Lentiviral Delivery of PPAR γ shRNA Alters the Balance of Osteogenesis and Adipogenesis, Improving Bone Microarchitecture

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Introduction: Skeletal aging is associated not only with alterations in osteoblast (OB) and osteoclast (OC) number and activity within the basic metabolic unit, but also with increased marrow adiposity. Peroxisome proliferator-activated receptor gamma (PPAR γ) is commonly considered the master transcriptional regulator of adipogenesis, however, it has known roles in osteoblast and osteoclast function as well. Here, we designed a lentiviral delivery system for PPAR γ shRNA, and examined its effects *in vitro* on bone marrow stromal cells (BMSC) and in a mouse intramedullary injection model.

Methods: PPAR γ shRNA was delivered by a replication-deficient lentiviral vector, after *in vitro* testing to confirm purity, concentration, and efficacy for *Pparg* transcript reduction. Next, control green fluorescent protein lentivirus or PPAR γ shRNA expressing lentivirus were delivered by intramedullary injection into the femoral bone marrow of male SCID mice. Analyses included daily monitoring of animal health, and postmortem analysis at 4 weeks. Postmortem analyses included high resolution microcomputed tomography (microCT) reconstructions and analysis, routine histology and histomorphometric analysis, quantitative real time polymerase chain reaction analysis of *Pparg* transcript levels, and immunohistochemical analysis for markers of adipocytes (PPAR γ , fatty acid binding protein 4 [FABP4]), osteoblasts (alkaline phosphatase [ALP], osteocalcin [OCN]), and osteoclasts (tartrate-resistant acid phosphatase [TRAP], Cathepsin K).

Results: *In vitro*, PPAR γ shRNA delivery significantly reduced *Pparg* expression in mouse BMSC, accompanied by a significant reduction in lipid droplet accumulation. *In vivo*, a near total reduction in mature marrow adipocytes was observed at 4 weeks postinjection. This was accompanied by significant reductions in adipocyte-specific markers. Parameters of trabecular bone were significantly increased by both microCT and histomorphometric analysis. By immunohistochemical staining and semi-quantification, a significant increase in OCN+ osteoblasts and decrease in TRAP+ multinucleated osteoclasts was observed with PPAR γ shRNA treatment.

Discussion: These findings suggest that acute loss of PPAR γ in the bone marrow compartment has a significant role beyond anti-adipose effects. Specifically, we found pro-osteoblastogenic, anti-osteoclastic effects after PPAR γ shRNA treatment, resulting in improved trabecular bone architecture. Future studies will examine the isolated and direct effects of PPAR γ shRNA on OB and OC cell types, and it may help determine whether PPAR γ antagonists are potential therapeutic agents for osteoporotic bone loss.

Introduction

SKELETAL AGING IS ASSOCIATED not only with alterations in osteoblast (OB) and osteoclast (OC) number and activity within the basic metabolic unit, but also with an increase in

marrow adiposity. Historically, intramarrow adipocytes have been considered a passive, inert cell type, however, recent literature has identified such cells as elaborators of “adipokines”—cytokines that negatively affect bone health.¹⁻³ For example, high levels of adiponectin have been

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associated with low bone mineral density in those with disuse bone loss after spinal cord injury.⁴ Therefore, negative regulation of intramarrow adipogenesis may be a novel mechanism in the promotion of bone maintenance and prevention of age-related bone loss.

Peroxisome proliferator-activated receptors (PPAR) are members of the steroid/thyroid hormone receptor gene superfamily⁵ and were initially named for PPAR α .⁶ Subsequently, structural analogs, PPAR δ and PPAR γ have been discovered and all are activated by polyunsaturated fatty acids.⁷ All three PPARs are found in mammals and interact with binding sites on targeted genes by forming heterodimers with the retinoid X receptor to recruit transcriptional coactivator proteins.⁸ While both PPAR α and PPAR δ are expressed during adipogenesis, PPAR γ is adipocyte cell type restricted and rapidly increases in expression during early adipogenesis.^{9,10} Indeed, PPAR γ is considered the master regulator of adipogenesis, for no other factor can rescue adipocyte formation in the event of PPAR γ knockout, and generally all cell signaling pathways leading to adipogenesis converge with PPAR γ .^{11–13} In fact, PPAR γ haploinsufficient mice have reduced ability to differentiate into adipocytes, depicting PPAR γ as an essential factor in adipogenesis.^{14,15}

Moreover, PPAR γ also has important roles in cell types other than adipocytes, including both osteoblasts and osteoclasts.¹⁶ In terms of osteoblasts, ectopic or increased expression of PPAR γ is required to convert immature osteoblasts, and a subset of mature osteoblasts, into adipocytes.^{17,18} Conversely, suppression of PPAR γ inhibits adipogenesis and stimulates osteoblastogenesis.¹⁹ Similarly, PPAR γ haploinsufficiency results in increased bone mass and increased bone marrow stromal cells (BMSC) osteoblastic differentiation.²⁰ Thus, PPAR γ activity modulates osteoblastic maturation as seen in changes in alkaline phosphatase (ALP) activity, Runx2/Cbfa1 activity, and osteocalcin (OCN) expression.²⁰ In terms of osteoclasts, gain in PPAR γ function via ligand activator rosiglitazone promotes both osteoclast lineage commitment and maturation by maintaining levels of c-fos in monocyte precursors and osteoclasts.²¹ Conversely, loss of function through PPAR γ deletion impairs osteoclast differentiation and bone resorption.²¹ Wei *et al.* showed that the pro-osteoclastogenic effects are mediated by PPAR γ coactivator 1 β , and estrogen-related receptor α along with PGC1 β .²² In a subsequent study, Wei *et al.* identified GATA2 as a novel and critical PPAR γ target gene in osteoclast progenitors.²³ In aggregate, these data clearly demonstrate that PPAR γ transcriptional activity has a significant role beyond the confines of adipogenic differentiation.

