Published in final edited form as:

J Cell Physiol. 2015 February ; 230(2): 296–307. doi:10.1002/jcp.24705.

Intrinsic Sex-Linked Variations in Osteogenic and Adipogenic Differentiation Potential of Bone Marrow Multipotent Stromal Cells

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Abstract

Bone formation and aging are sexually dimorphic. Yet, definition of the intrinsic molecular differences between male and female multipotent mesenchymal stromal cells (MSC) in bone is lacking. This study assessed sex-linked differences in MSC differentiation in 3-, 6-, and 9-month-old C57BL/6J mice. Analysis of tibiae showed that female mice had lower bone volume fraction and higher adipocyte content in the bone marrow compared to age-matched males. While both males and females lost bone mass in early aging, the rate of loss was higher in males. Similar expression of bone- and adipocyte-related genes was seen in males and females at 3 and 9 months, while at 6 months, females exhibited a two-fold greater expression of these genes. Under osteogenic culture conditions, bone marrow MSCs from female 3- and 6-month-old mice expressed similar levels of bone-related genes, but significantly greater levels of adipocyte-related genes, than male MSCs. Female MSCs also responded to rosiglitazone-induced suppression of osteogenesis at a 5-fold lower (10 nM) concentration than male MSCs. Female MSCs grown in estrogen-stripped medium showed similar responses to rosiglitazone as MSCs grown in serum containing estrogen. MSCs from female mice that had undergone ovariectomy before sexual maturity also were sensitive to rosiglitazone-induced effects on osteogenesis. These results suggest that female MSCs are more sensitive to modulation of differentiation by PPAR γ and that these differences are intrinsic to the sex of the animal from which the MSCs came. These results also may explain the sensitivity of women to the deleterious effects of rosiglitazone on bone.

Keywords

Aging; bone; osteogenesis; adipogenesis; sexual dimorphism; rosiglitazone

1. INTRODUCTION

It is well established that most mammals exhibit sexual dimorphism in bone mass, with males accumulating greater bone mass and showing a larger size of their skeletal elements compared to females. Differences in sex hormones, the growth hormone-insulin-like growth factor axis and responsiveness to mechanical loading have all been mechanistically related to the sexual dimorphism of skeletal tissue development (as reviewed in (Callewaert et al., 2010a)). With aging, there is a loss of bone mass in both males and females. The sex-linked nature of the development of bone loss also is well established and shown in both human and animal studies to be related to the loss of estrogen (as reviewed in (Cui et al., 2013)). In humans, trabecular bone remodeling increases in women at menopause, and osteoporosis results from an imbalance between bone formation and turnover. Women are at greater risk for osteoporotic fractures, in part because increased remodeling after menopause is not effectively counteracted by periosteal apposition, as it is in men (as reviewed in (Seeman, 2002)). While it has been recognized for some time that loss of estrogen increases bone turnover (Wronski et al., 1988), estrogen-dependent control of bone remodeling is complex and results from the concerted interactions among osteoclasts, osteoblasts and osteocytes (as reviewed in (Melville et al., 2014)).

At the cellular level, bone formation is mediated by the differentiation of multipotent mesenchymal stromal cells (MSCs) into osteoblasts, while resorption is dependent on hematopoietic stem cells that give rise to osteoclasts. Interestingly, aging-associated osteoporosis is coupled with the development of fatty marrow, and the loss of bone has been linked to a shift in bone marrow MSC differentiation from osteogenesis to adipogenesis (Justesen et al., 2001; Meunier et al., 1971; Rodriguez et al., 2000; Rosen and Bouxsein, 2006; Shen et al., 2012). A key transcriptional regulator of adipogenesis is peroxisome proliferator-activated receptor- γ (PPAR γ) (Tontonoz et al., 1994), which belongs to the nuclear receptor family whose members interact with prostaglandins, steroids, vitamin-D3, retinoids and triiodothyronine (Mangelsdorf et al., 1995). This family is known to have important roles in the development and maintenance of skeletal tissues. Additionally, PPAR γ ligands (thiazolidinediones (TZDs), e.g., rosiglitazone) have been shown to increase fracture risk in post-menopausal women (Bilik et al., 2010) and produce secondary osteoporosis in an age- and sex-dependent manner in rodent models (Ali et al., 2005; Lazarenko et al., 2007; Sardone et al., 2011).

Although it is documented that skeletal tissues in mice are sexually dimorphic, show sex-dependent and age-related changes (Glatt et al., 2007), and show strain-dependent effects after ovariectomy (Bouxsein et al., 2005), few studies have focused on how sex mechanistically mediates these differences. Therefore, in the current study, we first characterized bone phenotype and performed molecular and histological assessments of osteoblast, osteoclast, and adipocyte contents in male and female mice during the early phases of aging (3 to 9 months of age). This time period spans the point at which peak bone mass is reached and skeletal growth is completed up to the onset of early age-dependent changes in bone quantity and quality. Using bone marrow MSCs from these same animals, we examined the osteogenic and adipogenic differentiation potential to determine if aging male- and female-derived MSCs showed definable differences that were mechanistically

relatable to the *in vivo* changes. Lastly, we assessed the role that estrogen plays in osteogenic and adipogenic differentiation *in vitro* separate from those effects solely related to the sex-origin of the cells.

2. MATERIALS AND METHODS

2.1. Materials

Rosiglitazone was from Cayman Chemical (Ann Arbor, MI). Perilipin antibody was from Cell Signaling Technology (Danvers, MA). 17β -Estradiol, human insulin, Nile Red, and p-nitrophenyl phosphate (pNPP) reagent were from Sigma-Aldrich (St. Louis, MO). All other reagents were from Thermo Fisher Scientific (Suwanee, GA) unless noted otherwise.

