

Mechanical Stimulation of Mesenchymal Stem Cell Proliferation and Differentiation Promotes Osteogenesis While Preventing Dietary Induced Obesity

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Abstract

Mesenchymal stem cells (MSCs) are defined by their ability to self-renew and differentiate into the cells which form mesodermal tissues such as bone and fat. Low magnitude mechanical signals (LMMS) have been shown to be anabolic to bone, and recently reported to suppress the development of fat in normal animals fed a regular diet. Using male C57BL/6J mice, the ability of LMMS (0.2 g, 90 Hz signal applied for 15 mins/day, 5 day/wk) to simultaneously promote bone formation and prevent diet induced obesity was correlated to mechanical influences on the molecular environment of the bone marrow, as indicated by the population dynamics and lineage commitment of MSCs. Six weeks of LMMS increased the overall marrow-based stem cell population by 37% and the number of MSCs by 46%. Concomitant with the increase in stem cell number, the differentiation potential of MSCs in the bone marrow was biased towards osteoblastic and against adipogenic differentiation, as reflected by up-regulation of the transcription factor Runx2 by 72% and down-regulation of PPAR γ by 27%. The phenotypic impact of LMMS on MSC lineage determination was evident at 14w, where visceral adipose tissue formation was suppressed by 28%, while trabecular bone volume fraction in the tibia was increased by 11%. Translating this to the clinic, a one year trial in young women (15-20y; n=48) with osteopenia showed that LMMS increased trabecular bone in the spine and kept visceral fat at baseline levels, while control subjects showed no change in bone density yet an increase in visceral fat. Mechanical modulation of stem cell proliferation and differentiation indicates a unique therapeutic target to aid in tissue regeneration and repair, and may represent the basis of a non-pharmacologic strategy to simultaneously prevent obesity and osteoporosis.

Key words: mesenchymal stem cells, osteoblasts, osteoporosis, mechanical loading, adipocytes, obesity, adipogenesis, osteoblastogenesis

Introduction

The relationship between bone and fat is complex, and highly contingent on a multitude of factors including genetic, nutritional, biochemical, metabolic and physical inputs that coordinate the overall interaction and resulting phenotype. At the clinical level, the control of bone and fat formation are critically relevant to the increasing prevalence of, and costs associated with, osteoporosis and obesity. Predominantly, treatments are designed to treat obesity and osteoporosis once symptoms have already manifested, and such strategies, pharmacologic in particular, are primarily designed to target the extant fat cells (obesity) or bone cells (osteoporosis) to modify their activity, whether to reduce the lipid accumulation in adipocytes or to suppress activation of osteoclasts.

With the ever-increasing knowledge of the etiology of these diseases, new targets for primary prevention are being considered. And rather than target the extant cell population residing in bone and fat, strategies are being designed to control the fate of their progenitors, mesenchymal stem cells, that have yet to commit and differentiate into the mature cell types.^(1;2) Pluripotent mesenchymal stem cells (MSCs) are considered ideal therapeutic targets for both regenerative medicine and strategies intended to mitigate disease pathogenesis, as they hold the capacity to differentiate into osteoblasts, adipocytes, fibroblasts, chondrocytes, and myocytes. In the case of obesity and osteoporosis, biasing the fate of these precursors would help control the phenotypic outcomes of bone and fat mass, yet the difficulty in identifying the key environmental cues that regulate the lineage selection of MSCs has made it difficult to translate this to the clinic.⁽³⁻⁵⁾

While the interest in MSCs and their application to regenerative therapies and molecular therapeutics has been intense, the understanding of these cells remains in the nascent stages. Even gaining consensus on the criteria which serve to identify the MSC population has proven difficult, as stem cells and their neighboring cells within tissues are difficult to delineate and distinguish as based on current histological methods. Illustrating the inherent difficulty of high-

fidelity cellular identification, a combination of markers employed to distinguish another stem cell type, hematopoietic stem cells (HSCs) from 99.9% of the other cells in the bone marrow yields a population estimated at only 3% purity.⁽⁶⁾ The expression of surface markers is not well characterized, and to date, those which can be used to exclusively define MSCs have not been identified. Cell populations obtained in current isolation methods inevitably include heterogeneous mixtures of several cell types.⁽⁷⁾ Although a consensus has not yet been reached, there are several markers such as stem cell antigen (Sca-1) that are commonly used for identification of stem cells in murine models.^(8;9) For example, Sca-1 is an antigen previously associated with hematopoietic cells, but more recently cells expressing Sca-1 have been demonstrated to exhibit adipogenic, chondrogenic and osteogenic potential.⁽⁸⁾ Pref-1 (also known as Fetal antigen 1 (FA1) and delta-like 1 (Dlk-1)) of the epidermal growth factor (EGF) like protein family, has been identified as promoting the maintenance of a bipotential stem cell population.⁽¹⁰⁾ In addition, the molecule is robustly expressed on preadipocytes, and regulates the ability of this precursor cell to achieve the mature adipocyte phenotype.⁽¹⁰⁾

When the bone marrow stem cell population is driven to differentiate towards one cell fate, the establishment of another cell type is inherently suppressed. More specifically, MSCs express small amounts of both adipogenic and osteogenic factors, which cross regulate to retain the cell in an undifferentiated state.⁽¹¹⁾ When oncostatin, a member of the IL-6 family, is used to promote osteogenesis, it simultaneously inhibits adipogenesis.⁽¹²⁾ Conversely, anti-diabetic thiazolidinediones such as Rosiglitazone, a potent activator of PPAR γ , promotes adipogenesis while suppressing osteogenesis.⁽¹³⁻¹⁵⁾ This inverse relationship is also evident in the physical realm; hyper-physiologic levels of tensile strain will increase proliferation of bone marrow cells,⁽¹⁶⁾ and down-regulate PPAR γ in the local bone marrow, thus favoring osteoblastogenesis over adipogenesis.⁽²⁾ Further, the ability of different mechanical signals, including fluid flow and compression, to alter the differentiation patterns of MSCs has been explored and utilized by tissue engineers to promote the growth of both bone and cartilage.⁽¹⁷⁻²⁰⁾

The potential to harness MSCs as a means of prevention and treatment of disease is dependent on an improved understanding of the means by which exogenous signals regulate cell activity, and the ability of these stimuli to influence either/both proliferation and differentiation.⁽²¹⁾ Pharmacologic enhancement of stem cell proliferation has recently been demonstrated *in vivo*,⁽²²⁾ while extremely low magnitude mechanical signals (LMMS) have been shown to promote bone formation⁽²³⁾ as well as suppress adipogenesis in the growing animal without the use of drugs.⁽²⁴⁾

Materials & Methods

Animal Model to Prevent Diet Induced Obesity. All animal procedures were reviewed and approved by the Stony Brook University animal care and use committee. The overall experimental design consisted of two similar protocols, differing in the duration of treatment to assess mechanistic responses of cells to LMMS (6w of LMMS compared to sham control, n=8 per group) or to characterize the phenotypic effects (14w of LMMS compared to sham control). Further, two models of DIO were employed: 1. to examine the ability of LMMS to *prevent* obesity, a “High Fat Diet” condition (n=12 each, LMMS and CON) was evaluated where LMMS and DIO were initiated simultaneously, and 2. to examine the ability of LMMS to *reverse* obesity, an “Obese” condition (n=8 each, LMMS and CON) was established, whereby LMMS treatment commenced 3 weeks after the induction of DIO, and was compared to sham controls who were fed a high fat diet of this same extended time period.

Mechanical enhancement of stem cell proliferation and differentiation in DIO. Beginning at 7w of age, C57BL/6J male mice were given free access to a high fat diet (45% kcal fat, # 58V8, Research Diet, Richmond, IN). The mice were randomized into two groups, defined as LMMS (5d/w of 15min/d of a 90Hz, 0.2g peak acceleration mechanical signal, where 1.0g is earth’s gravitational field), and placebo sham controls (CON). The LMMS protocol⁽²⁴⁾ provides

low magnitude, high frequency mechanical signals by a vertically oscillating platform,⁽²⁵⁾ and for this acceleration and frequency generates strain levels in bone tissue of less than five microstrain, several orders of magnitude below peak strains generated during strenuous activity.^(23;26) Animal mass and food consumption were monitored weekly.

