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Leptin Functions Peripherally to Regulate Differentiation of Mesenchymal Progenitor Cells

ERICA L. SCHELLER,[a] JUNHUI SONG,[a] MICHAEL I. DISHOWITZ,[b] FABIANA NAOMI SOKI,[a] KURT D. HANKENSON,[b] PAUL H. KREBSBACH[a]

[a]Department of Biologic and Materials Sciences, University of Michigan School of Dentistry, Ann Arbor, Michigan, USA; [b]Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA

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ABSTRACT

Leptin functions through a well-documented central neuroendocrine pathway to regulate bone mass. However, the ability of leptin to modulate bone mass through a peripheral mechanism has been debated due to conflicting *in vitro* results and lack of sufficient *in vivo* models. We utilized mice with LoxP sites introduced into the long-form leptin receptor (ObRb) gene to determine how leptin regulates mesenchymal progenitor cell (MPC) differentiation and osteoblast function *in vitro* and *in vivo*. Rapid phosphorylation of Stat3 after leptin treatment of bone marrow stromal cells (BMSCs) from mice with conditional deletion of ObRb in macrophages ($LysM^{Cre+F/F}$) confirmed expression of functional leptin receptors by BMSCs. Adenovirus-Cre mediated disruption of ObRb in primary stromal cells decreased mineralization and increased adipogenesis. In contrast, BMSCs harvested from leptin-signaling deficient Ob/Ob or Db/Db mice showed increased mineralization. To determine the physiologic relevance of these differences, mice with cell-specific deletion of ObRb in mesenchymal precursors ($3.6^{Cre+F/F}$) or osteoblasts ($2.3^{Cre+F/F}$) were generated. Although the $2.3^{Cre+F/F}$ mice were grossly normal, the $3.6^{Cre+F/F}$ mice displayed mild obesity that was not attributed to food intake. Femurs of $3.6^{Cre+F/F}$ animals showed a 58%–61.9% increase in trabecular bone volume and a 65.5%–74% increase in bone mineral density. Cortical volume and mineral content were also increased 18%–22%. Primary $3.6^{Cre+F/F}$ BMSCs recapitulated the high mineralization phenotype of Ob/Ob and Db/Db BMSCs. We conclude that leptin may have multiple peripheral roles depending on the differentiation state of MPC. Leptin (a) helps maintain MPCs in an undifferentiated state and (b) promotes mineralization of more differentiated osteoblasts. STEM CELLS *2010;28:1071–1080*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Leptin, named after the Greek "*leptos*" meaning "thin," was discovered in 1994 during the analysis of a colony of spontaneously obese mice [1]. This 16 kDa cytokine was later found to play a prominent role in regulation of energy metabolism and appetite [2–4]. Many secondary functions of leptin have also been uncovered [5], including modulation of immune cell responses [6] and maintenance of bone mass [7–9]. The ability of leptin to regulate bone formation through peripheral mechanisms has been debated due to conflicting *in vitro* results and a lack of sufficient *in vivo* models. The challenge to study leptin's peripheral effects with conditional regulated gene recombination (Cre-LoxP) systems was proposed in 1998 [10], but these experiments were not possible until the recent generation of a mouse model with LoxP sites flanking the Jak2 signaling component of the long-form leptin receptor (ObRb) [11]. To help clarify the controversy of leptin's peripheral actions on bone through regulation of mesenchymal progenitor cell (MPC) differentiation and osteoblast function, we have used this model and generated three cell-specific knockouts of ObRb using LysM-Cre (macrophage), Col2.3-Cre (osteoblast), and Col3.6-Cre (MPC) mice.

Previous research demonstrates two mechanisms of leptin regulation of bone mass. First, elegant *in vivo* studies support the central hypothesis which states that binding of leptin to ObRb in the hypothalamus can stimulate bone loss by regulating osteoblast activity through the sympathetic nervous system [7, 12, 13]. Conversely, the peripheral hypothesis postulates that there are mechanisms through which local leptin can directly regulate bone formation by acting on cells in the

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Correspondence: Kurt D. Hankenson, D.V.M., Ph.D., 311 Hill Pavilion, 380 S. University Ave, University of Pennsylvania, Philadelphia, Pennsylvania 19103, USA. e-mail: kdhank@vet.upenn.edu Received February 19, 2010; accepted for publication April 14, 2010; first published online in STEM CELLS *EXPRESS* April 22, 2010. 1066-5099/2009/$30.00/0 doi: 10.1002/stem.432

bone marrow. The existence of such mechanisms has been explored by multiple groups who have compiled key pieces of evidence. For example, leptin receptors are expressed on the surface of human bone marrow stromal cells (BMSCs) [14] and recombinant leptin stimulation of human BMSCs *in vitro* has been shown to promote mineralization and partly inhibit adipogenic differentiation [15]. It has further been demonstrated *in vitro* that BMSC fate decisions can be regulated by suppression of leptin expression after addition of adipogenic factors [16]. Last, *in vivo*, peripheral (subcutaneous or intraperitoneal) delivery of supra-physiologic doses of leptin to leptin-deficient Ob/Ob mice resulted in a >30% increase in fluorochrome-labeled tibial endosteal surface and an 84.2% increase in distal femoral trabecular mineral content, respectively [8, 9]. Despite this evidence, the existence of physiologically relevant peripheral mechanisms is debated due to conflicting studies that have failed to demonstrate pro-osteogenic effects of leptin stimulation of primary mouse osteoblast cultures [7] and one *in vivo* publication with osteoblast-specific ObRb deletion that lacked significant differences in vertebral bone mass [17]. Although conflicting, these studies have laid the groundwork for a more in-depth examination of leptin's peripheral actions with additional delineation of effects on primitive MPCs and more differentiated osteoblast populations.

The true mesenchymal stem cell (MSC) is an elusive *in vivo* precursor to mesenchymal lineage cells such as osteoblasts, adipocytes, and chondrocytes. MSCs have been identified in tissues including bone marrow [18], adipose [19], and dental pulp [20]. Although many groups have focused on characterizing the MSC, specific cell surface markers have not been identified [21, 22]. Thus, our methods for *in vitro* isolation date back to the 1970s [18, 23] and rely on the adherence of a subpopulation of cells to tissue culture plastic and subsequent passaging to enrich for MSCs [24]. *in vivo* the MSC is defined as a multipotent cell that undergoes self-renewal until stimulated to differentiate into a daughter lineage. Culturing these cells *in vitro* results in cells that are more differentiated and proliferative, yet not fully lineage committed. Thus, studies which disrupt gene expression *in vitro* may result in a phenotype that is different than effects found when the gene is deleted *in vivo*. In recognition of the limited knowledge of the "true" MSC, this report will use the term MPC to refer to the *in vivo* precursor populations targeted by Col3.6-Cre and BMSCs or adipose-derived stromal cells (ADSCs) to refer to MSCs that have been isolated and cultured *in vitro*.

Analysis of current models of leptin signaling deficiency such as the Ob/Ob mouse (leptin deficient) and the Db/Db mouse (ObRb deficient) are limited by secondary complications of diabetes and obesity. Mutant mice display a phenotype of uncontrolled eating and rapid weight gain. They also exhibit hyperphagia, glucose intolerance, elevated plasma insulin, subfertility, impaired wound healing, and both low (femoral) and high (vertebral) trabecular bone mass [25, 26]. To circumvent these systemic complications, we utilized a mouse model with LoxP sites flanking exon 17 of the ObRb gene, deletion of this gene segment by Cre recombinase terminates receptor function [11]. This study was designed to critically evaluate the ability of leptin to modulate differentiation of MPCs and the function of their osteoblast progeny. We hypothesized that leptin exerts differential effects on lineage committed cells such as osteoblasts when compared with their more primitive MPC precursors. To clarify these differences, we have used *in vitro* adenovirus Cre and *in vivo* Col2.3-Cre (osteoblast) and Col3.6-Cre (MPC) mediated recombination of ObRb to explore the inherent role of physiologic, circulating leptin in bone formation and maintenance.