2.2. Animal Studies

All animal studies were approved by the Institutional Animal Care and Use Committee at Boston University. All studies were carried out in C57BL/6J mice. Male and female mice (3, 6 and 9 months of age) were from a colony of C57BL/6J mice that was maintained at Boston University and housed under a 12-hour light-dark cycle. At euthanasia, the right tibia was fixed in 4% paraformaldehyde for 1 week at 4°C, after which the bones were stored in phosphate buffered saline at 4°C. This bone was used for micro-computed tomography (micro-CT), after which it was processed for histological analyses. Humeri were immediately flash frozen, stored at -80°C, and used for RNA analyses. The left tibia and both femora were dissected under sterile conditions, and marrow cells were collected for culturing.

2.3. Micro-Computed Tomography (micro-CT)

Samples were scanned at a resolution of 6 $\mu\text{m}/\text{voxel}$ (Scanco μCT 40, Scanco Medical, Brütisellen, Switzerland). Trabecular bone was analyzed in the proximal tibia, with the region of interest beginning 0.3 mm distal to the physis and extending 0.6 mm distally. The trabecular compartment was manually segmented from the cortical shell. For cortical bone, the proximal boundary of the region of interest began 5 mm proximal to the distal tibial-fibular junction and extended 0.3 mm distally. Any trabecular bone present was manually segmented for exclusion from the region of interest, while the periosteal boundary of cortex was identified using an automated script. Global thresholding was used with thresholds determined by an iterative method (Scanco Medical). Standard trabecular and cortical parameters then were calculated (Bouxsein et al., 2010). Tissue mineral density was calculated with the aid of a standard curve obtained from a scan of a hydroxyapatite phantom consisting of five different mineral densities.

2.4. Histology and Immunohistochemistry

After completion of the micro-CT scans, bones were decalcified in 14% w/v EDTA at 4°C. The entire tibia was embedded in paraffin such that each tibia was positioned into the block in a similar sagittal plane. Seven μm sections were cut across the whole bone until comparable mid region sections were obtained, in which both cortices, the joint surface, the secondary center of ossification, and the epiphyseal growth plate were all visualized. Five groups of 20 serial sections each were then taken at 100 μm increments across this mid-

region for each bone. Sections were stained with Aniline Blue and Safarin O and Tartrate resistant acid phosphatase (TRAP) as previously described (Gerstenfeld et al., 2003; Jepsen et al., 2008). Microscopy was carried out on an Olympus BX51 light microscope (Olympus America, Inc., Center Valley, PA). Photomicrographs were constructed from composite images of the entire tibia that were collected using an automated stage. Image analysis was performed using CellSense Dimensions Version 4.1.0.0 for Windows (Olympus America, Inc). Static histomorphometric measurement for the percent of osteoclast surface was determined by measuring the osteoclast surface and the trabecular surface perimeter. Percentage was then calculated. All assessments were made by taking a fixed region of interest immediately under the proximal growth plate. Trabecular and cortical bone were separately considered. Five sections across the entire mid diaphyseal region from each animal were used to determine each animal's mean values.

For immunohistochemical analyses, rehydrated sections were incubated with Rodent Decloaker Reagent (Biocare Medical, Concord, CA) for antigen retrieval. Sections were incubated with monoclonal rabbit-anti perilipin (D1D8), then with Rabbit on Rodent HRP Polymer (Biocare Medical). To visualize the immune reactions, slides were incubated with 3,3'-diaminobenzidine.

2.5. Cell Culture

Primary bone marrow cultures were prepared for Experiment 1 from C57BL/6J mice (females and males, 12 weeks of age, Jackson Laboratories, Bar Harbor, ME), for Experiment 2 from C57BL/6J mice (females and males, 3, 6 and 9 months of age, Boston University colony), for Experiment 3 from C57BL/6J mice (intact females, females ovariectomized at 4 weeks of age, and males, 12 weeks of age, Jackson Laboratories), and Experiment 4 from C57BL/6J mice (females, 12 weeks of age, Jackson Laboratories). Bones marrow was removed by reaming the space with a 23-gauge needle and flushing with RPMI media containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. Bone marrow cells were passed through a 70 µm cell strainer. For Experiments 1–3, bone marrow cells were diluted in MSC medium (α-MEM with phenol red containing 10% FBS and 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml Amphotericin B), and seeded at 6×10^6 /ml in 2 ml per well in a 6-well plate. For Experiment 4, bone marrow cells were diluted in modified MSC medium (α-MEM without phenol red containing 10% FBS or 10% charcoal/dextran-stripped FBS and 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml Amphotericin B, with or without 1 nM estradiol (E2)). Half of the medium was replaced 4 days after plating, and the cultures were continued for 3 days. Prior to dosing, the MSC or modified MSC medium was supplemented with osteogenic additives: ascorbate (12.5 µg/ml), β-glycerol phosphate (8 µM), dexamethasone (1 nM), and insulin (500 ng/ml), except for Naïve wells. Cultures received no treatment (Naïve) or were treated with vehicle (Vh; DMSO, 0.1% final concentration) or rosiglitazone (10–50 nM). Following treatment, the cells were cultured for 7–14 days. Medium was changed, and the cultures were re-dosed three times per week, over the 14 day period.