Status of MSC pool by flow cytometry. Cellular and molecular changes in the bone marrow resulting from 6w LMMS (n=8 animals per group, CON or LMMS) were determined at sacrifice from bone marrow harvested from the right tibia and femur (animals at 13w of age). Red blood cells in the bone marrow aspirate were removed by room temperature incubation with Pharmlyse (BD Bioscience) for 15 mins. Single cell suspensions were prepared in 1% sodium azide in PBS, stained with the appropriate primary and (when indicated) secondary antibodies, and fixed at a final concentration of 1% formalin in PBS. Phycoerythrin (PE) conjugated rat anti-mouse Sca-1 antibody and isotype control were purchased from BD Pharmingen and used at 1:100. Rabbit anti-mouse Pref-1 antibody and FITC conjugated secondary antibody were purchased from Abcam (Cambridge, MA) and used at 1:100 dilutions. Flow cytometry data was collected using a Becton Dickinson FACScaliber flow cytometer (San Jose, CA).

RNA extraction and real-time RT-PCR. At sacrifice, the left tibia and femur were removed and marrow flushed into RNAlater solution (Ambion, Foster City, CA). Total RNA was harvested from the bone marrow as described previously.⁽²⁷⁾ Briefly, TRIzol reagent (Life Technologies, Gaithersburg, MD) was added to the total bone marrow cell suspension and the solution homogenized. Phases were separated with chloroform under centrifugation. RNA was precipitated via ethanol addition and applied directly to an RNeasy Total RNA isolation kit (Qiagen, Valencia, CA). DNA contamination was removed on column with RNase free DNase. Total RNA was quantified on a Nanodrop spectrophotometer and RNA integrity monitored by agarose electrophoresis. Expression levels of candidate genes was quantified using a real-time

RT-PCR cycler (Lightcycler, Roche, IN) relative to the expression levels of samples spiked with exogenous cDNA.⁽²⁸⁾ A “one-step” kit (Qiagen) was used to perform both the reverse transcription and amplification steps in one reaction tube.

Body habitus established by *in vivo* microcomputed tomography (CT) Phenotypic effects of DIO, for both the “prevention” and “reversal” of obesity test conditions were defined at both 12 and 14w of LMMS. At 12w, *in vivo* μ CT scans were used to establish fat, lean, and bone volume of the torso (VivaCT 40, Scanco). Scan data was collected at an isotropic voxel size of 76 μ m (45 kV, 133 μ A, 300 ms integration time), and analyzed from the neck to the distal tibia for each animal. Threshold parameters were defined during analysis to segregate and quantify fat and bone volumes. Detailed CT scanning protocol and analysis techniques are reported elsewhere.^(29;30) At 14w, following euthanasia, the epididymal fat pads and a subcutaneous fat pad from the mesenteric region were harvested and used to validate CT volume data generated at 12w. Correlations between *ex vivo* and *in vivo* fat measurements (reported elsewhere) were high ($R^2 > 0.90$).⁽²⁹⁾

Bone phenotype established by *ex vivo* microcomputed tomography. Trabecular bone morphology of the proximal left tibia of each mouse was established by μ CT at 12 μ m resolution (MicroCT 40, Scanco). The metaphyseal region spanned 600 μ m, beginning 300 μ m distal to the growth plate. Bone volume fraction (BV/TV), connectivity density (Conn.D), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and the structural model index (SMI) were determined.⁽³¹⁾

Serum and tissue biochemistry. Blood collection was performed after overnight fast by cardiac puncture with the animal under deep anesthesia. Serum was harvested by centrifugation. Mice were euthanized by cervical dislocation, and the different tissues (i.e.,

epididymal fat pad and subcutaneous fat pads from the lower torso, liver, and heart) were excised, weighed, frozen in liquid nitrogen, and stored at -80°C. Total lipids from white adipose tissue (epididymal fat pad) and liver were extracted and purified based on a chloroform–methanol extraction. Total triglycerides (TG) and non-esterified free fatty acids (NEFA) were measured on serum (n=10 per group) and lipid extracts from adipose tissue (n=5 or 6 per group) and liver (n=10 per group) using enzymatic colorimetric kits (TG Kit from Sigma, Saint Louis, MO; and NEFA C from Wako Chemicals, Richmond, VA). ELISA assays were utilized to determine serum concentrations of leptin, adiponectin, resistin (all from Millipore, Chicago, IL), osteopontin (R&D Systems, Minneapolis, MN), and osteocalcin (Biomedical Technologies Inc, Stoughton, MA), using a sample size of n=10 per group.

Human trial to examine inverse relationship of adipogenesis and osteoblastogenesis. A trial designed to evaluate if 12 months of LMMS could promote bone density in the spine and hip of women with low bone density relative to controls,⁽³²⁾ was evaluated retrospectively to examine if there were concurrent changes in visceral fat volume. All procedures were reviewed and approved by the Childrens Hospital of Los Angeles Committee on Research in Human Subjects.

Forty-eight healthy young women (aged 15-20 years) were randomly assigned into either LMMS or CON groups (n=24 in each group). The LMMS group underwent brief (10 min requested), daily treatment with LMMS (30 Hz, 0.3 g signal) for one year. Computed tomography (CT) scans were performed at baseline and at one year, with the same scanner (model CT-T 9800, General Electric Co., Milwaukee, WI), the same reference phantom for simultaneous calibration, and specially designed software for fat and muscle measurements. Identification of the abdominal site to be scanned was performed with a lateral scout view, followed by a cross-sectional image obtained from the mid-portion of the third lumbar vertebrae at 80 kVp, 70 milliamperes, and 2S.

Cancellous bone of the 1st, 2nd and 3rd lumbar vertebrae was established as measures of the bone density in milligrams per cubic centimeter (mg/cm^3). Visceral fat area (cm^2) was defined at the mid-portion of the third lumbar vertebrae (L3) as the intra-abdominal adipose tissue surrounded by the rectus abdominus, external oblique, quadratus lumborum, and psoas muscles and the lumbar spine, and consisted mainly of perirenal, pararenal, retroperitoneal and mesenteric fat. The average area of paraspinous musculature (cm^2) was defined as the summed area of the erector spinae, psoas major and quadratus lumborum muscles at the midportion of L3.⁽³²⁾ All analyses of bone density, and muscle and fat area were performed by an operator blinded as to subject enrollment.

Statistical analyses. All data are shown as mean \pm standard deviation, unless noted. To determine significant differences between LMMS and CON groups, two tailed t-tests were used throughout. *Ex vivo* trabecular bone data were analyzed with body mass of the animals as a covariate. Animal outliers were determined based on animal mass at baseline (before the start of any treatment) as animals falling outside of two standard deviations from the total population, or in each respective group at the end of 6 or 14 weeks LMMS (or sham CON) by failure of the Weisberg one-tailed t-test ($\alpha = 0.01$). The Weisberg test has been shown as an objective tool for showing consistency within small data sets.⁽³³⁾ No outliers were identified in the 6w CON and LMMS groups. Two outliers per group (CON and LMMS) were identified in the Fat Diet model (14w LMMS study) and removed. Data from these animals were not included in any analyses, resulting in a sample size of $n=10$ per group for all data, unless otherwise noted. No outliers were identified in the 14w Obese model ($n=8$). Data presented from the human trial are based on the intent to treat data set (all subjects included in the evaluation). The significance of absolute changes in bone density, muscle and visceral fat area, relative to baseline, were determined for LMMS and CON subjects using a two tailed t-test. Statistical significance was considered at the 5% level.

