogenic murine bone marrow transplantations, while also reducing adverse effects compared to single lethal doses of radiation (212).

Well-established protocols for *in vivo* transplantation of tagged bone marrow cells can be found in the literature, and administration via tail vein injection is a widely used methodology with applications that include bone marrow cell tracking. Following the delivery of whole bone marrow into the circulation of mice exposed to whole body irradiation, donor cells are actually found in most tissues, including lung, intestine, liver, heart, and brain, making it difficult to distinguish a true physiologic migration of cells from relocation solely due to injection into the circulation or the damaged state of the organs. A cell tracking study conducted by Taguchi *et al.* in 2013 demonstrated that tail vein injected bone marrow cells following lethal irradiation do in fact need to home in the bone marrow cavity prior to migrating to peripheral tissues (213). Since the homing of bone marrow cells in the marrow cavity occurs within 48 hours (214), the ten week time lapse suggests that the detection of GFP+ cells outside of the bone is due to true migration and engraftment. Additionally, recipient animals did not receive respective diets until the day following bone marrow transplantation. Because at the time of injection all recipient animals were receiving the same regular chow diet, the differences observed in migration patterns ten weeks following the procedure must be due to long-term diet effects rather than physiologic/biologic differences at the time of transplantation.

While there is no definitive measure for obesity in the mouse, a true DIO phenotype did not develop due to irradiation exposure and ten week timeline, evidenced by no differences between diets in body weight or visceral fat pad weights. All irradiated animals gained less of their original body weight compared to non-irradiated controls. However, differences between diets began to occur after two weeks of recovery and immediately prior to LIV treatment, when pooled regular diet animals had a trend towards having lost less of their original body weight than pooled high-fat diet groups. After two weeks of specified diets, age-matched, healthy controls had grown an additional 6.7% of their original body weight while regular diet animals had lost 1.6% and high-fat animals 3.6%. At the conclusion of the study and after ten weeks of diet, both RD and RDV had gained less weight than age-matched controls while there were no differences in HD or HDV relative to sham-irradiated animals. These data demonstrate the high-fat diet protocol influenced body development. Most importantly, because the

overall aim was to identify cellular changes rather than phenotype, the model used was sufficient to detect physiological changes on the cellular and molecular levels, as measured with cell migration.

The impact of high-fat diet was also evident in the mesenteric fat pad, as measured with adipogenic gene expression. As expected, key regulators of adipogenesis, including PPARγ and PPARλ, were upregulated in both HD and HDV groups relative to RD. No differences were detected in expression of FABP4, which is downstream of PPARγ and involved in lipid transport. FABP4 is abundantly expressed in mature adipocytes (136), is involved in insulin sensitivity and inflammation (171), and also indicates changes in fatty acid metabolism (119). While the lack of a robust obese phenotype and heavier fat pad in the high-fat diet groups (and possibly no increase of mature adipocytes relative to regular diet) may explain why FABP4 was not upregulated, there is evidence that FABP4 is also expressed adipocyte progenitor populations (215). The upregulation of PPAR genes in the visceral fat pad is indicative of an altered environment due to diet and is likely to affect inflammation, cellular/endocrine signaling, and cell recruitment or migration patterns.

Similar increases in PPARγ and PPARλ were seen in the soleus muscle of the OVX mouse model compared to age-matched controls, and intermuscular fat was also more prevalent in the skeletal muscle of animals on the ten weeks of high-fat diet. Six weeks of LIV treatment successfully suppressed adipogeneic gene expression in the musculature of OVX mice, while no differences were seen with eight weeks of LIV treatment in the high-fat diet model. Despite an additional two weeks of treatment and extended treatment time (30 min/day versus 15 min/day), the mechanical stimulation protocol was unable to suppress the fat gene program. Differences are likely to be due to location of gene expression analysis (skeletal muscle tissue versus visceral fat tissue), also evidenced by the ability of LIV to mitigate intermuscular fat accumulation in the soleus of this same study.

Whether bone marrow-derived cells contribute to visceral or subcutaneous fat formation or function is still debated. Animal models have both supported (216) and invalidated (210, 211, 217) that bone marrow-derived cells have a significant presence in adipose tissue. Interestingly, our data show that the migration of bone marrow cells was accelerated to the visceral fat pads following ten weeks of high-fat diet. HD and HDV animals had a 38% and 43% increase, respectively, in bone marrow cell engraftment relative to RD animals, emphasizing the significance of diet influence on cellular fate. Crossno *et al.* also demonstrated that bone marrow derived cells contribute to the formation of visceral fat tissue and adipocytes using both a 20% fat high-fat diet and roziglitazone (210). The increase in bone marrow cells to the fat pads may recruitment for MSC differentiation to the adipocyte lineage and fat

pad expansion. Recruitment of bone marrow cells could also be contributed to the inflammatory response caused by the state of chronic inflammation due to excess fat. Novotny *et al.* identified an accumulation of macrophages in the fat pads of obese mice (218), of which were involved in activated inflammatory pathways. LIV did not influence the migration of bone marrow cells to the visceral fat pad in the HDV group.

The impact of diet on donor cell migration is of particular importance when it comes to clinical bone marrow transplants and patient physiological response to the procedure. Recently, Ryden *et al.* demonstrated that body mass heavily influences bone marrow cell contribution to fat tissue in patients who underwent either bone marrow or peripheral blood stem cell transplants. Specifically, obese individuals had a significant increase in donor cell fat pad engraftment (209). Our results in a murine model were consistent with this finding and suggest fat pad formation and function are not derived strictly from local progenitor pools. Recruitment of bone marrow cells (mesenchymal and hematopoietic stem cells and progenitors, immune cells, etc.) in the presence of high-fat diet or obesity could be attributed to the physical expansion of the fat pad or state of inflammation.