In the present study, we designed a lentiviral delivery system for PPAR γ shRNA to examine its effects on both adipogenesis and bone metabolism *in vitro* and *in vivo*, using a mouse intramedullary injection model. PPAR γ shRNA delivery not only led to a dramatic reduction in intramarrow adiposity, but also led to a significant improvement in trabecular bone architecture and alteration in osteoblast and osteoclast numbers.

Materials and Methods

Antibodies and reagents

Primary antibodies used in this study were anti-PPAR γ (sc-7273; Santa Cruz Biotechnology), anti-OCN (sc-18322;

Santa Cruz Biotechnology), anti-Cathepsin K (ab19027; Abcam), anti-fatty acid-binding protein 4 (anti-FABP4, AF1443; R&D Systems), anti-proliferating cell nuclear antigen (anti-PCNA, (M0879; Dako), and anti-ALP (sc-166261; Santa Cruz Biotechnology). Antibodies used for western blot analysis were anti-PPAR γ (sc-7273; Santa Cruz Biotechnology) or anti-GAPDH (GTX-627408; GeneTex). All other reagents were purchased from Dako unless otherwise specified.

Viral production and purification

The lentiviral vector encoding PPAR γ shRNA or green fluorescent protein (GFP) was generated by co-transfection of 293T cells with the PPAR γ -shRNA plasmid vector (SC-29455-SH; Santa Cruz Biotechnology) or the FG12 vector (14884; Addgene),²⁴ with helper plasmids pCMV-dR8.2-vprX and pCMV-VSVG. The viral vector was collected from transfected cell cultures and cell debris was removed through 0.22 μ m filtration and concentrated by ultrafuge at 4°C, 17,000 rpm for 60 min using Beckman SW32 rotors. The pellets that contained the lentiviral vector were resuspended with Dulbecco's Modified Eagle's Medium (DMEM) medium to bring the concentration to 5×10^7 TCID₅₀/mL. Lentiviral vector titer was estimated by measuring the gag p24 protein, 1 pg of p24 reading was assigned as 10 tissue culture infective dosage (TCID₅₀) for freshly isolated lentiviral vectors.

BMSC isolation and analysis

Femurs from 8-week-old C57BL/6 mice were dissected and marrow was harvested by inserting a syringe needle (27-gauge) into one end of the bone and flushing with DMEM (Gibco). The bone marrow cells were filtered through a 70 mm nylon mesh filter (BD, Falcon). Cells were plated into six-well plastic cell culture plate at a density of 25×10^6 cells per well in DMEM containing 15% fetal bovine serum (FBS; Sigma-Aldrich), 2 mm L-glutamine (Gibco BRL), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco). Cultures were kept at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. Cells from passage 2 only were used for further experiments. The generated lentiviral vector was used to infect mouse BMSC cells with multiplicity of infection of 10 (MOI=10). Adipogenic differentiation was performed using Mouse Adipogenic Stimulatory Supplements in conjunction with MesenCult™ Basal Medium (Stemcell Technologies). Next, Oil Red O staining was performed. Monolayers were rinsed with PBS, fixed with buffered formalin for 60 min at room temperature, and stained with 3% Oil Red O solution in 60% isopropyl alcohol/40% ddH₂O for 10 min. The intensity of staining was analyzed using commercial software Image-Pro Plus 6 and presented as relative mean optical density.

Protein isolation and western blot

Total protein was extracted from BMSC with RIPA lysis buffer. Lysate was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the gel was blotted onto PVDF membrane (Millipore). The membrane was blocked in 5% nonfat milk, and then incubated with either anti-PPAR γ or anti-GAPDH antibody at a 1:1000 dilution. The bands were quantified using the Image J software by measuring the band intensity (area $\times$ optical density) for

each group, and normalized with GAPDH. The final results are expressed as fold changes by normalizing the data to the GFP group.

Intramedullary injection

Animals were housed in a light and temperature-controlled environment and given food and water *ad libitum*. All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the University of California and approved by the Chancellor's Animal Research Committee. Male SCID mice (8-week-old, six animals per group) were kept and handled under pathogen-free conditions and fed on sterile food and acidified water. Knee arthrotomy and intramedullary injection of the femur were performed for virus delivery.

Briefly, a 5 mm-longitudinal incision was made along the medial aspect of the quadriceps–patellar complex. The patella was dislocated laterally to expose the intercondylar groove. A 0.9 mm k-wire on a trephine drill was used to create a trephination defect. A 26-gauge needle was inserted through the defect, and 15 μ L of PPAR γ lentivirus (4×10^7 TCID₅₀/mL) was injected into the intramedullary cavity. The quadriceps–patellar complex was then repositioned, and the medial arthrotomy was carefully repaired with 5-0 vicryl suture. Fifteen microliters of GFP lentivirus (5×10^7 TCID₅₀/mL) as control was injected into the contralateral femur, following the same surgical procedure as above.

Microcomputed tomography analysis

Femur samples were harvested 4 weeks after viral injection, fixed in formalin, and scanned using high-resolution microcomputed tomography (microCT; Skyscan 1171F) at an image resolution of 17.81 μ m (55 kV and 181 mA radiation source, using a 0.5 mm aluminum filter). For analysis of trabecular bone formation, regions of interest were drawn to select only metaphyseal intramedullary trabecular bone from the distal end, with a length of 1.225 mm (35 slices at 0.035 mm each). Trabecular bone formation was analyzed using CTAn software, with a threshold value of 75. The microCT scans were reconstructed using CTVol software.

