Parathyroid Hormone (PTH)/PTH-related Peptide Type 1 Receptor (PPR) Signaling in Osteocytes Regulates Anabolic and Catabolic Skeletal Responses to PTH

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*Running title: Osteocytes regulate anabolic and catabolic responses to PTH

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Keywords: Osteocyte; PTH; anabolic; catabolic

Background: Osteocytes, the most abundant cells in adult bone, express PPR.

Results: Mice with constitutive PPR deletion in osteocytes demonstrate blunted anabolic and catabolic bone responses, and inability to recruit osteoblasts and osteoclasts upon PTH administration.

Conclusion: PPR in osteocytes is needed for a full skeletal response to PTH administration

Significance: PPR signaling in osteocytes is necessary for PTH-driven anabolic effects during osteoporosis therapy.

Introduction

Parathyroid hormone (PTH) is the only FDA approved anabolic agent to treat osteoporosis; however cellular targets of PTH action in bone remain controversial. PTH modulates bone turnover by binding to the PTH/PTH-related Peptide (PTHrP) type 1 receptor (PPR), a G-protein-coupled-receptor highly expressed in bone and kidneys. Osteocytes, the most abundant cells in adult bone, also express PPR. However, physiological relevance of PPR signaling in osteocytes remains to be elucidated. Towards this goal, we generated mice with PPR deletion in osteocytes (Ocy-PPRKO). Skeletal analysis of these mice revealed a significant increase in bone mineral density, and trabecular and cortical bone parameters. Osteoblast activities were reduced in these animals, as demonstrated by decrease Col1α1 mRNA and RANKL expression. Importantly, when subjected to anabolic or catabolic PTH regimen, Ocy-PPRKO animals demonstrated blunted skeletal responses. PTH failed to suppress SOST/Sclerostin or induce RANKL expression in Ocy-PPRKO animals compared to controls. In vitro osteoclastogenesis was significantly impaired in Ocy-PPRKO upon PTH administration, indicating that osteocytes control osteoclast formation through a PPR-mediated mechanism. Taken together, these data indicate that PPR signaling in osteocytes is required for bone remodeling and receptor signaling in osteocytes is needed for anabolic and catabolic skeletal responses.

Summary

Parathyroid hormone (PTH) is an 84 amino acid single-chain polypeptide synthesized and secreted by the parathyroid glands in a calcium-regulated manner. PTH maintains serum calcium homeostasis, controls renal phosphate resorption, and vitamin D 1α-hydroxylation by activating the PTH/PTH-related Peptide (PTHrP) type 1 receptor (PPR), a G-protein coupled receptor capable of activating multiple G protein-coupled pathways, including those signaling through cAMP/Protein kinase A (PKA), phospholipase C (PLC)/Protein kinase C (PKC), and non-PLC-dependent PKC and Ca++ (1). The first 34 amino-acids of PTH are necessary and sufficient to fully activate the receptor (1), and the amino-terminal region received FDA approval in 2002 as the first anabolic agent to treat osteoporosis, a health disorder estimated to affect 200 million people worldwide (2)(3). In a 2002
prevalence report by the National Osteoporosis Foundation, it was estimated that over 10 million people suffered from osteoporosis in the US, and ~80% of these were women. According to the World Health Organization, osteoporotic fractures are a major cause of morbidity and disability in older people, and hip fractures could lead to premature death (3). Therefore, understanding the cellular targets of PTH action in bone is central for the development of future osteoporosis therapeutics.

The PPR is abundantly expressed in bone and kidney, and its expression has also been reported in a variety of other tissues where it likely reflects the local paracrine role of PTHrP (4). In bone, PPR is expressed in cells of the osteoblast lineage, including osteocytes. Osteocytes are terminally differentiated osteoblasts and comprise 90-95% of all cells in adult bone (5). They survive for decades in their mineralized microenvironment and have the longest lifespan among all skeletal cells. Recent evidence suggests direct or indirect interactions of osteocytes with organs, such as kidneys, muscles, heart, and bone, through various molecules, such as FGF-23 and sclerostin (5, 6). Moreover, osteocytes might play important roles in diseases, such as hypophosphatemic rickets, osteopenia, and osteopetrosis (5, 6). Emerging literature also supports crucial roles of osteocytes in normal physiological processes, such as lactation and bone modeling and remodeling (4, 7, 8).

It has been previously proposed that osteocytes may mediate, in part, the anabolic effects of PTH by suppressing Sclerostin expression through a Mef2C-mediated pathway (9–12). Sclerostin, encoded by the SOST gene, is secreted by osteocytes and inhibits osteoblast function and bone formation by binding to the low density lipoprotein receptor-5 and 6 and suppressing the Wnt signaling pathway (13–16). Moreover, PPR on osteoblasts is known to regulate RANKL expression, and thereby control osteoclast formation and bone resorption (17, 18). Importantly, activities of both osteoblasts and osteoclasts are needed for bone remodeling, and anabolic and catabolic bone responses.

Recently osteocytes have been shown to modulate osteoclast function(s) through a RANKL-mediated mechanism (19, 20). Mice lacking RANKL specifically in osteocytes showed an increase in bone mineral density and osteopetrosis (19, 20), suggesting a role of osteocyte-derived RANKL in bone remodeling.

We previously generated transgenic mice in which the PPR was conditionally ablated from osteocytes post-natally upon tamoxifen injection (10Kb DMP1-Cre-Ert2). These mice demonstrated mild osteopenia by 4-6 weeks of age, characterized by a reduction in trabecular bone, accompanied by a tonic elevation of SOST and Sclerostin, and a lack of PTH-induced SOST and Sclerostin suppression (4). To further investigate the physiological role of PPR in osteocytes, we have generated mice with constitutive PPR ablation in osteocytes by using the constitutive 10Kb DMP1 promoter to drive Cre recombinase expression in PPR-floxed mice. Osteocyte-PPR KO mice (Ocy-PPRKO) showed a significant increase in bone mineral density (BMD), and trabecular and cortical bone parameters at 12 weeks of age, indicating that PPR on osteocytes is required for normal bone remodeling. Interestingly Ocy-PPRKO displayed normal serum calcium, phosphate, and PTH, suggesting that under physiological conditions PPR signaling in osteocytes is not needed to maintain normal mineral homeostasis. When subjected to intermittent or continuous PTH administration, Ocy-PPRKO generated blunted anabolic and catabolic skeletal responses, indicating that PPR signaling in osteocytes is necessary for full skeletal responses. Upon PTH administration, Ocy-PPRKO failed to increase Collagen-1α1 (Col1α1) mRNA expression in osteoblasts or suppress SOST/Sclerostin expression in osteocytes. Moreover, reduced RANKL expression in osteocytes was observed in Ocy-PPRKO, and PTH failed to induce a catabolic response in these animals. All together, these data highlight the necessity of PPR signaling in osteocytes for proper bone remodeling and full skeletal responses to PTH.

**Experimental procedures**

**Mice**

To generate Ocy-PPRKO animals, mice in which 10Kb DMP1 promoter drives the expression of Cre-recombinase (10Kb DMP1-Cre, kindly provided by Dr. J. Feng) were mated with mice in which the E1 exon of the PPR gene is flanked by lox-P sites (control, kindly provided by Dr. T.
Kobayashi). The genotypes of the mice were determined by PCR analysis of the genomic DNA extracted from tail biopsies. For the 10Kb Dmp1-Cre transgene, the forward Cre primer (5’-CGCGTCTGGCAGTAAAACATATC-3’) and the reverse Cre primer (5’-CCCACCCTCAGTGAGATATC-3’) were used to generate a PCR product of approximately 400bp. 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 GCT GAC CTC TCT GAA AGA ATG T -3’) were used, which recognized the sequence spanning the 3’lox-P site, as previously reported (4). Wild-type and mutant alleles give ~ 210 bp and 290 bp products, respectively.