Bone marrow, blood, and fat tissues all had approximately 20-40% of their cell populations originating from the transplanted bone marrow cells, demonstrating that bone marrow contributes to the formation and likely the function of these tissues. Interestingly, despite its close proximity to bone tissue, GFP engraftment in skeletal muscle was much lower than other tissues analyzed, ranging from only 0.01-0.03%; the contributions of bone marrow-derived cells to muscle tissue in this study are minimal. While others have demonstrated bone marrow-derived cells migrate and engraft into muscle tissue (219), it was not without initiation of tissue damage and cytokine mediators. The presented insult of high-fat diet in this study may not have been strong enough to elicit the same response from the marrow niche. Because of low engraftment, we can assume that bone marrow-derived progenitors are not likely contributors to the increased lipid content seen in the soleus of animals on the high-fat diet. It is probable that fibro/adipogenic progenitors (FAPs) are the cells responsible for accumulating the large quantities of neutral lipids. These cells accumulate lipid, readily differentiate into adipocytes, lack myogenic potential, and are influenced by cues in the muscular niche (103, 220).

Work by Ferrari *et al.* showed that bone marrow cells do contribute to the formation and regeneration of muscle tissue (221). Differences in our results may be due to the fact their muscle had chemically induced degeneration and the presented insult of high-fat diet in this study was not strong enough to elicit the same response from the bone marrow. The data presented in Chapter 3 showed no

changes in absolute number of satellite cells with high-fat diet, suggesting the regenerative capabilities of the skeletal muscle in this study were not considerably impaired. Nonetheless, the interactions between muscle and bone are comprised of a complex mechanical and chemical relationship (222) and further investigation into the contribution of bone marrow cells to skeletal muscle in the presence of high-fat diet is needed.

High-fat diet not only accelerated bone marrow cell migration visceral fat pads, but quantification of donor MSCs via flow cytometry also showed that high-fat diet specifically accelerated donor MSC migration to the visceral fat. Visualization of donor cell engraftment in the gonadal fat pad of injected animals via immunohistochemistry confirmed the flow cytometric analysis that bone marrow cells migrated to adipose depots outside of the bone. Considering no adipocytes were injected during bone marrow transplantation (all adipocytes were lysed in bone marrow preparation), all GFP+ adipocytes resulted from the migration and differentiation of bone marrow stem cells. MSCs are known to enter the blood stream and travel to peripheral tissues, and this increase in recruitment with high-fat diet is likely utilized for fat pad expansion or part of the immune response to the fatty diet. The same increase of donor MSCs was not seen in HDV, suggesting LIV altered migration patterns. This idea is paralleled in the bone marrow, where HDV had lower MSC proportions in the marrow cavity relative to age-matched controls. RDV also had a trend toward lower MSC proportions, suggesting LIV is affecting either MSC migration from the marrow or is increasing stem cell differentiation into higher order tissues.

Flow cytometry is a sensitive assay and the endogenous GFP signal in this study was easily distinguishable from non-fluorescent cells, however, the assay does not distinguish host from donor cells without flaw. All GFP+ proportions in the AC group should be 0% since these animals received a saline injection and not GFP+ whole bone marrow. However, due to the nature of the assay, gated GFP+ populations contain some non-GFP expressing cells. This error is due to noticeable shifts in the cell populations of irradiated animals that were not consistent with non-irradiated controls. Rather than excluding GFP+ cells in our analysis, especially when quantifying significantly small populations such as MSCs, we ensured all GFP+ cells in irradiated groups were accounted for and that gates were consistent across all experimental groups. This resulted in the quantification of “donor cells” in age-matched control group (particularly apparent in the visceral fat donor MSC quantification). In actuality, no donor cells were injected and this value should be considered 0. Lastly, the appropriate identification of an MSC is a highly debated topic and the way we defined the stem cell (Sca-1+, C-Kit+, CD44+, and CD90.2+)

does not align with all other research groups. While the International Society for Cellular Therapy provides minimal criteria for defining MSCs (223), the characterization of this cell type is not straightforward.

In seeing changes in bone marrow cell migration and skeletal muscle composition (intermuscular fat in the soleus, Chapter 3) in animals on a high-fat diet, it was plausible that the bone marrow environment was altered. We investigated adipocyte encroachment into the marrow using histological analysis of the proximal tibia and saw that adipocyte size was increased in both groups on high-fat diet relative to RDV. Additionally, total adiposity was greater in HDV compared to RDV and trended towards being greater than RD animals. Using a pre-diabetic model, the time point at which we ceased experiment was likely to be before full adipocyte encroachment occurred in the marrow. High-fat diet is known to cause increases in marrow adiposity (224, 225), and if our experiment were carried out as a true DIO model, it is likely differences in diet would have been more robust. Importantly, our ORO staining in the soleus muscle confirms that intermuscular fat accumulation occurs prior to adipocyte encroachment in the marrow, and although changes in the marrow space are not drastic, diet alone, not changes in body habitus, does alter both the marrow space and skeletal muscular niche.

Exercise in high-fat diet rats suppresses the accumulation of marrow adipose tissue (225) and after six weeks of LIV treatment, OVX mice exposed to LIV had a 55% reduction in apparent adiposity, 33% reduction in adipocyte size, and a 38% reduction in number of adipocytes per area compared to non-vibrated controls (226). Our animals did not have this same response to mechanical signal exposure despite two weeks of additional treatment. Small reductions in adiposity in the regular diet animals with vibration treatment resulted in significant differences between RDV and high-fat groups that were not seen between RD, perhaps indicating vibration is effective in reducing adiposity in the regular animals to a small extent.

