Original Article

ORIGINAL ARTICLE

ANSARI ET AL.

MURINE SKELETON AUTO/PARACRINE REGULATION BY OSTEOCYTE-DERIVED PTHrP

Autocrine and Paracrine Regulation of the Murine Skeleton by Osteocyte-Derived Parathyroid Hormone-Related Protein[1]

Niloufar Ansari,[1,2]* Patricia WM Ho,[1]* Blessing Crimeen-Irwin,[1] Ingrid J Poulton,[1] Athena R Brunt,[3] Mark R Forwood,[3] Paola Divieti Pajevic,[4] Jonathan H Gooi,[2] T John Martin,[1,2] and Natalie A Sims[1,2]

[1]St. Vincent's Institute of Medical Research, Fitzroy, Victoria, Australia

[2]The University of Melbourne, Department of Medicine at St. Vincent's Hospital, Fitzroy, Victoria, Australia

[3]School of Medical Science and Menzies Health Institute Queensland, Griffith University, Gold Coast, Queensland, Australia

[4]Department of Molecular and Cell Biology, Boston University Goldman School of Dental Medicine, Boston, MA, USA

[1] This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi:10.1002/jbmr.3291

ABSTRACT

Parathyroid hormone–related protein (PTHrP) and parathyroid hormone (PTH) have N-terminal domains that bind a common receptor, PTHR1. N-terminal PTH (teriparatide) and now a modified N-terminal PTHrP (abaloparatide) are US Food and Drug Administration (FDA)-approved therapies for osteoporosis. In physiology, 00PTHrP does not normally circulate at significant levels, but acts locally, and osteocytes, cells residing within the bone matrix, express both PTHrP and the PTHR1. Because PTHR1 in osteocytes is required for normal bone resorption, we determined how osteocyte-derived PTHrP influences the skeleton. We observed that adult mice with low PTHrP in osteocytes (targeted with the *Dmp1(10kb)-Cre*) have low trabecular bone volume and osteoblast numbers, but osteoclast numbers were unaffected. In addition, bone size was normal, but cortical bone strength was impaired. Osteocyte-derived PTHrP therefore stimulates bone formation and bone matrix strength, but is not required for normal osteoclastogenesis. PTHrP knockdown and overexpression studies in cultured osteocytes indicate that osteocyte-secreted PTHrP regulates their expression of genes involved in matrix mineralization. We determined that osteocytes secrete full-length PTHrP with no evidence for secretion of lower molecular weight forms containing the N-terminus. We conclude that osteocyte-derived full-length PTHrP acts through both PTHR1 receptor-mediated and receptor-independent actions in a paracrine/autocrine manner to stimulate bone formation and to modify adult cortical bone strength. © 2017 American Society for Bone and Mineral Research

KEY WORDS: OSTEOCYTES; GENETIC ANIMAL MODELS; PTHRP; ANABOLICS; CELL/TISSUE SIGNALING; PARACRINE PATHWAYS

Received in original form June 28, 2017; revised form August 28, 2017; accepted September 6, 2017. Accepted manuscript online September 15, 2017.

Address correspondence to: Natalie A Sims, BSc (Hons), PhD, St. Vincent's Institute of Medical Research, Fitzroy, Victoria, Australia. E-mail: nsims@svi.edu.au

*NA and PWMH contributed equally to this work.

Additional Supporting Information may be found in the online version of this article.

Journal of Bone and Mineral Research, Vol. XX, No. X, Month 2017, pp XXXX–XXXX

DOI: 10.1002/jbmr.3291

Introduction

Parathyroid hormone related protein (PTHrP, gene name: *Pthlh*) is a multifunctional cytokine,[1] initially discovered as the cause of the humoral hypercalcemia of malignancy.[2] Soon after its discovery, PTHrP was found to be expressed widely in the body, where it acts in many tissues in a paracrine, autocrine, or intracrine manner to regulate endochondral bone formation,[3] breast cancer metastasis,[4] placental calcium transport,[5] mammary gland development,[6] and vascular relaxation.[7] In bone, PTHrP is expressed by osteoblasts (bone-forming cells) and osteocytes.[8] Two loss-of-function models identified that physiological osteoblast-derived PTHrP promotes bone formation and resorption: (i) adult mice globally haploinsufficient for PTHrP and (ii) immature mice lacking PTHrP in the osteoblast lineage (*Col1(2.3kb)Cre.Pthlh*$^{f/f}$).[9] Both models exhibited low bone mass and impaired osteoclast and osteoblast differentiation.[10]

The physiologic action of local PTHrP to promote bone formation has been applied therapeutically to osteoporosis treatment, where intermittent parathyroid hormone (PTH, teriparatide) injection promotes bone formation.[11–14] This occurs because PTHrP and PTH are related in the N-terminal region, in which eight of the first 13 residues are identical. This homology allows the two to act with equal potency through a common G-protein-coupled receptor, PTH receptor 1 (PTHR1), to activate signaling predominantly through cyclic AMP (cAMP).[15] This action has recently been exploited further by a second clinical agent (abaloparatide), which has high homology to the PTHrP N-terminal sequence. Like PTH, abaloparatide both acts through the PTHR1[16] and prevents fractures in osteoporosis.[17] Abaloparatide was approved recently by the US Food and Drug Administration (FDA) as TYMLOS™.