2.6. Adipogenesis and Osteogenesis Assays

Lipid accumulation was quantified by Nile Red staining (Yanik et al., 2011). After Nile Red staining, cells were fixed in 2% paraformaldehyde. To quantify alkaline phosphatase activity, cells were incubated with pNPP solution (Sigma). After quenching with sodium hydroxide, absorbance (405 nM) was measured. Following the pNPP assay, cells were stained with Alizarin Red (Osteogenesis Quantitation Kit, Millipore, Billerica, MA). Cells were washed extensively and then photographed using the UVP Bioimaging System (UVP, Inc., Upland, CA). The images were analyzed for bone nodule number using Image-Pro Plus (Media Cybernetics, Rockville, MD). After image capture, Alizarin Red staining was quantified according to the manufacturer's instructions. All absorbance and fluorescence measurements were determined using a Synergy2 multifunction plate reader (Biotek, Inc., Winooski, VT). For lipid accumulation, fluorescence in experimental wells was normalized by subtracting the fluorescence measured in untreated cultures and reported as "Nile Red RFUs." For bone assays, absorbance in experimental wells was normalized by dividing by the absorbance measured in untreated cultures and reported as "Fold Change."

2.7. mRNA Expression

For gene expression analyses from bone, all muscle was carefully removed. Total RNA was extracted from whole bone and genomic DNA was removed by double TRIzol® extractions (Life Technologies, formerly Invitrogen, Grand Island, NY). Gel electrophoresis was used to visualize the integrity of the RNA. cDNA was synthesized from total RNA using Taqman® Reverse Transcription Reagents (Applied Biosystems, Carlsbad, CA), with random hexamer primers. qPCR reactions were performed using Taqman® Universal PCR Master Mix (Applied Biosystems). Validated primers were purchased from Applied Biosystems and labeled with 6-carboxyfluorescein (FAM). All qPCR reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems): Hot-Start activation at 95°C for 2 min, 40 cycles of denaturation (95°C for 15 sec) and annealing/extension (55°C for 60 sec). A comparative C_T method was used for quantification, using the C_q value for 18s ribosomal RNA (*Rn18s*) for normalization. The average C_q value from unrelated, 12 week old, female C57BL/6J mice was used as the reference.

For gene expression analyses from cell cultures, total RNA was extracted and genomic DNA was removed using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). cDNA was prepared from total RNA using the GoScript™ Reverse Transcription System (Promega), with a 1:1 mixture of random and Oligo (dT)₁₅ primers. qPCR reactions were performed using the GoTaq® qPCR Master Mix System (Promega). Validated primers were purchased from Qiagen Inc. (Valencia, CA). All qPCR reactions were performed as described above. Relative gene expression was determined using the Pfaffl method (Pfaffl, 2001), using the threshold value for *Rn18s* for normalization. The C_q value from undifferentiated bone marrow cultures prepared from 9 week old male C57BL/6 mice was used as the naïve, reference.

2.8. Statistics

Statistical analyses were performed with Prism 5 (GraphPad Software Inc., La Jolla, CA). For *in vivo* and *in vitro* analyses, data are presented as mean ± standard error (SE). For *in*

vivo analyses, each n represents an individual mouse. For *in vitro* analyses, each n represents an independent bone marrow preparation. Gene expression data from the *in vitro* experiments was log transformed prior to analysis. The n for each analysis is indicated in each figure legend. One-factor ANOVA (Dunnett's) and Two-factor ANOVA (Bonferroni) were used to analyze data.

3. RESULTS

3.1. In Vivo Assessments of Sex-Related Differences in Bone Mass and Rate of Bone Loss During Early Aging

Differences between male and female long bones were identified at 3, 6, and 9 months of age using micro-CT analysis of trabecular bone in the proximal tibia and cortical bone at the tibial mid-diaphysis (Figure 1A–C). The selected age range assessed the period after sexual maturity was reached and skeletal growth was completed to the initial onset of aging. At 3 months of age, young female mice showed a significant 40% lesser bone volume fraction (BV/TV) compared to age-matched males and over the next six months lost an additional 20% of BV/TV, although this bone loss did not reach statistical significance. In contrast, males lost 34% of their bone mass in this six-month-long period and had a significantly lower BV/TV at 9 months of age than at 3 months of age. Interestingly, by 9 months of age, female and male bone mass were no longer significantly different.

Microarchitecture analysis of the bone demonstrated multiple differences between the sexes (Figure 1B). Within the trabecular compartment, females possessed fewer trabeculae with greater trabecular separation compared to males, and these differences were maintained through 9 months of age. The trabecular number significantly decreased by 29% (females) and 37% (males), trabecular separation significantly increased by 45% (females) and 53% (males), and connective density (ConnD) significantly decreased by 74% (females) and 61% (males) from 3 to 9 months. Trabecular thickness was one parameter where the sexes diverged with age. While 3-month-old males and females had similar trabecular thickness, only females showed a significant 37% increase by 9 months of age. Males showed no significant difference due to age, thus females had greater trabecular thickness compared to males at 6 and 9 months of age. The cortices, however, showed few differences between the sexes and or during aging (Figure 1C). As expected, the females showed a ~20% lower total cross-sectional area (Tt.Ar) compared to males at all three time points. Cortical thickness and cortical area fraction increased in females over age, with no significant change occurring in males.