Histological and immunohistochemical analyses

Tissue from the femur and its overlying periosteum adjacent to the injection site were harvested after sacrifice, fixed in formalin, and decalcified using 19% ethylenediaminetetraacetic acid (EDTA). Five- μ m-thick paraffin sections, of decalcified samples cut along the coronal plane, were stained with hematoxylin & eosin (H&E) and Masson's Trichrome staining. Using H&E sections, histomorphologic assessments of random 200 cell counts were performed to assess the treatment effects on bone marrow composition ($n=6$ samples per group). Histomorphometric analysis of serial H&E stained sections was performed using random 400 $\times$ H&E images with Adobe Photoshop quantitation, with parameters including mean bone area (B.Ar), bone perimeter (B.Pm), trabecular width (Tb.Wi), trabecular number (Tb.N), and trabecular separation (Tb.Sp). Marrow adiposity of random 400 $\times$ H&E sections was quantified using Adobe Photoshop, by comparing the area of adipose tissue to the area of the whole bone marrow. Additional

sections were analyzed by indirect immunohistochemistry. Briefly, unstained sections were deparaffinized and incubated with anti-OCN, anti-Cathepsin K, anti-PPAR γ , anti-PCNA, anti-FABP4, and anti-ALP primary antibodies and then by the appropriate biotinylated secondary antibodies (Dako). PCNA primary antibody was used at a 1:1000 dilution. All other primary antibodies were used at a dilution of 1:100, while all secondary antibodies were used at a dilution of 1:200. Positive immunoreactivity was detected following ABC complex (PK-6100, Vectastain Elite ABC Kit; Vector Laboratories, Inc.) incubation and development with AEC chromagen (K346911-2; Dako). Controls for each antibody consisted of incubation with secondary antibody in the absence of primary antibody. Sections were counterstained with hematoxylin. Photomicrographs were acquired using Olympus BX51 (400 $\times$ magnification lens, UPLanFL; Olympus). The relative intensity of PPAR γ , FABP4, and Cathepsin K staining was analyzed using commercial software Image-Pro Plus 6 and quantified by the mean optical density of staining signal $\times$ per percent area positively stained $\times 100$.²⁵ For ALP and OCN immunohistochemical staining, the number of positively stained osteoblasts along the bone perimeter were determined by three blinded observers. For PCNA immunohistochemical staining, the number of positively stained cells along the bone perimeter was determined by three blinded observers. Reported results were average of data collected from six random fields per sample.

Tartrate resistant acid phosphatase staining

For tartrate-resistant acid phosphatase (TRAP) staining, the acid phosphatase, leukocyte (TRAP) kit (387A; Sigma-Aldrich) was used per the manufacturer's protocol. Sections were immersed in TRAP stain solution (12.5 mg/mL Naphthol AS-BI phosphoric acid + 2.5 M acetate buffer + diazotized Fast Garnet GBC solution + deionized water) at 37°C, for 60 min protected from light. Subsequently, sections were thoroughly rinsed in deionized water and counterstained in hematoxylin. TRAP staining was analyzed by three blinded observers and quantified by the number of osteoclasts per trabecular bone perimeter in millimeters. The observers analyzed and averaged the results from six random fields per sample.

TUNEL assay for detection of apoptosis

A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method was used for the detection of apoptotic cells. For this purpose, we used the DeadEnd™ Colorimetric TUNEL Assay kit (G7360; Promega) according to the manufacturer's instructions. Briefly, paraffin-embedded slides were labeled with biotinylated nucleotide at the 3'-OH DNA ends, using the terminal deoxynucleotidyl transferase, recombinant (rTDT) enzyme, for 60 min in a humidified chamber. The slides were incubated with streptavidin horseradish peroxidase and diaminobenzidine was used as the chromagen. TUNEL staining was analyzed by three blinded observers and quantified by the percentage of positively stained cells per field from six random fields per sample.

Real-time polymerase chain reaction

To analyze the PPAR γ expression in SCID mice with intramedullary injection of PPAR γ shRNA or GFP lentivirus,

BMSC were isolated 1 week postinjection using the aforementioned method. Total RNA from tissue or cells for real-time polymerase chain reaction (PCR) evaluation was extracted using TRIzol reagent (Invitrogen). Bone tissue was homogenized in ~2 mL of TRIzol reagent per femur using mortar with liquid nitrogen. After homogenization, insoluble material from the homogenate was removed by centrifuging at 10,000 rpm for 10 min at 4°C. $n=3$ mice per treatment group were used for RNA studies. To determine osteogenic differentiation marker expression levels in PPAR γ shRNA or GFP transfected BMSC, BMSC was cultured in osteogenic differentiation medium (alpha MEM + 15% FBS with 50 μ g/mL ascorbic acid and 3 mM β -glycerophosphate) for 3, 6, and 9 days. Total RNA was isolated using TRIzol reagent. Following isopropyl alcohol precipitation, total RNA was treated with DNase per the manufacturer's protocol. cDNA was synthesized from 1 μ g of total RNA using the SuperScript III Reverse-Transcriptase Kit (Invitrogen) in a final volume of 20 μ L. Real-time PCR reactions were routinely performed in triplicate in 96-well plates using the 7300 Real-Time PCR System instrument (Applied Biosystems). The reactions were incubated in 96-well optical plates at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and at 60°C for 10 s. The threshold cycle (Ct) data were determined using default threshold settings. The Ct was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Mix primer sequences of mouse PPAR γ were sense: GGAAAGACAACGGACAAATCA and anti-sense: TACGGATCGAACTGGCAA; those of mouse Runx2 were sense: CGGTC TCCTTCCAGGATGGT and anti-sense: GCTTCCGTCA

CGGTCAACA, those of mouse ALP were sense: GTTGC CAAGCTGGGAAGAACAC and anti-sense: CCCACCC CGCTATTCCAAAC, and those of mouse OCN were sense: GGGAGACAACAGGGAGGAAAC and anti-sense: CAG GCTTCCTGCCAGTACCT. The relative expression level of the transcripts was compared to GAPDH. The primer sequences of the mouse GAPDH control were sense: TGC ACCACCAACTGCTTAGC and anti-sense: CCACCACC CTGTTGCTGTAG.

