Targeted ablation of the PTH/PTHrP receptor in osteocytes impairs bone structure and homeostatic calcemic responses

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Abstract

Parathyroid hormone (PTH) is a major physiologic regulator of calcium, phosphorous, and skeletal homeostasis. Cells of the osteoblastic lineage are key targets of PTH action in bone, and recent evidence suggests that osteocytes might be important in the anabolic effects of PTH. To understand the role of PTH signaling through the PTH/PTHrP receptors (PPR) in osteocytes and to determine the role(s) of these cells in mediating the effects of the hormone, we have generated mice in which PPR expression is specifically ablated in osteocytes. Transgenic mice in which the 10 kb-Dmp1 promoter drives a tamoxifen-inducible Cre-recombinase were mated with animals in which exon 1 of PPR is flanked by lox-P sites. In these animals, osteocyte-selective PPR knockout (Ocy-PPR cKO mice) could be induced by administration of tamoxifen. Histological analysis revealed a reduction in trabecular bone and mild osteopenia in Ocy-PPR cKO mice. Reduction of trabecular number and thickness was also detected by micro-computed tomography analysis whereas bone volume fraction (BV/TV%) was unchanged. These findings were associated with an increase in Sost and sclerostin expression. When Ocy-PPR cKO mice were subjected to a low-calcium diet to induce secondary hyperparathyroidism, their blood calcium levels were significantly lower than littermate controls. Moreover, PTH was unable to suppress Sost and sclerostin expression in the Ocy-PPR cKO animals, suggesting an important role of PTH signaling in osteocytes for proper bone remodeling and calcium homeostasis.

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Introduction

Osteocytes are the most abundant cells in bone, outnumbering osteoblasts by 10-fold and osteoclasts by 1000-fold (Aguirre et al. 2006), and yet their function is still incompletely understood. Osteocytes act as mechanosensors of bone, and recent evidence has indicated a role for these cells in bone modeling and remodeling and phosphate homeostasis (Feng et al. 2006, Lorenz-Depiereux et al. 2006, Tatsumi et al. 2007). Studies on osteocytes have been hampered by the inaccessibility of these cells and by the lack of molecular and cell surface markers that could be used to isolate and characterize them. In the last decade, however, our knowledge of osteocytes has expanded dramatically, mostly as a result of the identification of several selective osteocyte markers, such as dentin matrix protein 1 (DMP1; George et al. 1994, Ye et al. 2004, Yang et al. 2005), matrix extracellular phosphoglycoprotein/ostecyte-factor 45 (MEPE/OF45; Petersen et al. 2000, Gowen et al. 2003), membrane-type matrix metalloproteinase (MT1-MMP; Holmbeck et al. 1999, 2005), phosphate regulating endopeptidase homolog (PHEX; Westbroek et al. 2002, Liu et al. 2006), and sclerostin (Sost; Winkler et al. 2003, van Bezooijen et al. 2004) that have allowed, for the first time, a more direct analysis of the molecular and cellular biology of osteocytes.

Osteocytes express receptors for several extracellular ligands, including the parathyroid hormone (PTH) type 1 receptor (PPR; Fermor & Skerry 1995), estrogen receptors (both α and β; Ehrlich et al. 2002, Jessop et al. 2004, Lanyon et al. 2004, Lee et al. 2004), prostaglandin receptor (EP2), and a novel, as yet uncloned, receptor that specifically recognizes the carboxyl-terminal region of PTH, the carboxyl-terminal PTH receptor (Divieti et al. 2001, 2005).

PTH, a single-chain polypeptide comprised of 84 amino acids, is synthesized and secreted by the parathyroid glands in a calcium-regulated manner. PTH maintains serum calcium homeostasis and controls renal phosphate reabsorption and vitamin D 1α-hydroxylation. PTH modulates bone turnover by actions that are mediated by a G protein-coupled receptor, the PPR (Juppner et al. 1991). The PPR is highly expressed in bone and kidney, but is also found in a variety of other tissues not regarded as classical PTH targets. This likely reflects the local paracrine role of PTHrP in tissues such as breast, skin, heart, blood vessels, and others. The N-terminal 34 amino

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acids of PTH are necessary and sufficient to fully activate the PPR, which, in turn, can activate multiple G protein-coupled pathways, including those that signal through cAMP/protein kinase A, phospholipase C (PLC)/protein kinase C (PKC), and nonPLC-dependent PKC and Ca$^{2+}$. It has been shown that PTH can prevent apoptosis of osteocytes (and osteoblasts) and that this mechanism may contribute to the anabolic action of intermittently administered PTH (Jilka et al. 1998, 1999). Moreover, recent studies from O’Brien et al. (2006) revealed a critical role of PTH (and PPR) in osteocytes. They generated transgenic mice expressing a constitutively active PPR specifically in osteocytes by placing the H223R mutant receptor (Schipani et al. 1995, 1997) under the control of the 8 kb-Dmp1 promoter (named DMP1-cAPTHR1). In these mice, the levels of Sost mRNA in bone were threefold lower than wild-type littermates at 8 weeks of age. Strikingly, DMP1-cAPTHR1 mice also exhibited a 42% and an 84% increase in bone mineral density (BMD) in the spine and femur respectively as determined by dual-energy X-ray absorptiometry (DEXA) (O’Brien et al. 2006), suggesting an important role for PPR/cAMP-mediated pathways in the anabolic effect of PTH.