Gene expression analysis in the bone marrow cavity showed no differences in major MSC differentiation regulators, PPARγ and RUNX2. Also in the bone marrow space, HDV animals had fewer MSCs than non-irradiated controls while RDV had a trend towards a smaller population. These data suggest LIV is increasing differentiation of the stem cells to higher order tissues or increasing migration out of the marrow space. Because there were no differences in expression of PPARγ and RUNX2, we hypothesize LIV is increasing migration of MSCs out of the marrow and to other tissues. Our gene expression analysis in the marrow was performed on the femur while flow cytometric analysis of MSC populations was done on the pooled femur and tibia. Considering the marrow space differs with bone

and anatomic location (*i.e*. diaphysis, epiphysis, metaphysis), future work should strictly compare gene expression data and flow cytometry from the same sites and bones.

The data presented here demonstrated that bone marrow cells contribute to the formation of tissues outside of the bone. The presence of a high-fat insult in the form of a 45% kcal from fat diet accelerated bone marrow cell migration, specifically bone marrow-derived MSCs, to visceral fat pads for differentiation into the adipocytes. High-fat diet also increases adipocyte size in the marrow niche, preceding the changes in body habitus caused by high-fat diet, and paralleling the increases in intermuscular fat seen in the soleus.

CHAPTER 5: IMPACT, LIMITATIONS, AND CONCLUSIONS

Gaps in the literature and need for this work

The role of adipose tissue as an active endocrine organ is essential in biological function, as it plays a primary role in endocrine production, metabolism, and energy storage. Its vital function is evidenced by findings that visceral fat depots can be responsible for 100% of circulating estrogen in postmenopausal women (173) and that its endocrine signaling regulates processes in multiple tissue systems, including bone and muscle (48, 54). However, in excess, adipose tissue can cause significant dysfunction and lead to debilitating complications, including insulin resistance, dyslipidemia, and hyperglycemia (227). The significant economic costs and decreases in quality of life associated with menopause, obesity, and type II diabetes only emphasize the need for a better understanding of these conditions and the use of a non-pharmacological treatment modality that is both safe and efficacious for the aging, frail, and physically incapable populations.

With obesity and type II diabetes expanding epidemics and menopause a condition that affects 50% of the worldwide population, these conditions are being extensively studied and are areas of high interest. Still, there are many unanswered questions regarding how these conditions affect the muscular niche or the molecular mechanisms by which a lack of estrogen or excess fat can damage tissue systems. It is not established in the literature to what extent OVX and high-fat diet impact the satellite cell population or the stem cell's ability to become activated and respond to injury. The molecular mechanisms by which skeletal muscle becomes insulin resistant are also still debated (197) and it is unknown if an insulin resistant environment directly affects resident stem cell populations. It is established that the bone marrow is a dynamic environment of cells continually entering circulation (63), but it is unclear to what extent bone marrow-derived cells migrate and engraft in visceral fat and muscle tissue, how systemic insults alter these behaviors, or if bone marrow-derived cells are aiding in the acceleration of fat induced pathologies. Lastly, low intensity mechanical signals have been used as a therapeutic intervention in a range of conditions, providing benefits to the musculoskeletal system in both humans and animal models (57, 79, 134, 136, 173). Further investigation into the impact of LIV on satellite cell populations, insulin resistance, bone marrow and muscle adiposity, and bone marrow cell migration is needed for this intervention to become of use in the clinic to treat systemic fat burdens and the subsequent complications.

To address the gaps in the literature, this dissertation investigates the hypothesis that bone marrow cells contribute to the formation of bone, fat, and muscle, and that this commitment is disrupted by systemic distress and normalized by mechanical stimulation. Using murine models of

ovariectomy and high-fat diet, we investigated the impact of an adipose burden on bone, skeletal muscle, and fat, on the genetic, molecular, and tissue levels. Additionally, we investigated the use of mechanical stimulation in the form of LIV (<0.4*g* where g is earth's gravitational field at 9.81m/s^2) to act as a surrogate treatment for exercise and mitigate the negative consequences caused by a systemic adipose burden.

Animal models of a systemic fat burden

In the presented work, we used two different types of adipose insults, one resulting from a lack of ovarian hormone production and the other from diet high in fat content. While the negative consequences associated with excess adiposity can be similar across insults, the high-fat diet and OVX models are vastly different and each have their own limitations. The high-fat diet model used in this work is 45% kcal from fat (vanHeek Series, TestDiet), which matches protein content in low-fat chow and is one of the most widely used diets to model DIO, allowing for easy comparison across the literature. While not necessarily mimicking the dietary intake of obese adults (likely to be high in sucrose and/or carbohydrates), the chosen high-fat diet follows the same progression of the development of glucose intolerance in the C57BL/6 mouse as is seen in humans (176). Because one of our primary goals was to investigate indications of insulin resistance and its mechanism of action, we used a model and experimental timeline that allowed us to investigate the pre-obese and pre-diabetic state of the muscle, bone, and fat. This can be considered a limitation of this work because the diet was not robust enough- or the experimental timeline was not long enough- to see extreme differences with high-fat diet, and thus limiting the ability of LIV to show improvement.