Statistical analysis

Statistical analysis was performed using an appropriate Student's *t*-test when two values were being compared. No collected data or experimental animals were excluded from analysis. All analyses were performed in a blinded fashion when feasible. All "center values" represent the median, while all error bars represent one standard deviation. In general, $*p<0.05$ and $**p<0.01$ were considered significant. A Bonferroni correction was performed for tests with multiple comparisons.

Results

Lentiviral PPAR γ shRNA inhibits BMSC adipogenesis in vitro

We first sought to verify the effectiveness of lentiviral delivered PPAR γ shRNA *in vitro*. To do this, primary mouse BMSC were cultured in the presence of lentivirus encoding PPAR γ shRNA or control (GFP). Oil red O staining and quantification of BMSC adipogenic differentiation

FIG. 1. BMSC adipogenic differentiation with PPAR γ shRNA. (A) Oil Red O staining performed after 12 days differentiation of mouse BMSC infected with PPAR γ shRNA lentivirus or GFP control lentivirus. (B) Photometric quantification of relative staining from (A). (C) Relative *Pparg* mRNA expression levels in BMSC after 3 days treatment. (D) Western blot analysis to determine expression levels of PPAR γ protein in BMSC after 3 days treatment. The two bands correspond to PPAR γ 1 and PPAR γ 2. (E) Quantification of protein bands using densitometry. Experiments were independently run three times. The data presented in this figure are from one representative experiment. $**p<0.01$. BMSC, bone marrow stromal cells; GFP, green fluorescent protein; PPAR γ , peroxisome proliferator-activated receptor gamma. Color images available online at www.liebertpub.com/tea

confirmed a significant reduction in lipid droplet accumulation with lentiviral delivered PPAR γ shRNA (Fig. 1A, B). Similarly, a significant reduction in *Pparg* transcript expression and PPAR γ protein was confirmed in PPAR γ shRNA-treated samples by qRT-PCR analysis (Fig. 1C) and western blot (Fig. 1D, E). Furthermore, the PPAR γ shRNA construct used in this study had no significant effects on *Ppar α* or *Ppar δ* transcript levels, as confirmed by qRT-PCR analysis (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). Significant cytotoxicity was not observed, as assayed by Trypan blue staining (not shown). In summary, these findings verified the *in vitro* efficacy, specificity, and anti-adipogenic effects of lentiviral delivered PPAR γ shRNA.

Lentiviral PPAR γ shRNA significantly alters intramedullary histology

Next, the ramifications of *in vivo* lentiviral delivery of PPAR γ shRNA were assessed. To directly affect BMSC, an intramedullary injection of lentivirus was performed in SCID mice. This was achieved via an intramedullary femoral injection, using a transpatellar (distal) approach. No postsurgical morbidity or mortality was encountered. Four weeks postinjection, animals were sacrificed and femurs with accompanying soft tissue were harvested for analysis. By routine H&E staining, a dramatic difference in marrow contents was observed at low and high magnifications (Fig. 2A, B). GFP-treated limbs showed a mixture of marrow elements, including mature adipocytes, and a combination of erythroid, myeloid, and megakaryocytic cell types. In marked contrast, PPAR γ shRNA-treated limbs exhibited an

essential absence of mature adipocytes. This was quantified, showing a mean marrow adiposity of 43.3% in control limbs as compared to 0.67% in PPAR γ shRNA-treated limbs (Fig. 2C). To quantify the hematopoietic marrow elements, morphologic assessments were performed using high-powered analysis of 200 random cells per sample (Table 1). Little statistically significant differences were observed in hematopoietic cell content between GFP and PPAR γ shRNA-treated samples.

Lentiviral PPAR γ shRNA inhibits intramedullary adipogenesis

Having observed a striking reduction in marrow lipid content with PPAR γ shRNA treatment, we next sought to extend these findings to adipocyte specific cytokines. First, reduction of *Pparg* transcript abundance was confirmed, using qRT-PCR analysis of total marrow cells 1 week postlentiviral injection (Fig. 3A). Immunohistochemical staining was next performed, confirming a near total reduction in observed positive staining for PPAR γ at 4 weeks postinjection (Fig. 3B, C). Similarly, FABP4 showed a marked reduction in immunohistochemical staining and semi-quantification (Fig. 3D, E). In summary, lentiviral delivered PPAR γ shRNA resulted in a significant reduction in the intramarrow lipid content, associated with reduction of multiple adipocyte specific markers.