A histomorphometric analysis of bone resorption was carried out to identify if differences in resorption contributed to either the initial sexually dimorphic differences in the male and female mice or to the age associated bone loss seen most significantly in male mice (Figure 2A–B). At 3 and 6 months of age, females showed an 89% and 146% greater osteoclast surface percentage compared to males. However, by 9 months of age the percentage of bone surface covered by osteoclasts was similar for the two sexes. While females maintained a high, but constant level of osteoclast surface from 3–9 months, males showed a significant 122% increase in osteoclast surface over this six month period. These data suggest that

young females have higher rates of resorption than males; but increases in resorption are more strongly associated with the age-related bone loss in males than that seen in females.

In order to assess the bone marrow adipocyte content, histological sections of bone were differentially stained with Alanine Blue and Safarin O (Figure 3A). Interestingly, these studies showed that the female mice had a substantially higher bone marrow fat content than the males, which was confirmed by immunohistological staining for perilipin (Figure 3B). The histological analyses also demonstrated that females had fewer trabeculae compared to males, corroborating the micro-CT analyses.

Differences in anabolic bone formation and adipocyte accumulation between male and females was assessed by probing the expression of two cassettes of mRNAs associated with the progression of either osteoblast (runt related transcription factor 2 (*Runx2*), osterix (*Sp7*), and dentin matrix protein 1 (*Dmp1*)) or adipocyte differentiation (peroxisome proliferator activated receptor γ (*Pparg*), fatty acid binding protein 4 (*Fabp4*), and adiponin (*Cfd*))(Figure 3C). RNAs were extracted from the forelimbs of the same animals used for the micro-CT and histological studies. Aging males showed no statistical changes in expression of osteogenic markers. Females showed a lower expression of osteogenic markers at 3 months of age compared to males. However, by 6 months age, females showed a significant peak in the expression of osteogenic markers, with a robust expression of *Runx2* (114%) and *Dmp1* (220%) over that observed in males. At 9 months of age, females and males again showed similar levels of osteogenic gene expression. Expression of adipocyte-associated genes also showed a peak at 6 months for females. Males demonstrated a greater expression of *Pparg* in young mice, but its expression decreased from 3 to 9 months of age. By 9 months males and females again showed similar gene expression profiles for all three adipocyte associated genes. Taken together, these data suggest that bone marrow of female mice has greater MSC differentiation activity, with a greater fat content, than male mice.

3.2. Osteogenic and Adipogenic Potential of Male and Female Bone Marrow MSCs

In order to examine sex linked differences in bone marrow MSC differentiation capacity at the cellular level, which may correlate to the observed differences in bone mass as the mice age, bone marrow was harvested from male and female C57BL6 mice at 3, 6, and 9 months of age. Cells were cultured in media absent of differentiation inducers to assess the basal level (BL) of gene expression associated with osteogenic and adipogenic differentiation. This was then compared to the gene expression of cells cultured in media with factors to induce osteogenic differentiation (OI). Under basal conditions, expression of transcription factors that control osteoblast differentiation (*Runx2*, *Sp7*) was evident, but the mature osteoblast marker *Dmp1* was not measurably expressed. Little difference in basal expression of osteogenic factors between cultures from male and female mice was evident. When the bone marrow MSCs were cultured in OI media, gene expression patterns diverged for males and females at young ages. In cultures from male mice, OI increased expression of *Sp7* and *Dmp1* at 3 and 6 months of age and increased expression of all osteoblast-related genes at 9 months of age, relative to BL conditions (Figure 4A). Cultures from female mice at 3 and 6 months of age responded to OI media with increased expression of *Sp7* and *Dmp1*, but those from 9 months of age failed to respond, in contrast to cultures from male mice. At 3 months

of age, cultures from female mice expressed a significantly greater level of *Dmp1* than cultures from male mice.

Examination of adipocyte-associated genes showed that under BL conditions *Pparg* along with its immediate target, *Fabp4*, were expressed in cultures from male and female mice; however, expression of the mature adipocyte marker, *Plin1* (perilipin 1), was not detectable (Figure. 4B). Bone marrow MSCs did not exhibit differential expression of adipocyte-related genes between the sexes under BL conditions. Interestingly, when the cultures were grown under OI conditions, the cultures from male and female mice strongly diverged in their expression of adipogenic mRNAs. At 3 and 6 months of age, OI decreased expression of *Pparg* and *Fabp4* in cultures from male mice, but had no effect at 9 months of age. Expression of *Plin1* was not induced at 3 months of age, but increased at 6 months of age and increased further at 9 months of age. In comparison, at 3 and 6 months of age, cultures from female mice responded to OI with increased expression of adipocyte-related genes, and the expression of these genes was greater than in cultures from male mice. At 9 months of age, cultures from female mice did not respond to OI. A peak in expression of adipocyte-related genes was evident in cultures from female mice at 6 months of age, similar to the peak in expression observed *in vivo*. In aggregate, these results are suggestive that male bone marrow MSC are less adipogenic than females and that the overall metabolic activity of the cells when placed in cultures is reflective of their *in vivo* state.

3.3. Female Bone Marrow MSCs Are More Susceptible to Rosiglitazone-Induced Suppression of Osteogenesis

In order to assess further if there were a sex dependent bias of MSC differentiation towards adipogenesis, bone marrow cultures from 3-month-old male and female C57BL/6J mice were grown under OI conditions and treated with vehicle (Vh) or a PPAR γ ligand, rosiglitazone (Rosi) at two sub-pharmacological doses (10 and 50 nM). Rosi stimulated adipogenesis in OI media in cultures from both male and female mice (Figure 5A), with a statistically significant increase in lipid accumulation with both 10 and 50 nM Rosi. Cultures from male mice showed a greater lipid accumulation compared to those from females at 50 nM. Rosi (10 nM) did not affect osteoblast differentiation in cultures from male mice as indicated by alkaline phosphatase activity, mineralization, or the number of osteoblast nodules (Fig. 5B–C). Suppression of osteogenesis was observed only at 50 nM Rosi in cultures from male mice. In striking contrast, osteogenesis was significantly suppressed in cultures from female mice by both 10 and 50 nM Rosi (Figure 5B–C). Further, the extent of suppression was significantly greater in cultures from female mice than cultures from male mice at both doses, but particularly at the 10 nM dose.