Results

Bone marrow stem cell population is promoted by LMMS. Flow cytometric measurements using antibodies against Stem cell antigen-1 (Sca-1) indicated that in animals in the “prevention” DIO group, 6w of LMMS treatment significantly increased the overall stem cell population (including hematopoietic and mesenchymal stem cells) relative to controls. Analysis focused on the primitive population of cells with low forward (FSC) and side scatter (SSC), indicating the highest Sca-1 staining for all cell populations. Cells in this region demonstrated a 37.2% ($p=0.024$) increase in LMMS stem cell numbers relative to sham CON animals. Mesenchymal stem cells as represented by cells positive for both Sca-1 and Preadipocyte factor-1 (Pref-1),⁽³⁾ represented a much smaller percentage of the total cells. Identified in this manner, LMMS treated animals had a 46.1% ($p=0.022$) increase in specifically mesenchymal stem cells relative to CON (Fig 1).

LMMS biases marrow environment and lineage commitment towards osteogenesis. After six weeks, cells expressing only the Pref-1 label, considered committed preadipocytes, were elevated by 18.5% ($p=0.25$) in LMMS treated animals relative to CON (Fig 2). Osteoprogenitor cells in the bone marrow population, identified as Sca-1 positive with high FSC and SSC, were 29.9% ($p=0.23$) greater in the LMMS group. This cell population can synthesize alkaline phosphatase, collagen, and osteocalcin and form a mineralized matrix in culture.⁽³⁴⁾ This trend indicating that differentiation in the marrow space of LMMS animals had shifted towards osteogenesis was confirmed by gene expression data, which demonstrated that transcription of Runx2 in total bone marrow isolated from LMMS animals was up-regulated 72.5% ($p=0.021$) relative to CON. In these same LMMS animals, expression of PPAR γ was down-regulated by 26.9% ($p=0.042$) relative to CON (Fig 3).

LMMS enhancement of bone quantity and quality. The ability of LMMS induced changes in proliferation and differentiation of MSCs to elicit phenotypic changes in the skeleton was first measured at 12w by *in vivo* μ CT scanning of the whole mouse (neck to distal tibia). Animals subject to LMMS showed a 7.3% ($p=0.055$) increase in bone volume fraction of the axial and appendicular skeleton (BV/TV) over sham CON. Post-sacrifice, μ CT scans of the isolated proximal tibia of the LMMS animals showed 11.1% ($p=0.024$) greater bone volume fraction than CON (Fig 4). The micro-architectural properties were also enhanced in LMMS as compared to CON, as evidenced by a 23.7% greater connectivity density ($p=0.037$), a 10.4% higher trabecular number ($p=0.022$), a 11.1% smaller separation of trabeculae ($p=0.017$) and a 4.9% lower structural model index (SMI, $p=0.021$; Table 1).

Prevention of obesity by LMMS: At 12w, neither body mass gains nor the average weekly food intake differed significantly between the LMMS or CON groups (Table 2). At this point, (19w of age), CON weighed $32.9\text{g} \pm 4.2\text{g}$, while LMMS mice were 6.8% lighter at $30.7\text{g} \pm 2.1\text{g}$ ($p=0.15$). CON were 15.0% heavier than mice of the same strain, gender and age that were fed a regular chow diet,⁽²⁴⁾ and the increase in body mass due to the high fat diet was comparable to previously reported values.⁽³⁵⁾ Adipose volume from the abdominal region (defined as the area encompassed by lumbar vertebrae L1-L5) was segregated as either subcutaneous or visceral adipose tissue (SAT or VAT, respectively). LMMS animals had 28.5% ($p=0.021$) less VAT by volume, and 19.0% ($p=0.016$) less SAT by volume. The epididymal fat pad weight was 24.5% ($p=0.032$) less in LMMS than CON, and the subcutaneous fat pad from the lower back region weighed 26.1% ($p=0.018$) less in LMMS animals (Table 2).

LMMS prevents increased biochemical indices of obesity. Triglycerides (TG) and non-esterified free fatty acids (NEFA) measured in plasma, epididymal adipose tissue, and liver were all lower in LMMS as compared to CON (Table 3). Liver TG levels decreased by 25.6%

($p=0.19$) in LMMS animals, paralleled by a 33.0% ($p=0.022$) decrease in NEFA levels. Linear regressions of adipose and liver TG and NEFA values to μ CT visceral volume (VAT) demonstrated strong positive correlations for CON animals, with $R^2 = 0.96$ ($p=0.002$) for adipose TG, $R^2 = 0.85$ ($p=0.027$) for adipose NEFA, $R^2 = 0.64$ ($p=0.006$) for liver TG and $R^2 = 0.80$ ($p=0.003$) for liver NEFA (Fig 5). LMMS resulted in weaker correlations between all TG and NEFA levels to increases in VAT.

At sacrifice, fasting serum levels of adipokines were lower in LMMS as compared to CON. Circulating levels of leptin were decreased by 35.3% ($p=0.05$), adiponectin by 21.8% ($p=0.009$), and resistin by 15.8% ($p=0.20$) compared to CON (Table 3). Circulating serum osteopontin (-7.5%, $p=0.41$) and osteocalcin (-14.6%, $p=0.22$) levels were not significantly affected by the mechanical signals.

LMMS fails to reduce existing adiposity. In the “reversal” model of obesity, 4w old animals were started on a high fat diet for 3w prior to beginning the LMMS protocol at 7w of age. These “obese” animals were on average 3.7 grams heavier ($p < 0.001$) than the age-matched “prevention” animals at the start of the protocol. The early-adolescent obesity in these obese animals manifested into adulthood, such that by the end of the 12w protocol, they weighed 21% more than the CON animals who began the fat diet at 7w of age ($p < 0.001$). In stark contrast to the “prevention” animals, where LMMS realized a 22.2% ($p=0.03$) lower overall adipose volume relative to CON (neck to distal tibia), no differences were seen for fat (-1.1%, $p=0.92$) or bone volume (-0.2%, $p=0.94$) between LMMS and CON groups after 12w of LMMS for these already obese mice (Fig 6).

LMMS promotes bone and muscle and suppresses visceral fat in humans. To determine whether the ability of LMMS to suppress adiposity and increase osteogenesis in mice can translate to the human, young women with low bone density were subject to daily exposure to

LMMS for 12 months. The study cohort ranged from 15-20 years old, and was originally designed to evaluate if LMMS could enhance the musculoskeletal system. Detailed descriptions of this study population are provided elsewhere,⁽³²⁾ and the results reported here represent a retrospective evaluation of the data to assess visceral adiposity.

At the end the of one year study, women (n=24) in the CON group had no significant change in cancellous bone density of the spine ($0.1 \pm \text{s.e.} 1.5 \text{ mg/cm}^3$; $p=0.93$), as compared to a $3.8 \pm 1.6 \text{ mg/cm}^3$ increase in the spine of the LMMS treated cohort ($p=0.025$; Fig 7). Further, at the level of the umbilicus, the average area of paraspinous muscle failed to change in CON ($1.2 \pm 1.9 \text{ cm}^2$; $p=0.43$), but was sharply elevated in the LMMS women ($10.1 \pm 2.5 \text{ cm}^2$; $p<0.001$). Visceral fat area measured at the lumbrosacral region of CON subjects increased significantly from baseline ($5.6 \pm 2.4 \text{ cm}^2$, $p=0.03$). In contrast, visceral fat area in LMMS subjects saw a small, non-significant increase from baseline ($1.8 \pm 2.3 \text{ cm}^2$, $p=0.45$).

Discussion

The interaction between bone and fat is complex, and highly dependent on a multitude of factors including genetic, biochemical, and mechanical inputs to coordinate an appropriate adaptive response. The experiments reported here focus on the mechanical contribution to this environment, and indicate that extremely low magnitude mechanical signals, well below those generated during locomotion, can promote the number of stem cells residing in the marrow. Further, these subtle mechanical signals biased the differentiation of the MSC population towards osteoblastogenesis over adipogenesis, such that obesity was prevented while bone formation was promoted. Translating the ability of LMMS to bias bone formation over fat formation to a group of young osteopenic women, these low magnitude mechanical signals promoted bone and muscle mass, and concurrently suppressed the accumulation of visceral fat

in the treated group, while controls failed to gain either bone or muscle over the course of the year, and showed a significant increase in visceral fat.