Lentiviral PPAR γ shRNA increases intramedullary trabecular bone

Recent studies have documented the anti-osteoblastic effects of PPAR γ signaling,¹⁶ and the negative effects of

FIG. 2. Histologic appearance after PPAR γ shRNA intramedullary injection. (A, B) H&E stained sections of the intramedullary space of the femur at low magnification (A) and high magnifications (B) demonstrate dramatic histologic changes after PPAR γ shRNA lentivirus injection. GFP lentivirus-injected limbs showed a mixture of marrow elements, including mature adipocytes, and a combination erythroid, myeloid, and megakaryocytic cell types. In marked contrast, with PPAR γ shRNA there was an essential absence of mature adipocytes in all samples. (C) Quantification of marrow adiposity among GFP control or PPAR γ shRNA lentiviral treatment. Representative 100 $\times$ and 400 $\times$ images shown. $n=6$ samples per treatment group. $**p<0.01$. H&E, hematoxylin & eosin. Color images available online at www.liebertpub.com/tea

TABLE 1. BONE MARROW DIFFERENTIAL COUNT AFTER PPAR γ shRNA INTRAMEDULLARY INJECTION

Cell type	GFP (mean% $\pm$ SD)	PPAR γ shRNA (mean% $\pm$ SD)	p-Value
Erythroblast	0.51 $\pm$ 0.87	0.66 $\pm$ 0.63	0.819
Orthochromatic erythroblast	9.86 $\pm$ 3.96	13.46 $\pm$ 2.41	0.263
Polychromatic erythroblast	8.30 $\pm$ 2.09	9.66 $\pm$ 6.82	0.767
Myeloblast	1.84 $\pm$ 1.90	1.14 $\pm$ 1.09	0.618
Promyelocyte	4.01 $\pm$ 4.34	6.45 $\pm$ 3.91	0.510
Myelocyte	9.76 $\pm$ 2.00	7.15 $\pm$ 7.25	0.601
Metamyelocyte	11.20 $\pm$ 2.50	8.90 $\pm$ 3.12	0.378
Bands	14.37 $\pm$ 0.77	14.62 $\pm$ 3.36	0.913
Polymorphonuclear leukocyte	30.84 $\pm$ 0.68	30.38 $\pm$ 3.51	0.842
Lymphocyte	5.57 $\pm$ 2.09	0.83 $\pm$ 1.44	0.038
Megakaryocyte	3.75 $\pm$ 0.90	6.76 $\pm$ 0.66	0.012

Bone marrow differential counts were performed after GFP or PPAR γ shRNA intramedullary injection, using 200 cell counts in random high-powered fields of $n=3$ samples from each treatment group. Overall, a similar cellular differential was observed between treatment groups. Numbers expressed as mean percentage $\pm$ SD and p -value.

GFP, green fluorescent protein; SD, standard deviation.

intramarrow adipocytes on bone health.³ This led us to examine the effects of PPAR γ shRNA treatment on femoral bone formation. High-resolution microCT cross sections of the distal femoral metaphysis showed a relative increase in trabecular bone among PPAR γ shRNA-treated limbs (Fig. 4A). In contrast, cortical bone thickness appeared unaffected. Three-dimensional microCT reconstructions of the distal femur trabecular bone again showed a qualitative increase in trabecular bone with PPAR γ shRNA treatment (Fig. 4B). Next, microCT quantifications of metaphyseal trabecular bone were performed (Fig. 4C–H). Significant increases in parameters including bone volume (BV) and percentage BV/tissue volume (BV/TV) were observed with PPAR γ shRNA treatment (Fig. 4D, E). Trabecular analyses

were likewise significantly improved with PPAR γ shRNA treatment, including a significant increase in trabecular thickness (Tb.Th) and Tb.N, and a nonsignificant trend toward reduced trabecular separation (Tb.Sp) (Fig. 4F–H). In summary, these data suggested that lentiviral delivered PPAR γ shRNA treatment had not only anti-adipocytic effects, but also exerted significant and positive effects on intramedullary trabecular bone.

Lentiviral PPAR γ shRNA alters the balance of osteoblast: osteoclast numbers

To further define the positive effects of PPAR γ shRNA treatment on intramedullary bone, histologic analyses were

FIG. 3. Adipogenic marker expression after PPAR γ shRNA intramedullary injection. **(A)** Relative intramarrow *Pparg* mRNA expression levels 1 week postinjection, assessed by qRT-PCR. **(B, C)** Immunohistochemical staining and semi-quantification of PPAR γ . Semi-quantification is expressed as the relative integrated intensity of intramarrow staining. **(D, E)** Immunohistochemical staining and semi-quantification of fatty acid-binding protein 4 (FABP4). Semi-quantification is expressed as the relative integrated intensity of intramarrow staining. Representative images shown at 400 $\times$. All semi-quantitation based on six random images per sample. $n=6$ samples per treatment group. ** $p < 0.01$. qRT-PCR, quantitative real time polymerase chain reaction. Color images available online at www.liebertpub.com/tea

FIG. 4. Trabecular bone formation after PPAR γ shRNA intramedullary injection. **(A)** Representative two-dimensional microCT images of femoral transverse sections from GFP and PPAR γ shRNA treatment groups. Images were taken from the distal femur. **(B)** Representative three-dimensional microCT reconstructions of distal femoral intramedullary trabecular bone from the GFP and PPAR γ shRNA treatment groups. **(C–H)** microCT quantification data, including bone marrow density (BMD), bone volume (BV), Percent BV/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). $n=6$ samples per treatment group. * $p<0.05$; ** $p<0.01$. microCT, micro-computed tomography. Color images available online at www.liebertpub.com/tea

performed (Fig. 5A). Histomorphometric analyses of serial histologic sections confirmed microCT findings, including significant increases in Trabecular B.Ar, trabecular perimeter (T.Pm), Tb.Wi, and Tb.N, and a significant reduction in Tb.Sp with PPAR γ shRNA treatment (Fig. 5B–F). Altera-

tions in trabecular bone could be the result of increased osteoblastic activity, decreased osteoclast-mediated bone resorption, or both. To address this, we first examined osteoprogenitor/osteoblast specific markers, by performing immunohistochemical staining for OCN (Fig. 5G) and ALP