Institutional Animal Care and Use Committee, Subcommittee on Research Animal Care, at Massachusetts General Hospital approved all animal protocols.

Allele specific DNA recombination was performed on DNA isolated from osteocyte-enriched long bones (generated by sequential collagenase and EDTA digestions as previously described in (4)), calvaria, kidney, liver, lung, heart, skeletal muscle, and spleen. Multiplex PCR analysis was performed using allele specific primers, P1, P2 and P3 (5’ACA TGG CCA TGC CTT CTT CTA AGG CTA -3’) and following the manufacturer protocol (Quiagen™ Multiplex PCR kit, Quiagen Valencia, CA USA)

**Histology**

Tibiae and vertebrae were fixed in 10% formalin/PBS solution at 4°C, ON, decalcified in 20% EDTA pH 8.0 for 7-15 days, processed, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin, or used for immunohistochemistry. Undecalcified femurs, fixed in 70% ethanol, were embedded in methylmethacrylate (Aldrich Chemical Co, Milwaukee, WI, USA), sectioned with a diamond-embedded wire saw, and stained by the Von Kossa method.

**Immunohistochemistry (IHC)**

Immunohistochemical RANKL expression detection was performed as described in (19) with minor modifications. On deparaffinized tibiae, endogenous peroxidase activity was inhibited by 3% H₂O₂ treatment for 10 min. Subsequently, antigen retrieval was performed with Tris-EDTA buffer (10mM Tris base, 1mM EDTA, 0.05% Tween-20, pH 9.0) in a boiling water bath at 95°C for 12 min. Slides were cooled to RT and blocked with TNB (TSA ™ Biotin Tyramide Kit, Perkin Elmer, Waltham MA, USA) for 30 min, RT, and incubated with anti-RANKL antibody (N-I9, Santa Cruz Biotechnology), 4°C, ON in Can Get Signal immunostain Solution A (Toyobo, Japan). Slides were washed, and incubated with Biotin-SP-conjugated AffiniPure Rabbit Anti-Goat IgG (H+L), (Jackson ImmunoResearch) for 30 min at RT. Afterwards, the slides were washed and incubated for 30 min with streptavidin (SA) conjugated horseradish peroxidase (HRP) and tyramide following the manufacturer’s protocol (TSA™ Biotin Tyramide Kit), developed with a 3,3’-diaminobenzidine substrate-chromogen system (Vector laboratories, Burlingame, CA, USA), and counterstained with methyl green. Brightfield microscopy was performed and images were acquired with a 40x objective (Nikon Eclipse E800). For quantification, ≥8 fields were imaged from ≥4 tibiae per group (control vehicle, control PTH, Ocy-PPRKO vehicle, and Ocy-PPRKO PTH). Subsequently, ImageJ (21) was used to quantify the RANKL-stained osteocyte area and normalized to total number of osteocytes per field. Statistical analysis was performed using 2-way ANOVA and Tukey’s HSD test.

Similar staining protocol was followed for Periostin IHC on tibiae except the anti-Periostin antibody (AF2955, R&D Systems) was diluted in Can Get Signal immunostain Solution B (Toyobo, Japan).

IHC for sclerostin expression was performed on deparaffinized vertebrae. Antigen retrieval was performed using proteinase K for 15 min at RT. The subsequent steps were similar to those described for RANKL IHC above, except no secondary antibody was used because we used the biotinylated anti-mouse sclerostin antibody (1:50 dilution; R&D Systems, Inc., Minneapolis, MN, USA) which was diluted in tris-NaCl blocking buffer. Brightfield microscopy was performed and images were acquired with a 20x objective (Nikon Eclipse E800). For quantification, ≥12 fields were imaged from ≥5 vertebrae per group (control vehicle, control PTH, Ocy-PPRKO vehicle, and Ocy-PPRKO PTH), sclerostin positive osteocytes were counted and normalized to total number of...
osteocytes per field. 2-way ANOVA was performed and the significance of p values was determined based on the Q scores obtained using Tukey's HSD test.

In Situ Hybridization

*In situ* hybridization was carried out as previously described (22). The antisense probe for *col1α1* has been reported (22).

Serology

Serum was isolated from the blood collected by retro-orbital bleeding. Serum mouse TRAP5b (Mouse TRAP Assay), PINP (Rat/Mouse PINP EIA), CTX (RatLaps EIA) and RANKL (Quantikine ELISA Kit, R&D systems) were measured using ELISA (Immunodiagnostic Systems Limited, UK). Serum total calcium was measured by calcium liquicolor arsenazo kit (Stanbio Laboratory, USA) and inorganic phosphate was quantified by phospho liquid-UV kit (Stanbio Laboratory, USA) as per the manufacturer’s instructions. Serum PTH was measured using mouse intact PTH ELISA kit (Immutopics, USA) according to the manufacturer’s protocol.

Anabolic and Catabolic PTH treatment

Human PTH(1–34) (MGH Peptide Core Facility) was dissolved in 0.1% TFA, aliquoted, stored frozen at –80°C, and subsequently diluted to the appropriate concentration in vehicle (2% heat-inactivated mouse serum, 0.1N HCl and 0.9% sodium chloride) immediately before administration. For anabolic treatment, control and Ocy-PPRKO female mice, randomly subdivided into sham or treatment groups, were injected with 100μL of either vehicle or PTH peptide. We treated mice with 80μg/kg PTH(1–34) anabolic dose sc 5 days per week for 4 consecutive weeks. For catabolic treatment, control and Ocy-PPRKO male mice, randomly subdivided into sham or treatment groups, were sc implanted with Alzet Micro-Osmotic Pump Model 1002 (Durect corporation, USA) to deliver 100μL of either vehicle or PTH peptide (100μg/kg/day) for 2 consecutive weeks. Upon sacrifice, left femurs and left tibiae were cleaned of soft tissue, fixed in 10% phosphate-buffered formalin (pH 7.2) for 48h and stored in 70% ethanol until further use. Left femur was used for microCT and histomorphometry analysis, and left tibia was used for histology. Right femurs and right tibiae were used to isolate RNA.

Histomorphometric Analysis

Mice were intraperitoneally injected with 20 mg/kg calcein and 40 mg/kg demeclocycline on days 9 and 2 before sacrifice, respectively. The distal femur metaphyses were removed and fixed in 70% ethanol. They were then dehydrated, infiltrated, and embedded in methyl methacrylate. Undecalcified 4-μm-thick sections were cut using a microtome (Leica RM 2255, Heidelberg, Germany) and mounted unstained for dynamic measurements. Consecutive sections were toluidine blue stained for static measurements. Histomorphometric parameters were measured using an Osteomeasure image analysis system (OsteoMetrics, Atlanta, GA) coupled to a photomicroscope and personal computer and the results were expressed according to standardized nomenclature (23). A sampling site of 2 mm² was established in the secondary spongiosa at 400 μm below the growth plate. Trabecular bone volume was assessed as a percentage of total tissue volume (BV/TV, %). Tb.Th (μm), Tb.N (1/mm), and Tb.Sp (μm) were calculated. Mineral apposition rate (MAR, μm/day) was derived from the mean distance between fluorescent labels divided by the labeling interval. The cancellous bone surface lined by osteoblasts and osteoclasts were measured. Osteoblasts were expressed per bone surface (Ob.S/BS,%) bone perimeter (N.Ob/B.Pm, /mm) and tissue area (N.Ob/T.Ar, /mm²). Osteoclasts were expressed per bone surface (Oc.S/BS,%), bone perimeter (N.Oc/B.Pm, /mm) and tissue area (N.Oc/T.Ar, /mm²). The data in Table 2 comparing control vs. Ocy-PPRKO were analyzed using an unpaired t-test. The data in Table 5 comparing catabolic response among 4 groups (Control vehicle, Control PTH, Ocy-PPRKO vehicle, and Ocy-PPRKO PTH), were analyzed using ANOVA with Tukey/Kramer.