Experimental Procedures

Primary Cell Culture

BMSCs were harvested as described previously [27] with slight modification. Femora, tibiae, and humeri were dissected free of surrounding muscle. Marrow was removed with phosphate buffered saline (PBS) and filtered through a 70-μm cell filter. The marrow content of 6–9 bones was plated into a 75-cm^2 culture flask in BMSC growth medium (α-Modified Eagle's Medium [α-MEM; Invitrogen, Carlsbad, CA, http://www.invitrogen.com/Gibco, Grand Island, NY, http://www.invitrogen.com], 10% fetal bovine serum [Gibco, Grand Island, NY, http://www.invitrogen.com; Lot no. 451459], 100 U/ml penicillin, 100 mg/ml streptomycin sulfate [Gibco, Grand Island, NY, http://www.invitrogen.com; Cat: 15140], 100 nm dexamethasone [Sigma, St. Louis, MO, www.sigmaaldrich.com; Cat: D8893]). To harvest ADSCs intra-abdominal and paralumbar fat pads were collected, washed with PBS, and minced. Minced adipose was incubated for 1 hour at 37°C in α-MEM + 0.2% CollagenaseII (Gibco, Grand Island, NY, http://www.invitrogen.com; Cat: 17101). ADSCs were pelleted at 1,100 rpm for 10 minutes at 4°C and plated at 6–8 million cells per 10 cm plate in ADSC growth medium (Dulbecco's Modified Eagle Medium [DMEM; Invitrogen/Gibco, Grand Island, NY, http://www.invitrogen.com], 10% fetal bovine serum [Gibco, Grand Island, NY, http://www.invitrogen.com; Lot no. 451459], 100 U/ml penicillin, 100 mg/ml streptomycin sulfate). Cells were cultured at 37°C in an atmosphere of 100% humidity and 5% CO_2. Colonies of adherent cells were formed by 11–14 days. The colonies were harvested and the subcultured cells were replated at 15,000 cells per square centimeter.

PCR

RNA was harvested using Trizol reagent (Invitrogen, Frederick, MD, www.invitrogen.com; Cat: 15596) and 0.3–1 μg of total RNA was processed using the SuperScript First-Strand RT-PCR kit (Invitrogen, Frederick, MD, www.invitrogen.com; Cat: 12371) to generate cDNA. Leptin receptor expression was analyzed as previously reported [28]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were used as a loading control, GAPDH_F: TTCCAGTATGACTCCACTCA CGGCAAAT; GAPDH_R: TGGTGAAGACACCAGTAGAC TCCACGAC. Genomic DNA was harvested using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany, http://www.qiagen.com; Cat: 69506). Expression of osteocalcin was determined with computed threshold method qPCR using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, www.appliedbiosystems.com; Cat: 4309155) with GAPDH as a loading control. OCN_F: CAAGCAGGGT-TAAGCTCACA; OCN_R: GGTAGTGAAC AGACTCCGGC; GAPDH_F: TGAAGCAGGCATCTGAGGG; GAPDH_R: CGAAGGTGGAAGAGTGGGAG.

Western Blot

Protein was harvested using Nonidet (TM) P40 lysis buffer (10% glycerol, 1% Nonidet P40, 50 mM Tris pH 7.4, 200 mM NaCl, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1× Protease Inhibitor Cocktail [Sigma, St. Louis, MO, www.sigmaaldrich.com; Cat: P8340]) + phosphatase inhibitors (Roche. Indianapolis, IN, www.roche.com; Cat: 04906845001). Cells were washed with cold PBS + phosphatase inhibitors, collected and pelleted at 4°C. Cell pellets were resuspended in 50–100 μl of lysis buffer and incubated for 30 minutes on ice. Protein concentration was measured at

595 nm with Bio-Rad protein assay dye concentrate (Cat: 500-0006). Protein extracts (50 μg) were boiled in 2× sodium dodecyl sulfate (SDS) sample buffer 10 minutes and separated on a 10% Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA, http://www.bio-rad.com). Protein was transferred to polyvinylidene fluoride (PVDF) membranes using a wet transfer system (Bio-Rad; Cat: 170-3930). Membranes were blocked in 5% milk (Bio-Rad Cat: 170-6404) 1 hour and probed overnight with 1:500 Stat3 (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com; Cat: 9132), 1:500 P-Stat3 (Cell Signaling Cat: 9131), or 1:1,000 GAPDH (Chemicon International, Temecula, CA, http://www.chemicon.com; Cat: MAB374). Signals were amplified with 1:1,500 horse radish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Pierce Product, Rockford, IL, www.piercenet.com) and developed using film exposure.

Adenoviral Transduction

P1 BMSCs were plated at a density of 15,000 cells per square centimeter in T75 flasks. After 4 hours, virus was added at 800 multiplicity of infection (MOI) for BMSC or 400 MOI for ADSC in 4 ml α-MEM + 0.5% FBS. Complete media was added after 4 hours. Adenovirus was used in accordance with NIH guidelines and obtained from the University of Michigan vector core (Ann Arbor, MI). Adenovirus-expressing cre recombinase, harvest date February 19, 2009, viral titer 2.40E11 plaque forming unit (PFU) per milliliter. Adenovirus CMVpLpA.dlE3 #1 (AdBlank), harvest date February 18, 2001, viral titer 1.30E11 PFU per milliliter.

in vitro Mineralization Assay

Primary BMSCs in passage 2–3 were used for this assay. Cells were plated in 12-well or 6-well plates at 25,000–30,000 cells per square centimeter. Plated cells were allowed to grow to confluence in complete medium for 2–4 days. After reaching confluence cells were cultured in osteogenic medium (BMSC Growth Medium + 100 nm dexamethasone [Sigma; Cat: D8893], 10 mM β-glycerophosphate [Sigma; Cat: G9891], and 50 μm ascorbic acid 2-phosphate [Sigma; Cat: A8960]) for up to 14 days. Cells were fixed with 70% ethanol at 4°C for 1 hour, rinsed with water, stained for 10 minutes in 40 mM, pH 4.2 alizarin red, and washed extensively with water. Dye was eluted in 10% w/v hexadecylpyridinium chloride monohydrate (Wako, Chemical, Osaka, Japan, http://www.wako-chem.co.jp/english) in 10 mM sodium phosphate pH 7.0 and concentration determined by absorbance measurement at 562 nm.

in vitro Adipogenic Differentiation

ADSCs were plated at 30,000 cells per square centimeter and grown to confluence for 2 days. Cells were induced with adipogenic medium (ASC Growth Medium + 50 μM Isobutylmethylxanthine [Sigma; Cat: I7018], 1 μM dexamethasone [Sigma; Cat: D8893), 167 nM insulin [Sigma; Cat: I5500], and 5 μg/ml Troglitazone [Cayman Chemical, Ann Arbor, MI, www.caymanchem.com; Cat: 71750]) for 2 days. Adipogenic maintenance media (ADSC Growth Medium + 167 nM insulin [Sigma; Cat: I5500]) was then added for 2 days. This process of induction media followed by maintenance media was repeated once. Cells were then fixed in zinc buffered formalin (Z-fix, Anatech LTD, Battle Creek, MI, www.anatechltdusa.com) for 1 hour and stained with Oil Red O solution (Sigma; Cat: O0625). Dye was eluted in 100% isopropanol and quantified at 500 nm.