FIG. 5. Osteoblast markers after PPAR γ shRNA intramedullary injection. **(A)** Representative images of Masson's Trichrome staining after GFP or PPAR γ shRNA treatment. **(B–F)** Trabecular bone histomorphometric quantification performed on serial H&E sections of GFP or PPAR γ shRNA-treated samples, including bone area (B.Ar), bone perimeter (B.Pm), Tb.Wi, Tb.N, and Tb.Sp. **(G, H)** Immunohistochemical staining and semi-quantification of osteocalcin (OCN). Semi-quantification is expressed as the number of OCN+osteoblasts per B.Pm. **(I, J)** Immunohistochemical staining and semi-quantification of alkaline phosphatase (ALP). Semi-quantification is expressed as the number of ALP+osteoblasts per B.Pm. Arrowheads indicate positively stained cells. Images shown at 400 $\times$. All semi-quantitation based on six random images per sample. $n=6$ samples per treatment group. ** $p < 0.01$. Color images available online at www.liebertpub.com/tea

activity (Fig. 5I). Semi-quantification of immunohistochemical stains revealed a significant increase in both the number of OCN+ and ALP+ osteoblasts per B.Pm (Fig. 5H, J). Next, an *in vitro* correlate was performed in which BMSC were cultured under osteogenic conditions. To assess osteogenic differentiation, qRT-PCR was used to measure the mRNA expression levels of osteogenic differentiation markers, including Runx2, OCN, and ALP. Results showed that at 3, 6, and 9 days, PPAR γ shRNA significantly upregulated the expression of all three osteogenic gene markers (Fig. 6A–C).

Next, the effects of PPAR γ shRNA treatment on osteoclastic activity were assessed. Osteoclast-specific markers were examined by assessment of TRAP activity (Fig. 7A, B) and immunohistochemical staining for Cathepsin K (Fig. 7C, D). Both osteoclast markers showed a significant reduction in activity or immunohistochemical staining when treated with PPAR γ shRNA. Lastly, cellular proliferation and apoptosis was assessed via PCNA and TUNEL staining, respectively. No difference in either parameter was assessed by staining and semi-quantitation (Fig. 7E–H).

Discussion

In summary, we have observed that PPAR γ shRNA delivery via a lentiviral vector not only led to a dramatic reduction in intramarrow adiposity, but also led to a significant improvement in trabecular bone architecture and alteration in osteoblast and osteoclast numbers. These findings suggest that loss of PPAR γ in the bone marrow compartment has a significant role beyond anti-adipose effects. These effects were acute in onset, with the development of a long bone phenotype in only 4 weeks post-treatment.

As mentioned, the reduction in intramarrow adipocytes was quite dramatic and seen across all animals examined. This led us to inquire as to the mechanism of such a relatively rapid change in marrow composition. We examined *in vivo* intramarrow proliferation and apoptosis, finding no difference in either cell parameter with PPAR γ shRNA treatment. Likewise, cell toxicity was not observed under any condition or in any cell type examined with PPAR γ shRNA. Several potential explanations for this striking observation exist. First, although unlikely, PPAR γ shRNA treatment may have led to a brisk and resolving pro-apoptotic

effect on intramarrow adipocytes that was not seen in our 4 week time point. We consider this unlikely as cell death such as this would have likely resulted in residual inflammation, edema, or fibrosis—none of which were observed with PPAR γ shRNA treatment. Along these lines, most sources consider PPAR γ signaling to have pro-apoptotic effects itself,²⁵ and so PPAR γ silencing as in our study would unlikely have the same effect. A second and more likely explanation for this relatively rapid disappearance of intramarrow differentiation is transdifferentiation of mature fat cells induced by PPAR γ shRNA treatment. Theoretically, the transdifferentiation of mature mesenchymal cells to that of another lineage has been repeatedly described. For example, mature white adipocytes have been described to transdifferentiate into osteoblastic cells²⁶ or adopt a brown adipocyte phenotype under specific conditions.²⁷ Other examples include the transdifferentiation of fully differentiated osteoblasts²⁸ or mature myocytes into mature adipocytes.²⁹ Certainly a similar phenomenon of transdifferentiation of mature adipocytes may have been observed in our study, although the elucidation of this phenomenon requires further study.

Interesting similarities and differences exist between the phenotype induced by PPAR γ shRNA treatment and the skeletal phenotype induced by deficiency in PPAR γ . The effects of complete knockout of PPAR γ has been difficult to study, as homozygous knockout mice demonstrate lethality in the midembryonic stage.³⁰ However, heterozygosity in PPAR γ (PPAR γ ^{+/-} mice) or lineage-specific PPAR γ knockout mice have been studied in terms of both skeletal and adipose tissue phenotypes. For example, culture of PPAR γ ^{+/-} BMSC revealed not only reduced adipogenic differentiation (as anticipated), but also increased ALP activity and von Kossa staining.³¹ An increased expression of Runx2, Osterix, and Lrp5 was also observed.³¹ This is in direct agreement with our findings, in which PPAR γ shRNA treatment led to increased expression of Runx2, ALP, and OCN expression in BMSC. However, this increase in osteogenic differentiation was not observed with PPAR γ ^{+/-} calvarial osteoblasts.²⁰ Thus, it appears that loss of PPAR γ signaling has its osteoblastogenic effects on less differentiated osteoprogenitor cells. Interesting future studies would involve using PPAR γ shRNA treatment on various osteoprogenitor cell

FIG. 6. Osteogenic differentiation of BMSC after PPAR γ shRNA treatment. (A) Relative *Runx2* mRNA expression levels at 3, 6, and 9 days in osteogenic differentiation medium, as assessed by real-time PCR. (B) Relative *ALP* mRNA expression levels at 3, 6, and 9 days in osteogenic differentiation medium, as assessed by qRT-PCR. (C) Relative osteocalcin mRNA expression levels at 3, 6, and 9 days in osteogenic differentiation medium, as assessed by qRT-PCR. The cell culture experiments were independently run three times. The data presented in this figure are from one representative experiment. ** $p < 0.01$ compared to GFP control at that time point.