RNA extraction and purification

RNA was extracted from whole bones (femur and tibia) and from osteocyte-enriched calvaria. Calvariae from control and Ocy-PPRKO mice were subjected to sequential collagenase and EDTA digestions to remove endosteal and periosteal osteoblasts and bone marrow cells as
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detailed in (4). For basal mRNA expression analysis, osteocyte-enriched calvariae were frozen in liquid nitrogen. RNA was extracted by homogenization in Trizol (Invitrogen, USA) as per the manufacturer’s instructions. RNA quality and quantity was ascertained by UV spectrophotometry (NanoDrop 8000, Thermo Fisher, USA).

Quantitative RT-PCR
Reverse transcription was performed on 0.5-1 µg of DNase-treated total RNA and oligoDT primers using Omniscript (Invitrogen, USA) or QuantiTect (Qiagen, USA) according to the manufacturer’s instructions. Quantitative PCR for PPR, SOST, RANKL, OPG, and β-actin was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, USA) on StepOnePlus (Applied Biosystems, USA) as described previously (4). Primer sequences are available upon request. The data were normalized to beta actin, and statistical significance was determined using a t-test in Excel and p≤0.05 was considered significant.

Cyclic AMP measurement
Tibiae, femurs, and calvariae were isolated from 8-12-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 2-3mL of α-MEM supplemented with 0.1% bovine serum albumin, BSA and 25mM 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 (Dulbecco Modified Essential Medium containing 10mM HEPES, 0.1% heat-inactivated BSA and 1mM 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), 100nM human PTH (1-34) or 0.1µM 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 0.5M 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 (50mM sodium acetate/0.05% sodium azide, pH 6.2) for measurement of cAMP by a specific RIA, as previously described (38). Bones were washed twice with 0.5mL acetone and once with 0.5mL 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.

Total Body Weight and dual-X-ray absorptiometry (DXA)
Total body weight in g was recorded at the ages mentioned in the results. Mouse BMD was measured by DXA using a Lunar PIXImus II densitometer (GE Medical System Luna, Madison, WI). In brief, animal were briefly anesthetized by sc. administration of tri-bromoethanol, placed on a tray and total body mineral density was acquired using manufactures protocols. Post-acquisition skeletal analysis was performed by excluding the mouse head from the final BMD (g/cm²) determination. For total body weight and BMD, statistical significance was determined using a t-test and p≤0.05 was considered significant.

MicroCT analysis
Bone morphology and microarchitecture were analyzed using a desktop high-resolution µCT (µCT40, Scanco Medical, Brüttisellen, Switzerland), as described previously (24). Briefly, the distal femoral metaphysis, femoral midshaft, and L5 vertebral body were scanned using an X-ray energy of 70keV, integration time of 200ms, and a 12µm isotropic voxel size. Longitudinal sections were scanned using an X-ray energy of 70keV, a current of 114µA, integration time of 200ms, and a 18µm isotropic voxel size; subsequently the half of total femur length was measured, and distal to mid-diaphysis in the femurs were used for measuring trabecular parameters. For the trabecular bone region BV/TV (%), Tb.N (/mm), Tb.Sp (mm), and Tb.Sp (mm); and for the cortical bone region Cort.Th (mm), Cort.A (mm²), MA (mm²), Cort.Por (%), Cort. Den (mgHA/ccm), and pMOI (mm⁴) were assessed.

Ex Vivo Osteoclastogenesis
We followed the protocol described in (19) with modifications. Briefly, non-adherent
spleen cells isolated from ~12-week-old control mice were cultured with 10 ng/ml mouse M-CSF (R&D system) for 3 days and were used as osteoclast precursors. Osteocyte-enriched long bones were isolated from ~10-week-old Ocy-PPRKO and control, weighed, cut into small pieces using aseptic technique, added to 24-well plates in 500µl complete medium (αMEM with L-Glutamine, 10% FBS, and Pen-Strep) containing 0.1µM dexamethasone, and incubated overnight at 37°C in a humidified atmosphere with 5% CO₂. The next day, 20,000 osteoclast precursors were added to each well of the 24-well plates and cultured with 100nM hPTH(1-34), 10nM 1,25-dihydroxyvitamin D3, and/or 100ng/mL murineOPG/Fc chimera (R&D Systems) in the presence of dexamethasone and M-CSF for 13 days with fresh ingredients added every 3-4 days. Next, these were stained for TRAP, and multinucleate cells with 2 or more nuclei were scored as TRAP-positive cells using an inverted microscope. The number of TRAP-positive cells per well were normalized to the weight of the bone pieces added to that well. Statistical significance was determined using a t-test in Microsoft Excel with p≤0.05 as significant. Photographic evidence was recorded with a Nikon Eclipse E800 microscope at a 10x objective.

Results

Generation of mice lacking PPR expression in osteocytes

To generate mice lacking PPR expression primarily in osteocytes (Ocy-PPRKO), mice with 10kb-DMP1-Cre were mated with mice in which exon E1 of PPR was flanked by Lox-P sites (Fig. 1A). Receptor ablation was demonstrated by genomic recombination as assessed by PCR analysis of genomic DNA. Presence of the ~600 bp band, expected with successful Cre-based recombination, confirmed PPR gene deletion in calvaria and the osteocytes from long bones, but not in other tissues (Fig. 1B). PPR gene knockout resulted in significantly reduced PPR mRNA expression in osteocyte-enriched long bones from Ocy-PPRKO as compared with littermate controls, as we have recently reported (25). In contrast, PPR mRNA expression in primary calvarial osteoblasts derived from control and Ocy-PPRKO was unchanged (control = 100±6.9 %, Ocy-PPRKO = 112.1±6.9 %, expression normalized for β-actin; p=0.25, NS) confirming that receptor expression was not altered in osteoblasts. In addition, osteocalcin mRNA expression was also similar (control = 100±22.1 %, Ocy-PPRKO = 54.2±13.0 %, expression normalized for β-actin; p=0.14, NS) confirming the osteoblastic nature of these cells. Functional ablation of the receptor was ascertained by assessing cAMP accumulation in response to PTH in osteocyte-enriched long bone explants (OEBe). OEBe were treated with 100nM hPTH(1-34) or 10µM forskolin. Ocy-PPRKO bone explants had a statistically significant reduction (86.5% reduction) in cyclic AMP accumulation in response to PTH compared to controls (Ocy-PPRKO = 15.0±4.7 picomol/mg bone vs. controls = 110.8±20.5 picomol/mg bone; p<0.001) whereas both Ocy-PPRKO and control mice demonstrated a robust cyclic AMP response to forskolin (Ocy-PPRKO = 94.2±58.3 picomol/mg bone vs. controls = 66.8±10.6 picomol/mg bone; p=0.359) (Fig. 1C) demonstrating that Ocy-PPRKO mice retain intact adenylate cyclase responsiveness, but lack cAMP accumulation following PTH administration due to reduced PPR expression in osteocytes (Fig. 1C). Moreover, primary calvarial osteoblasts from Ocy-PPRKO and control mice showed robust cAMP production when treated with either PTH or forskolin (Fig. 1D), demonstrating intact PPR signaling in osteoblasts.