With this in mind, we aimed to specifically ablate the PPR in osteocytes to directly assess the role of receptor activation in these cells. We have generated transgenic mice in which the 10-kb promoter of Dmp1 drives a tamoxifen-inducible bacteriophage Cre-recombinase. These mice were crossed with mice in which exon 1 of the PPR gene was flanked by lox-P sites to generate animals that specifically lack PPRs in osteocytes. Initial histological analysis demonstrated that lack of PPR expression in osteocytes induces a reduction in trabecular bone, accompanied by a tonic elevation of Sost and sclerostin and a lack of PTH-induced Sost and sclerostin suppression.

Moreover, ablation of PPR in osteocytes impairs skeletal responses to a low-calcium diet, indicating a critical role of this receptor in controlling calcium homeostasis.

Materials and Methods

Generation of the 10 kb-Dmp1-Cre-ERT2 mice

The 14-kb Dmp1 promoter fragment (−9624 to +4439) containing a 9624-bp promoter region, a 95-bp exon 1 (E1), 4326-bp intron 1, and 17-bp initial non-coding region of exon 2 (E2) was cloned into the Cre-ERT2-LacZ-MH vector, which contains the Cre-ERT2 DNA. The Dmp1-Cre-ERT2 transgene was released from the vector backbone with the use of the unique restriction enzymes Notl and SalI, purified by Qiaquick gel extraction kit (Qiagen), quantified, and microinjected into the pronucleus of B6C3F1 hybrid mice (Taconic, Hudson, NY, USA) to generate founder mice. Injections were performed on site at the Massachusetts General Hospital transgenic core facility (CBRC MGH mouse facility). Three rounds of pronuclear injections were performed, and a total of 68 offspring were obtained. Eighteen pups died at birth, and 14 of the remaining 50 were positive for Cre-recombinase integration (23%), as assessed by genomic PCR of tail DNA using Cre-specific primers (Fig. 1B). Six independent transgenic founders were mated with wild-type C57BL/6 mice, and the F1 offspring were analyzed. All transgenic lines produced pups at the expected 1:1 ratio, and the pups appeared normal, grew indistinguishably from wild-type, and were fertile. To further analyze the characteristics of these mice, a Southern blot analysis of genomic DNA was performed. A Cre-recombinase-specific probe was cloned using PCR primers, as shown in Fig. 1A. Genomic DNA was digested using the restriction enzyme EcoRI that cleaves the insert in one site, and Southern blot analysis confirmed the germline transmission and single site of insertion. In addition, to assess that the transgene was transmitted through generations, Southern blot analysis of genomic DNA from F2 progenies was performed, as shown in Supplementary Figure 1, see section on supplementary data given at the end of this article.

PPR-floxed mice were described previously (Kobayashi et al. 2005). 10 kb-Dmp1-Cre-ERT2 mice were crossed with homozygous PPR-floxed (PPR<sup>fl/fl</sup>) mice to obtain doubly heterozygous mice 10 kb-Dmp1-Cre-ERT2:PPR<sup>fl/+</sup>, which were in turn mated with PPR<sup>fl/fl</sup> to obtain the desired 10 kb-Dmp1-Cre-ERT2:PPR<sup>fl/+</sup> mice and littermates which include 10 kb-Dmp1-Cre-ERT2:PPR<sup>fl/fl</sup>, or osteocyte-selective PPR knockout (OctycPPR<sup>KO</sup>) mice, and littermates which were all of which were used as controls in all experiments. We crossed the Dmp1-Cre-ERT2 mice with ROSA26R mice (Soriano 1999), kindly provided by Dr Henry Kronenberg (Massachusetts General Hospital, Boston, MA, USA), to obtain the ROSA26R and Dmp1-Cre-ERT2 double transgenic mice for monitoring Cre-recombinase expression. For tamoxifen injections, 10 mg of free base tamoxifen (MPBio, Solon, OH, USA) was dissolved in 100 μl of dimethylformamide (Fisher Scientific, Waltham, MA, USA) and then diluted to 10 mg/ml in corn oil (Sigma). Mice were injected with 50–150 μl, depending on their age.

Genotyping of mice

The genotypes of the mice were determined by PCR analysis of genomic DNA extracted from tail biopsies. For the 10 kb-Dmp1-Cre-ERT2 transgene, the forward Cre primer (5’-CGCGGTCTGGCAGTAAAACACTATC-3’) and the reverse Cre primer (5’-CCCACCGTCAGTCGAGAGAT-3’) were used to generate a PCR product of ~400 bp. For the floxed PPR allele, the P1 primer (5’-ATG AGG TCT GAG GTA CAT GGC TCT GA-3’) and the P2 primer (5’-CCT GCC TAC TCT TCT GAA AGA ATG T-3’) were used, which recognized the sequence spanning the 3’ lox-P site, as previously reported (Kobayashi et al. 2002). Wild-type and mutant alleles give ~210 and 290 bp products respectively. Mice were primarily kept in mixed genetic backgrounds with dominance of the C57Bl6, and all
experiments were performed with littermates as controls. Procedures that involved mice were approved by the Institutional Animal Care and Use Committee, Subcommittee on Research Animal Care, at Massachusetts General Hospital.