Animals

All procedures were approved by the University Committee on the Use and Care of Animals (UCUCA). Col2.3-Cre and Col3.6-Cre mice were obtained from Dr. Fei Liu (University of Michigan) [29]. LysM-Cre mice were obtained from Jackson Laboratory (Bar Harbor, Maine, www.jax.org; Cat: 004781) [30]. Flox/Flox Jak2-ObRb mice were obtained from Dr. Martin Myers (University of Michigan) with permission of Dr. Streamson Chua (Columbia University) [11]. Breeding cages contained one male with two females. Pups were genotyped via PCR analysis of tail biopsy DNA with primers as reported previously [11]. Deletion of the floxed allele was determined using the three-primer system designed by McMinn et al. [11]. Analysis of food intake was performed daily for 1 week. Mice were housed 2–3 animals per cage, mass of chow was determined every 24 hours, chow intake was averaged per housed animals to determine daily intake.

Blood Collection and Serum Analysis

Blood was collected from the mice by nicking the lateral tain vein and pooling blood drops into BD Microtainer tubes (Franklin Lakes, NJ, www.bdbiosciences.com). Blood was allowed to clot at room temperature for 30–45 minutes. Samples were spun at 3,000 rpm for 10 minutes at 4°C and serum supernatant removed. Analysis of serum chemistries was performed by the Animal Diagnostic Laboratory of the University of Michigan (Ann Arbor, MI). For glucose and cholesterol analyses, animals were placed on a 6 hours fast before serum collection. Leptin analysis was performed using an ELISA assay kit as described for serum leptin (R&D Systems Inc., Minneapolis, http://www.rndsystems.com; Cat: MOB00).

MicroCT

Femurs were wrapped in 70% ethanol-soaked gauze and scanned using a vivaCT 40 μCT system (Scanco Medical, Switzerland) with an isotropic voxel size of 10.5 μm (55 kVp, 145 μA, 1,000 projections per 180°, 200 milliseconds integration time). 2D transverse slices were reconstructed into 2,048 × 2,048 pixel matrices. Cortical bone parameters were measured by analyzing 50 slices (0.525 mm) of the mid-diaphysis. This region was defined to be the central portion between the proximal and distal ends of the femur. A semi-automated contouring method was used to determine the outer cortical bone perimeter. Briefly, a user-defined contour was drawn around the cortical bone perimeter of the first slice. This initial estimate is then subjected to automated edge detection. This semi-automated contour then serves as the initial estimate for the second slice, and the automated contouring process continues for all 50 slices. A fixed, global threshold of 375 (1/1,000 gray scale) corresponding to 635.9 mg of hydroxyapatite (HA) per milliliter was used to distinguish cortical bone from soft tissue and marrow. Trabecular bone parameters were measured by analyzing 101 slices (1.06 mm) of the distal metaphysis. Briefly, the distal end of the analysis region was chosen to be 0.105 mm proximal to the primary spongiosa in the marrow cavity. This assured that only trabecular bone was analyzed. Starting at this image, a user-defined contour was drawn to include only the marrow cavity and exclude any cortical bone. User-defined contours were drawn every 10 images (0.105 mm) and an automated morphing program was used to interpolate the contours for all images in between. A fixed, global threshold of 230 (1/1,000 gray scale) corresponding to 321.6 mg of HA per milliliter was used to distinguish trabecular bone from soft tissue and marrow. For both analyses, a Gaussian low-pass filter was used ($\sigma = 0.8$, support = 1).

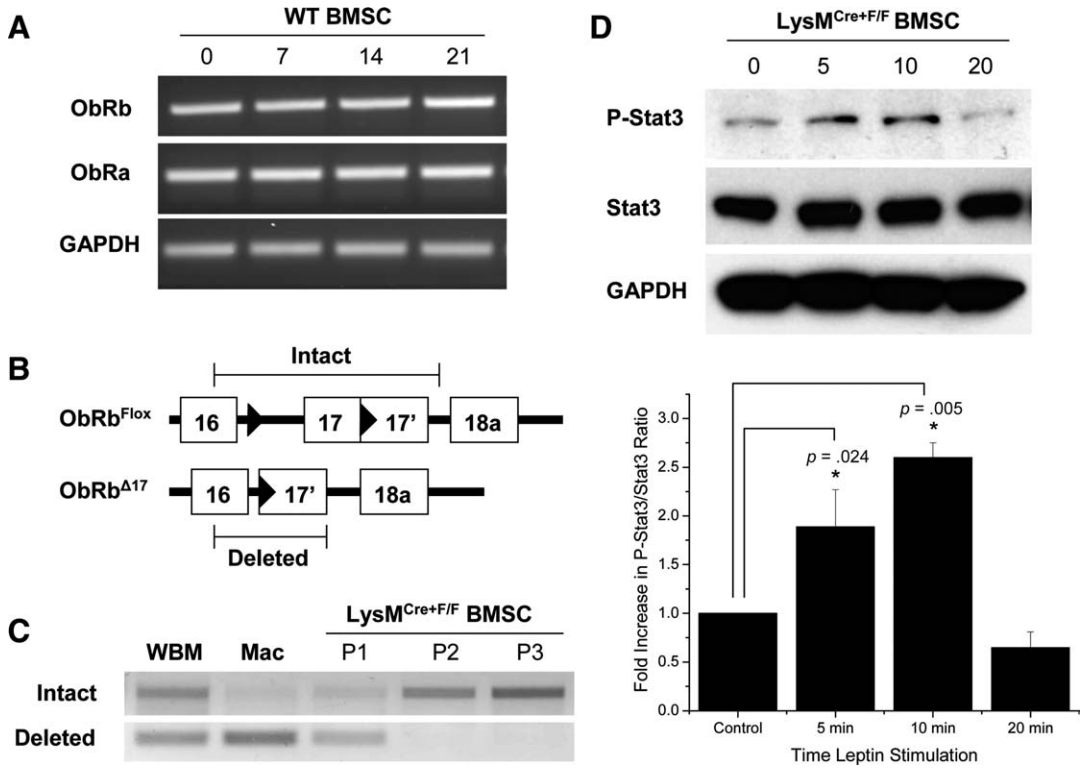


Figure 1. Primary BMSCs express functional ObRb. **(A):** PCR analysis of long-form (ObRb) and short-form (ObRa) leptin receptor. RNA from C57BL/6J (WT) P2 BMSCs. **(B):** Diagram of amplified PCR products from intact or cre-recombined (deleted) genomic DNA. **(C):** LoxP recombination in genomic DNA from whole bone marrow (WBM), primary Mac, and P1-P3 BMSC from $LysM^{Cre+F/F}$ mice. **(D):** P-Stat3 induction after 1,000 ng/ml leptin administration ($n = 3$). Abbreviations: BMSC, bone marrow stromal cell; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; Mac, macrophage; ObRb, long-form leptin receptor; WT, wild type.

Statistics and Image Editing

A two-tailed, homoscedastic *t* test was used to determine any difference between control and experimental groups. Values are reported as the mean ± the standard deviation. $p < .050$ was considered statistically significant. $p < .100$ was considered to represent a nonsignificant trend. In images with "intact" and "deleted" bands lanes were cropped from the same parent gel. Brightness and contrast were optimized equally across all lanes for improved visibility.