Ocy-PPRKO mice displayed increased trabecular and cortical bone

PTH is the most important hormonal regulator of calcium and phosphate homeostasis. It exerts its effects by binding PPR expressed on its target organs, namely bone and kidney. To investigate if receptor deletion in osteocytes altered mineral homeostasis, we measured serum calcium and phosphate concentration. Both serum calcium (control = 7.24±0.35 mg/dL, Ocy-PPRKO = 6.88±0.62, mg/dL; n=5, NS) and phosphate (control = 9.3±0.89 mg/dL, Ocy-PPRKO = 8.4±0.41, mg/dL; n=5, NS) were indistinguishable between Ocy-PPRKO and controls, suggesting that other PPR-expressing cells, likely osteoblasts or kidney cells, regulate serum calcium and phosphate concentrations. Moreover, intact circulating PTH was also similar between Ocy-PPRKO and control (control = 111.5±25.1 pg/mL,
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Ocy-PPRKO = 104.6±25.2, pg/mL; NS), indicating that the skeletal phenotype observed in Ocy-PPRKO mice is not a consequence of an altered parathyroid state.

We then analyzed the bone phenotype of Ocy-PPRKO mice. Considering that bone weight, fat tissue, and lean tissue contribute towards total body weight, we measured it at different ages. Total body weight was comparable between Ocy-PPRKO and control at 4 and 8 weeks of age (Fig. 2A). However, significantly increased body weight was recorded at 12 weeks for Ocy-PPRKO as compared with control (Fig. 2A). Increased body weight was not present in control (PPRfl/fl mice) and DMP1-Cre expressing mice (Fig. 2A), indicating that the higher weight observed in Ocy-PPRKO animals was associated with the loss of PPR expression in osteocytes. As assessed by DXA, fat tissue (control = 839.5±37.6 g/Kg, Ocy-PPRKO = 845.3±27.4, g/Kg; n≥6, NS) and lean tissue (control = 160.6±35.9 g/Kg, Ocy-PPRKO = 154.1±25.7, g/Kg; n≥6, NS) weights were similar between 12-week-old Ocy-PPRKO and control. BMD was similar between Ocy-PPRKO and control mice at 8 weeks in both females (Fig. 2B) and males (Fig. 2C). However, at 12-15 weeks a statistically significant increase in BMD was observed in both sexes and it persisted at 20 weeks (Fig. 2B, C). DMP1-Cre only mice at 12 weeks of age were indistinguishable from controls (Fig. 2B).

H&E staining (Fig. 2D) of tibiae showed increased trabecular bone in Ocy-PPRKO as compared with control, starting at 8 weeks. Similarly, Von Kossa staining of femurs showed increased trabecular bone at 14 weeks (Fig. 2E).

To further assess the skeletal phenotype of these animals, we performed microCT analysis of both vertebrae (L5) and distal femurs in 5-6 week old Ocy-PPRKO and controls. At this age, there were no statistically significant differences between Ocy-PPRKO and control mice, as shown in Table 1. MicroCT analysis at 12 weeks, on the other hand, showed a significant increase in trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), and thickness (Tb.Th), and decrease in separation (Tb.Sp) in the vertebral body (L5) of Ocy-PPRKO as compared with control (Table 2). Analysis of femur midshafts showed a significant increase in cortical thickness (Cort.Th), area (Cort.A), and polar moment of inertia (pMOI), in Ocy-PPRKO as compared with control (Table 2). Cortical density (Cort. Dens), medullary area (MA) and cortical porosity (Cort.Por) were comparable in Ocy-PPRKO and control (Table 2).

To investigate whether the increase in BMD was driven by reduced bone resorption or increased bone formation, or a combination of both, we measured, in these animals, serum markers of bone formation and bone resorption. PINP (control = 232.8±26.8 ng/mL, Ocy-PPRKO = 185.1±11.5, ng/mL; 12-week-old females, n≥4, NS), CTX (control = 9.5±1.9 ng/mL, Ocy-PPRKO =7.0±0.3, ng/mL; 12-week-old females, n≥4, NS), and TRAP5b (control = 2.35±0.31 U/L, Ocy-PPRKO = 2.17±0.18, U/L; 12-week-old females, n≥4, NS) were unchanged, as compared to controls.

Osteoblasts and osteoclasts number and function were reduced, although not significantly, in Ocy-PPRKO animals compared to controls (Table 5, vehicle treated animals), as demonstrated by a decrease in Ob.S/BS, N.Ob./T.Ar, N.Ob./B.Pm, osteoid surface (OS/BS) and osteoid volume (OV/TV), Oc.S/BS, N.Oc./T.Ar, N.Oc./B.Pm, and erosion surface (ES/BS). These findings were associated with a significant increase in both SOST mRNA expression (Fig. 2G) and in the number of Sclerostin-positive osteocytes (Fig. 3E, top row, and 3F) in bones from these animals, suggesting that the suppression of the Wnt/β-catenin pathways might drive the reduced osteoblasts number and activity. Reduced osteoblast function was also demonstrated by a dramatic reduction in Col1α1 mRNA expression, as shown by in situ hybridization in both trabecular and cortical bone (Fig. 3C), in Ocy-PPRKO animals compared to controls. Interestingly, comparable number of Sclerostin- expressing osteocytes was observed in the cortical region of the vertebrae from Ocy-PPRKO and control, suggesting that cellular responses to PTH are site specific (Fig. 3G).

To examine cells of the osteoclast lineage, we analyzed RANKL and OPG expression. There was a significant decrease in both OPG (Fig. 2H) and RANKL (Fig. 2I) mRNA expression in osteocyte-enriched calvariae from Ocy-PPRKO compared with control whereas the ratio RANKL/OPG remained unchanged. A significant 85% decrease in RANKL protein expression was
also observed in osteocytes from Ocy-PPRKO as compared with control (Fig. 5E, top row, and 5F).

To investigate whether the decrease in osteoclast activity was associated with the presence of unresorbed calcified cartilage, as recently reported for chondrocyte and osteoblast-specific RANKL knockout mice (20), we performed safranin O staining on tibiae from Ocy-PPRKO and control. Growth plates in Ocy-PPRKO were indistinguishable from controls (Fig. 2F), indicating that PPR activation in osteocytes is not required for proper bone growth and cartilage resorption. Moreover, the presence of a normal growth plate demonstrates normal PPR expression and signaling in other bone cells, such as chondrocytes and osteoblasts.

Altogether, these findings indicate that in the absence of PPR signaling in osteocytes there is an age-dependent increases in BMD, and in trabecular and cortical bone parameters, that is associated with a decrease in osteoblast and osteoclast functions but not number.

**Blunted skeletal response to anabolic and catabolic PTH administration in Ocy-PPRKO**

Ocy-PPRKO and control were then subjected to intermittent or continuous PTH treatment. As expected, intermittent hPTH(1-34) at a dose of 80µg/Kg/day, induced a significant increase in vertebral BV/TV and Tb.N, and decrease in Tb.Sp (Table 3) in control. Analysis of midshaft femurs showed a significant increase in Cort.Th and Cort.A, and pMOI in PTH-treated controls compared to vehicle treated animals (Table 3). In contrast, these trabecular and cortical parameters remained unchanged between vehicle- and PTH-treated Ocy-PPRKO (Table 3). No significant changes were observed in the other bone parameters between vehicle- and PTH-treated control or Ocy-PPRKO mice (Table 3). These data demonstrate that osteocyte-mediated signals are required for anabolic skeletal responses to PTH.

To evaluate the role of PPR signaling in osteocytes in modulating osteoblast and osteoclast activities during anabolic PTH treatment, PINP and CTX serum levels were measured. Intermittent administration of PTH significantly increased circulating PINP, a marker of osteoblast activity, in control whereas had no effect in Ocy-PPRKO (Fig. 3A). Serum CTX, a marker of osteoclast activity, remained unchanged in both groups (Fig. 3B). In Ocy-PPRKO, intermittent PTH failed to stimulate ColI1 mRNA expression (Fig. 3C). Periostin, a regulator of SOST/Sclerostin expression (26), was unchanged upon intermittent PTH administration both in control and Ocy-PPRKO mice (Fig. 3D). SOST/Sclerostin expression has been reported to return to baseline by 24 hours after PTH treatment (27), which limited out ability to analyze Sclerostin expression in intermittent PTH-treated bone samples because, as per our anabolic protocol, these were harvested 24 hours after the last PTH injection. Therefore, to study Sclerostin regulation, we treated control and Ocy-PPRKO with vehicle or 50nmol/Kg hPTH(1-34) for 1.5 hours. While PTH induced a decrease in Sclerostin expression in the osteocytes from control, it failed to suppress Sclerostin expression in Ocy-PPRKO (Fig. 3E, F). Interestingly, similar sclerostin expression was observed in the cortical bone of vertebrae from both vehicle- and PTH-treated Ocy-PPRKO and control (Fig. 3G), suggesting a differential PPR-mediated regulation of sclerostin expression in trabecular and cortical sites.