Allele-specific DNA recombination was performed on DNA isolated from long bones (femurs), calvaria, kidney, liver, skeletal muscle, and spleen. Briefly, pups were treated with tamoxifen (400–500 mg) at days 3, 5, 7, 14, and 21. Mice were killed at 3 weeks of age by CO2 inhalation, tissues were quickly removed, and DNA was isolated following standard protocol. Multiplex PCR analysis was performed using allele-specific primers, P1, P2, and P3 (5'-ACA TGG CCA TGC CTG GGT CTG AGA-3') and following the manufacturer protocol (Qiagen Multiplex PCR kit, Qiagen).

Histology and immunohistochemistry

Tissues were fixed in 10% formalin/PBS solution at 4 °C ON, decalcified in 20% EDTA pH 8 for 7–15 days, processed, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin, or used for immunohistochemistry. In some experiments, undecalcified femurs were embedded in methyl methacrylate (Aldrich Chemical Co, Milwauk ee, WI, USA). Sections were cut using a diamond-embedded wire saw, and then stained by the Von Kossa method. Sclerostin expression was detected immunohistochemically using commercially available biotinylated anti-mouse sclerostin antibody (1:50 dilution; R&D Systems, Inc., Minneapolis, MN, USA) and dianimobenzidine detection (Vector, Burlingame, CA, USA). Briefly, sections were deparaffinized, and endogenous peroxidase activity was inhibited by 3% H2O2 treatment for 15 min. Subsequently, slides were blocked with tris-NaCl blocking buffer (TSA Biotin Tyramide kit, Perkin Elmer, Waltham, MA, USA) for 30 min at room temperature and then incubated for 1 h with biotinylated anti-mouse sclerostin antibody. After extensive rinsing, sections were incubated for 30 min with streptavidin (SA)-conjugated HRP and tyramide following the manufacturer's protocol (TSA Biotin Tyramide kit) and developed with a 3,3'-diaminobenzidine substrate chromogen system (Vector Laboratories).

Serology

Blood was collected by tail vein bleeding for ionized calcium and by carotid transection or retro-orbital bleeding for serum collection. Ionized calcium was measured by the Ciba–Corning 634 Ca2+/pH analyzer (Ciba–Corning Diagnostics Corp., Medfield, MA, USA). Intact immuno reactive PTH was measured in duplicate using ELISA (Immuno his, Inc., San Clemente, CA, USA). Serum mouse TRAP5B (Mouse TRAP Assay), PINP, and CTX (Rat-LAPS EIA) were measured in duplicate using ELISA (Immunodiagnostic Systems Ltd, Boldon, UK). Statistical analysis was performed using the Student's t-test, and P values <0.05 were accepted as significant.
Low-calcium diet and PTH injections

In some experiments, mice were fed a low-calcium diet (0.02% calcium, 0.4% phosphorous, Teklad, Harlan, Madison, WI, USA) for 2 weeks prior to calcium measurement. Mice were injected s.c. with 300 µg/kg of human PTH (1–34; MGH Peptide Core Facility). Blood was collected by tail bleeding for ionized calcium measurement before and 1 h after PTH injections using a Ca²⁺/pH analyzer.

RNA extraction and purification

RNA was isolated from long bones of Ocy–PPRcKO mice and littermate controls following sequential collagenase and EDTA digestions to remove endosteal and periosteal osteoblasts and bone marrow cells. For detailed protocol, see Supplementary Methods, see section on supplementary data given at the end of this article. Femurs were washed with 1 ml of RNA-later solution (Ambion, Austin, TX, USA) prior to RNA isolation by Trizol. RNA was extracted from the bone by homogenizing it in Trizol (Invitrogen) using a tissue homogenizer for 1 min on ice. The RNA was then extracted from the homogenate according to the manufacturer’s recommendations. RNA quality and quantity were ascertained by u.v. spectrophotometry (NanoDrop 8000, Thermo Fisher, Waltham, MA, USA).

Quantitative reverse transcription-PCR

Reverse transcription was performed on 0.5–1 µg of DNase-treated total RNA and oligoDT primers using Omniscript (Invitrogen) according to the manufacturer’s instructions. Quantitative PCR (qPCR) was performed using the QuantiTect SYBR Green PCR kit (Qiagen) and the DNA Engine Opticon 2 qPCR system (MJ Research, Inc., Waltham, MA, USA). The comparative method, using the ΔΔCt formula, was used to determine the RNA relative expression, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) being used for normalizing. Statistical analysis was performed using the Student’s t-test, and P values <0.05 were accepted as significant. Primer sequences are described in Supplementary Methods, see section on supplementary data given at the end of this article.

cAMP measurement

Tibiae were isolated from 4- to 6-week-old mice. Briefly, tibiae were dissected and cleaned of adherent tissues. Distal and proximal epiphyses were removed, and the bone marrow was flushed out using 2–3 ml of ocMEM supplemented with 0.1% BSA and 25 mM HEPES (pH 7.4). The remaining diaphyseal-enriched region of the bones was cut into three pieces and sequentially digested as described above for RNA isolation. Each piece was then placed in ice-cold cAMP-assay buffer (DMEM containing 10 mM HEPES, 0.1% heat-inactivated BSA, and 1 mM isobutylmethylxantine). Bone pieces were then incubated in cAMP-assay buffer with the appropriate treatment at 37 °C for 15 min. The three pieces of each tibia were incubated with vehicle alone (assay buffer), 100 nM human PTH (1–34), or 0.1 µM forskolin. At the end of the incubation, the reaction was terminated by quickly removing the bones and placing them in 0.3 ml of cold 90% 2-propanol in 0.5 M HCl. Bones were then incubated for 16–18 h at 4 °C. Propanol extraction was repeated, and the combined extracts were evaporated by vacuum centrifugation. The dried extracts were redissolved in acetate buffer (50 mM Na acetate/0.05% Na azide, pH 6.2) for measurement of cAMP by a specific RIA, as previously described (Divi et al. 2005). Bones were washed twice with 0.5 ml acetone and once with 0.5 ml ether and were air-dried and weighed. The results were normalized for the bone weight, and the data were expressed as picomole of cAMP produced per mg of dry bone. Each experiment was repeated at least three times.