Results

Primary Bone Marrow Stromal Cells Express Functional ObRb

Primary BMSCs were harvested from wild-type C57BL/6J mice and analyzed for expression of leptin receptor mRNA at passage two (P2). Consistent with previous reports in human BMSCs [14] and mouse clonal MC3T3-E1 osteoblasts, [28] both short-form ObRa and long-form ObRb leptin receptors were expressed as the progenitor cells differentiated to mature osteoblasts for 7, 14, or 21 days (Fig. 1A). As primary BMSCs are a heterogeneous population that is contaminated by cells of the macrophage lineage, we generated a mouse model with floxed ObRb deletion driven by the LysozymeM locus ($LysM^{F/F}$) to disrupt ObRb function in macrophages [30]. Cre-lox recombination is a tool that is used to mediate site-specific recombination of genomic DNA [31]. DNA sequences that are flanked by LoxP nucleotide sequences or "floxed" are susceptible to deletion and recombination by the enzyme Cre recombinase. When expression of Cre is placed under the control of a specific promoter, such as LysM, gene deletion can be limited to the cell populations where that promoter is expressed. In our experiments, the flox sites were designed to flank exon 17 of ObRb. When probed with primers designed to surround this exon, cre-mediated deletion of the segment was determined based on the appearance of a shorter ~200 bp PCR band when compared with the intact 646 bp band (Fig. 1B). Stimulation of marrow-derived monocytes from these mice with 50 ng/ml macrophage colony stimulating factor (M-CSF) for 2 days resulted in recombination of the ObRb locus in macrophages *in vitro* (Fig. 1C). As expected, culture of primary BMSCs from $LysM^{F/F}$ mice demonstrated that macrophage contamination of BMSC cultures decreased with passaging of the cells (Fig. 1C). Primary BMSCs (P2) derived from $LysM^{F/F}$ mice were stimulated with 1,000 ng/ml leptin for 0, 5, 10, or 20 minutes and downstream phosphorylation of Stat3 (P-Stat3) was analyzed. In response to leptin stimulation, rapid induction of P-Stat3 was observed after 5–10 minutes that could be attributed to the non-macrophage portion of the primary BMSC preparation (Fig. 1D).

Knockout of ObRb *in vitro* Decreases Mineralization and Increases Adipogenesis of Primary Stromal Cells

To evaluate the significance of endogenous leptin production in culture, primary BMSCs were harvested from long bones of control mice harboring the ObRb LoxP mutation. Cells were treated at P1 with adenovirus-expressing Cre

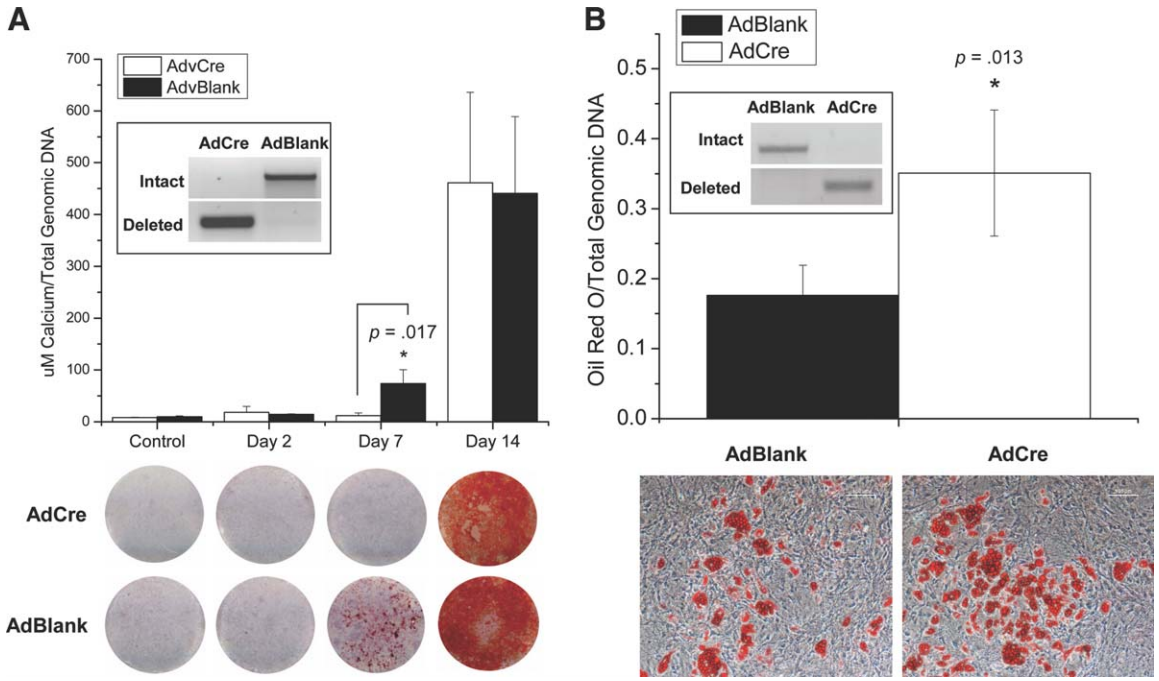


Figure 2. Knockout of ObRb *in vitro* decreases the mineralization of primary bone marrow stromal cells (BMSCs) and increases adipogenesis of primary adipose-derived stromal cells (ADSCs). **(A):** Alizarin red stain and stain quantification of BMSCs treated with AdCre or AdBlank 800 MOI ($n = 3$). **(B):** Adipogenesis, Oil Red O stain, of ADSCs treated with AdCre or AdBlank 400 MOI ($n = 4$). Insets: Genomic DNA analysis of LoxP recombination.

recombinase (AdCre) or a nonfunctional poly-L-poly-A sequence (AdBlank) and monitored for ObRb gene deletion. Complete recombination of the LoxP site occurred after AdCre treatment (Fig. 2A,B: insets). Treated BMSCs were harvested and replated at a density of 25,000 cells per square centimeter in 12-well plates and induced to differentiate toward osteoblasts. AdCre-treated cells exhibited significantly less *in vitro* mineral apposition at day 7 of osteogenic culture that was equalized by day 14 (Fig. 2A). This deletion study is the converse of previous experiments that have added leptin directly to primary BMSCs *in vitro* and observed an increase in mineralization [15]. Similarly, primary ADSCs were harvested from para-lumbar and intra-abdominal fat pads and treated with AdCre or AdBlank as described above. Ablation of ObRb function increased the adipogenic differentiation of the confluent cell monolayers by 99.8% ± 25% (Fig. 2B). This is also consistent with previous results that have demonstrated inhibition of adipogenesis *in vitro* by recombinant leptin [32].

BMSCs Obtained From Mice With an Absence of Total Body ObRb Signaling Show Increased Mineralization Potential *in vitro*

Surprisingly, although the *in vitro* disruption of leptin signaling resulted in reduced mineralization, primary BMSCs from leptin-deficient Ob/Ob and ObRb-deficient Db/Db mice showed a high mineralization phenotype. At day 14, mineralization of Ob/Ob and Db/Db cultures was increased by 50.7 ± 6.4% or 45.1 ± 5.4% respectively (Fig. 3A). Addition of 100 ng/ml recombinant leptin to the medium failed to rescue the high mineralization phenotype (Fig. 3A). Osteocalcin expression at day 14 was also increased by 2.9- ± 0.08-fold (Ob/Ob) or 2.4- ± 0.23-fold (Db/Db) (Fig. 3B). Alkaline phosphatase activity was significantly higher per total protein in Ob/Ob but not Db/Db cells (Fig. 3C).