To examine this difference in sensitivity to PPAR γ -mediated suppression of bone formation across ages, osteoblast and adipocyte gene expression was assessed in bone marrow cultures from 3-, 6- and 9-month-old mice. Bone marrow cultures from male and female C57BL/6J mice were cultured under OI conditions and treated with Vh or Rosi (10–50 nM). In cultures from male mice, Rosi (50 nM) decreased expression of *Runx2* and *Sp7* at 3, 6 and 9 months of age (Figure 6A). *Dmp1* expression was increased by Rosi at 3 months of age, but was not significantly affected by Rosi at 6 or 9 months of age. All adipogenic genes were

significantly upregulated by both doses of Rosi at all time points in cultures from male mice (Figure 6B). In cultures from female mice at 3 and 6 months of age, Rosi significantly suppressed expression of all osteoblast genes, with expression of *Sp7* sensitive to 10 nM Rosi (Figure 6A). Rosi induced few significant changes in osteoblast gene expression at 9 months. All adipogenic genes were significantly upregulated by both doses of Rosi at all time points in cultures from female mice (Figure 6B). At 3 and 6 months of age, cultures from female mice responded to Rosi with greater suppression of osteoblast-related genes than cultures from males and greater induction of adipocyte-related genes. However, by 9 months of age, few differences in response to Rosi between cultures from male and female mice were evident.

3.4. Female Bone Marrow MSC Phenotype is Maintained in MSCs from Ovariectomized Females and is Minimally Modified by Exogenous Estrogen

Estrogen plays an important, yet complex, role in bone formation and homeostasis and is known to contribute to the sexually dimorphic formation of bone. Therefore, the early effect of estrogenic programming *in vivo* was removed by ovariectomy (OVX) of 4 week old female mice. Bone marrow cultures from 3-month-old female, OVX and male C57BL/6J mice were grown under OI conditions, treated with Vh or Rosi (10 nM) and assessed for adipogenic and osteogenic differentiation. Adipogenesis, as indicated by lipid accumulation, was significantly increased by Rosi in cultures from female, OVX, and male mice (Figure 7A). In contrast, Rosi treatment suppressed osteogenic differentiation in female and OVX cultures, as shown by decreased alkaline phosphatase activity, mineralization and osteoblast nodule number (Fig. 7B–C). No significant differences in estrogen receptor α or β were observed between sexes or between treatments (data not shown). The removal of estrogen at the time that they approached sexual maturity did not result in a male-type phenotype in OVX MSC cultures, suggesting that the sex-linked differences between response to Rosi was intrinsic to the sex of the animal from which the cells were derived and not dependent upon the estrogen surge at puberty.

In order to further assess if responsiveness of cultures from female mice was estrogen-dependent, bone marrow cultures from 3-month-old female C57BL/6J mice were incubated in either OI medium with serum, with serum from which estrogen was stripped (SS), or with stripped serum supplemented with 1nM E2 (SS+E2). The cultures were then treated with vehicle or Rosi (10 nM) and assessed for adipogenic and osteogenic differentiation (Figure 7D–G). Once again, Rosi treatment increased lipid accumulation and adipocyte gene marker expression in cultures grown in medium containing either control or steroid hormone-stripped serum (Figure 7D–E). Cultures grown in medium containing steroid-stripped serum, however, had significantly greater lipid accumulation and exhibited the greatest *Pparg* expression relative to those grown in medium supplemented with estrogen. Osteoblast differentiation also was inhibited by Rosi treatment in all three media conditions as indicated by alkaline phosphatase activity (Figure 7E), and showed the greatest suppression in the media supplemented with estrogen. However the removal or supplementation of E2 did not alter the expression of any of the assessed osteoblast genes (Figure 7F). Together, these results show that, while the presence of estrogen dampened the adipogenic response to Rosi, it did not significantly modify the sensitivity of cultures from

female mice to Rosi-mediated suppression of osteogenesis, suggesting that estrogen signaling did not directly participate in the Rosi-induced effects on osteogenesis.

4. DISCUSSION

Skeletal sexual dimorphism is well characterized in multiple mammalian species including mice; however, a mechanistic understanding of how sex-dependent differences contribute to age-dependent bone loss is lacking. Consistent with all prior studies, our results show that young C57BL/6J female mice have smaller bones with lower bone volume fraction compared to age-matched males. Interestingly, as the male mice aged, they showed a continual decrease in bone volume fraction and by 9 months of age showed a significant bone loss, which correlated with an increased osteoclast surface fraction. Although females had a high osteoclast surface fraction, bone volume fraction did not decrease over this six-month-long period of time. These results suggest that sexual dimorphic bone maintenance in young adult mice is related to a higher bone turnover in females. While bone resorption increased in males resulting in bone loss with age, bone turnover in females was high but resorption and osteogenesis remained more closely balanced, apparently blunting bone loss with age.