Next, we examined the ability of Ocy-PPRKO to respond to continuous PTH administration. Mice were subjected to 2 weeks of continuous administration of 100µg/Kg/day of hPTH(1-34). MicroCT (Fig. 4C and Table 4) and Von Kossa staining (Fig. 4D) showed a catabolic response upon PTH treatment in control, but not Ocy-PPRKO. PTH treatment induced a significant decrease in Tb.N, and increase in Tb.Sp in control mice, as shown by microCT analysis (Table 4). In contrast, these parameters remained unchanged between vehicle- and PTH-treated Ocy-PPRKO (Table 4), demonstrating that mice lacking PPR expression in osteocytes are resistant to continuous infusion of PTH. No significant changes were observed in other parameters between vehicle- and PTH-treated control or Ocy-PPRKO mice (Table 4).

Histomorphometric analysis demonstrated a significant increase in both osteoblasts and osteoclasts number and function, upon PTH treatment in controls, as demonstrated by a significant increase in Ob.S/BS, N.Ob./T.Ar, N.Ob./B.Pm, osteoid surface (OS/BS), osteoid volume (OV/TV), Oc.S/BS, N.Oc/T.Ar, N.Oc/B.Pm, and erosion surface (ES/BS) (Table
None of these parameters were significantly changed in Ocy-PPRKO animals upon PTH administration (Table 5), indicating that deletion of PPR in osteocytes blunts the skeletal response to continuous PTH. Interestingly, PTH did significantly decrease Tb.N in Ocy-PPRKO mice and not in controls; however this change was not associated with a decrease (or change) in any other bone parameter (Table 5). Moreover, histomorphometry further confirmed the increased BV/TV, Tb.N and decreased in Tb.Sp in Ocy-PPRKO compared to controls (Table 5).

Serum marker of osteoblast activity, PINP, was significantly elevated in PTH-treated control, but remained unchanged in Ocy-PPRKO, further confirming the lack of PTH-responsiveness in Ocy-PPRKO (Fig. 4A). Interestingly, serum levels of CTX, a marker of bone resorption, were significantly increased in Ocy-PPRKO (p<0.05) whereas in controls the increase (167%) did not reach significance (p=0.0508) (Fig. 4B). These results suggest that PTH-induced CTX elevation is independent of PPR signaling in osteocytes and most likely reflect a osteoblast-mediated, PTH dependent mechanism(s).

To investigate if the lack of response to continuous PTH was due to an osteoclast defect, we analyzed the osteoclast-forming ability of osteocytes from Ocy-PPRKO and control in vitro. OEBE from Ocy-PPRKO and control were cocultured with osteoclast precursors derived from wild type spleens. Upon PTH treatment, multinucleate osteoclasts (MNC) were observed in both cultures (Fig. 5A). However, the number of MNC formed in Ocy-PPRKO cultures was significantly reduced (Fig. 5B; Control MNC/mg OEBE = 4.6±0.2, Ocy-PPRKO MNC/mg OEBE = 1.8±0.2, p=0.001). Importantly, OPG completely abrogated the osteoclastogenic ability of both Ocy-PPRKO and control OEBE (Fig. 5A,B), suggesting a RANKL-dependent mechanism. The number of multinucleated osteoclasts formed upon 1,25-dihydroxyvitamin D treatment was similar in both Ocy-PPRKO and control cultures (Fig. 5), demonstrating reduced osteoclast formation in Ocy-PPRKO because of ablated PPR signaling.

To further investigate the reduced osteoclast activity in response to PTH observed by histomorphometry (Table 5), we analyzed RANKL expression in osteocytes upon catabolic PTH administration. ANOVA showed a significant difference in genotypes, but not the PTH treatments or genotype and PTH treatment interaction. Importantly, Tukey HSD identified a significantly higher RANKL expression in both vehicle- and PTH- treated control osteocytes as compared with both vehicle- and PTH-treated Ocy-PPRKO osteocytes (Fig. 4E and F). To further assess the role of RANKL in these animals, we measured its level in the serum. There was no difference in circulating RANKL between control and Ocy-PPRKO animals and PTH treatment had no effect (control-veh = 129.7±10.9 pg/mL, control-PTH = 101.7±18.6 pg/mL, p=0.19, NS; Ocy-PPRKO-veh =98.8±7.8, pg/mL, Ocy-PPRKO-PTH =107.4±20.5, pg/mL; p=0.65, NS) suggesting that circulating RANKL might not reflect the local effects of this cytokine. RANKL levels in bone marrow supernatant of femurs and tibias of Ocy-PPRKO and controls were below the limit of detection of the assay used. Moreover, RANKL mRNA expression in RNA isolated from intact bones (including both bone marrow and osteoblasts) showed no changes in vehicle- or PTH-treated control and Ocy-PPRKO (Relative % RANKL mRNA expression: control-veh = 100.0±12.0%, control-PTH = 83.0±2.9%, p=0.198, NS; Ocy-PPRKO-veh =53.4±5.2%, Ocy-PPRKO-PTH =86.2±22.7%; p=0.270, NS). These findings are in agreement with previous studies (20) and suggest that the contribution of RANKL produced by osteocytes in small relative to the total RANKL produced, yet its biological effects can be detected as lack of PTH catabolic actions. In the absence of PPR signaling in osteocytes, continuous PTH administration fails to increase both osteoblasts and osteoclasts and induce a skeletal effect.

**Discussion**

It has been known for over a decade that PTH can exert its anabolic or catabolic effect on bone via activation of the PPR widely expressed on cells of the osteoblast lineage; however, the exact cellular target of its action on bone has remained elusive. Several mechanisms have been proposed and they comprise recruitment and proliferation of osteoprogenitors cells, activation of bone lining cells (28, 29), inhibition of osteoblast and osteocyte apoptosis (30), and suppression of SOST/Sclerostin expression (27). Osteocytes, the most abundant cells in bone, have
recently emerged as important modulator of bone modeling and remodeling (5). In this regard, the molecular roles of sclerostin and RANKL, two predominantly osteocytic skeletal factors, have been reported (5). Interestingly, both molecules are known targets of PTH actions with SOST/Sclerostin being suppressed and RANKL being increased upon receptor activation.

Herein, using a murine model of PPR ablation in osteocytes, we provide, for the first time, evidences that receptor signaling in these cells is important to control both osteoblast and osteoclast functions via SOST/Sclerostin and RANKL-mediated mechanisms. Importantly, our studies demonstrated that PPR signaling in osteocytes is required for generating full skeletal responses to anabolic and catabolic PTH administration. During PTH anabolic regimen, Sclerostin suppression is needed to allow a full hormonal effect and, in the absence of PPR signaling in osteocytes, PTH has little, if any, skeletal effects.