Fluorescence-activated cell sorting analysis

In some experiments, homozygous Dmp1-GFP transgenic mice (provided by Drs Ivo Kalajzic and David W Rowe, University of Connecticut Health Center, Hartford, CN, USA) were crossed with PPRcKO mice to generate Ocy–PPRcKO and WT mice that express green fluorescent protein (GFP) under the 8 kb–Dmp1 promoter. Calvarial cells obtained from mice exhibiting the same genotype were pooled, and GFP-expressing cells (enriched in osteocytes) were separated from GFP-negative cells (enriched in osteoblasts) by immediately subjecting the cell suspension to fluorescence-activated sorting using an Aria flow cytometer (BD Biosciences, San Jose, CA, USA) at the Massachusetts General Hospital Flow Cytometry Core Facility.

Bone mineral density DEXA

Mouse BMD was measured by DEXA using a Lunar PIXImus II densitometer (GE Medical System Luna, Madison, WI, USA). In brief, mice were killed; right femurs and vertebral bodies (region L4–L5) were used to determine bone mineral content (g) and BMD (g/cm²). Mouse BMD was measured by DEXA using a Lunar PIXImus II densitometer (GE Medical System Luna, Madison, WI, USA). In brief, mice were killed; right femurs and vertebral bodies were fixed overnight in 10% buffered formalin and then preserved in 70% ethanol. The excised right femurs and vertebral bodies (region L4–L5) were used to determine bone mineral content (g) and BMD (g/cm²).

Micro-computed tomography (µCT) analysis

Assessment of bone morphology and microarchitecture was performed using a desktop high-resolution µCT (µCT40, Scanco Medical, Brütisellen, Switzerland), as described previously (Bouxsein et al. 2010). In brief, the distal femoral metaphysis and L5 vertebral body were scanned using an X-ray energy of 70 keV, integration time of 200 ms, and a 12 µm isotropic voxel size. For the cancellous bone region, we assessed bone volume fraction (BV/TV, %), trabecular
thickness (Tb.Th, μm), trabecular separation (Tb.Sp, μm), trabecular number (Tb.N, 1/mm), connectivity density (Conn.D, 1/mm³), and structure model index (SMI).

**Statistical analysis**

All data are presented as mean ± s.d. The statistical significance of differences between groups was determined by Student’s t-test. P values < 0.05 were accepted as significant.

**Results**

*Generation and characterization of 10 kb-Dmp1-Cre-ERT2 transgenic mice*

10 kb-Dmp1-Cre-ERT2 animals were generated as described in Materials and Methods. To test the efficiency and specificity of the lines generated, the 10 kb-Dmp1-Cre-ERT2 animals were intercrossed with the homozygous ROSA26R reporter mice (Soriano 1999). ROSA26R mice have a floxed stop-cassette upstream of the LacZ gene. In the presence of Cre-recombinase activity, the stop cassette is excised, LacZ is expressed, and β-galactosidase (β-gal) activity can be detected using the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining procedure. Three-day-old 10 kb-Dmp1-Cre-ERT2/ROSA26R mice were exposed to i.p. injections of tamoxifen (50–60 μg/g) at days 3, 5, and 7, and then maintained on a weekly injection of 5–10 μg/g of tamoxifen for an additional 2 weeks. After X-gal staining, calvarial bones and hindlimbs were embedded in paraffin and stained with eosin. In some experiments, kidneys were also evaluated for X-gal staining. As shown in Fig. 2A–C for calvaria (Dp1-cre positive: A top panel and B at 40× and DMP1-Cre negative: A bottom panel and C at 40×) and Fig. 2D–F for long bone (femur of DMP1-Cre positive: D top panel and E at 40× and DMP1-Cre negative: D bottom panel and F at 40×) for one founder, most of the osteocytes were positive for the X-gal staining (Fig. 2A and D, top panels and B and E), while no staining was present in Dmp1-Cre-negative controls (Fig. 2A and D, bottom panels and C and F), in kidneys, bone marrow, or skeletal muscle of DMP1-Cre positive (Supplemental Figure 2A, C, and D, see section on supplementary data given at the end of this article), or in Dmp1-Cre-positive animals not treated with tamoxifen (data not shown). Under this regimen, we calculated that 77 ± 9%
of osteocytes stained blue for X-gal activity (81.3 ± 3% in calvaria; 72.6 ± 6% in tibiae, and 78.5 ± 1.1% in femurs), indicating excision of the flox-stop codon in the lacZ promoter region in these cells. In addition, to further assess the specificity of the promoter, we calculated the percentage of X-gal-positive endosteal and periosteal osteoblasts. Under the regimen described above, 8.6 ± 6.8% of calvaria osteoblasts and 1.1 ± 1% of femoral osteoblasts were positive for X-gal staining, indicating that only a minority of mature osteoblasts express the 10 kb Dmp1-promoter. Initial analysis showed that two of the six lines demonstrated highly penetrant excision of the ROSA26R stop cassette, and we focused our experiments on these two lines.