Generation of Transgenic Mice Expressing Col2.3-Cre or Col3.6-Cre and Harboring the $ObRb^{F/F}$ Site

To determine the physiologic relevance of the divergent *in vitro* mineralization patterns, we generated osteoblast (Col2.3) and MPC (Col3.6) cell-specific knockout mice. The Col3.6 promoter is active in osteoblast precursors, whereas the 2.3 promoter is active when cells differentiate to become osteoblasts [29]. Mice harboring the $ObRb^{F/F}$ site were mated with Col2.3-Cre $ObRb^{F/-}$ or Col3.6-Cre $ObRb^{F/-}$ mice to generate litters consisting of Cre-F/F, Cre-F/–, Cre+F/–, and Cre+F/F mice in a 1:1:1:1 ratio. Breeding conformed to expected Mendelian ratios, however, complete gene disruption was observed in a significant number of female pups and some of the males for both Col3.6-Cre and Col2.3-Cre litters (observation, not quantified). This is consistent with previous reports of sporadic germline recombination [33] when using the Col2.3 and Col3.6 promoters. Complete knockout (KO) mice were easily identified phenotypically by their frank obesity and genetically by 100% band excision in tail tip DNA (Supporting Information Fig. 1A). Tissue analysis of genomic DNA revealed some recombination in all tissues of $3.6^{Cre+F/F}$ mice with the highest deletion rate in adipose, tendon, and calvaria (Fig. 4A). Consistent with previous reports, the majority of *in vitro* primary $3.6^{Cre+F/F}$ BMSCs harbored the deleted form of the ObRb locus [29] (Fig. 4B). We speculate that the background recombination in $3.6^{Cre+F/F}$ whole tissues can be attributed to expression of Col3.6 in tissue-associated MPC or vascular endothelial cells. Recombination in $2.3^{Cre+F/F}$ mice was highest in muscle, calvaria, and whole marrow (Fig. 4A).

The gross phenotype of $2.3^{Cre+F/F}$ mice was identical to 2.3^{Cre-} controls. The $3.6^{Cre+F/F}$ mice demonstrated mild obesity that remained statistically significant after approximately 12 weeks of age (Fig. 4C). Mice were examined for alterations in fasting serum chemistries including cholesterol, calcium, alanine aminotransferase (ALT), albumin, alkaline phosphatase, amylase, blood

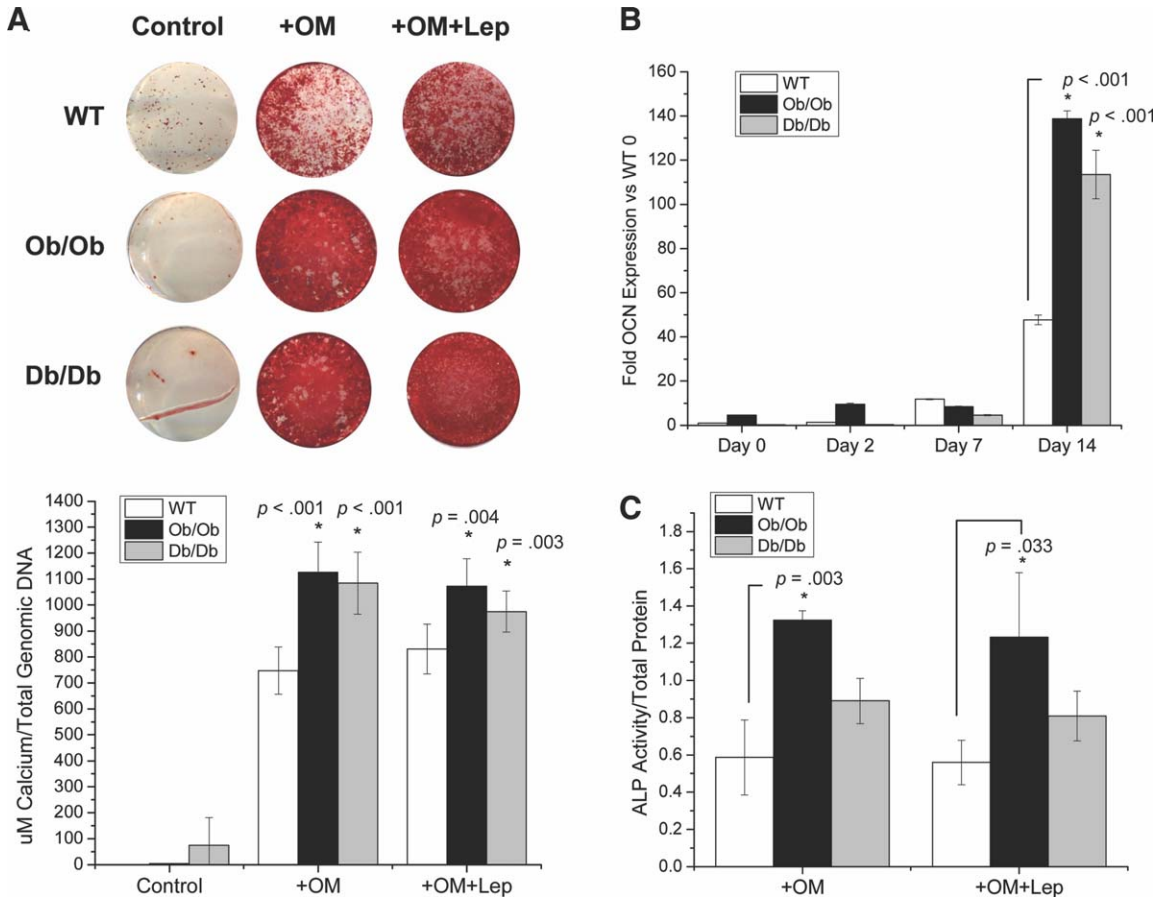


Figure 3. BMSCs from Ob/Ob and Db/Db mice show increased mineralization. **(A):** Day 14 OM with or without 100 ng/ml leptin, alizarin red stain, and quantification, P2 BMSCs ($n = 5$–6). **(B):** Quantitative PCR for osteocalcin expression ($n = 3$). **(C):** Alkaline phosphatase activity as measured by conversion of *p*-nitrophenol phosphate to nitrophenol, normalized to total protein ($n = 3$). Abbreviations: ALP, alkaline phosphatase; Db, Db/Db leptin-receptor deficient mice; Ob, Ob/Ob leptin-deficient mice; OCN, osteocalcin; OM, osteogenic media; WT, wild type.

urea nitrogen, creatinine, hemoglobin, phosphate, total bilirubin, total protein, glucose, and leptin (Fig. 4D, 4E). The only significant differences in these values between the 3.6^{Cre-} and $3.6^{Cre+\ F/F}$ mice were a slight increase in serum calcium (within normal range), a small increase in total protein driven by an increase in albumin and a significant elevation of serum leptin (Fig. 4D). Thus, unlike the complete KO mice, $3.6^{Cre+\ F/F}$ mice were not diabetic and had no significant alterations in liver or kidney function. Next, food intake of 42-week-old mice was monitored for 1 week. No significant differences were observed between the three groups (Data Not Shown).