It has been previously reported that mice expressing a constitutively active PPR in osteocytes (DMP1-caPTHR1) have increased trabecular and cortical bone (8, 31), suggesting that receptor signaling in osteocytes drives bone formation. Interestingly, mice lacking receptor expression in osteocytes, the Ocy-PPRKO mice, also displayed increased trabecular and cortical bone. The apparently similar phenotype, i.e., increase in trabecular and cortical bone parameters, observed in murine models of constitutive expression (DMP1-caPTHR1) or deletion (Ocy-PPRKO) of PPR in osteocytes is driven by two distinct mechanisms. The increased trabecular and cortical bone in DMP1-caPTHR1 is a result of increased osteoblast and osteoclast activities, increased bone formation rate, and high bone turnover. In contrast, the increased bone in the Ocy-PPRKO is the net result of reduced osteoblast and osteoclast function, and low bone turnover state. Bone formation rate in Ocy-PPRKO mice is not changed, as is the mineral apposition rate (data not shown). Histomorphometric analysis showed a reduced, although not significant, number of both osteoblasts and osteoclast, and, upon PTH administration, this cellular defect became evident (see Table 5). We can hypothesize that, under normal conditions, the lack of receptor expression in osteocytes does not significantly alter the number of osteoblasts and osteoclast but reduces their function with a net increase in BMD, as demonstrated by both DXA, microCT and histomorphometric analysis. On the other hand, when Ocy-PPRKO animals were subjected to a skeletal perturbation (such as intermittent or continuous PTH administration), they failed to properly increase both osteoblast and osteoclast number and activity as shown by histomorphometric analysis (Table 5), expression of Col1α1(Fig. 3C) and Sclerostin (Fig. 3E). Interestingly, continuous administration of PTH failed to induce trabecular bone loss or increase osteoclast number in Ocy-PPRKO animals despite a significant increase in serum CTX. We can speculate that CTX might derive from skeletal sources other than the one analyzed or from the modest increase in osteoclast numbers detected by histomorphometry (Table 5).

We previously reported that mice with inducible PPR deletion (postnatally, Ocy-PPRKO (4) have mild osteopenia and homeostatic defects whereas constitutive ablation of the receptor, as achieved in Ocy-PPRKO induce an increase in BMD. We can speculate that the difference between Ocy-PPRKO and Ocy-PPRKO could be ascribed mostly to the timing of receptor ablation (postnatally vs. embryonic), different promoter activities or tamoxifen effects independent of PPR activation. Moreover, in the conditional PPR knock-out the severity of the phenotype might depend on the age of mice at which tamoxifen treatment was started, the dose of tamoxifen administered, and the duration of receptor ablation. To investigate whether a transient osteopenia was present in the Ocy-PPRKO as well, we performed microCT analysis on L5 vertebrae and femurs of mice at ~5 weeks of age (similar age as for the inducible animals). As reported in Table 1, there were no detectable skeletal differences between controls and Ocy-PPRKO animals, suggesting that timing of receptor ablation, promoter activity, and tamoxifen effects might drive the osteopenic phenotype in the inducible model.

Increased osteoblast function in DMP1-caPTHR1 was associated with decreased Sclerostin expression (31). Similarly, decreased osteoblast function in Ocy-PPRKO was associated with increased Sclerostin expression, supporting
the concept that regulation of Sclerostin expression is an important effector of PPR signaling in osteocytes.

Osteocytic regulation of osteoclasts through osteoblast-mediated mechanism is well understood (5). Emerging literature, however, suggested that osteocytes could directly regulate osteoclasts. Apoptotic osteocytes release apoptotic bodies expressing RANKL, which, in turn, activate osteoclasts (6). Further, targeted ablation of osteocytes using the 10Kb DMP1 promoter to drive the expression of diphtheria toxin receptor expression followed by a single injection of diphertheria toxin caused necrosis of 70% osteocytes in cortical bone and activated osteoclasts (6), demonstrating osteocytes mediated osteoclast-regulation. Moreover, murine model of osteocyte-specific RANKL ablation showed osteopetrosis, demonstrating a critical role for osteocyte-derived RANKL in osteoclasts function and ultimately bone remodeling (19, 20). In Ocy-PPRKO mice, we observed significantly reduced RANKL expression in osteocytes and reduced osteoclastogenesis in PTH-treated OEBEs from these animals, suggesting that the reduced osteoclasts number upon continuous PTH administration and their activity is likely RANKL-dependent. Interestingly, upon continuous PTH administration, RANKL mRNA was not significantly increased in intact bones (including osteoblasts and bone marrow) of both controls and Ocy-PPRKO nor was in osteocyte-enriched bones.

To investigate the role of PPR signaling in osteocytes in anabolic and catabolic skeletal effects, we subjected the Ocy-PPRKO mice to intermittent or continuous PTH regimens. Interestingly, both the anabolic and catabolic skeletal responses to PTH were blunted in Ocy-PPRKO mice, indicating an important role of PPR signaling in osteocytes in mediating the skeletal responses to the hormone. Immunohistochemical analysis of tibiae from Ocy-PPRKO mice revealed a lack of Sclerostin suppression or Coll-1α1 expression in response to PTH administration. We can hypothesize that in the absence of receptor signaling in osteocytes, PTH failed to suppress sclerostin and to increase osteoblast activity and, ultimately, induce an anabolic response. Moreover, lack of PPRs also significantly reduced RANKL expression in osteocytes and impaired osteoclast activities and ultimately bone remodeling (Fig. 6).

In summary, we have shown here, for the first time, that PPR signaling in osteocytes is required for anabolic and catabolic responses to PTH.

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Acknowledgements

We would like to thank Dr. Henry M. Kronenberg, Endocrine Unit, MGH for critical review of this manuscript, Drs. Tomoki Nakashima and Hiroshi Takayanagi from Tokyo Medical and Dental University for technical recommendation for the RANKL IHC, and Dr. Mary Bouxsein and Leeann Louis from MGH for suggestions on microCT analysis. Research reported in this publication was supported by the National Institute of Diabetes and Digestive and Kidney Diseases, part of the National Institutes of Health, under Award Number DK079161 to PDP.
Figure Legends:

Figure 1: Generation of Ocy-PPRKO mice. (A) Mating scheme. (B) Allele-specific DNA recombination PCR showing specific PPR deletion, ascertained by the presence of a ~600bp band, in long bone and calvaria. The presence of the larger band (~1.5 kb) is due to contamination with other bone cells (i.e. bone marrow cells, osteoblasts, and osteoclasts) that were not removed during DNA preparation. in long bone and calvaria. C) cAMP response in osteocyte-enriched long bones and D) calvarial osteoblasts. Gray bars represent vehicle, white bars represent 100nM hPTH(1-34), black bars represent 10µM forskolin, values are mean ± SEM.

Figure 2: Increased body weight and BMD in Ocy-PPRKO. Control represents “PPR fl/fl” mice, and littersmates were analyzed here and throughout. (A) Total body weight at 4 weeks (Control n=5, Ocy-PPRKO n=6), 8-9 weeks (Control n=12, Ocy-PPRKO n=11), and 12-14 weeks (DMP1-Cre only n=5, Control n=13, Ocy-PPRKO n=15) in females. (B) Total BMD at 8 weeks (Control n=8, Ocy-PPRKO n=9), 12-13 weeks (DMP1-Cre only n=7, Control n=6, Ocy-PPRKO n=9), and 19-21 weeks (Control n=3, Ocy-PPRKO n=3) in females. (C) Total BMD at 8 weeks (Control n=7, Ocy-PPRKO n=6), and 15 weeks (Control n=10, Ocy-PPRKO n=12) in males. (D) H&E stained tibiae from females, images acquired with a 4x objective. (E) Von Kossa stained femurs from males. (F) Safranin O stained tibiae from females, images acquired with a 4x objective. Real-time RT PCR for (G) SOST, (H) OPG, and (I ) RANKL mRNA expression in osteocyte-enriched calvariae from 16-week-old control and Ocy-PPRKO males. Values are mean ± SEM, *p≤0.05.