In another set of experiments, 3- to 4-week-old mice were injected with 5–10 μg/g of tamoxifen three times a week for 2–4 weeks before analyzing PPR expression by real-time qPCR. The efficiency of PPR ablation, as assessed by qPCR, in mRNA derived from collagenase-digested long bones, with this tamoxifen regimen was again around 60–70%, and was comparable to the one described above (Fig. 3A), indicating that the receptor can be successfully ablated in adult mice.

**DMP1-Cre-ERT2 model effectively and specifically decreases PPR expression in osteocytes**

10 kb–Dmp1-Cre-ERT2 mice were crossed with homozygous PPR-floxed mice, as described in Materials and Methods, to obtain double heterozygotes that were in turn mated with PPR+/− to obtain 10 kb–Dmp1-Cre-ERT2:PPR+/− or “Ocy-PPR−/−” mice and littermate controls.

10 kb–Dmp1-Cre-ERT2:PPR+/− females were time mated with PPR+/− males and injected i.p. with 2 μg of tamoxifen at 17.5 days post-coitus. Pups were killed 1 day after birth, genotyped, and long bones were excised and analyzed histologically. As anticipated, given the short interval between tamoxifen induction and killing, we did not observe any macroscopic phenotype in the Ocy-PPR−/− mice and long bones of these mice compared to littermate controls. Immunohistochemical analysis using anti-sclerostin antibody showed no differences in expression between Ocy-PPR−/− and littermate calvaria and long bones after 3 days of exposure to tamoxifen.

To ascertain the successful deletion of PPR from osteocytes in response to the tamoxifen regimen, we first carried out qPCR analysis for PPR expression. Mice, both Ocy-PPR−/− and littermate controls, were injected with tamoxifen at days 3, 5, and 7 and then weekly, as described above. We performed real-time qPCR for the PPR using primers specific for the floxed E1 region. As shown in Fig. 3A, Ocy-PPR−/− femurs had a 64% reduction in PPR expression compared to littermate controls (results are expressed as percentage of control and are normalized by GAPDH). To further evaluate PPR ablation in a pure osteocyte population, we generated Ocy-PPR−/− and control mice that express GFP under the 8 kb-DMP1 promoter by mating the PPR+/− mice with the 8 kb-DMP1-GFP mice. Newborn pups were genotyped and then injected with 50 μg/g of tamoxifen at days 2 and 4 postnatally. Primary calvarial cells were then isolated by eight sequential collagenase and EDTA digestions, and cells derived from fractions 3 to 8 were sorted, as described in Materials and Methods. As shown in Fig. 3B, PPR expression was reduced by 80% in GFP-positive cells from Ocy-PPR−/− animals compared to littermate controls (control 100 ± 8.8%, Ocy-PPR−/− 23.1 ± 0.27%, control-GFP 48.1 ± 17.5%, and Ocy-PPR−/−:GFP neg 52.7 ± 8.8%, results are expressed as percentage of control and are normalized for GAPDH expression). There was no significant difference in PPR expression in GFP-negative cells compared to littermate controls (Ctrl, n = 3) and Ocy-PPR−/− (n = 3) mice. Data are expressed as relative mRNA levels, normalized by GAPDH, and expressed as percentage of controls. PPR expression in the Ocy-PPR−/− is decreased to 36.1 ± 7.7% of controls. Data are expressed as mean ± S.D. of triplicates. * P < 0.05. Representative experiment.

**Figure 3** PPR ablation in osteocytes. Real-time qPCR from (A) RNA extracted from femurs of 5-week-old littermate controls (Ctrl, n = 3) and Ocy-PPR−/− (n = 3) mice. Data are expressed as relative mRNA levels, normalized by GAPDH, and expressed as percentage of controls. PPR expression in the Ocy-PPR−/− is decreased to 36.1 ± 7.7% of controls. Data are expressed as mean ± S.D. of triplicates. * P < 0.05. Representative experiment. (B) Real-time qPCR of RNA extracted from sorted GFP-positive and GFP-negative calvarial cells derived from Ocy-PPR−/−:PPR−/− and control littermates (Ctrl). Data are expressed as percentage of controls and are corrected for GAPDH. PPR expression in GFP-negative Ocy-PPR−/− osteocytes (PPR-cKO) is decreased by 80% compared to littermate control GFP-positive osteocytes (Ctrl) (two left-most bars). PPR expression in GFP-negative cells (osteoblasts) from controls and Ocy-PPR−/− animals is not significantly different (two right-most bars). Data are expressed as mean ± S.D. of triplicates. * P < 0.05. Representative experiment. (C) cAMP accumulation in bone marrow-deprived, collagenase-digested tibial explants. Right tibiae of 5-week-old Ocy-PPR−/− (n = 3, black bars) and control littermates (n = 4, gray bars) were assayed for their in vitro responsiveness to 100 nM hPTH (1–34) or 10 μM forskolin (Fsk). Data are expressed as pmol of cAMP/mg of bone. Data are expressed as mean ± S.D. of triplicates. ** * P < 0.002 Ocy-PPR−/− versus WT. (D) cAMP accumulation in primary calvarial cells. Primary calvarial cells from 4- to 5-day-old pups were assayed for their in vitro responsiveness to 100 nM hPTH (1–34) or 10 μM forskolin (Fsk). Data are expressed as pmol cAMP/well. Data are expressed as mean ± S.D. of triplicates.
compared to Ocy-PPR\(^{\text{cKO}}\) and control animals (osteoblasts). These results demonstrate that the PTH receptor ablation is specific for osteocytes and that it can be induced upon tamoxifen administration. Interestingly, receptor expression in control GFP-positive cells (osteocytes) is twofold that of GFP-negative cells (osteoblasts), suggesting higher receptor expression in osteocytes than osteoblasts.