These data provide support for the growing body of evidence of an inversely coupled relationship between pre-osteoblasts and pre-adipocytes in the marrow cavity, and osteogenesis and adipogenesis overall.⁽³⁶⁾ It has been reported that overweight individuals tend to have higher bone mineral density than normal weight individuals, and are less prone to osteoporosis⁽³⁷⁾ as the load bearing challenges inherent to obesity should have a beneficial impact on the skeleton. Conflicting evidence, however, indicates this increase in bone quantity is not proportional to the increase in weight. In a review of risk factors for fractures in normally active children and adolescents, it was seen that obesity increased the incidence of fracture from 15.5% in normal weight children to 33.3% in obese children.^(38;39)

Sca-1 was used to give a general indication of the status of the bone marrow derived stem cell population, representing both hematopoietic and mesenchymal stem cells as the marker does not distinguish between the two cell types. The relative increase in the overall bone marrow stem cell population induced by LMMS reflected an enhancement of stem cells of both hematopoietic and mesenchymal lineages. The method utilized for bone marrow harvesting (flushing of bones) does not remove the significant portion (33%) of hematopoietic stem cells (HSCs) that resides in proximity to the endosteal surface of the bone,⁽⁴⁰⁾ and thus the influence of LMMS on HSC's is likely underestimated in this model. MSCs have been reported as expressing a series of surface markers, including both Sca-1 and Pref-1.⁽³⁾ The use of both markers should be more specific in identifying mesenchymal stem cells, and while accordingly these cells occur less frequently, this population demonstrated a larger increase in response to LMMS than that measured in the overall stem cell population. In all, these data indicate that the stem cell pool has been positively influenced by mechanical signals, resulting in an increase in total number of cells.

At the molecular level, LMMS induced a clear shift in the biological balance of the key osteogenic and adipogenic factors, with a marked increase in the expression level of Runx2, and a significant decrease in the level of PPAR γ . Together, this change in balance may conspire to bias stem cells in the undifferentiated state preferentially towards the formation of bone and away from fat.⁽³⁶⁾ Reduced levels of PPAR γ are permissive to osteoblastogenesis and can lead to higher trabecular bone volume,⁽⁴¹⁾ by promoting the osteoblastic lineage decision of MSCs.⁽⁴²⁾

Ultimately, it will be important to perform *in vitro* assays to more comprehensively validate the commitment of stem cells to a given lineage, despite the inherent limitations even of using culture systems to define stem cell development.⁽⁴³⁾ The plasticity of MSCs and their ability to transdifferentiate make attempts at *in vitro* functional characterization difficult,⁽⁴⁴⁾ and subtle shifts in differentiation potential such as we have observed in these studies may be difficult to detect. And of course, even given the many advantages of a culture system, an inherent limitation is that the “system” level interactions of stem cells, bone marrow, blood flow, mechanical signals, the hormonal milieu, etc., cannot serve as an agent of change. While not statistically significant, the increased percentage of cells in the osteoprogenitor and preadipocyte populations in bone marrow from the LMMS animals showed *in vivo* trends that support a conclusion that lineage selection of cells has been altered by the mechanical signal. There is evidence suggesting that preadipocytes, through expression of the plasma membrane protein Pref-1, are responsive to differentiation signals from the extracellular environment,⁽⁴⁵⁾ with Pref-1 expression being *inhibitory* of adipogenesis and terminal adipocyte differentiation.⁽⁴⁶⁾ The ultimate fate of marrow preadipocytes and their ability to migrate to other adipose tissue depots has not been definitively addressed and ultimately may highlight the inherent difficulty in harnessing stem cell plasticity as a therapeutic endpoint.^(47;48)

While the impact of the LMMS signal was evaluated in bone marrow derived stem cells, the systemically delivered stimulus is certain to influence other cell populations as well. As

such, the phenotypic changes measured in fat and bone are likely not the exclusive result of regulating the bone marrow stem cell population, and more realistically result from influencing many interacting cell populations, including bone and fat cells. What is apparent, however, is that LMMS increases the size of the precursor pool in the bone marrow, and biases them away from adipogenesis and towards higher order connective tissues. We believe these data support a conclusion that the mechanical biasing of MSC lineage selection towards osteoblastogenesis inherently suppresses adipogenesis because the stem cell ultimately can only make a “single pathway” commitment. Indeed, even though adipose tissue mass was more than twenty percent greater in the controls relative to the LMMS animals, animal weights differed by less than seven percent, as the reduced fat mass of the LMMS animals was to a degree, compensated by the increase in bone mass, and further emphasized the binary nature of the differentiation process.

While these data indicate a role of development in the etiology of obesity, they do not preclude a critical contribution of the overall metabolic state of the organism, as increased adiposity alters the systemic physiology by changing the endocrine and metabolic state of the fat tissue,⁽⁴⁹⁾ as well as susceptibility to diseases. The amount of visceral adipose tissue is an important risk factor to metabolic complications which afflict the obese, with adiposity positively correlating with fasting plasma insulin, triglyceride, low-density lipoprotein and apolipoprotein B levels, as well as the cholesterol/high-density lipoprotein ratio.⁽⁵⁰⁾ Increased abdominal adiposity is also known to be a significant risk factor for type 2 diabetes.⁽⁵¹⁾ Control animals presented with these same positive correlations of TG and NEFA levels in adipose tissue and the liver to visceral adipose accumulation, while adipose gains in LMMS treated animals did not translate into proportional increases, suggesting that suppression of adipogenesis and/or adiposity could ultimately inhibit associated sequelae. While also of significance, specimen limitations prevented a full characterization of marrow adiposity, specifically, for DIO and/or LMMS treated animals. Ultimately, while histology-based determinations of adipose infiltration of the marrow

space may provide qualitative data in this regard, various imaging techniques under development, such as high-field micro-MRI, may provide a more accurate quantification of marrow adiposity in a small animal model such as the one used here.⁽⁵²⁾

Serum levels of osteopontin were measured to assay systemic changes in bone tissue, as osteopontin is secreted by osteoblasts and acts to activate osteoclasts in the normal process of bone remodeling. Additionally, osteopontin has been reported as a potent constraining factor on hematopoietic stem cell proliferation.⁽⁵³⁾ That osteopontin is unaffected by LMMS treatment highlights the general promotion of stem cell proliferation (HSC and MSC), but the specificity of the proposed mechanism to MSC differentiation as biasing the formation of osteoprogenitors did not examine if osteoclasts, which derive from HSCs, were either elevated or activated. Circulating levels of the key adipokines leptin, adiponectin and resistin are known to be elevated under conditions of DIO, as these molecules are secreted by white adipose tissue.^(35;54) These molecules each exhibit pleiotropic effects, with implications for inflammatory and immune responses.⁽⁵⁵⁾ In light of the similar food intake between groups, the markedly reduced levels of circulating adipokines in LMMS animals may best reflect the reduced adipose burden in these mice.

In evaluating the ability of LMMS to prevent obesity, these data indicate that these mechanical signals were effective at the molecular, cellular and tissue level, as indicated by a distinct bias towards osteogenesis after 6w, translating at 12 and 14w into clear phenotypic differences in bone and fat volume. In contrast, mice allowed to become obese (4w of a high fat diet) before being subject to LMMS indicated not only the inability of these mechanical signals to reverse obesity in animals that were already fat, it failed to influence their bone mass, despite receiving the same mechanical signal as the “prevention” group. This could either mean that the stem cell population in the “pre-obese” mice was already committed towards adipogenesis and away from bone by the time the mechanical signal was introduced, or that a saturated, adipogenic environment catalyzed by a high fat diet persists and supersedes the ability of other

mechanical signals to drive MSCs towards specific lineages. Certainly, the inability to reduce the volume of adipose tissue in an already obese animal emphasizes the starkly different challenges of a developmental strategy to prevent obesity versus the metabolic realities of reversing it.