Consistent with previous studies, our results show that young, skeletally mature male C67BL/6J mice have larger bones with greater bone volume fraction compared to age-matched females (Amblard et al., 2003; Bab et al., 2007; Glatt et al., 2007). Bone mass was shown to peak in both male and female C57BL/6J mice at 2–3 months of age (Glatt et al., 2007). Similar to Halloran et al. (Halloran et al., 2002), our results also showed a significant and steep decline in trabecular bone in the tibia from 3 to 9 months of age in male C57BL/6J mice, reflected in a decrease in BV/TV, trabecular number and ConnD, and an increase in trabecular separation. A similar loss of trabecular bone in the tibia of male mice also was observed in the BALB/c strain (Willinghamm et al., 2010). Trabecular bone in the femora and vertebrae also has been shown to decrease in male C57BL/6J and C57BL/6N mice, beginning as early as 3 months of age (Bab et al., 2007; Glatt et al., 2007; Shahnazari et al., 2012). Our data showed that the females did not lose tibial trabecular bone as rapidly as the males, which is different from what was seen in the analysis of the femora (Glatt et al., 2007). Interestingly, in an aging study of bone loss in C57BL/6N female mice in which the tibia was examined, comparable results to our study were observed (Buie et al., 2008), suggesting that there is considerable variation in trabecular bone loss at various sites within the appendicular skeleton. Site-specific differences in bone loss are further suggested by differing rates of fractures at various sites in humans (Djuric et al., 2010).

Our results are consistent with prior studies that showed that young females demonstrate increased osteoclast surface fraction compared to males. Very young mice (4 weeks of age) showed no sex-dependent difference in the number of osteoclasts or resorption area; however, at 7 and 14 weeks females had greater osteoclast numbers and larger resorption area than males (Abe and Aoki, 1989). Assessment of *Rankl* and *Opg* expression in osteoblasts from young female and male C57BL/6 mice showed a higher ratio in females than males and could explain a greater rate of osteoclastogenesis in young female mice (Zanotti et al., 2014). Our data also show an overall age-associated increase in osteoclast

surface area in males. This is consistent with two other aging studies carried out in male C57BL/6N mice showing that the osteoclast precursor pool increases with age and is accompanied by a significant increase in osteoclastogenesis (Cao et al., 2005; Perkins et al., 1994).

While young (3-month-old) male and female mice had similar expression of osteoblast-related genes, a higher level of osteogenesis in mature, 6-month-old females was suggested by the higher expression of osteoblast-related genes. This result supports the idea that mature females in general may have a higher overall rate of coupled remodeling as compared to males. Indeed, 3- and 6-month-old female C57BL/6 mice were shown to have greater osteoblast numbers and surface than age- matched males (Zanotti et al., 2014). Greater bone formation was not evident in female mice, suggesting that the rates of bone formation and resorption favor resorption (Zanotti et al., 2014). Although, results from this study suggest that the processes in young adult female mice are relatively well balanced. Lower resorption and formation rates in male than female C57BL/6J mice have been observed previously (Amblard et al., 2003). It is interesting to note that these sex-related differences in bone maintenance may be related to the need to mobilize calcium stores during reproduction and lactation in females, in which a much higher rate of coupled turnover is maintained during their period of peak reproductive capacity. Indeed, lactating mice lose 20–30% of their bone mineral density, lose bone volume and have altered trabecular architecture (VanHouten and Wysolmerski, 2003). Remarkably, baseline bone mineral density is recovered within 12 days of weaning (Woodrow et al., 2006).

A higher bone turnover in mature females also is consistent with the sex-related differences in trabecular structure, with females developing thicker trabeculae than males. The most pronounced change in aging bone in humans and experimental animals is a reduction in trabecular number and decrease in connective density (as reviewed in (Gabet and Bab, 2011). In aging females, in the context of a higher rate of coupled formation and remodeling, the smaller trabeculae that are initially present at young ages are likely to be fully resorbed and fail to be regenerated because these surfaces are permanently lost (Gabet and Bab, 2011). In contrast, trabeculae that were initially thicker undergo continued reformation resulting in the remaining trabeculae showing an overall increase in average thickness. This overall change in trabecular structure was not observed in males for which there was a low constant rate of coupled bone turnover in which trabeculae were more uniformly thinned and decreased in number. It is of note that the observed differences in trabecular structure observed for the tibia was also observed in prior observations made in the assessment of sex-linked differences observed in the femur and the spine (Glatt et al., 2007).

A significantly greater fat content and adipocyte-related gene expression was observed in females than in males, particularly at 6 months of age. Aging is associated with an increased expression of *Pparg* in and increased fat content of bone marrow in both mice and humans (Jiang et al., 2008; Justesen et al., 2001; Lazarenko et al., 2007), with less change observed in the expression of *Runx2* (Jiang et al., 2008). The greatest increase in adipocyte content has been observed in women (Griffith et al., 2012) and in both men and women with osteoporosis (Griffith et al., 2005; Justesen et al., 2001; Meunier et al., 1971; Rosen and

Bouxsein, 2006; Shen et al., 2012). Consequently, MSCs isolated from osteoporotic patients are more efficient at differentiating into adipocytes than osteoblasts (Rodriguez et al., 2000).

Overall, the data support the conclusion that females have a higher rate of coupled turnover. In contrast, male mice start losing bone upon reaching sexual maturity as a result of increasing osteoclast activity as they continue to age. The conclusion that males lose bone continuously after reaching maturity and peak bone mass is further supported by the studies of Hollaran et al (Halloran et al., 2002), which concluded that while cortical bone area remained constant in males that rapid, early loss of trabecular bone in males occurs as a continuum from early life. In men, loss of trabecular bone is similar or only slightly less than loss in women (as reviewed in (Seeman, 2002)).