In addition, allele-specific DNA recombination for the PPR locus was evaluated by PCR using primer spanning the floxed E1 region. As shown in Fig. 1C, the DNA recombination (lower band) was observed only in the skeletal tissues such as long bones (bone marrow–depleted femurs) and calvaria, whereas no recombination was detected in kidney, spleen, muscle, and liver, indicating that the PPR ablation was specific for osteocytes.

**Functional consequences of deletion of the PPR gene in osteocytes**

To assess functional ablation of the PPR in osteocytes, we measured PTH-induced cAMP accumulation in osteoblast/marrow cell-depleted bone explants, enriched in osteocytes, from Ocy-PPR\(^{\text{cKO}}\) and littermate controls. Tibiae were isolated from 4- to 5-week-old Ocy-PPR\(^{\text{cKO}}\) and control littermates injected with tamoxifen (50–60 μg/g) at days 3, 5, and 7 postnatally and then weekly with 5–10 μg/g, as described for the ROSA26R experiments. As shown in Fig. 3C, there was a statistically significant reduction (86-8%) of cAMP accumulation in response to PTH in Ocy-PPR\(^{\text{cKO}}\) osteocyte-enriched bone fragments, compared to littermate controls (Ocy-PPR\(^{\text{cKO}}\) = 6.16 ± 9.6 picomol/mg bone versus control = 46.9 ± 16.2 picomol/mg bone; \(P<0.05\)). As a positive control, no difference was detected in the response to forskolin (Ocy-PPR \(^{\text{cKO}}\) = 43.4 ± 13.8 picomol/mg bone versus littermate controls = 36.7 ± 23.9 picomol/mg bone) indicating an intact adenylyl cyclase machinery in these osteocyte-enriched bone fragments. To assess that PPR ablation was confined to osteocytes and not to osteoblasts, we performed cAMP accumulation in response to PTH in primary calvarial cells (predominantly composed of osteoblasts) isolated from 4- to 5-day-old

![Figure 4](http://dx.doi.org/10.1530/JOE-10-0308)
Ocy-PPR<sup>cKO</sup> and littermate controls. As shown in Fig. 3D, cAMP accumulation in calvarial osteoblasts from Ocy-PPR<sup>cKO</sup> was indistinguishable from littermate controls, indicating that PPR ablation was specific for osteocytes.

Moreover, primary calvaria cells isolated from 4- to 5-day-old Ocy-PPR<sup>cKO</sup> and littermate controls were treated with 100 nM hPTH (1–34) for 4 h, and RANKL expression was assessed by qPCR. As expected, RANKL was significantly up-regulated in both Ocy-PPR<sup>cKO</sup> and littermate controls (7.6 ± 2.5- and 18.5 ± 1.8-fold respectively) indicating that osteoblasts in Ocy-PPR<sup>cKO</sup> do have intact PTH responsiveness.

To further assess the phenotype of mice lacking PPR in osteocytes, we proceeded to analyze adult mice. Four- to six-week-old mice were injected with tamoxifen (20–30 μg/g) three times a week for 4 weeks. At the end of the 4 weeks, mice were acutely challenged with a single s.c. injection of human PTH (1–34; 300 μg/kg) and killed after 1 h. As shown in Fig. 4A, sclerostin expression (assessed immunohistochemically) was markedly reduced in the tibiae of littermate control animals following PTH administration, but was readily detectable in tibiae of Ocy-PPR<sup>cKO</sup> animals (Fig. 4B), demonstrating no PTH-induced sclerostin inhibition in Ocy-PPR<sup>cKO</sup> mice. We also analyzed Sost mRNA expression by real-time PCR, with Ocy-PPR<sup>cKO</sup> mice showing no acute reduction of Sost expression after PTH injection, whereas controls showed more than a 40% acute down-regulation of Sost mRNA expression (Fig. 4C). Moreover, Ocy-PPR<sup>cKO</sup> also displayed a significant overall increase in Sost expression of more than 54%, as detected by real-time qPCR (control = 90 ± 20 versus Ocy-PPR<sup>cKO</sup> 147 ± 33%, n = 9; P < 0.01; Fig. 4D) as well as a suppression of Wnt-signaling pathways as demonstrated by a significant reduction of Axin-2 mRNA (control = 109 ± 47 versus Ocy-PPR<sup>cKO</sup> 65 ± 25%, n = 7; P < 0.05; Fig. 4E).

We then analyzed the bone phenotype of Ocy-PPR<sup>cKO</sup> and littermate controls. Hematoxylin and eosin staining of decalcified tibiae demonstrated a reduction of trabecular bone and a delay in the secondary ossification center. Furthermore, Von Kossa staining of plastic (Fig. 5A and B) sections confirmed a reduction of the trabecular bone. We measured BMD in isolated femurs and vertebral bodies (L4–L5) by DEXA (Piximus) in both females and males. In Ocy-PPR<sup>cKO</sup> females, there was a significant reduction of both vertebral and femoral BMD (Fig. 5C and D). Males also tended to have a lower BMD compared to littermate controls, but this was not significantly different.