A similar scenario may well be evident in preventing versus reversing age-related bone loss with mechanical signals, as the deterioration of the marrow based stem cell population which parallels aging may undermine the ability of any intervention to harness the potential of stem cells to treat disease.⁽⁵⁶⁾ Aging animals demonstrate a significant reduction in their stem cell population and their regenerative capacity,⁽⁵⁷⁾ while simultaneously predisposing this environment towards adipogenesis in the remaining MSCs.⁽⁵⁸⁾ Extending this “aging” related deterioration to disuse, inactivity and microgravity markedly reduce osteoblastogenesis in the MSC pool,⁽⁵⁹⁾ while the actual number of osteoprogenitor cells is also severely compromised.⁽⁶⁰⁾ Thus, both age and activity are determinants of the viability of the stem cell population, and independently or together may conspire towards a reduced regenerative capacity. This deterioration can be somewhat mitigated by replenishment of the bone marrow stem cell population, either directly or via exposure to a “young” environment, showing promise as a intervention to restore musculoskeletal health.^(61;62) When considering this in the context of the data presented here in osteopenic women, where LMMS increased bone and muscle mass while concurrently suppressing visceral adiposity, suggests that susceptibility to diseases such as obesity and osteoporosis may be more closely linked than previously thought, due – potentially - to a failure to drive stem cells towards the “right” fate, and provide some early support that early prevention for both diseases is ultimately easier than treating either.

It has been estimated that 80% of obese adolescents develop into obese adults,⁽⁶³⁾ contributing to the conclusion by the American Heart Association that primary prevention is the key to constraining the societal impact,⁽⁶⁴⁾ as treatments once an individual is obese are limited. The differential response to LMMS in the two animal models presented herein further highlights

this disparity, in that prevention of obesity via developmental control was achievable, but that reversal of an obese state, once cell fate had been pre-determined, was not realized. Rather than a metabolic pathway, these data indicate a developmentally mediated mechanism by which the suppression of fat and the enhancement of bone are coupled, as linked to mechanical influences on stem cell populations. Indeed, the mechanically mediated increase in the number of progenitor cells, taken together with the ability of these mechanical signals to drive commitment choices, indicates a viable means to enhance an organism's regenerative capacity and reduce susceptibility to disease, achieved by exploiting stem cell sensitivity to physical signals. Similar to bone, recent findings definitively demonstrate that adipocytes do indeed turn over in humans with approximately 10% of fat cells are renewed annually in adults, via a balance of adipocyte death and generation.⁽⁶⁵⁾ Based on the ability of LMMS to slow and deter the development of stem cells into fat, perhaps even adult obesity could be slowly repressed via capitalizing on the normal process of adipocyte death if the formation of new adipocytes could be stemmed.

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TABLE 1. MICRO-ARCHITECTURAL PARAMETERS OF TRABECULAR BONE IN FAT DIET ANIMALS MEASURED AT 14W (MEAN \pm S.D., N=10) DEMONSTRATE THE ENHANCED STRUCTURAL QUALITY OF BONE IN THE PROXIMAL TIBIA OF LMMS TREATED ANIMALS AS COMPARED TO CONTROLS.

	CON	LMMS	% diff	p-value
Conn.D (1/mm ³)	105.3 \pm 34.2	130.3 \pm 28.9	23.7	0.037
Tb.N (1/mm)	3.06 \pm 0.45	3.38 \pm 0.37	10.4	0.022
Tb.Th (mm)	0.029 \pm 0.001	0.030 \pm 0.001	1.0	0.398
Tb.Sp (mm)	0.304 \pm 0.046	0.270 \pm 0.035	-11.1	0.017
SMI	2.93 \pm 0.22	2.78 \pm 0.14	-4.9	0.021

TABLE 2: DESPITE SIMILAR BODY MASS AND WEEKLY FOOD CONSUMPTION, PHENOTYPIC PARAMETERS OF THE FAT DIET ANIMALS AFTER 12W OF LMMS OR AT SACRIFICE (14W, MEAN \pm S.D., N=10) DEMONSTRATE A LEANER BODY HABITUS, AS THE ADIPOSE BURDEN (VISCERAL AND SUBCUTANEOUS FAT) IS SIGNIFICANTLY LOWER IN THE LMMS ANIMALS.

	CON	LMMS	% diff	p-value
Animal Weight at 12 weeks (grams)	32.9 \pm 4.12	30.7 \pm 2.74	-6.8	0.152
Weekly Food Consumption (grams)	18.9 \pm 1.57	18.5 \pm 1.47	-2.5	0.406
Visceral Adipose Tissue (VAT, cm ³)	2.3 \pm 0.72	1.6 \pm 0.34	-28.5	0.021
Subcutaneous Adipose Tissue (SAT, cm ³)	0.84 \pm 0.16	0.68 \pm 0.08	-19.0	0.016
Epididymal Fat Pad (grams)	1.85 \pm 0.52	1.40 \pm 0.32	-24.5	0.032
Subcutaneous Fat Pad (grams)	0.67 \pm 0.17	0.50 \pm 0.12	-26.1	0.018
Liver (grams)	0.99 \pm 0.16	0.94 \pm 0.07	-4.9	0.399

TABLE 3: BIOCHEMICAL PARAMETERS OF THE FAT DIET ANIMALS (MEAN \pm S.D., N=10)
HIGHLIGHT LOWER LEVEL OF TG, NEFA, AND CIRCULATING ADIPOKINES FOLLOWING
14W OF LMMS STIMULATION AS COMPARED TO CONTROLS.

	CON	LMMS	% diff	p-value
TG Liver (total mg)	31.8 \pm 14.3	23.6 \pm 12.7	-25.6	0.195
NEFA Liver (total mol)	7.5 \pm 2.7	5.0 \pm 1.5	-33.0	0.022
TG Adipose (total mg)	91.6 \pm 34.6 (n=5)	72.9 \pm 18.1 (n=6)	-20.4	0.321
NEFA Adipose (total mmol)	18.1 \pm 5.8 (n=5)	15.3 \pm 2.4 (n=6)	-15.8	0.345
TG Serum (mg/dl)	46.2 \pm 17.0	47.0 \pm 18.4	1.6	0.928
NEFA Serum (mmol/l)	0.68 \pm 0.10	0.64 \pm 0.14	-5.3	0.526
Leptin Serum (ng/mL)	15.9 \pm 7.2	10.1 \pm 4.7	-37.6	0.049
Resistin Serum (ng/mL)	4.3 \pm 1.2	3.6 \pm 1.0	-15.8	0.200
Adiponectin Serum (μ g/mL)	9.2 \pm 1.7	7.0 \pm 1.4	-23.5	<0.01
Osteopontin Serum (ng/mL)	197.8 \pm 22.8	183.0 \pm 39.6	-7.5	0.409
Osteocalcin Serum (ng/mL)	55.7 \pm 17.2	47.6 \pm 7.8	-14.6	0.218

FIG 1. Representative density dot plots from flow cytometry experiments indicate the ability of LMMS to increase the number of stem cells in general (Sca-1 single positive, upper quadrants), and MSCs specifically (both Sca-1 and Pref-1 positive, upper right quadrant). Red = high cell density, blue = low cell density. As compared to Control animals (A), low level mechanical signals increase the number of stem cells in the bone marrow of LMMS animals (B). The actual increase in total bone marrow derived stem cell number (C) and MSC number (D) was calculated as % positive cells/total cells for the cell fraction showing highest intensity staining.