In vitro mechanistic studies revealed that MSC cultures derived from young and mature (3–6-month-old) female mice displayed upregulated genes associated with both adipocyte differentiation and osteoblast differentiation when cultured in osteogenic medium, consistent with the observed *in vivo* mRNA expression profiles. This phenotype is in contrast to cultures from male mice, which responded to the differentiation cocktail with suppression of adipogenic gene expression and upregulation of osteogenic gene expression. Classically, adipogenic and osteogenic differentiation are thought to be mutually exclusive (Akune et al., 2004; Jeon et al., 2003); thus the data from the cultures from male mice are consistent with prior data. However, the response of cultures from female mice is consistent with studies of fetal rat calvaria osteoblasts that showed the presence of a subset of osteoblasts which expressed high levels of PPAR γ that could switch fates to become adipocytes (Yoshiko et al., 2010). Since in these studies the sex of the starting animals from which the cells are derived is mixed, it is possible that the cells showing the higher PPAR γ were those cells from the female animals. Here, cultures from female mice appeared to be more anabolically active than males, which is in contrast with a recent study showing that bone marrow MSCs from C57BL/6, FVB and C3H HeJ females (1-month-old) were less osteogenic than those derived from males when stimulated to differentiate with ascorbate and β -glycerolphosphate (Zanotti et al., 2014). It is of interest to note however that the cell populations of the studies presented here were all from older mice (3–9-months-old) and that these differences also were seen in the *in vivo* anabolic activities seen in the different ages and sexes of the mice from which the cells are prepared. The conclusion that the *in vitro* osteogenic capacity is dependent on the sex from which the cells are obtained is further supported by the fact that steroid regulation of osteogenic differentiation in rat-derived bone marrow cultures also appears to be sex-dependent (Hong et al., 2009; Ishida and Heersche, 1997).

PPAR γ ligands are known to suppress osteogenesis, concomitantly with activating adipogenesis (Ali et al., 2005; Lazarenko et al., 2007; Sardone et al., 2011). Here we show that there is a sexually-dimorphic response to the suppression of osteogenesis by rosiglitazone. While activation of adipogenesis by rosiglitazone was similar in male and female MSCs, suppression of osteogenesis occurred at a five-fold lower concentration in cultures from female mice. The dimorphic response did not appear to be dependent upon estrogen present in the cultures, but rather to be programmed *in vivo*.

It is possible that the sexual imprinting of bone forming cells by estrogen is an element of the intrinsic sex-related differences in osteogenic differentiation *in vitro*. There are three life stages at which estrogen could exert a programming effect, during prenatal development, early postnatal development and during the hormone surge at puberty. Indeed, prenatal estrogen exposure results in reduced femur length and increased bone mass in adulthood (Migliaccio et al., 1996). Similarly, OVX at three weeks of age increased bone mass (Callewaert et al., 2010b). Exposure of OVX MSCs to rosiglitazone resulted in a female-like sensitivity to suppression of osteogenesis, suggesting prenatal programming of the sexually-dimorphic response. While estrogen is a likely player in bone cell imprinting, other pathways may be operating. For instance, prenatal programming of osteogenic potential also is evident in studies of dietary restriction and high fat diet consumption during the intrauterine period (Devlin et al., 2013; Lanham et al., 2008).

In conclusion, our data support the growing body of literature that suggests that osteogenesis is location-, age- and sex-dependent and that osteogenic capacity *in vivo* is reflected in the *in vitro* MSC differentiation potential. While both males and females lost a significant amount of bone in the tibia over an early period of aging, the male loss was steeper than in females, which had a slow and steady loss of bone. That female MSCs appear to more readily differentiate into adipocytes than osteoblasts contributes to our knowledge of mechanisms by which females are more susceptible to bone loss with aging. Importantly, PPAR γ ligands have been shown to increase fracture risk particularly in post-menopausal women (Bilik et al., 2010). Our results suggest that this susceptibility may be in part due to the greater susceptibility of MSCs to the bone suppressive effects of PPAR γ ligands, a novel explanation for the greater risk of fracture in women than men taking thiazolidinedione therapeutics.

Acknowledgments

Grant Information:

Ellison Foundation

Superfund Research Program [P42 ES007381]

National Institute of Diabetes and Digestive and Kidney Diseases [R56 DK090455].

This work was supported by an Ellison Foundation grant to LCG, JJS and GVD, the Superfund Research Program at the National Institutes of Health [P42 ES007381] to JJS, and the National Institute of Diabetes and Digestive and Kidney Diseases at the National Institutes of Health [R56 DK090455] to GVD. The authors would like to thank Ms. Faye Andrews, Ms. Danielle Salazar and Ms. Dana Dauks for their superb technical assistance.

Abbreviations

BL	basal
BM-MSC	bone marrow multipotent mesenchymal stromal cells
E2	estradiol
microCT	micro computer assisted tomography

MSC	multipotent mesenchymal stromal cell
OI	osteoinductive
OVX	ovariectomy
PPAR	peroxisome proliferator activated receptor
pNPP	p-nitrophenyl phosphate
Rosi	rosiglitazone
Vh	vehicle

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Figure 1. Female C57BL/6J mice have lower bone volume fraction compared to age-matched males, but males lose more bone over time

Tibiae were harvested and fixed from male and female mice (3, 6, and 9 months old, C57BL/6J). Bone parameters were analyzed by micro-CT. **(A)** Representative 3D micro-CT rendering of the trabecular bone in the proximal tibia and cortical bone at the mid-diaphysis of 3-, 6-, and 9-month-old male and female mice. **(B)** Analysis of trabecular parameters (bone volume fraction (BV/TV), trabecular thickness (Tb.Th*), trabecular number (Tb.N*), trabecular separation (Tb.Sp*) and connective density (ConnD.)). **(C)** Analysis of cortical parameters (the total area (Tt.Ar), cortical area (Ct.Ar), cortical bone fraction (Ct.Ar/Tt.Ar), and cortical thickness (Ct.Th). Data are presented as means $\pm$ SE (n=5–10). * $p < 0.05$ compared to 3 month and ^ $p < 0.05$ between males and females at each time point (Two-factor ANOVA, Bonferroni).