FIG. 7. Osteoclast markers and proliferation/apoptosis after PPAR γ shRNA intramedullary injection. (**A, B**) Histochemical staining and semi-quantification of tartrate-resistant acid phosphatase (TRAP). Semi-quantification is expressed as the number of TRAP + osteoclasts per B.Pm. (**C, D**) Immunohistochemical staining and semi-quantification of Cathepsin K. Semi-quantification is expressed as the relative intensity of Cathepsin K staining per high powered field. (**E, F**) Immunohistochemical staining and semi-quantification of PCNA. Semi-quantification is expressed as the number of PCNA + cells per B.Pm. (**G, H**) Histochemical staining and semi-quantification of TUNEL. Semi-quantification is expressed as the percentage of TUNEL stained cells per high powered field. Arrowheads indicate positively stained cells. Images shown at 400 $\times$. All semi-quantitation based on six random images per sample. $n=6$ samples per treatment group. $**p<0.01$. PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling. Color images available online at www.liebertpub.com/tea

types, to confirm the hypothesis that only uncommitted or early osteoprogenitor cells are responsive to PPAR γ loss.

Studies regarding PPAR γ deficiency in osteoclasts have been less comprehensive. For example, Kawaguchi *et al.* found no difference in PPAR $\gamma^{+/-}$ osteoclastic activity either when isolated or when co-cultured with PPAR $\gamma^{+/-}$ osteoblasts.²⁰ Wan *et al.* used osteoclast lineage-specific deletion of PPAR γ with Tie2Cre mice to show that PPAR γ deficiency results in increased bone density and reduced osteoclastic number and activity.²¹ Our results with PPAR γ shRNA mediated inhibition of osteoclast numbers parallel and confirm these results from osteoclast-specific PPAR γ deletion. Interestingly, the skeletal phenotype observed in our study, after only 4 weeks post- shRNA-mediated PPAR γ inhibition, was remarkably similar to that observed by other investigators after lifelong PPAR γ depletion. Further study is required to more fully ascertain the differences in acute versus chronic loss of PPAR γ expression.

Clear limitations exist in the model studied herein. For example, intramedullary lentiviral delivered PPAR γ shRNA is a relatively impractical approach for clinical translation, involving both the surgical risks from an invasive procedure, and the risks associated with insertional mutagenesis. However, we have shown that acute onset loss of PPAR γ activity in the bone marrow niche has dramatic repercussions on intramarrow adipocytes, osteoblasts, and osteoclasts. Whether these effects of PPAR γ shRNA can be sustained to improve bone strength have yet to be investigated. For example, these experiments were performed in young mice, while the clinical need for improved bone microarchitecture is primarily in the aged skeleton. Kawaguchi *et al.* found that the high bone mass phenotype of PPAR γ haploinsufficient mice becomes more pronounced with age,²⁰ potentially suggesting age-related changes in the response to PPAR γ loss. Future studies will plan to elucidate the differential responsive of young and aged mice to PPAR γ shRNA treatment.

An alternative approach would be the pharmacologic inhibition of PPAR γ , which indeed has been pursued. Duque *et al.* used the PPAR γ antagonist BADGE (bisphenol-A-diglycidyl ether) to improve BV and decrease intramarrow adiposity in skeletally mature mice.³² However, other research groups have used BADGE treatment in mice without a discernable benefit to bone parameters.³³ Thus, the efficacy of pharmacologic PPAR γ inhibition for improved bone health is not yet known.

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Disclosure Statement

No competing financial interests exist.