The impact of high-fat diet on tissue systems depends on food composition, source of fat, and duration of exposure, as evidenced in both humans (228) and animals (229, 230), and the data presented here are likey to be different if another diet were used, making clinical implications complex. While we have attributed the data presented here as a precursor to obesity or type II diabetes, it must be understood that the insult of a high-fat diet is not necessarily the same as the insult of obesity. While obesity is known to cause type II diabetes, diabetes can be present without the obese condition, and in some cases, pre-diabetes will only develop as a result of obesity over time as tissues become fatty and insulin signaling becomes impaired. Moreover, just as the content of the diet can influence tissue systems in different ways, total caloric intake can have physiological implications. A high-fat diet in mice has shown to have negative effects on bone structure and marrow populations (57), and conversely, a

calorie-restricted diet can lead to a reduction in muscle mass in mice (134) and bone density in mice and humans (134, 231). It is apparent that diet, both total caloric content and quality of diet, has a significant influence on the musculoskeletal system and that a balance in both fat quantity and adipokine release is essential in maintaining healthy tissues.

The ten week timeline used in this study did not allow for animals on the 45% kcal high-fat diet to become significantly heavier or develop larger fat pad mass than regular diet controls. While this allowed for identification of phenotypic changes in the muscle and bone during the early development of obesity, in hindsight, a longer experimental timeline where the obese phenotype developed would have provided more conclusive data regarding the influence of fat on the musculoskeletal system versus the influence of diet. A longer timeline may have also allowed for LIV to show greater effects in preventing the formation of fat in the bone marrow and reducing indications of insulin resistance in the muscle. Improving this experimental design for future studies would consist of extending the total experimental timeline to 18 weeks of diet and 16 weeks of LIV treatment, keeping the same two weeks of diet induction and recovery period. This would allow adequate time for high-fat diet animals to become significantly heavier than regular diet controls while simultaneously providing a burdened system in need for improvement and intervention. The 60% kcal high-fat diet is likely to overwhelm the system causing the subtle LIV signal to be ineffective, and the 45% kcal high-fat diet should be kept as a suitable diet regimen. Lastly, in regard to the bone marrow cell tracking aspect of the study, injection of tagged individual cell types rather than whole bone marrow cells would provide better insight as to which cell types are being affected by obesity. This can most successfully be achieved using multiple strains of transgenic mice that have bone marrow tagged with varying fluorophores. Using flow cytometry, one bone marrow cell type can be extracted and isolated from each transgenic mouse and pooled together for one transplantation. For example, GFP+ MSCs can be combined with tdTomato+ HSCs and appropriate accessory cells for successful rescue of lethally irradiated animals and appropriate cell tracking.

Although there is an evident increase of systemic adiposity in OVX, a significant limitation of this model is the confounding factor of depleted ovarian hormone. While we hypothesized the changes in satellite cell populations in the muscle of OVX were due to local increases in adiposity, it is likely that the lack of estrogen played a role in reducing this stem cell population. The OVX model is an abrupt insult that is detrimental to tissue systems in as little as two weeks following OVX surgery, as seen by extreme increases in abdominal adiposity and body weight and decreased bone volume fraction in the proximal

tibia (226). The immediate and complete removal of the ovaries does not model the gradual progression of ovarian hormone depletion seen in women in menopause. Although more labor intensive due to daily injections, other murine models of ovarian failure, such as chemical induction via 4-vinylcyclohexene diepoxide (VCD) (232), have a more gradual influence on tissue systems and may be less severe, also allowing for LIV to be a more effective intervention.

While more gradual alternatives to OVX are available for murine models of estrogen depletion, OVX is the most widely used murine model for menopause. The surgical procedure is easily repeatable between animals and results in the bone loss and weight gain seen in humans, among other symptoms. Future work in investigating the impact of ovarian hormone depletion on the musculoskeletal system should continue with the OVX model. However, changes in the LIV intervention timeline should be made to prevent the rapid destruction of bone and fat systems. Instead of beginning LIV treatment two weeks after the OVX procedure, treatment should begin within two days following an in-house surgery. While typically animals need a recovery period, the LIV signal is subtle and does not affect animal longevity, and earlier LIV intervention was shown to be more successful in improving the bone of OVX animals rather than waiting until after the 2 week recovery period (226).

Bone marrow transplantation methodology

Another major limitation of this work is the bone marrow transplantation methodology used to track bone marrow-derived cells in high-fat diet, specifically the exposure of animals to lethal irradiation. Following (6+6) Gy of ^{137}Cs gamma irradiation, animals were rescued 24 hours later with 10,000 GFP+ whole bone marrow cells. This particular methodology was chosen to track bone marrow cells because of its widespread use and well documentation of methods. Feasibility of the pilot project and successful rescue of the animals were both a concern and this method offered well laid out transplantation protocols with evidence of successful experiments in the literature, including our own laboratory (82). Previous work in our lab has also demonstrated the detrimental effects of sub-lethal radiation on tissue systems (233). Considering the focus of our study was on the cellular and molecular responses of cells to diet, inability to recover tissue phenotype or body weight due to irradiation was not a concern. Additionally, all of our analyses were performed 10 weeks following radiation exposure, allowing ample time for recovery. A large number of assays showed recovery from radiation and no differences between non-irradiated controls and regular diet animals, while animal body weight, fat tissue mass, microarchitecture of bone (data not included in this dissertation) and satellite cell

populations did not recover, making it extremely difficult to assess the effects of diet and demonstrating the tissue response to radiation significantly varies between tissue types. While lethal irradiation certainly adds a confounding variable into investigating the effect of diet on cellular migration, it provides a successful means of tracking cells. The fact that lethal irradiation causes healthy injected bone marrow cells to home in the marrow prior to migration (213) makes this work physiologically relevant. Additionally there were no issues detecting GFP+ cells vs non-GFP with flow cytometry in any tissues analyzed. For future studies, other injection sites such as tibia should be considered, as it may be more appropriate in claiming donor cells homed in the marrow prior to migration. Suitable lethal irradiation doses and cell injection numbers will have to be optimized to maintain animal survivability following the procedure.