Figure 2. Male and female C57BL/6J mice are dimorphic for changes in osteoclast activity with age

Tibiae were harvested and fixed from male and female mice (3, 6, and 9 months old, C57BL/6J). Following micro-CT scanning, tibiae were decalcified, embedded, and sectioned. Osteoclasts were identified by TRAP staining (A) and active osteoclast surface was analyzed (B). Data are presented as means $\pm$ SE (n=4). * $p < 0.05$ compared to 3 month and ^ $p < 0.05$ between males and females at each time point (Two-factor ANOVA, Bonferroni).

Figure 3. Marrow adiposity and adipocyte gene expression is higher in females than males across age

(A–B) Tibiae were harvested and fixed from male and female mice (3, 6, and 9 months old, C57BL/6J). Following micro-CT scanning, tibias were decalcified, embedded, and sectioned. Representative histological images are shown for Aniline Blue and Safranin-O staining (A) and anti-perilipin staining (B). (C) Humeri were harvested from the same mice that were analyzed by micro-CT, and RNA was isolated from whole bone. Expression analysis of bone- (*Runx2*, *Sp7*, and *Dmp1*) and adipocyte- (*Pparg*, *Fabp4*, and *Cfd*) related markers was determined by qRT-PCR. Data are presented as means $\pm$ SE (n=4–6). * $p < 0.05$ compared to 3 month and ^ $p < 0.05$ between males and females at each time point (Two-factor ANOVA, Bonferroni).

Figure 4. Comparison of osteogenic potential of male and female bone marrow MSCs
 Primary bone marrow cultures were established from male and female mice (3, 6 and 9 months old, C57BL/6J). Established cultures were either harvested on day 7 (BL) or underwent osteogenic induction (OI). Cultures were harvested after 7 days under osteogenic conditions. RNA was isolated, and RT-qPCR was performed for expression analysis of (A) osteoblast/osteocyte- and (B) adipocyte-related genes. Data are presented as means $\pm$ SE (n=3–4). * p<0.05 compared to BL and ^ p<0.05 between males and females at each time point (Two-factor ANOVA, Bonferroni).

Figure 5. Rosiglitazone has greater efficacy in suppressing bone formation in female-derived versus male-derived cultures

Primary bone marrow cultures were established from male and female mice (12 weeks old, C57BL/6J). Osteogenesis was initiated with the addition of: ascorbate, β -glycerol phosphate, insulin and dexamethasone. Cells were treated with Vh (DMSO, 0.1%) or rosiglitazone (10–50 nM), cultured for 12 days and analyzed for (A) lipid accumulation, (B) alkaline phosphatase activity, and (C) mineralization, and nodule number by Alizarin staining. Note that the Vh data are equivalent to the OI data in Fig. 3. Data are presented as means $\pm$ SE (n=4). * p<0.05 compared to Vh and ^ p<0.05 between males and females (Two-factor ANOVA, Bonferroni).

Figure 6. Female-derived MSCs maintain their adipogenic response during aging, but lose their osteogenic response

Primary bone marrow cultures were established from male and female mice (3, 6, and 9 months old, C57BL/6J). Osteogenesis was initiated with the addition of: ascorbate, β -glycerol phosphate, insulin and dexamethasone. Cells were treated with Vh (DMSO, 0.1%) or rosiglitazone (10–50 nM), cultured for 7 days. RNA was isolated, and RT-qPCR was performed for expression analysis of (A) osteoblast/osteocyte- and (B) adipocyte-related genes. Data are presented as means $\pm$ SE (n=4). ** p<0.01 compared to Vh and ^ p<0.05 between males and females (Two-factor ANOVA, Bonferroni).

Figure 7. Early ovariectomy and exogenous estrogen do not reverse female phenotype

(A–C) Primary bone marrow cultures were established from 12 week old, C57BL/6J mice (males, intact females, and females that had been ovariectomized at 4 weeks of age). Osteogenesis was initiated with the addition of: ascorbate, β -glycerol phosphate, insulin and dexamethasone. Cells were treated with Vh (DMSO, 0.1%) or rosiglitazone (10 nM), cultured for 12 days and analyzed for (A) lipid accumulation, (B) alkaline phosphatase activity, and (C) mineralization and nodule number by Alizarin staining. (D–G) Primary bone marrow cultures were established from 12 week old, female C57BL/6J mice. Cells were cultured in standard serum (Serum), steroid stripped serum (SS) or SS supplemented with 1nM estradiol (SS+E2). Osteogenesis was initiated accumulation, (E) adipocyte-related gene expression by qRT-PCR, (F) alkaline phosphatase activity, (G) osteoblast/osteocyte-related gene expression. Data are presented as means $\pm$ SE (n=3–4). * $p < 0.05$, ** $p < 0.01$ compared to Vh and ^ $p < 0.05$ compared to “serum” (Two-factor ANOVA, Bonferroni).