To investigate whether the low BMD in Ocy-PPR<sup>cKO</sup> mice was due to a reduction in osteoblast activity, an increase in osteoclast activity, or a combination of both, we measured markers of bone resorption and bone formation in control and Ocy-PPR<sup>cKO</sup> mice, as shown in Table 1. Serum levels of collagen type I fragment were indistinguishable between Ocy-PPR<sup>cKO</sup> and control littermates (PINP: controls, n = 17, 70.3 ± 51.2 ng/ml; Ocy-PPR<sup>cKO</sup>, n = 14, 74.9 ± 55.2 ng/ml), whereas CTX was slightly elevated in KO mice compared to controls (controls, n = 9, 9.07 ± 5.03 ng/ml; Ocy-PPR<sup>cKO</sup>, n = 11, 13.8 ± 6.7 ng/ml) although they were not statistically different. Finally, TRAP5b was not significantly different between control and Ocy-PPR<sup>cKO</sup> mice (TRAP5b: controls, n = 16, 1.51 ± 0.78 U/l; Ocy-PPR<sup>cKO</sup>, n = 12, 1.08 ± 0.44 U/l), suggesting that rates of bone modeling and remodeling are relatively normal in these mice. To further evaluate the bone architecture of...
Low-calcium diet

Table 1 Serum markers of bone formation and bone resorption in controls and Ocy-PPR<sup>cko</sup> animals. Data are expressed as average ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Ocy-PPR&lt;sup&gt;cko&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>PINP (ng/ml)</td>
<td>70.3 ± 51.2</td>
<td>74.9 ± 55.2</td>
</tr>
<tr>
<td>n = 17</td>
<td></td>
<td>n = 14</td>
</tr>
<tr>
<td>CTX (ng/ml)</td>
<td>9.07 ± 5.03</td>
<td>13.8 ± 6.7</td>
</tr>
<tr>
<td>n = 9</td>
<td></td>
<td>n = 11</td>
</tr>
<tr>
<td>TRAP5B (U/l)</td>
<td>1.51 ± 0.78</td>
<td>1.08 ± 0.44</td>
</tr>
<tr>
<td>n = 16</td>
<td></td>
<td>n = 12</td>
</tr>
<tr>
<td>Low-calcium</td>
<td>1.24 ± 0.03</td>
<td>1.07 ± 0.08*</td>
</tr>
<tr>
<td>diet (mol/l)</td>
<td>8.45 ± 0.295</td>
<td>7.93 ± 0.317†</td>
</tr>
<tr>
<td>n = 11</td>
<td></td>
<td>n = 12</td>
</tr>
</tbody>
</table>

*P < 0.05, †P < 0.0001.

Ocy-PPR<sup>cko</sup> mice, we performed μCT analysis of trabecular bone both in males and in females. As shown in Table 2, Ocy-PPR<sup>cko</sup> tended to have lower trabecular bone compared to controls (reduced connectivity density, SMI, and trabeculae number), although this was not statistically significant indicating that ablation of PPR in osteocytes induces mild skeletal abnormalities, as assessed by μCT.

Finally, to further analyze PPR KO in osteocytes, we subjected 6-week-old mice to 2 weeks of a low-calcium diet (0.02% calcium) to induce secondary hyperparathyroidism. Mice were pre-treated with tamoxifen (20–30 μg/g three times a week) 1 week before starting the diet and during the 2 weeks of the diet. Ionized calcium was measured at the beginning of the study (before starting tamoxifen injections) and was not significantly different between groups. At the end of the 3 weeks, mice were killed, and blood and skeletal specimens were collected and analyzed as described above. Plasma PTH levels in these mice were elevated (190.7 ± 82 and 294.8 ± 124 pg/ml for control and Ocy-PPR<sup>cko</sup> respectively), confirming that secondary hyperparathyroidism had been achieved. Interestingly, Ocy-PPR<sup>cko</sup> animals displayed significantly lower ionized and total calcium levels compared to littermate controls (which included DMP1-Cre:PPR<sup>fl/fl</sup> not treated with tamoxifen and tamoxifen-treated DMP1-Cre:PPR<sup>B/B</sup> and PPR<sup>B/B</sup>; Fig. 6A and B and Table 1). Similar results were also obtained in a separate set of experiments using younger mice treated with tamoxifen from day 3 postnatally to ablate PPRs at an earlier time when data were not shown. In these mice, tamoxifen was administered at days 3, 5, 7, 14, and 21 postnatally, and mice were fed a low-calcium diet at day 21 for an additional 2 weeks. In addition, PPR expression in the kidneys of these animals was evaluated to ascertain that the receptor was fully expressed in this organ. We did not observe any difference in PPR mRNA levels between Ocy-PPR<sup>cko</sup> and littermate controls, indicating that the lower serum calcium level was not likely due to altered renal responsiveness to circulating PTH.

Table 2 Trabecular bone architecture of vertebral body (L5) and femoral distal end was analyzed by μCT at 8–12 weeks of age in female and male mice. Data are expressed as average ± S.D.

Discussion

Osteocytes are believed to play a key role in skeletal mechanosensing whereby they modulate bone modeling and remodeling in response to changes in shear or strain forces. Moreover, recent studies (O’Brien et al. 2006) demonstrated that mice in which the constitutively active PPR is expressed in osteocytes display a dramatic increase in trabecular bone, indicating an important role of PTH and the PPR in osteocytes.