Figure 3: Defective osteoblast recruitment in response to intermittent PTH administration in Ocy-PPRKO. Anabolic response was analyzed in females injected with vehicle or 80 µg/Kg/day hPTH(1-34): Serum levels of (A) PINP and (B) CTX (Control, vehicle n=6; Control, PTH n=7; Ocy-PPRKO, vehicle n=7; Ocy-PPRKO, PTH n=7), (C) In situ for Collagen 1α1 mRNA on tibiae, (D) IHC for Periostin on tibiae. Images acquired with a 20x objective. (E, F) Lack of Sclerostin regulation in Ocy-PPRKO in response to PTH: (E) Representative images of sclerostin IHC in the trabecular bone in the vertebrae from females injected with vehicle or 50 nmol/Kg hPTH(1 -34) for 1.5 hours. Arrows indicate sclerostin expression in osteocytes. Sclerostin positive osteocytes per field in the trabecular region (F) and cortical ring (G) of the vertebrae were counted and normalized to total number of osteocytes per field. Images acquired with a 20x objective. Values are mean ± SEM, *p≤0.05.

Figure 4: Blunted catabolic response in Ocy-PPRKO upon continuous PTH administration. Catabolic response was analyzed in males injected with vehicle or 100 µg/Kg/day hPTH(1-34): Serum levels of PINP (A) and CTX (B), (Control, vehicle n=7; Control, PTH n=7; Ocy-PPRKO, vehicle n=7-8; Ocy-PPRKO, PTH n=4), *p<0.05. (C) Representative MicroCT images of mid-diaphysis to epiphysis of femurs. (D) Representative images of Von Kossa stained femurs. (E) Representative images of RANKL IHC from tibiae. Arrows indicate osteocytes immunostained for RANKL. Images acquired with a 40x objective. (F) RANKL stained osteocyte area was quantified by ImageJ and normalized to total number of osteocytes per field. Values are mean ± SEM, NS=not significant, *p≤0.01.

Figure 5: Reduced osteoclast formation in Ocy-PPRKO upon PTH stimulation in vitro. (A) Representative images of TRAP+ multinucleate osteoclasts induced by osteocyte-enriched bone explants (OEBE) from Ocy-PPRKO and control when co-cultured with osteoclast precursors (Oc precursors) with or without hPTH(1-34), OPG, and vitamin D (vitD). Images acquired with a 10x objective. (B) Number of TRAP+ multinucleate osteoclasts induced by OEBE from Ocy-PPRKO and control. Values are mean ± SEM, NS=not significant, *p≤0.01.

Figure 6: PPR signaling in osteocytes directly regulates osteoblasts and osteoclasts. (A) Osteocytes have been reported to regulate osteoblasts, which in turn regulate osteoclasts via RANKL expression. Recent evidence suggests that RANKL in osteocytes regulates osteoclasts. (B) In our murine model of...
constitutive PPR deletion in osteocytes, increased SOST mRNA and Sclerostin protein expression and decreased osteoblasts were observed. In addition, decreased RANKL mRNA and protein expression and decreased osteoclasts were observed.
Osteocytes regulate anabolic and catabolic responses to PTH

Tables:

Table 1: MicroCT analysis showed similar trabecular and cortical bone between 5-6-week-old Ocy-PPRKO and control females. Trabecular bone volume per total volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) in L5 vertebrae. Cortical thickness (Cort.Th), cortical density (Cort. Dens), cortical area (Cort. A), medullary area (MA), cortical porosity (Cort. Por), and polar moment of inertia (pMOI) in midshaft of femurs. Values are mean ± SEM, two-tailed t-test assuming equal variance, NS is not significant.

| Parameter       | Control         | Ocy-PPRKO       | p Value |
|-----------------|-----------------|-----------------|---------|
| **L5 vertebrae**| N=5             | N=6             |         |
| BV/TV (%)       | 24.4±0.96       | 27.2±1.93       | NS      |
| Tb.N (/mm)      | 5.5±0.2         | 5.7±0.1         | NS      |
| Tb.Th (mm)      | 0.05±0.001      | 0.05±0.003      | NS      |
| Tb.Sp (mm)      | 0.14±0.005      | 0.13±0.006      | NS      |
| **Midshaft femur** | N=4            | N=5             |         |
| Cort. Th (mm)   | 0.099±0.005     | 0.098±0.003     | NS      |
| Cort.Dens (mmHA/ccm) | 1053.9±5.7   | 1054.2±11.8     | NS      |
| Cort. A (mm²)   | 0.39±0.04       | 0.38±0.02       | NS      |
| MA (mm²)        | 0.97±0.07       | 0.94±0.03       | NS      |
| Cort. Por (%)   | 0.59±0.08       | 0.61±0.13       | NS      |
| pMOI (mm⁴)      | 0.15±0.03       | 0.14±0.01       | NS      |
Osteocytes regulate anabolic and catabolic responses to PTH

Table 2: MicroCT analysis showed increased trabecular and cortical bone in 12-week-old Ocy-PPRKO females. Trabecular bone parameters in L5 vertebrae. Cortical bone parameters in midshaft of femurs. Values are mean ± SEM, two-tailed t-test assuming equal variance, *p≤0.05, NS = not significant.

| Parameter       | Control   | Ocy-PPRKO | p Value |
|-----------------|-----------|-----------|---------|
| **L5 vertebrae**| N=4       | N=7       |         |
| BV/TV (%)       | 23.2±0.7  | 35.7±2.6  | 0.01    |
| Tb.N (/mm)      | 4.5±0.1   | 5.1±0.2   | 0.03    |
| Tb.Th (mm)      | 0.05±0.002| 0.07±0.004| 0.01    |
| Tb.Sp (mm)      | 0.17±0.006| 0.13±0.009| 0.01    |
| **Midshaft femurs**| N=7      | N=9       |         |
| Cort. Th (mm)   | 0.16±0.001| 0.18±0.004| 0.001   |
| Cort.Dens (mmHA/ccm) | 1213.3±7.9 | 1217.2±6.6 | NS      |
| Cort. A (mm²)   | 0.61±0.01 | 0.69±0.02 | 0.001   |
| MA (mm²)        | 0.82±0.03 | 0.85±0.02 | NS      |
| Cort. Por (%)   | 0.2±0.01  | 0.2±0.01  | NS      |
| pMOI (mm⁴)      | 0.23±0.01 | 0.28±0.01 | 0.01    |
Table 3: MicroCT analysis showed lack of anabolic response upon intermittent PTH administration in Ocy-PPRKO. Anabolic responses were analyzed in females injected with vehicle or 80 µg/Kg/day hPTH(1-34): Trabecular bone parameters in L5 vertebrae. Cortical bone parameters in midshaft of femurs. Values are mean ± SEM, two-tailed t-test assuming equal variance was performed to compare vehicle- vs. PTH-treated controls, and vehicle- vs. and PTH-treated Ocy-PPRKO, *p≤0.05, NS is not significant.