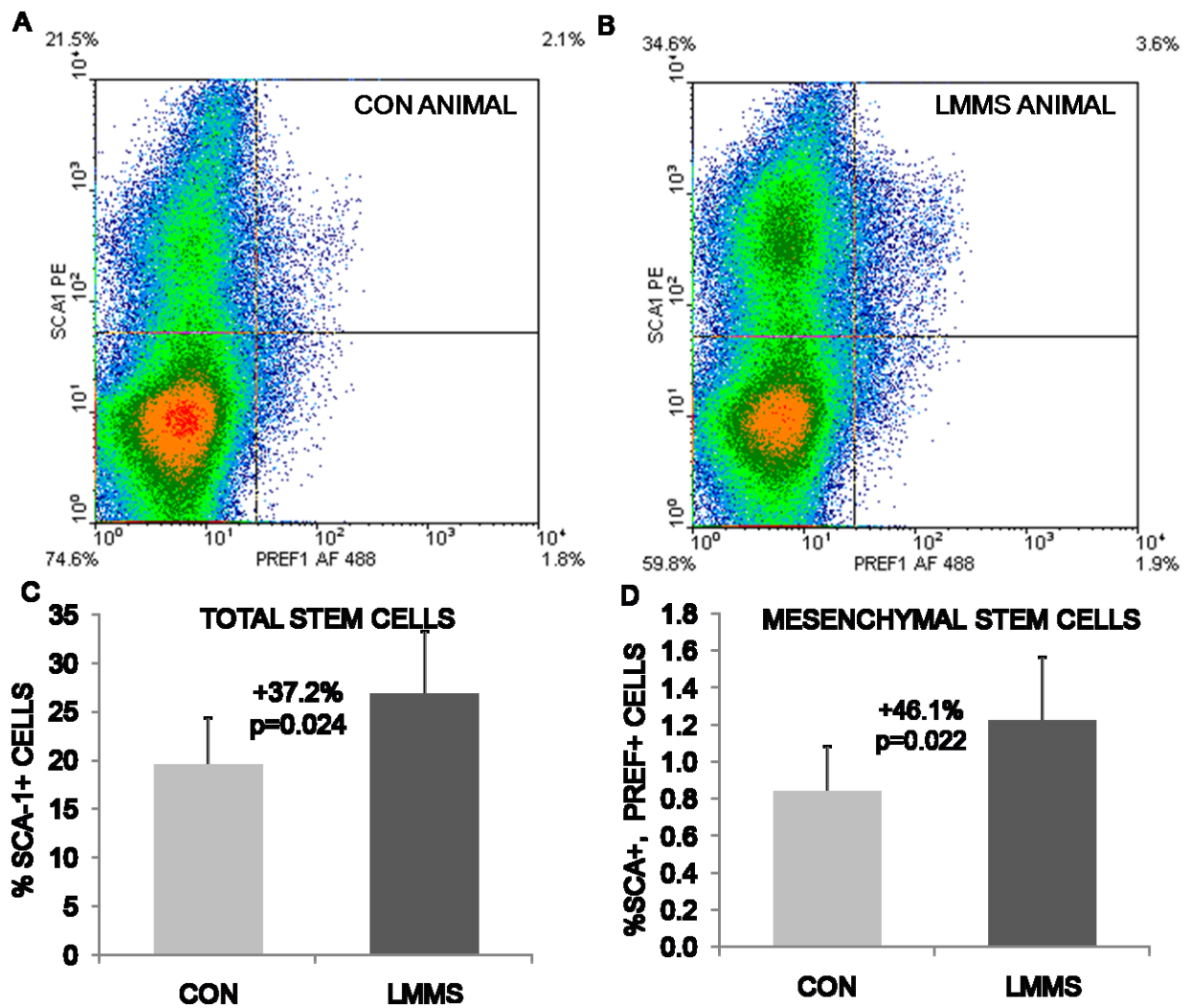


FIG 2. LMMS influences on stem cells was focused on the distinct cell populations identified in flow cytometry (A), with stem cells being identified as low forward (FSC) and side (SSC) scatter. Osteoprogenitor cells were identified as Sca-1(+) cells, residing in the region highlighted as high FSC and SSC, and were 29.9% (p=0.23) more abundant in the bone marrow of LMMS treated animals (B). The preadipocyte population, identified as Pref-1 (+), Sca-1 (-), demonstrated a trend (+18.5%; p=0.25) towards an increase in LMMS relative to CON animals (C).

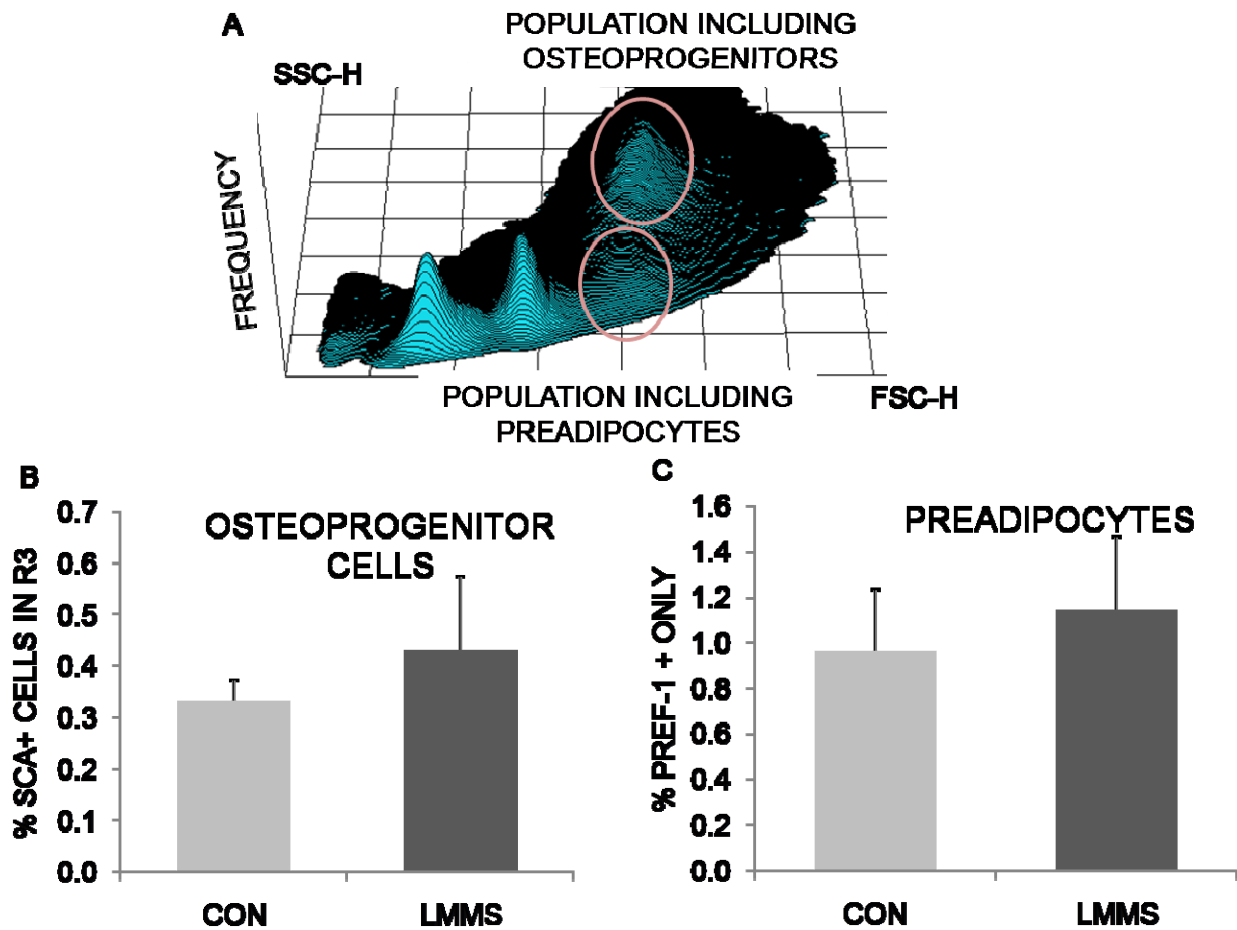


FIG 3. Relative to CON, LMMS biases the bone marrow environment towards osteogenesis and away from adipogenesis. Real Time RT-PCR analysis of bone marrow samples harvested from animals subject to 6 weeks LMMS treatment or sham control indicated a significant upregulation of the osteogenic gene Runx2 (A) and downregulation of the adipogenic gene PPAR γ (B). Data are show as expression levels relative to values for sham handled CON animals (represented as 1.0).