In light of this hurdle being detrimental to some tissue systems and masking the effect of high-fat diet, using this particular method has made this work clinically relevant for patients undergoing lethal irradiation or receiving bone and blood transplants. Our bone marrow tracking data demonstrated diet affects the behavior of healthy donor cells upon injection and should be of consideration for obese patients or those with unhealthy diets receiving transplants. Conversely, the effect of diet on cells could also be a concern for those receiving transplantations of cells from obese people. A “fatty” bone marrow may not be as functional as a “healthy” marrow.

The influence of systemic fat and mechanical stimulation on the muscular niche

As anticipated, OVX resulted in a significant adipose burden, seen in both the abdominal compartment and skeletal muscle. *In vivo* µCT analysis showed a marked increase in both TAT and VAT eight weeks following OVX surgery. Although only a trend, LIV mitigated total abdominal fat accretion by half. While it has been established that LIV biases stem cell differentiation away from adipogenesis (82), this finding was rather striking considering the subtlety of the signal, brief treatment period of only six weeks, and such drastic change in abdominal fat phenotype. An increase in adiposity was also realized in the musculature, where we observed an upregulation of adipogenic gene expression, again attenuated by mechanical signals. In this study, muscle tissue was not preserved for histological analysis, but would have been valuable in investigating the ability of LIV to mitigate fat in the cellular level. For appropriate analysis of the muscle, both gene expression and histological assays should be performed.

In switching to a high-fat diet model, we preserved the appropriate tissue and performed Oil red O staining and lipid quantification on frozen sections of the soleus, where we saw an upregulation of

intermuscular fat. Again, LIV was able to mitigate fat accumulation in skeletal muscle. Interestingly, large increases of accumulated lipid were seen in high-fat animals despite no differences in body weight or visceral fat pad weights. This is important in the pathogenesis of pre-diabetes, as changes in the musculature precede weight gain or any major indications of glucose intolerance. These data also demonstrate that LIV is an effective in mitigating the cellular changes caused by high-fat diet that may prove to be successful in delaying the onset of pre-diabetes.

We investigated the impact of high-fat diet and LIV on intermuscular fat, but intramyocellular lipids, which actually accumulate within the muscle fibers, are also an important component of muscular health and are associated with insulin sensitivity (234). While this type of fat could be seen in our histological sections, analytic methodologies were not adequate for accurate quantification. Intramyocellular lipid distribution can also indicate fiber type (type I fibers typically have more intramyocellular lipid) and changes caused by diet may initiate fiber type switching. While this was not within the scope of the study, functional assays of the muscle including contractile profile power generation should be investigated to determine if the noted changes in muscular composition are affecting functional capabilities. And, while we suspect the source of intermuscular lipid accumulation is FAPs, immunohistochemistry should be utilized to identify the cells accumulating excess lipid and e*x vivo* differentiation assays should be performed to test differences in adipocyte differentiation in these cells.

The accumulation of intermuscular adipose tissue seen in high-fat diet animals was likely to be associated with impaired insulin signaling. Overall, we saw only moderate indications of the onset of impaired insulin signaling in the high-fat diet model. We aimed to identify if there was increased DAG content and upregulated PKCθ, which is hypothesized to initiate downstream signaling leading to insulin resistance (197). Our data did not show increased DAG content in high-fat diet animals, although the ELISA used to assess DAG is not standard for this quantification of this metabolite and it is plausible we missed the transient increase of the molecule. Longitudinal quantification of DAG is not possible with ELISA without the excessive euthanasia of animals. Future investigation of DAG should be done with *in vivo* magnetic resonance spectroscopy, as there is overwhelming evidence in the literature that DAG is seen in elevated quantities in obese and diabetic models and these molecules are responsible for activating the PKCθ pathway (197). Upregulation in PKCθ in high-fat animals relative to RDV did indicate an initiation of the target molecular pathway. Interestingly, systemic evaluation of glucose intolerance also showed minor reductions in high-fat diet glucose tolerance using the GTT assay, which was not

expected considering high-fat diet animals were not heavier than their regular diet counterparts. This emphasizes that indications of insulin resistance can precede obesity.

Our work here attempts to draw a relationship between two related, but also independent, factors: intermuscular adiposity and insulin resistance. While the two are highly correlated to one another, our study design and assays do not address a direct or causative relationship. This is also the case in comparing satellite cells to adipogenic gene expression in OVX; only correlations can be assessed. Future work should attempt to draw causative relationships between the molecular mechanisms of insulin resistance, impairment in satellite cell proportions, and intermuscular adiposity. This will also aid in the understanding of how LIV is reducing adiposity in the muscle and protecting stem cell populations. Appropriate investigation of DAG or other culprits (*e.g.* ceramides) in activating target pathways should be considered in future work. *In vitro* analyses investigating the mechanism of action of molecular pathways and LIV may also be useful. It must also be noted that the "athlete's paradox" demonstrates that increases in fat in the muscle and bone are seen in well-trained athletes (235). While animals in these models are not on elaborate exercise regimes and local increases in fat are likely to be a negative consequence of the systemic fat burdens, this cannot be assumed.