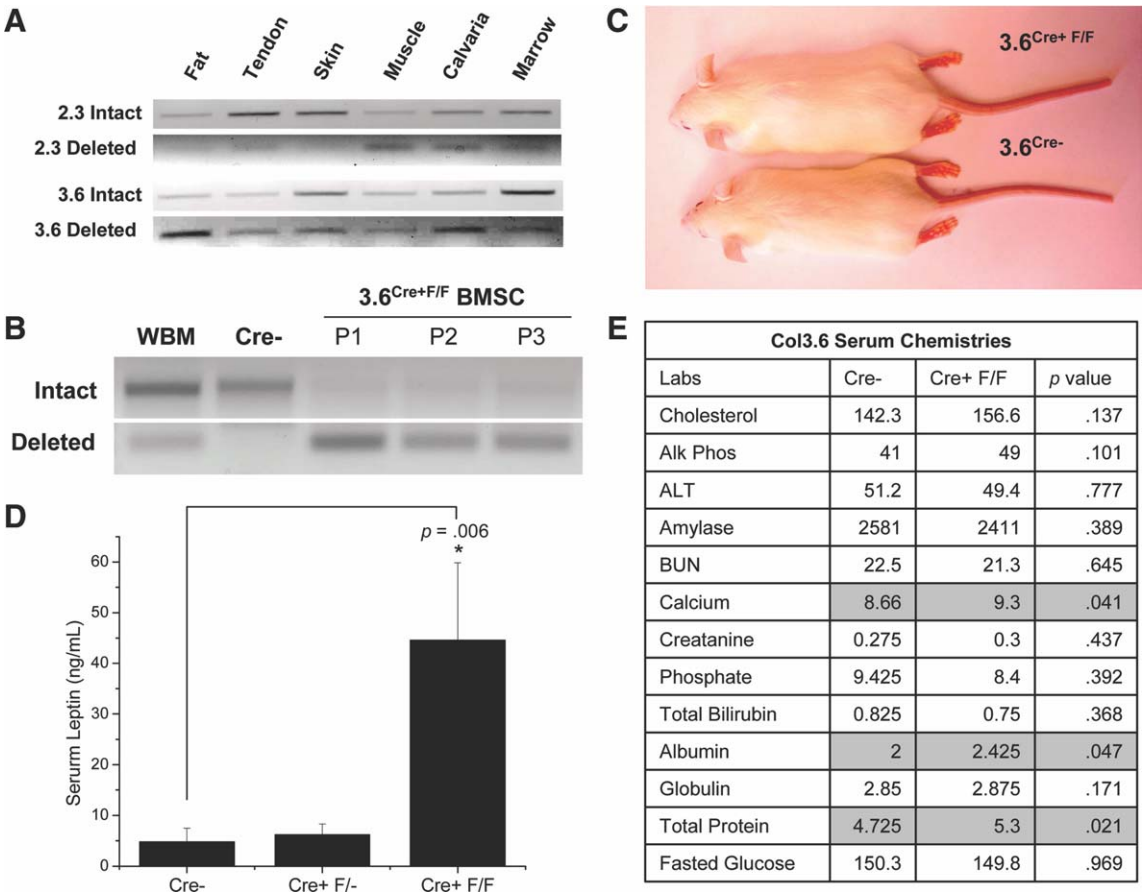
Contrasting Bone Phenotypes of $2.3^{Cre+F/F}$ and $3.6^{Cre+F/F}$ Mice

At 12 weeks, female $3.6^{Cre+F/F}$ mice maintained a 41% ± 5.3% body mass increase over controls compared with a 146% ± 6.2% body mass increase in the complete KO mice (Fig. 5A). Femurs were dissected free of muscle and radiographed at 32 kV for 45 seconds (Fig. 5B). Femoral microCT analysis revealed no difference in trabecular parameters of the $2.3^{Cre+F/F}$ femurs (Fig. 5C). However, cortical parameters showed a nonsignificant trend ($p < .100$) toward increased bone volume and bone area (Fig. 5D). The only significant change was a 1% decrease in cortical total volume mineral density (Fig. 5D).

Because of the presence of significant differences, femurs of $3.6^{Cre+F/F}$ mice were analyzed at both 12 and 42 weeks of age. At 42 weeks, femur length was increased by 0.78 ± 0.02 mm (5.5% ± 0.16%) in male $3.6^{Cre+F/F}$ mice when compared with 3.6^{Cre-} controls (Supporting Information Fig. 1B). At both ages, trabecular parameters including bone volume fraction (BVF) and mineral density of total volume (MD of TV) were significantly increased by 58%–61.9% and 65.5%–74%, respectively (Fig. 5C, Supporting Information Fig. 1C). Increased cortical parameters at 12 weeks mimicked those present in complete KO mice with an 18.3% ± 1.7% and a 21.7% ± 2.2% increase in bone volume and total mineral content, respectively (Fig. 5D). The decrease in femoral trabecular BVF and increased cortical parameters of our spontaneous complete KO mice recapitulates what has been reported previously for Ob/Ob mice [26] (Fig. 5C, 5D).

Primary BMSCs From Mice $3.6^{Cre+\ F/F}$ Mice Have Increased Osteoblast and Adipocyte Differentiation Potential *in vitro*

To verify that deletion of ObRb *in vivo* results in an increased differentiation *in vitro*, we harvested primary stromal cells from both $3.6^{Control}$ and $3.6^{Cre+\ F/F}$ mice. Mineralization of the BMSCs at P2 *in vitro* revealed a robust mineralization



Col3.6 Serum Chemistries			
Labs	Cre-	Cre+ F/F	*p* value
Cholesterol	142.3	156.6	.137
Alk Phos	41	49	.101
ALT	51.2	49.4	.777
Amylase	2581	2411	.389
BUN	22.5	21.3	.645
Calcium	8.66	9.3	.041
Creatanine	0.275	0.3	.437
Phosphate	9.425	8.4	.392
Total Bilirubin	0.825	0.75	.368
Albumin	2	2.425	.047
Globulin	2.85	2.875	.171
Total Protein	4.725	5.3	.021
Fasted Glucose	150.3	149.8	.969

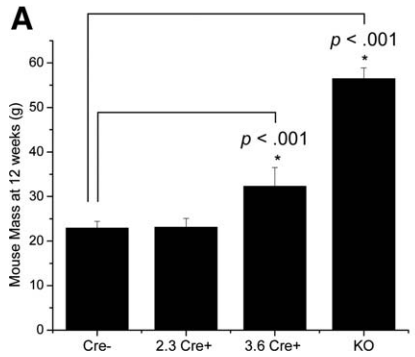
Figure 4. Characterization of mice expressing Col2.3-Cre or Col3.6-Cre and harboring the $ObRb^{F/F}$ site. **(A):** Representative PCR of tissue genomic DNA. **(B):** PCR of WBM and P1-P3 BMSCs from $3.6^{Cre+F/F}$mice. **(C):** Representative mouse photographs. **(D):** Serum leptin ELISA ($n = 6$). **(E):** Col3.6 mouse serum chemistries ($n = 3$–6). Abbreviations: ALT, alanine aminotransferase; BMSC, bone marrow stromal cell; BUN, blood urea nitrogen; WBM, whole bone marrow.

phenotype that mimicked that of BMSCs from Ob/Ob and Db/Db mice. Mineralization was increased by 422% ± 215% at day 7 and 192% ± 17% at day 14 (Fig. 6A). Differentiation of primary BMSCs also showed a 97.7% ± 23.1% increase in adipogenic potential (Fig. 6B).

Discussion

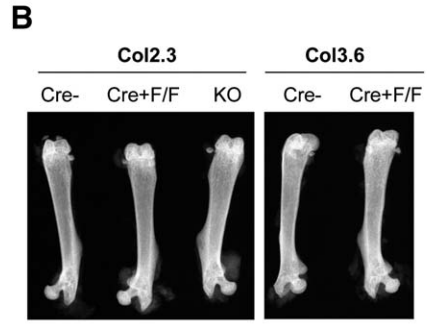
It is well-established that leptin acts primarily through the hypothalamus to regulate weight gain through food intake [2–4]. Generation of a mouse with conditional disruption of the long-form leptin receptor gene in the MPC and osteoblast has allowed us to begin to understand the physiologic contributions of peripheral leptin in bone. We used a mouse with LoxP sites flanking exon 17 of ObRb to critically evaluate physiologic regulation of bone formation by peripheral leptin *in vitro* and *in vivo*. To do this, three transgenic mouse lines were generated with cell-specific KO of ObRb function using the LysM, Col2.3, and Col3.6 promoters. Use of the Col2.3 and Col3.6 promoters is somewhat limited by high levels (15%–50%) of germline recombination that decreases the number of usable offspring [33] as well as nonspecific expression of Col3.6 in tendon, skin, and muscle [29]. However, both promoters have previously been used successfully [34, 35] and an alternative promoter that drives reliable recombination in the mesenchymal precursor compartment without affecting neighboring bone cells is not yet available.