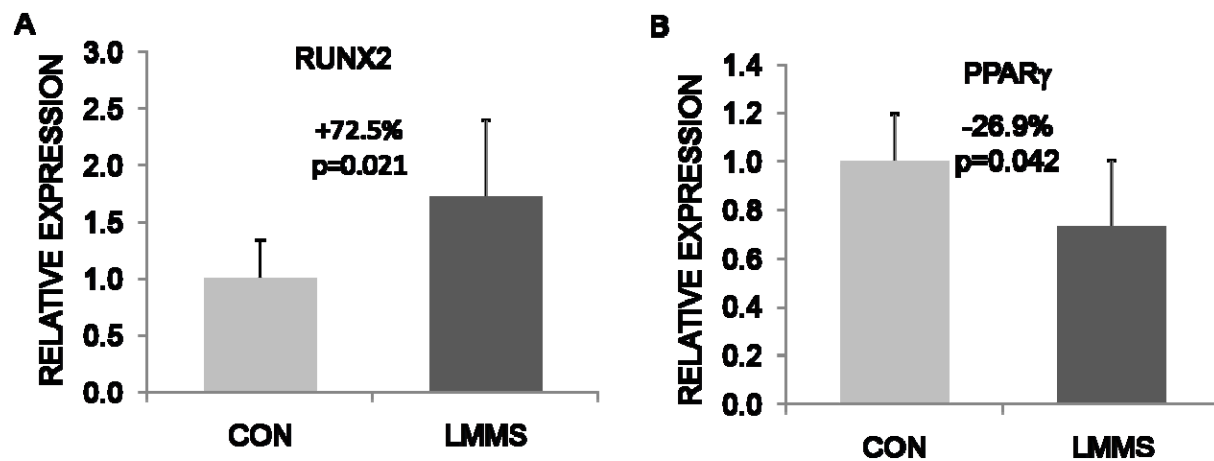


FIG 4. Bone volume fraction, as measured *in vivo* by low resolution μ CT, indicated that LMMS increased bone volume fraction across the entire torso of the animal (A). Post-sacrifice, high resolution CT of the proximal tibia indicated a significant increase in trabecular bone density (B). Body mass of the animal at sacrifice was used as a covariate in the statistical analysis. As compared to controls (C), representative μ CT reconstructions of the proximal tibia indicate the enhanced morphological properties of LMMS animals.

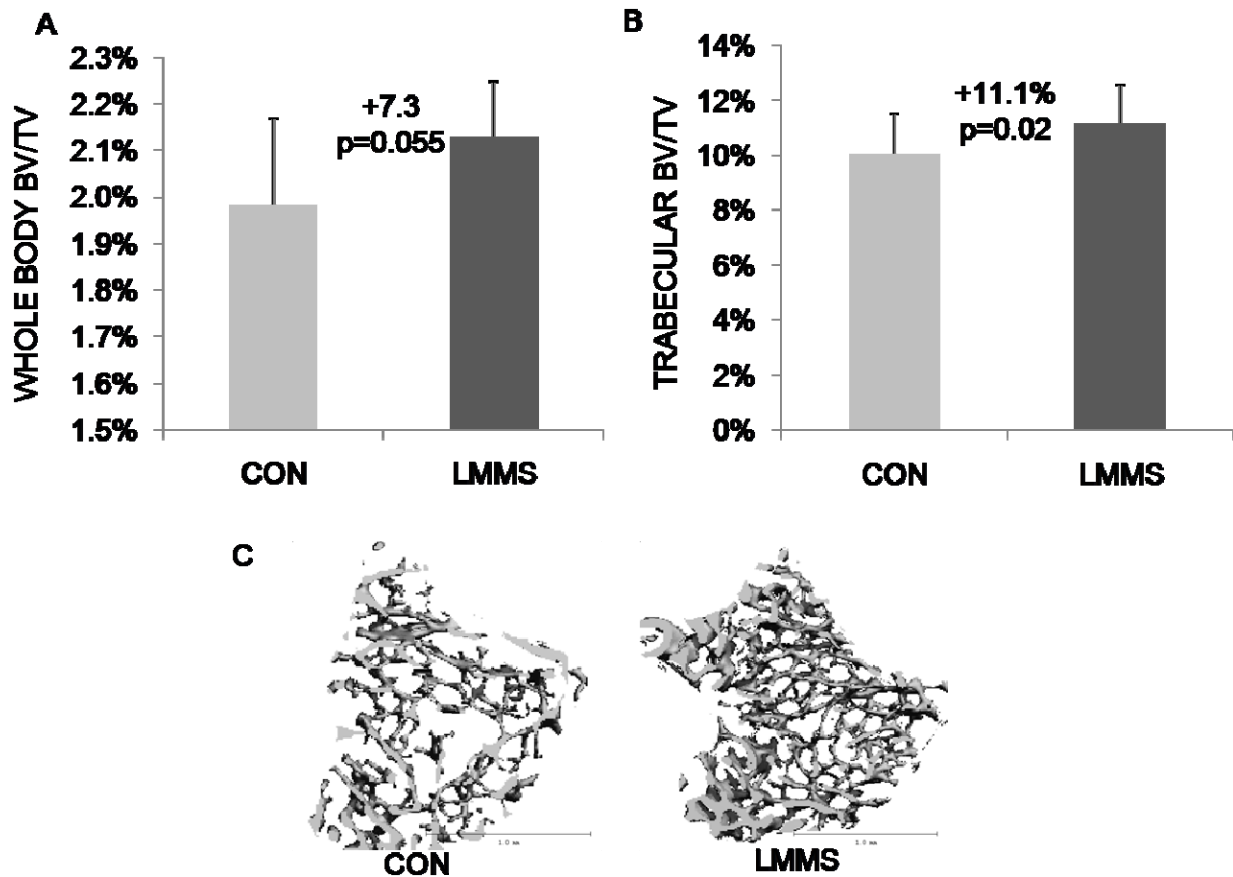


FIG 5. Representative *in vivo* μ CT images used to discriminate visceral and subcutaneous adiposity in the abdominal region of a CON and LMMS animal. Visceral fat is shown in red, subcutaneous fat in gray (A). Linear regressions of calculated visceral adipose tissue (VAT) volume against adipose and liver biochemistry values demonstrated strong positive correlations in CON, and weak correlations in LMMS groups, as well as generally lower levels for all LMMS biochemical values (n=6 for adipose, n=10 for liver). Regressions for adipose TG (p=0.002, B), adipose NEFA (p=0.03, C), liver TG (p=0.006, D) and liver NEFA (p=0.003, E) were significant for CON animals, but only liver NEFA (p=0.02) was significant for LMMS. Overall, LMMS mice exhibited lower, non-significant correlations in liver TG (p=0.06), adipose TG (p=0.19), and adipose NEFA (p=0.37) to increases in visceral adiposity. CON = \circ , LMMS = \blacksquare