Exercise is anabolic to muscle (107) and LIV has been shown to increase cross-sectional area of muscle fibers in mice (81) and muscular strength in post-menopausal women (236). Unexpectedly, LIV did not have a notable effect on myogenic gene expression in the muscle of OVX mice. We attribute this difference to the severe impact of OVX on the muscle tissue, which was not recoverable by mechanical stimulation. Estrogen directly binds to muscle on two sites (ERα and ERβ) and regulates skeletal growth (237), highlighting the potential impact of an abrupt and sustained depletion of ovarian hormone. The mentioned increase in fiber cross-sectional area with LIV was achieved in healthy, adolescent animals, and a severe systemic insult such as OVX, which is more abrupt than menopause in the human, may be too difficult to overcome by a subtle signal in six weeks. No histological analyses of the muscle could be performed in this study and change (or lack thereof) in fiber cross-sectional area could not be assessed. To gain a complete understanding of how OVX is impacting skeletal muscle and whether the anabolic potential of LIV can be useful in this model, a complete genetic profile should be analyzed, beyond what was investigated here, in addition to analysis of the muscular phenotype.

In parallel with the increased adipogenic gene expression in the muscle of OVX animals, we discovered satellite cell proportions were compromised. We hypothesized it was the adipose burden suppressing the satellite cell population due to the large increase of systemic fat and local increase of

adipogenic gene expression in the musculature. We sought to test this hypothesis using a high-fat diet model, effectively eliminating the variable of depleted ovarian hormone. To our surprise, no differences were seen between groups after ten weeks of high-fat diet. To add insult to injury, the data showed that the methodology used in the study, lethal irradiation, in fact reduced the satellite cell proportions in all groups that received bone marrow transplantations. If there were an impact of high-fat diet on this stem cell population, it may have been masked by the irradiation detriment. While it was expected that irradiation may impair satellite cell populations in the short-term, these findings were somewhat surprising to find ten weeks post-exposure.

Two subsequent analyses were performed to determine if high-fat diet impacts satellite cell populations, both high-fat diet models using no transplantation methods. Both 45% kcal and 60% kcal from fat high-diets showed high-fat diet does not reduce satellite cell proportions, disproving our hypothesis. Interestingly, our high-fat diet and satellite cell analyses were performed in both young and old mice, ranging from 13-25 wk of age at the time of euthanasia, suggesting the influence of high-fat diet on the size of the satellite cell pool is not dependent on age. Alternatively, high-fat diet did alter the stem cell ability to respond to mechanical stimulation; only RDV animals exposed to LIV treatment had a shift in CD34 ratio. Despite our findings that high-fat diet has no influence on satellite number, changes in activation may indicate alterations in functional capabilities (164).

Our conclusions that high-fat diet does not affect satellite cell populations are based solely off flow cytometry data and satellite cell counts relative to the total number of cells in the muscle. The gold standard for satellite cell quantification is using immunohistochemistry and the Pax7 marker, which is a widely accepted positive marker for satellite cells. While we are confident the flow cytometry marker profile used (CD45-/CD31-/Sca-1-/Integrin alpha 7+) is indeed identifying the target population (111), using immunohistochemistry methods may yield more sensitive and reliable results. Additionally, our work does not investigate the function of satellite cells, which can be done *ex vivo*. Multiple protocols have been developed to extract and isolate muscle fibers and quantify the proliferation and differentiation capabilities of satellite cells, which may be influenced by both OVX and high-fat diet. Future work should include these types of assays to gain a complete understanding of how the resident cells are affected in the muscular niche. Similarly, changes in stem cell proportions may not necessarily translate to any impairment of functional indices of the muscle, which should also be investigated.

High-fat diet and LIV impact on cell migration and the bone marrow niche

Systemic fat insults altered the phenotype of the muscle in both OVX and high-fat diet, but it was unknown whether bone marrow-derived cells contributed to these pathologies or if changes were due strictly to local cell populations. It is also unclear in the literature if bone marrow-derived cells contribute to the formation or function of tissues outside of bone and whether a systemic fat insult disrupts this commitment. Our data showed bone marrow-derived cells to be found in visceral fat, bone marrow, blood, and skeletal muscle tissues, suggesting the contribution of bone marrow cells in peripheral tissue systems may be significant. High-fat diet accelerated bone marrow-derived cells and MSCs to the gonadal fat pad as determined by flow cytometric analysis of GFP+ and (Sca-1+/C-Kit+/CD44+/CD90.2+) cells, respectively. High-fat diet is likely increasing migration of healthy, bone marrow-derived cells and donor MSCs to the fat to assist in expansion of the fat tissue through utilizing healthy cells in a system that was crippled by irradiation (visceral fat pad did not recover after ten weeks). Immunohistochemistry depicted adipocytes originating from the GFP tagged cells in all groups that received transplants. This demonstrates that after stem cell recruitment to the fat, cells differentiated into adipocytes and aided in the expansion of the fat pad. Because high-fat diet accelerated this migration, changes in migration patterns cannot solely be due to irradiation. Adipogenic differentiation markers were also upregulated in the fat in high-fat diet groups and endocrine signaling to the bone via adipokine release could be responsible for accelerated recruitment. These data are important in emphasizing the impact of a systemic insult on cellular processes, and that in the case of high-fat diet, visceral fat utilizes bone marrow cells more so than another tissues analyzed. LIV had no effect on cellular migration. If experimental conditions were different (*e.g.* experimental timeline was carried out longer and visceral fat pad mass recovered from irradiation) LIV may have influenced cellular migration through mitigating visceral fat accumulation, as seen in OVX. It is plausible that a reduction in fat mass would translate to alterations in adipokine release and endocrine signaling, and subsequent changes in migration patterns.