The Col3.6 promoter is known to be expressed in multiple tissues including tendon, skin, calvaria, and long bone [29]. Analysis with ROSA reporter mice shows widespread expression in the bone cavity that encompasses the osteocyte, osteoblast, and stromal fibroblast population with additional expression observed in some clustered areas of proliferative and hypertrophic chondrocytes [29]. Adipose tissue was not analyzed in this previous publication, although our current study reveals high levels of recombination in the fat pads (Fig. 4A). As the Col3.6 promoter mediates LoxP recombination in osteoblasts, adipocytes, and some chondrocytes, we believe that it likely deletes the gene a precursor population for these lineages, the MPC. BMSC from Col3.6-Cre mice crossed with ROSA Cre-reporter mice in low-density colony forming unit-fibroblast (CFU-F) cultures show high expression of Cre-recombinase in primitive colonies which is additional evidence that Col3.6 forces recombination at an early MPC-like level [29]. Thus, we propose that recombination driven by Col3.6-Cre is one method that can be used to study effects of gene deletion on MPC function *in vivo*. However, it is still possible that an unidentified population of neurons expresses Col3.6 and that ObRb deletion in these cells is contributing to the $3.6^{Cre+F/F}$ mouse phenotype. When leptin receptors are completely deleted in the hypothalamus *in vivo*, this results in decreased length and trabecular bone of the femur [26]. As the $3.6^{Cre+F/F}$ mice have longer femurs with increased



C

	2.3Cre+F/F		3.6Cre+ F/F		Complete KO	
Trabecular	**3mo**	***p* value**	**3mo**	***p* value**	**3mo**	***p* value**
Bone Volume	–12.0%	.245	80.0%	.006	–12.0%	.170
Bone Volume Fraction	–14.0%	.131	61.9%	.011	–17.0%	.050
Trabecular Number	–6.0%	.183	20.8%	.056	–9.3%	.071
Trabecular Thickness	–3.0%	.393	15.9%	.005	–3.1%	.332
MD of TV	–14.0%	.171	65.5%	.008	–10.0%	.190
Total Mineral Content	–12.0%	.288	84.3%	.004	–4.4%	.582
Bone Mineral Content	–12.0%	.239	80.9%	.006	–13.2%	.124



D

Cortical	**3mo**	***p* value**	**3mo**	***p* value**	**3mo**	***p* value**
Total Volume	4.0%	.160	11.3%	.060	15.0%	.008
Bone Volume	4.0%	.099	18.3%	.012	19.7%	.015
MD of TV	–1.0%	.023	9.0%	.104	1.7%	.061
Total Mineral Content	3.0%	.220	21.7%	.013	23.8%	.002
Bone Mineral Content	3.0%	.221	18.8%	.015	21.6%	.005
Total Area	4.0%	.183	11.6%	.057	14.9%	.009
Bone Area	4.0%	.098	4.8%	.012	19.6%	.014

Figure 5. $2.3^{Cre+F/F}$, but not $3.6^{Cre+F/F}$ mice show increased trabecular bone. **(A):** Mass of mice ($n = 3$–6). **(B):** Representative faxitron x-ray at 32 kV, 45-second of femurs. **(C, D):** Percent change in trabecular or cortical bone parameters as determined by microCT ($n = 3$–6). Abbreviations: KO, knockout; MD of TV, mineral density of total volume; mo, month.

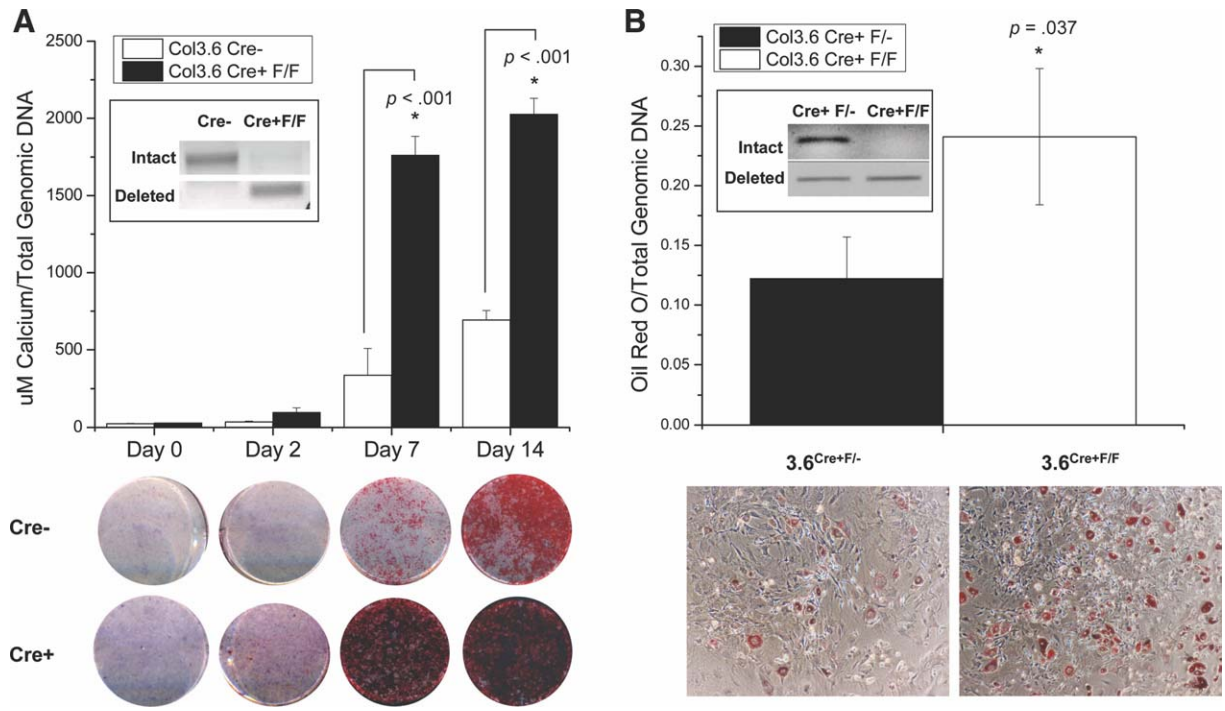


Figure 6. Knockout of ObRb in mesenchymal progenitor cells (MPCs) *in vivo* with Col3.6-Cre results in increased *in vitro* mineralization and adipogenic potential. **(A):** Mineralization, alizarin red stain, and quantification, P2 bone marrow stromal cells (BMSCs) ($n = 3$). **(B):** Adipogenesis, oil red o stain, P2 BMSCs ($n = 3$). Insets: Genomic DNA analysis of LoxP recombination.

trabecular bone, it is not yet clear how nonspecific brain recombination would contribute to the bone phenotype. However, future studies will continue to explore this possibility. Col2.3 promoter expression is more restricted and shows deletion primarily in more differentiated osteoblasts [29]. We have used the above premise to explore the contrasting *in vitro* mineralization phenotypes found in leptin-signaling deficient BMSCs that were generated *in vitro* (AdCre) or *in vivo* (Ob/Ob and Db/Db) (Figs. 2, 3).