| Parameter | Control Vehicle | Control PTH | p Value | Ocy-PPRKO Vehicle | Ocy-PPRKO PTH | p Value |
|-----------|-----------------|-------------|---------|-------------------|--------------|---------|
| L5 vertebrae | N=6 | N=5 | N=8 | N=7 |
| BV/TV (%) | 22.52±1.2 | 26.96±0.9 | 0.02 | 29.33±1.3 | 31.29±1.8 | NS |
| Tb.N (/mm) | 4.60±0.05 | 4.98±0.06 | 0.001 | 4.94±0.16 | 5.19±0.19 | NS |
| Tb.Th (mm) | 0.049±0.002 | 0.054±0.002 | NS | 0.059±0.001 | 0.060±0.002 | NS |
| Tb.Sp (mm) | 0.169±0.004 | 0.146±0.003 | 0.002 | 0.145±0.008 | 0.135±0.009 | NS |
| Midshaft femurs | N=6 | N=5 | N=7 | N=6 |
| Cort. Th (mm) | 0.14±0.006 | 0.16±0.002 | 0.04 | 0.15±0.003 | 0.16±0.005 | NS |
| Cort. Dens (mmHA/ccm) | 1193.7±4.7 | 1190.5±6.7 | NS | 1200.3±4.9 | 1200.1±5.1 | NS |
| Cort. A (mm$^2$) | 0.54±0.02 | 0.64±0.02 | 0.01 | 0.63±0.02 | 0.65±0.03 | NS |
| MA (mm$^2$) | 0.86±0.02 | 0.95±0.05 | NS | 0.98±0.03 | 0.99±0.03 | NS |
| Cort. Por (%) | 0.24±0.02 | 0.21±0.02 | NS | 0.21±0.01 | 0.22±0.00 | NS |
| pMOI (mm$^4$) | 0.20±0.009 | 0.27±0.02 | 0.01 | 0.27±0.01 | 0.28±0.02 | NS |
Table 4: MicroCT analysis showed lack of catabolic response to continuous PTH administration in Ocy-PPRKO. Catabolic response was analyzed in males treated with vehicle or 100 µg/Kg/day of hPTH(1-34). Trabecular bone parameters in mid-to-distal-diaphysis of femurs. Cortical bone parameters in midshaft of femurs. Values are mean ± SEM, 2-way ANOVA with TukeyHSD. *p<0.01 compared to control Vehicle; +p<0.01 compared to control PTH; #p<0.01 compared to Ocy-PPRKO.

| Parameter                     | Control Vehicle | Control PTH | Ocy-PPRKO Vehicle | Ocy-PPRKO PTH | ANOVA p Value |
|-------------------------------|-----------------|------------|-------------------|--------------|---------------|
| **Mid to distal diaphysis femurs** | N=9             | N=7        | N=12              | N=9          |               |
| BV/TV (%)                    | 13.3±1.5        | 7.1±0.8    | 17.1±0.7          | 13.9±1.4     | 0.076         |
| Tb.N (/mm)                   | 2.28±0.19       | 1.37±0.13  | 2.61±0.75         | 2.15±0.16    | 0.042         |
| Tb.Th (mm)                   | 0.06±0.003      | 0.05±0.002 | 0.07±0.003        | 0.06±0.003   | 0.253         |
| Tb.Sp (mm)                   | 0.41±0.04       | 0.71±0.07  | 0.32±0.01         | 0.43±0.04    | 0.002         |
| **Midshaft femurs**          | N=7             | N=5        | N=9               | N=10         |               |
| Cort. Th (mm)                | 0.18±0.005      | 0.16±0.002 | 0.19±0.005        | 0.18±0.004   | 0.068         |
| Cort. A (mm²)                | 0.72±0.02       | 0.63±0.03  | 0.75±0.01         | 0.71±0.03    | 0.194         |
Table 5: Blunted catabolic response to continuous PTH administration in Ocy-PPRKO detected by histomorphometric analysis in femurs. Osteoid volume per total volume (OV/TV), osteoid thickness (O.Th), osteoid surface per bone surface (OS/BS), erosion surface over bone surface (ES/BS). Values are mean ± SEM, ANOVA with Tukey/Kramer, *p<0.05 compared to control Vehicle; +p<0.05 compared to control PTH; # p<0.05 compared to Ocy-PPRKO.

| Parameter | Control Vehicle N=9 | Control PTH N=8 | Ocy-PPRKO Vehicle N=8-9 | Ocy-PPRKO PTH N=8 | ANOVA p value |
|-----------|---------------------|-----------------|--------------------------|------------------|---------------|
| BV/TV (%) | 11.17±0.97          | 10.29±1.23      | 15.39±0.62*              | 13.19±1.24       | 0.02          |
| Tb.Th (µm) | 33.78±1.70         | 34.05±2.21      | 37.24±1.29               | 36.85±2.07       | 0.4           |
| Tb.N (/mm) | 3.27±0.16          | 3.17±0.17       | 4.13±0.10**              | 3.53±0.18*       | 0.000         |
| Tb.Sp (µm) | 278.8±18.1         | 287.8±18.3      | 206±56.5**               | 252.1±17.5       | 0.003         |
| Ob.S/BS (%) | 8.35±1.83          | 24.94±5.54*     | 2.45±1.19*               | 7.37±1.64*       | 0.000         |
| N.Ob/T.Ar (/mm²) | 41.40±8.30    | 129.59±32.59*   | 15.22±7.03*              | 38.80±8.49*      | 0.000         |
| N.Ob/B.Pm (/mm) | 6.51±1.32      | 19.89±4.60*     | 1.80±0.79*               | 5.72±1.27*       | 0.000         |
| OV/TV (%) | 0.062±0.013         | 0.230±0.076*    | 0.010±0.003*             | 0.036±0.012*     | 0.002         |
| OS/BS (%) | 3.30±0.7           | 10.62±3.5*      | 0.47±0.1*                | 1.71±0.5*        | 0.002         |
| O.Th (µm) | 3.00±0.20          | 3.32±0.21       | 2.46±0.48                | 2.94±0.36        | 0.348         |
| Oc.S/BS (%) | 1.26±0.28          | 3.43±0.56*      | 0.72±0.23*               | 1.27±0.23*       | 0.000         |
| N.Oc/T.Ar (/mm²) | 2.72±0.52        | 8.51±1.68*      | 2.42±0.73*               | 3.37±0.67*       | 0.000         |
| N.Oc/B.Pm (/mm) | 0.44±0.09        | 1.29±0.21*      | 0.30±0.09*               | 0.49±0.10*       | 0.000         |
| ES/BS (%) | 0.43±0.10          | 1.26±0.26*      | 0.26±0.12*               | 0.57±0.14*       | 0.001         |
Figure 1

A

10Kb DMP1-Cre

B

CRE

Control

DMP1-Cre

LoxP PPRKO LoxP

C

D

cAMP (pmol/ml)

Control  Ocy-PPRKO

Control  Ocy-PPRKO
Osteocytes regulate anabolic and catabolic responses to PTH

Figure 2

A

Total Body Weight (g)

| Age (weeks) | 4 | 8 | 12 |
|-------------|---|---|----|
| 0           |   |   |    |
| 5           |   |   |    |
| 10          |   |   |    |
| 15          |   |   |    |

B

BMD (g/cm²)

| Age (weeks) | 8 | 12 | 20 |
|-------------|---|----|----|
| 0           |   |    |    |
| 0.02        |   |    |    |
| 0.04        |   |    |    |
| 0.06        |   |    |    |

C

BMD (g/cm²)

| Age (weeks) | 8 | 15 | 12 |
|-------------|---|----|----|
| 0           |   |    |    |
| 0.02        |   |    |    |
| 0.04        |   |    |    |
| 0.06        |   |    |    |

D

Control vs. Ocy-PPRKO

4 w

8 w

12 w

G

Relative % of SOST mRNA

H

Relative % of OPG mRNA

I

Relative % of RANKL mRNA

E

Control vs. Ocy-PPRKO

14 w

12 w
Figure 3

A

B

C

D

E

F

G

Osteocytes regulate anabolic and catabolic responses to PTH
Osteocytes regulate anabolic and catabolic responses to PTH
Figure 5

Osteocytes regulate anabolic and catabolic responses to PTH
Figure 6

Figure showing the regulation of anabolic and catabolic responses to PTH by osteocytes.
Parathyroid Hormone (PTH)/PTH-related Peptide Type 1 Receptor (PPR) Signaling in Osteocytes Regulates Anabolic and Catabolic Skeletal Responses to PTH
Vaibhav Saini, Dean J. Marengi, Kevin J. Barry, Keertik S. Fulzele, Erica Heiden, Xiaolong Liu, Christopher Dedic, Akira Maeda, Sutada Lotinun, Roland Baron and Paola Divieti Pajevic

J. Biol. Chem. published online June 2, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M112.441360

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