Those administrating bone marrow transplantations in the clinic should be aware that injected cells could contribute to the obese pathology, particularly in obese patients. Our findings also lead us to wonder if the source of marrow (a “fatty” marrow versus a “healthy” marrow) will respond in the same manner when injected into non-obese persons. An interesting study would be to inject high-fat diet bone marrow or regular diet bone marrow into animals on a high-fat diet to see if the same recruitment patterns occur in both models.

Analyses of GFP+ cells using flow cytometry showed clear GFP+ populations, distinct from cells not expressing the endogenous fluorophore. Determining the MSC populations via flow cytometry was a much more complex process. The markers used for analysis do not yield clustered populations, making gating difficult and somewhat subjective. Flow cytometric gating of age-matched controls showed the presence donor MSCs in the fat even though these animals received sham-injections (*i.e.* no GFP). This is a significant error considering the already small population of MCSs in the fat. Another limitation of this study is the lack of the quantification of bone-marrow adipocytes. Adipocytes cannot be quantified using flow cytometry because of their large lipid droplets and immunohistochemistry sections did not show full adipocyte membranes, making counting actual cells impossible. Total fluorescence is also not an accurate representation of adipocyte differentiation. Future studies should quantify pre-adipocytes via flow cytometry and optimize a staining protocol that will allow for the quantification of fat cells using confocal microscopy.

Bone marrow cells contributed minimally to skeletal muscle, at least comparatively to the other tissues analyzed. It is not clear if whether this small population (<0.05% of total cells) plays a significant role in any muscular functions or, if there were a greater or different insult to the muscle, such as a physical injury, the proportion of bone marrow cells would increase. In the case of OVX where satellite cell proportions were reduced, it would have been interesting to see if the migration and engraftment of bone marrow cells in the muscle was greater than that of our high-fat diet model, where satellite cell proportions were unimpaired. It is plausible that as muscle injury or dysfunction becomes more severe, the tissue relies more heavily on cell recruitment from the bone marrow or elsewhere for muscle maintenance. Future work should utilize bone marrow tracking methods and investigate bone marrow engraftment in muscle in models of muscle injury or satellite cell dysfunction. Pax7-/- mice completely lack satellite cells and would be an appropriate model for determining the need for bone marrow-derived cells in a state of impairment; however, these mice typically die within two weeks after birth (238, 239). It would be helpful to make use of other knockout models to identify the extent, if at all, muscle tissues relies on bone marrow-derived cells. Chemically induced muscle injury or less severe models of impaired muscular regeneration may be more appropriate when adding the insult of irradiation and bone marrow transplantation in order to track the engraftment of bone marrow cells.

The bone marrow niche is a complex network of multiple cell types, including bone cells (osteoclasts and osteoblasts), adipocytes, and stem cells (hematopoietic and mesenchymal). The health of the MSC population is largely dependent on the status of the bone marrow niche and whether the

microenvironment is one suitable for the population to thrive (62). Additionally, the quality of the marrow space may be a contributor to changes seen in bone marrow cell migration. We evaluated bone marrow adiposity and saw an increase in average adipocyte size in both HD and HDV while these groups had a trend towards increased total adiposity, again demonstrating that local increases in tissue adiposity precede changes in total abdominal fat or body weight. In this pre-obese model, LIV was more effective in reducing adiposity in the musculature rather than the bone marrow early on. If the experimental timeline were carried out for a longer period or if a more robust diet were used, LIV may have had a greater influence on bone marrow adiposity, if the marrow were becoming fatter at a faster rate. PPARγ gene expression showed no major indication biased MSC differentiation in the bone marrow, although these data were variable. Trends show small reductions in PPARγ expression using LIV. LIV was also successful in reducing bone marrow adiposity in OVX animals (226). MSC proportions in the bone marrow also do not show an impact of high-fat diet on this cell population. However, trends toward a reduction in MSC proportions in both vibration groups may suggest LIV is altering MSC migration. No changes in PPARγ and RUNX2 indicate there are no changes in MSC differentiation. While bone marrow adiposity is only one indication of the health of the marrow space, these data do not show drastic changes in bone marrow adiposity; alterations in bone marrow cellular migration are likely a result of endocrine signaling, rather than strictly local changes in the marrow niche.

Although not quantified in the presented work, it is likely that other bone marrow cells besides adipocytes were altered by high-fat diet and that changes in these cell populations influenced the MSC pool and bone marrow cell migration. MSCs and HSCs reside in a closely knit environment and both cell populations are partially regulated by the other, creating a unique partnership. MSCs play a critical role in the HSC niche, expressing HSC maintenance genes and providing better homing capabilities for HSCs following lethal irradiation in mice (240). MSCs also regulate T-cells (241) and play a role in immunomodulation, influencing B-cells, natural killer cells, and dendridic cells (242). High-fat diet alters both HSC and immune cell populations in the bone marrow of mice (57, 148) and obesity is known to affect the immune system. Alterations in these bone marrow populations and downstream complications in immunity may in part result from alterations in MSCs. Additionally, because marrow immune cells are susceptible to the high-fat insult, these changes in the marrow niche may affect the MSC pool and the ability of these stem cells to respond to mechanical signals. MSC function relies in part on local cues from the marrow microenvironment (240) and disruption of the niche is likely to influence osteoblast or adipocyte lineage commitment.

Applications of LIV

We attempted to control the differentiation of MSCs using LIV, which, as expected, proved to be more effective in some situations than others. For example, LIV was successful in reducing adipogenic gene expression and intermuscular fat in the skeletal muscle, but not in preventing accelerated bone marrow cell migration to visceral fat pads or reducing bone marrow adiposity. LIV is a very subtle signal that makes it desirable as a treatment modality for those at risk of fracture and other pathologies that can make exercise difficult or unsafe. However, in some cases, its subtlety may not be capable of overcoming significant systemic burdens used in animal models.