When determining the ability of Ob/Ob and Db/Db cells to mineralize *in vitro*, an unanticipated finding was that despite decreases in femoral trabecular bone content *in vivo* [26]

(Fig. 5C) the cells actually mineralized more than their controls *in vitro* (Fig. 2A). This was matched by increases in osteocalcin expression and for Ob/Ob, increases in alkaline phosphatase activity (Fig. 2B, 2C). ALP activity is variable in murine BMSC cultures and we do not yet understand the significance, if any, of the nonsignificant differences between wild type (WT) and Db/Db BMSCs. This finding of high mineralization was initially assumed to be due to unknown systemic complications of diabetes and obesity. Addition of recombinant leptin did not reduce the mineralization of the cells to baseline levels (Fig. 2A) implying that the starting population derived from Ob/Ob mice is unique when compared with WT. Previous publications have shown that leptin is able to enhance mineralization at higher concentrations (0.6–1.2 μg/ml) [15]. The systemically healthy $3.6^{Cre+F/F}$ ObRb mouse model which exhibited an opposite *in vivo* phenotype of increased trabecular BVF and bone mineral density (BMD) in the femur (Fig. 5C) also showed increased mineralization potential *in vitro* similar to the Ob/Ob and Db/Db mice (Fig. 6A). This suggests that *in vivo* the systemic environment is driving differentiation of the MPCs in opposite directions in the Ob/Ob (increased medullary fat) and $3.6^{Cre+F/F}$ (increased trabecular bone) mice. Despite these differences *in vivo*, culture of the cells *in vitro* revealed uniform increased sensitivity to osteoblast differentiation. In contrast, deletion of ObRb in cultured and passaged P2 BMSCs *in vitro* decreased mineralization (Fig. 2A). We believe that this *in vitro* deletion occurs when mesenchymal lineage cells are fundamentally very different and more differentiated than the *in vivo* deletion that occurs in primitive in MPC of Ob/Ob, Db/Db and $3.6^{Cre+F/F}$ mice. Although an absence of leptin signaling in primitive cells results in MPC with enhanced differentiation potential, ObRb disruption in differentiated cells *in vitro* is inhibitory to osteoblast differentiation but promotes adipogenic differentiation. As observed with the $2.3^{Cre+F/F}$ mouse and one previous publication [17], inhibition of leptin's actions on osteoblasts does not appear to be physiologically relevant to trabecular bone parameters during states of healthy equilibrium *in vivo* (Fig. 5C).

Even in the presence of intact leptin signaling, obesity can have significant effects on bone cell function *in vitro* and bone mass *in vivo* [36, 37]. Studies that have analyzed bone parameters in mice due to diet induced obesity observed increases in cortical parameters of the femur [36] with no change [36] or decreases [37] in trabecular parameters. These studies observed changes in obese mice of age 14–25 weeks that were approximately 32–38 g. Despite being of comparable age and body mass to the diet-induced obese mice, our $3.6^{Cre+F/F}$ mice presented with statistically significant increases in trabecular bone parameters instead of decreases (Fig. 5C). Consistent with previous results [36], we also observed cortical changes in our $3.6^{Cre+F/F}$ mice at 12 weeks that failed to persist and equalized by 42 weeks (Fig. 5D, Supporting Information Fig. 1C). Thus, we conclude that the trabecular bone phenotype is due to ObRb deletion and not increased load bearing.

As systemic leptin is increased in the $3.6^{Cre+F/F}$ animals due to adipose accumulation, one must also consider the effects of leptin on osteoclast function. It is possible that the high bone mass observed in these animals is related to inhibition of osteoclast differentiation or activity. However, *in vivo* KO of ObRb in osteoclast precursor macrophages with the LysM-Cre promoter did not change body mass or bone parameters at 12 weeks (data not shown). In addition, published data do not support a consistent role for leptin in mature osteoclast function. For example, although Ob/Ob and Db/Db mice have over twofold increase in osteoclast number, the excretion of urinary deoxypyridinoline crosslink, a biochemical marker of bone resorption, was not impaired when compared with controls [7]. Conversely, leptin deficient rats have increased osteoclast surface, but no increase in numbers [38]. Isolation of primary bone marrow osteoclast progenitors from leptin-deficient mice showed no deficiency in osteoclast differentiation or their ability to form resorption lacunae on dentin [7]. Other *in vitro* studies demonstrated that leptin partially inhibits osteoclastogenesis of primary mouse marrow cultures but did not affect bone resorption of mature osteoclasts [39].

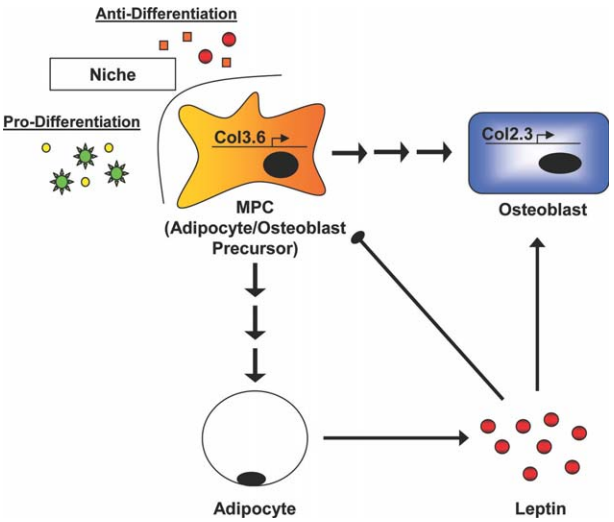


Figure 7. Peripheral model of leptin's action on MPCs and osteoblasts. Leptin (a) helps to maintain MPCs in an undifferentiated state and (b) promotes differentiation and mineralization of *in vitro* preosteoblasts and osteoblasts. Abbreviation: MPC, mesenchymal progenitor cell.

Conclusions

Based on our data we propose a working model for peripheral leptin regulation of bone mass that includes divergent mechanisms depending on the differentiation state of the MPC (Fig. 7). Generally, it is thought that a factor that controls MPC differentiation to adipocytes will inhibit differentiation toward osteoblasts and vice versa. However, in some situations, a factor may block differentiation of primitive MPCs without regard for the downstream possibilities, in effect maintaining stemness. Here, we demonstrate that leptin may be one of the factors that functions to maintain MPCs in an undifferentiated state. Thus, in mice with leptin signaling deficient MPC there is enhanced bone formation and increased adipogenesis, and cells *in vitro* show increased differentiation to these fates. Future studies will pursue this hypothesis and work to uncover any potential Col3.6 expressing neurons that may contribute to the bone and metabolic phenotype. Although we do not yet understand the full implications of this finding, there are multiple clinical scenarios that should be tested to uncover its relevance. For example, it has been previously reported that sites with high marrow fat content (and thus higher local leptin concentration) have a decreased rate of bone turnover [40]. It is possible that leptin's inhibition of MPC differentiation may play a role in this process. Or, perhaps limited fracture healing potential in obese individuals [41] with increased systemic leptin may be related to leptin's limitation of de novo mesenchymal activation, osteoblast recruitment, and subsequent matrix synthesis. In the future, we hope to address these questions and expand our molecular understanding of leptin's regulation of MPC function. In summary, we conclude that although the effect of leptin on

mature osteoblast function may not be physiologically significant *in vivo*, leptin has previously unrecognized peripheral roles in bone that include regulation of MPC differentiation.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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