To investigate the role of PTH and related molecules in osteocytes in vivo, we have generated mice in which the receptor is selectively and temporary ablated in these cells. We used the 9.6 kb of the DMP1-promoter to drive a tamoxifen-inducible Cre-recombinase in mice in which exon-1 of the PPR is flanked by lox-P sites. Ocy-PPR<sup>cko</sup> mice were born with the expected Mendelian ratio, were viable, and were indistinguishable from littermate controls, indicating that the Cre-recombinase was not expressed in the absence of tamoxifen induction. The 10 kb-DMP1-CreERT2 recombinase was exclusively and specifically expressed in osteocytes and in rare bone lining cells (<8% of total endosteal osteoblasts), as demonstrated by ROSA26 experiments. The promoter was readily inducible, and the efficiency of Cre-recombinase excision was around 70–80%. The transgene was heritable as assessed by Southern blot analysis of F2 generation of the original founders.

Initial characterization of the Ocy-PPR<sup>cko</sup> mice revealed a slight growth retardation (data not shown) that was associated with a mild osteopenic phenotype, as assessed by histological analysis of long bones and by DEXA measurement. Interestingly, vertebral and femoral BMD (as assessed by
and 6) and inhibits \( \text{wnt}^{-}/\text{catenin}^{-} \) signaling. In our mouse model, lack of PPR completely blocks the osteopenia of osteocytes. These findings are in agreement with recent studies of O’Brien et al. (2008), who demonstrated that mice expressing a constitutively active PPR receptor (Schipani et al. 1995, Calvi et al. 2001) specifically in osteocytes have a dramatic decrease in \( \text{Sost} \) expression and a 40% increase in BMD. Moreover, these transgenic mice could potentially rescue the osteopenic phenotype of \( \text{Lrp5}^{-/-} \) animals, if Lp5 and/or 6 play direct roles in osteocyte biology. This would clearly indicate that osteocyte-derived sclerostin is indeed a negative regulator of \( \text{wnt}^{-}/\text{beta-catenin}^{-} \) signaling. Recently, Yadav et al. (2008) reported that Lrp5 acts on bone via the regulation of serotonin synthesis in the gut and that the main action of Lrp5 is in the duodenum. The authors demonstrated that LRP5 acts on serotonin-producing cells in the gut by blocking an enzyme that converts the amino acid tryptophan to serotonin. This new connection between bone and gut-derived serotonin, although shifting the action of LRP5 from osteoblasts to the duodenum, does not exclude that osteocyte-secreted sclerostin functions as a negative regulator of the \( \text{wnt}^{-}/\text{beta-catenin}^{-} \) pathways in osteoblasts. Additional studies will be needed to investigate the role of serotonin in osteocytes.

Interestingly, the Ocy-PPR\(^{-/-} \) mice also demonstrated hypocalcemia when challenged with a low-calcium diet, suggesting an important role of osteocytes in regulating calcium ion homeostasis. Normally, this low-calcium diet is easily corrected by increased PTH production and increased remodeling to bring the calcium back to normal. However, when osteocytes lack a functional PPR, this does not happen. This suggests that the osteocyte might be important in regulating calcium through PTH signaling. The role of osteocytes in controlling calcium homeostasis is controversial. Osteocytes clearly express high levels of receptors for PTH, and the osteocyte lacuno-canalicular network constitutes a microcirculatory system for calcium-containing periosteocytic fluid that is distinct from blood plasma and lymph fluid (Knothe Tate 2003). The osteocyte network represents an enormous surface area over which the cells interface with the surrounding matrix, useful for matrix mineral access (Cullinane 2002). Osteocytes can serve as key regulators of calcium homeostasis, are distributed widely in bone matrix, and are ideally situated to engage in systemic calcium homeostasis. Here, we report that mice lacking the PPR specifically in osteocytes have an impaired calcium homeostasis when subjected to a low-calcium diet and fail to properly respond to secondary hyperparathyroidism. These data support this hypothesis. However, it should be considered that three organs participate in supplying calcium to the blood: i) the small intestine, through the absorption of calcium from the diet (vitamin D-dependent); ii) the kidney, through the reabsorption of calcium from the glomerular filtrate; and iii) the skeleton, with the release of calcium from the bone. PTH acts directly, or indirectly, on these target organs, but the mechanism by which the hormone rapidly (within 1–2 h) increases blood calcium concentration is not completely understood. Several hypotheses have been
postulated, which include an increase in renal calcium reabsorption from the kidney, an increase in bone resorption, and/or an increase in intestinal calcium absorption. Finally, our data support the theory that a release of calcium from the osteocytic lacuno-canalicular network may also be very important in this process, and regulated by PTH.

In summary, we have generated mice in which the PPR can be specifically ablated from osteocytes upon tamoxifen administration. These mice displayed mild osteopenia, tonic increase in Sost and sclerostin expression, and lack of PTH-induced Sost and sclerostin suppression. Moreover, Ocy-PPR-ko mice were unable to maintain a normal calcium plasma level when subjected to a low-calcium diet.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-10-0308.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

P D P, W F P, and F R B planned and designed the experiments; W F P, K J B, and P D P performed the experiments; I T performed the immunohistochemical analysis. T K provided the PPRΔ1−Δ16 mice; S H E provided the 10 kb-Dmp1-Cre-ERT2 plasmid; P D P and W F P analyzed the data. P D P, W F P, and F R B wrote the manuscript.

References