References

1. Liu, Y., Song, C.Y., Wu, S.S., Liang, Q.H., Yuan, L.Q., and Liao, E.Y. Novel adipokines and bone metabolism. *Int J Endocrinol* **2013**, 895045. 2013.
2. Sadie-Van Gijsen, H., Crowther, N.J., Hough, F.S., and Ferris, W.F. The interrelationship between bone and fat: from cellular see-saw to endocrine reciprocity. *Cell Mol Life Sci* **70**, 2331, 2013.
3. Kawai, M., de Paula, F.J., and Rosen, C.J. New insights into osteoporosis: the bone-fat connection. *J Intern Med* **272**, 317, 2012.
4. Doherty, A.L., Battaglini, R.A., Donovan, J., Gagnon, D., Lazzari, A.A., Garshick, E., *et al.* Adiponectin is a candidate biomarker of lower extremity bone density in men with chronic spinal cord injury. *J Bone Miner Res* **29**, 251, 2014.
5. Tontonoz, P., and Spiegelman, B.M. Fat and beyond: the diverse biology of PPARgamma. *Annu Rev Biochem* **77**, 289, 2008.
6. Issemann, I., and Green, S. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* **347**, 645, 1990.
7. Gottlicher, M., Widmark, E., Li, Q., and Gustafsson, J.A. Fatty acids activate a chimera of the clofibrate acid-activated receptor and the glucocorticoid receptor. *Proc Natl Acad Sci U S A* **89**, 4653, 1992.
8. Glass, C.K., Rose, D.W., and Rosenfeld, M.G. Nuclear receptor coactivators. *Curr Opin Cell Biol* **9**, 222, 1997.
9. Chawla, A., and Lazar, M.A. Peroxisome proliferator and retinoid signaling pathways co-regulate preadipocyte phenotype and survival. *Proc Natl Acad Sci U S A* **91**, 1786, 1994.
10. Adams, M., Montague, C.T., Prins, J.B., Holder, J.C., Smith, S.A., Sanders, L., *et al.* Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation. *J Clin Invest* **100**, 3149, 1997.
11. Imai, T., Takakuwa, R., Marchand, S., Dentz, E., Bornert, J.M., Messaddeq, N., *et al.* Peroxisome proliferator-activated receptor gamma is required in mature white and brown adipocytes for their survival in the mouse. *Proc Natl Acad Sci U S A* **101**, 4543, 2004.
12. Rosen, E.D., Walkey, C.J., Puigserver, P., and Spiegelman, B.M. Transcriptional regulation of adipogenesis. *Genes Dev* **14**, 1293, 2000.
13. Rosen, E.D., and MacDougald, O.A. Adipocyte differentiation from the inside out. *Nat Rev Mol Cell Biol* **7**, 885, 2006.
14. Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., *et al.* PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol Cell* **4**, 597, 1999.
15. Barak, Y., Nelson, M.C., Ong, E.S., Jones, Y.Z., Ruiz-Lozano, P., Chien, K.R., *et al.* PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol Cell* **4**, 585, 1999.
16. Wahli, W. PPAR gamma: ally and foe in bone metabolism. *Cell Metab* **7**, 188, 2008.
17. Kim, S.W., Her, S.J., Kim, S.Y., and Shin, C.S. Ectopic overexpression of adipogenic transcription factors induces transdifferentiation of MC3T3-E1 osteoblasts. *Biochem Biophys Res Commun* **327**, 811, 2005.
18. Yoshiko, Y., Oizumi, K., Hasegawa, T., Minamizaki, T., Tanne, K., Maeda, N., *et al.* A subset of osteoblasts expressing high endogenous levels of PPARgamma switches

- fate to adipocytes in the rat calvaria cell culture model. *PLoS One* **5**, e11782, 2010.
19. Takada, I., Suzawa, M., Matsumoto, K., and Kato, S. Suppression of PPAR transactivation switches cell fate of bone marrow stem cells from adipocytes into osteoblasts. *Ann N Y Acad Sci* **1116**, 182, 2007.
 20. Kawaguchi, H., Akune, T., Yamaguchi, M., Ohba, S., Ogata, N., Chung, U.I., *et al.* Distinct effects of PPAR-gamma insufficiency on bone marrow cells, osteoblasts, and osteoclastic cells. *J Bone Miner Metab* **23**, 275, 2005.
 21. Wan, Y., Chong, L.W., and Evans, R.M. PPAR-gamma regulates osteoclastogenesis in mice. *Nat Med* **13**, 1496, 2007.
 22. Wei, W., Wang, X., Yang, M., Smith, L.C., Dechow, P.C., Sonoda, J., *et al.* PGC1beta mediates PPARgamma activation of osteoclastogenesis and rosiglitazone-induced bone loss. *Cell Metab* **11**, 503, 2010.
 23. Wei, W., Zeve, D., Wang, X., Du, Y., Tang, W., Dechow, P.C., *et al.* Osteoclast progenitors reside in the peroxisome proliferator-activated receptor gamma-expressing bone marrow cell population. *Mol Cell Biol* **31**, 4692, 2011.
 24. Qin, X.F., An, D.S., Chen, I.S., and Baltimore, D. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci U S A* **100**, 183, 2003.
 25. Krishnan, A., Nair, S.A., and Pillai, M.R. Biology of PPAR gamma in cancer: a critical review on existing lacunae. *Curr Mol Med* **7**, 532, 2007.
 26. Schilling, T., Noth, U., Klein-Hitpass, L., Jakob, F., and Schutze, N. Plasticity in adipogenesis and osteogenesis of human mesenchymal stem cells. *Mol Cell Endocrinol* **271**, 1, 2007.
 27. Cinti, S. Transdifferentiation properties of adipocytes in the adipose organ. *Am J Physiol Endocrinol Metab* **297**, E977, 2009.
 28. Song, L., and Tuan, R.S. Transdifferentiation potential of human mesenchymal stem cells derived from bone marrow. *FASEB J* **18**, 980, 2004.
 29. Slack, J.M., and Tosh, D. Transdifferentiation and metaplasia—switching cell types. *Curr Opin Genet Dev* **11**, 581, 2001.
 30. Miles, P.D., Barak, Y., and Evans, R.M. Metabolic characterization of mice heterozygous for PPAR γ deficiency. *Diabetes* **48**:supplement 1, A68, 1999.
 31. Akune, T., Ohba, S., Kamekura, S., Yamaguchi, M., Chung, U.I., Kubota, N., *et al.* PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. *J Clin Invest* **113**, 846, 2004.
 32. Duque, G., Li, W., Vidal, C., Bermeo, S., Rivas, D., and Henderson, J. Pharmacological inhibition of PPARgamma increases osteoblastogenesis and bone mass in male C57BL/6 mice. *J Bone Miner Res* **28**, 639, 2013.
 33. Botolin, S., and McCabe, L.R. Inhibition of PPARgamma prevents type I diabetic bone marrow adiposity but not bone loss. *J Cell Physiol* **209**, 967, 2006.

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