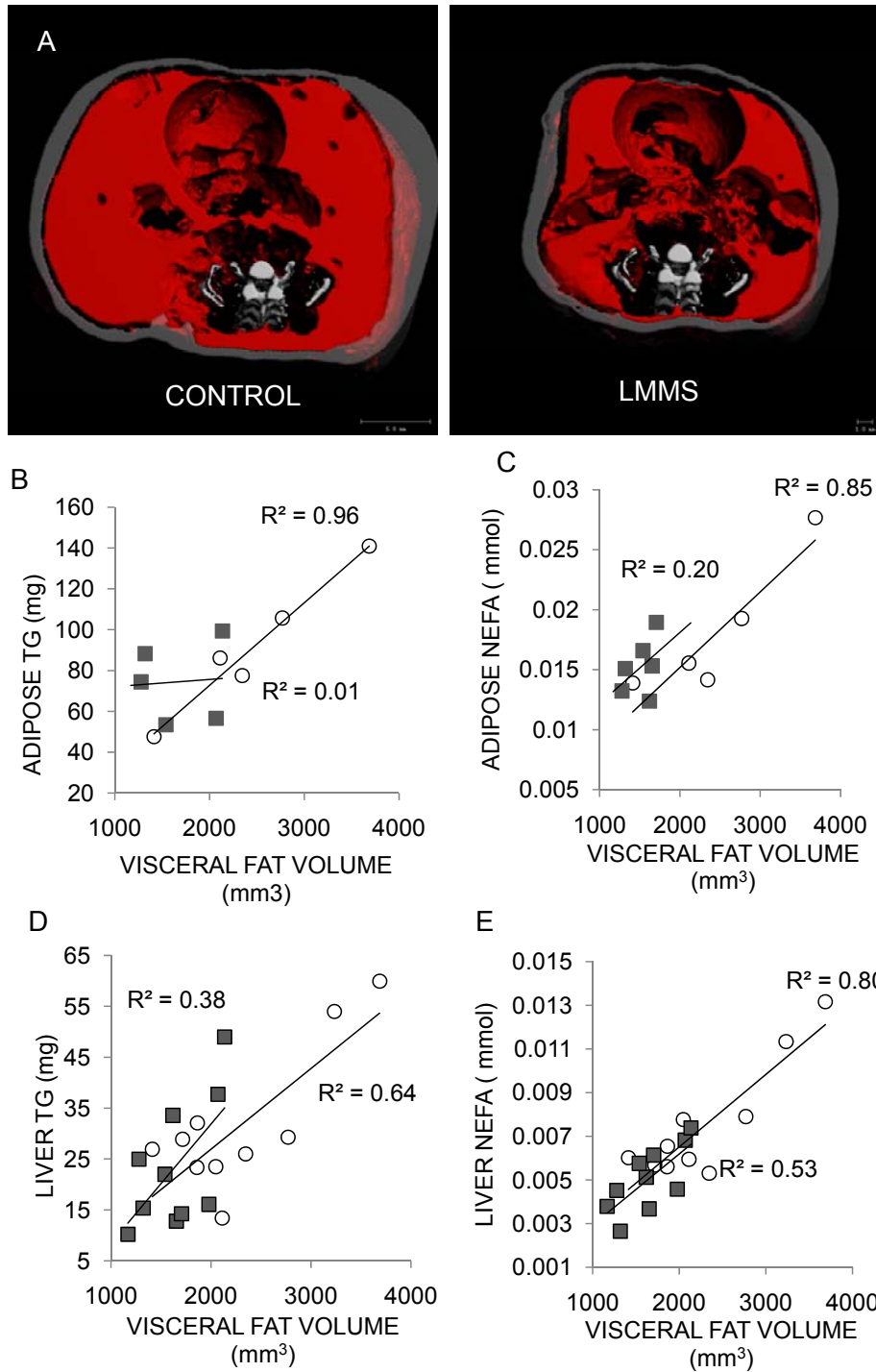


FIG 6. Suppression of the obese phenotype was achieved to a degree by stem cells preferentially diverting from an adipogenic lineage. Reconstructed *in vivo* μ CT images of total body fat (red; A) indicate that following 12w, animals which began LMMS at the time that the high fat diet was introduced exhibited 22.2% less fat volume as compared to control. In contrast, animals allowed a high fat diet for 4w prior to LMMS failed to demonstrate any reduction of fat volume (B). Shown as a relative percentage of fat to total animal volume, LMMS reduced the percent animal adiposity by 13.5% ($p=0.017$), while the lack of a response in the already obese animals reinforces a conclusion that the mechanical signal works primarily at the stem cell development level, as existing fat is not metabolized by LMMS stimulation.

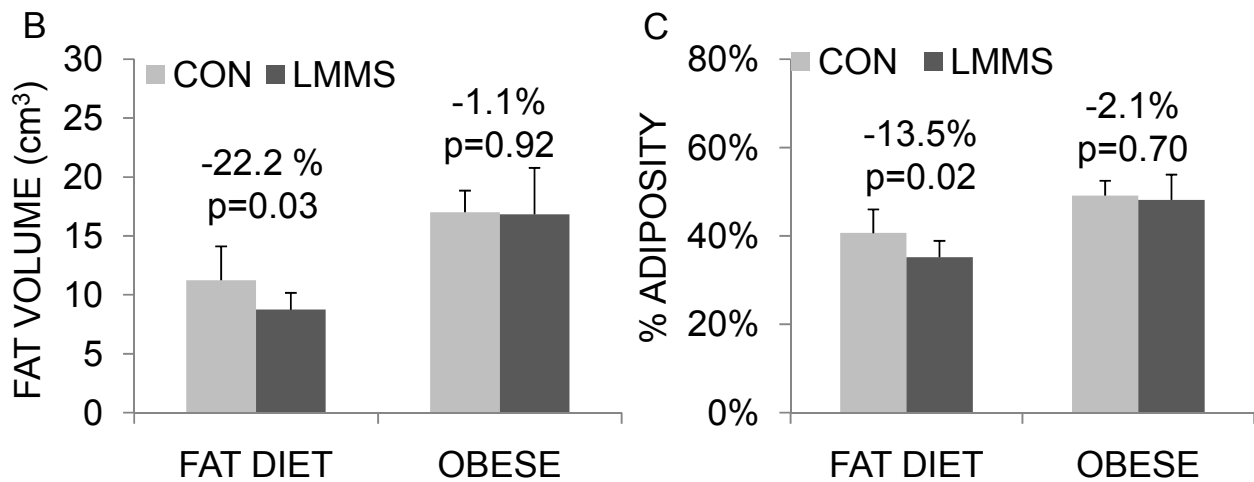
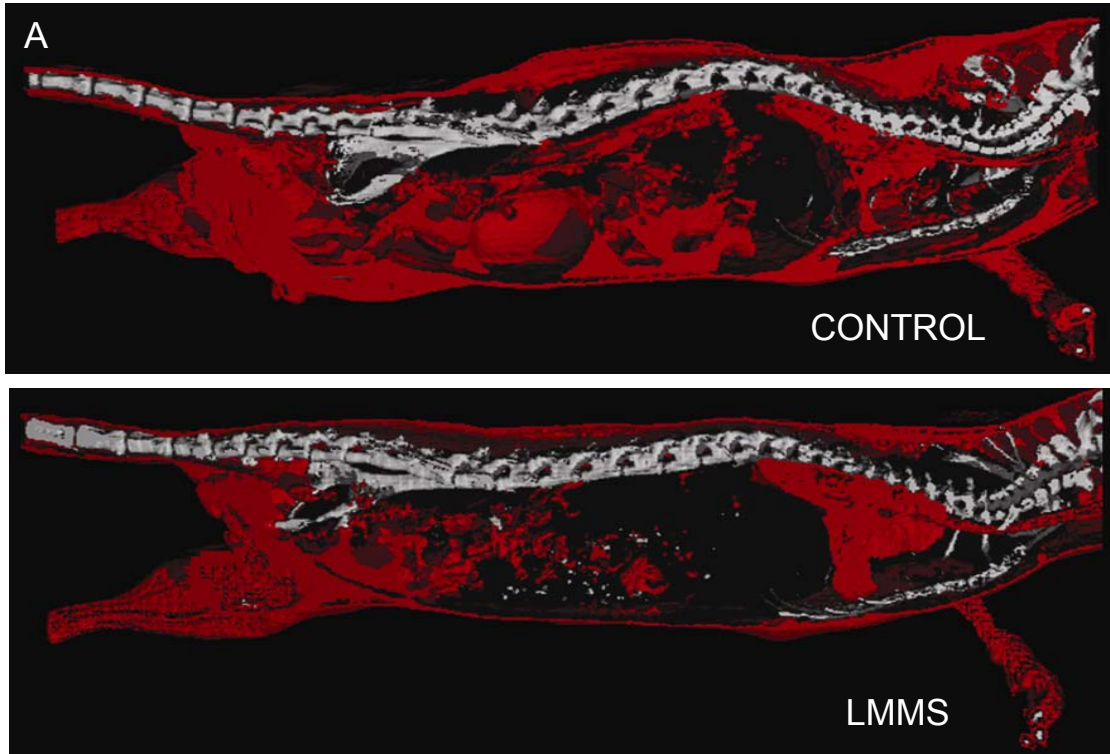


FIG 7. As measured by CT scans in the lumbar region of the spine, a group of young osteopenic women subject to LMMS for 12 months (n=24; gray bars \pm s.e.) increased both bone density ($p=0.025$ relative to baseline; mg/cm^3) and muscle area ($p<0.001$; cm^2), changes which were paralleled by a non-significant increase in visceral fat formation ($p=0.45$; cm^2). Conversely, women in the CON group (n=24; white bars \pm s.e.), while failing to increase either bone density ($p=0.93$) or muscle area ($p=0.43$), realized a significant increase in visceral fat formation ($p=0.03$). Changes that are significantly different from baseline are identified by *.