Both OVX and high-fat diet are systemic detriments that are arguably not physiologically similar to the human post-menopausal and obese conditions. Because the animal models and experimental methodologies are severe, it can make distinguishing the benefits from treatment difficult or may overwhelm the system by decreasing the responsiveness of cells to mechanical stimulation. Alterations in target signaling pathways thought to be utilized by vibration may prevent successful intervention in these disrupted systems. For example, mechanotransduction with the LIV signal is believed to involve the Wnt signaling pathway (243). Wnts are proteins involved in various developmental processes and Wnt-10b specifically mediates adipogenesis (244). Wnt regulation of adipocyte crosstalk and tissue remodeling (245) is likely to be altered in the presence of a systemic adipose burden and perhaps disrupts the same pathway in which LIV biases MSC differentiation away from adipogenesis. Mechanosensitive cells, such as osteoblasts, MSCs, and satellite cells may also be inherently affected by OVX and high-fat diet. Alterations in mechanoreceptors, including focal adhesions, integrins, gap junctions, or other plasma membrane modifications in these cells all may contribute to decreased sensitivity or impaired intracellular signaling. These complications emphasize the idea that LIV alone may not be a complete replacement for exercise and it may be best to be used as an adjunct treatment. Additionally, the prevention of obesity-related complications using LIV may be more achievable than the reversal of them.

While it is well-understood that a mechanical signal of low magnitude is necessary for the safe application of vibration, there is currently no general consensus on the particular signal parameters that are “most beneficial” in improving the musculoskeletal system. It is likely that the frequency and magnitude of the vibration and duration of the exposure period is somewhat specific to the target tissue–the signal most anabolic to bone may differ from the signal most effective in improving postural stability. The incorporation of a refractory period may be more important in influencing cellular

outcomes rather than the signal magnitude or total duration of exposure. A three hour refractory period was more successful in suppressing MSC adipogenesis than either a one hour or absent refractory period (246). Allowing a period of rest may allow for appropriate cellular adaptations to become more sensitive to or primed for responding to the mechanical signal.

While the mechanism how LIV influences bone, muscle, and fat is not completely understood, mechanical stimulation elicits an acute cellular response by regulating the organization of the cytoskeleton and intracellular signaling in the nucleus (247, 248), biasing stem cell differentiation (76) and providing a foundation for a range of clinical applications. The ability of LIV to bias the stem cell away from adipogenesis provides the foundation for this signal to be effective in preventing indications of obesity or type II diabetes (57). In humans, LIV has been an affective therapeutic intervention for a variety of musculoskeletal disorders, including building trabecular bone in the tibia of children with Duchenne muscular dystrophy (249), reducing low back pain (250) and improving balance (134) in patients who underwent bed rest, and increasing both muscle and bone mass in young women with low bone mineral density (173).

These clinical trials successfully utilized LIV intervention without any severe adverse events, highlighting the potential for LIV to be used in patients with severe disabilities or pain. A potential new target population is patients suffering from chronic low back, where current treatment methodologies, including pharmacological interventions, exercise, spinal manipulation, or spinal surgery, are expensive, invasive, too difficult or painful to do, or often not adequate in maintaining pain in the long-term. The brief application period of 10 minutes is achievable for most to stand on the plate, and a safe, non-invasive intervention would certainly be beneficial for those unable to successfully manage their chronic back pain.

In addition to LIV being a therapeutic intervention that could improve debilitating musculoskeletal conditions, the LIV signal could also be helpful in non-critical circumstances, such as alleviating cellulite. Cellulite is a cosmetic concern where the appearance of skin develops a rippled or bumpy affect due to hypertrophy and hyperplasia of adipocytes in the subcutaneous fat tissue in women (251). Current treatments include shock-wave therapy, massage, laser therapy, and topical agents, among others, and none of these have proven to be effective in the long-term (252). Similar to how LIV is effective in reducing adipocyte size in the bone marrow of OVX (226), a reduction in cell size in the subcutaneous fat region may reduce puckering of the skin and the appearance of cellulite. A benefit of LIV is that the signal is easily manipulated and be delivered through alternative devices. It is

plausible that placing a vibrating pad that delivers the LIV signal directly onto skin would reduce adipocyte hypertrophy and hyperplasia, and consequential rippled appearance.

Summary

In summary, this work demonstrates the complex interactions between bone, muscle, and fat tissues. Systemic adipose burdens in the form of OVX and high-fat diet both negatively impacted tissue systems, specifically through increasing muscular adiposity, which may contribute to the progression of type II diabetes. Other alterations in the muscular niche include the compromised satellite cell populations in OVX and alterations in insulin signaling in high-fat diet. Bone marrow-derived cells did not contribute to the intermuscular adiposity seen in high-fat diet animals, however, both bone marrow-derived cells and donor MSCs were accelerated to the visceral fat pads of high-fat diet animals, contributing to the expansion of the fat pad. These findings emphasize the notable changes in the musculature and bone marrow populations that precede changes in body habitus leading to obesity. Mechanical stimulation in the form of LIV was affective in mitigating muscular adiposity in both OVX and high-fat animal models and demonstrates the ability to be useful as a therapeutic intervention in preventing the onset of obesity or type II diabetes. Taken together, these data aid in the understanding of the relationship between muscle, bone, and fat tissues in the presence of a fat burden.

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