Establishment and maintenance of a strong skeleton depends upon many intercellular communication processes that operate among resident cells in bone, on the bone surface, and in bone marrow.[18,19] Attention has recently shifted to include signals from osteocytes, which are terminally differentiated osteoblasts, embedded within the mineralized matrix, and the most abundant cells in bone.[20] Situated within lacunae, their

dendrite-like processes extend through canaliculi within the mineralized matrix, connecting osteocytes with each other and with cells on the bone surface, including osteoblasts, bone lining cells, and osteoclasts[21]; such actions are crucial for bone modeling and remodeling.[22–24] Osteocytes express PTHrP[8] and their expression of PTHR1 is required for normal bone mass,[25,26] but whether this reflects physiological action of the hormone PTH, or the locally expressed cytokine PTHrP is not known.

We sought to establish whether osteocyte-secreted PTHrP acts in an autocrine/paracrine manner through the PTHR1 to influence normal bone mass. This led us to prepare mice null for *Pthlh* in osteocytes, yielding a low bone mass phenotype in adult mice with decreased bone formation and increased cortical fragility, yet normal osteoclast numbers compared with control mice. Further, we employed an osteocyte cell line to show by multiple methods that PTHrP has autocrine/paracrine actions on osteocytes, and that full-length PTHrP is released from these cells. We conclude that osteocyte-derived PTHrP is required for normal adult bone formation and that the secreted form is full-length PTHrP, with no evidence of shorter peptides capable of PTHR1-mediated action.

Materials and Methods

Materials

Synthetic human PTH(1-34) was purchased from Bachem (Torrance, CA, USA). Recombinant human PTHrP(1-141) was expressed in *Escherichia coli* (*E. coli*) and purified material was equipotent on a molar basis with PTHrP(1-34) when assayed by cAMP response in UMR106 cells.[27]

Cell culture

We used the osteocytic cell line, Ocy454, which is maintained in permissive conditions (33°C), then differentiated at 37°C to reach a stage of osteocytic gene expression after 10 to 14 days.[28] Ocy454 cells were cultured as described, in α-MEM supplemented with 10% FBS and 1% Penicillin-Streptomycin-Amphotericin B and Glutamax.[28] Briefly, for two-dimensional (2D) cell cultures, Ocy454 cells were plated at 2.5×10^5 cells per well of a six-well plate. Three-dimensional (3D) culture was performed using Reinnervate® Alvetex scaffold six-well inserts (Reinnervate, Pittsburgh, PA, USA). The scaffolds were made of highly porous, cross-linked polystyrene discs with 200 μm thickness and 22 mm

diameter. The Alvetex inserts were prepared for seeding by a 70% ethanol wash, followed by two washes with complete culture media. Cells were seeded at the density of 1.6×10^6 cells per insert. Cells were grown at the permissive temperature (33°C) for 3 days prior to transferring to (37°C) for differentiation. Cells were differentiated for up to 14 days as indicated in individual experiments carried out either using either Alvetex scaffolds or 2D surface growth as indicated.

Pthlh knockdown

shRNA with sequence 3′CCG-GCC-AAT-TAT-TCC-TGT-CAC-TGT-TCT-CGA-GAA-CAG-TGA-CAG-GAA-TAA-TTG-GTT-TTT-TG-5′ was used to knock down *Pthlh* in Ocy454 cells. This shRNA was synthesized by Sigma and cloned into a lentiviral vector PLKO, as we reported.[29] Undifferentiated Ocy454 cells were infected with virus and selected with puromycin (5 μg/mL), then cultured at permissive temperature (33°C) before transfer to 37°C for differentiation. Knockdown was assessed by quantitative real-time PCR (qRT-PCR).

RNA extraction, cDNA synthesis, and qRT-PCR

RNA was extracted by RNA extraction kits with on-column DNase digestion (Qiagen, Limburg, Netherlands; Bioline, London, UK), or TriSure reagent (Bioline, London, UK). Extracted RNA was DNase treated with Ambion TURBO DNA-free kit (Life Technologies) and quantified on a NanoDrop ND1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized from total RNA with AffinityScript cDNA synthesis kits (Agilent Technologies, Santa Clara, CA, USA). Gene expression was quantified on a Stratagene Mx3000P QPCR system (Agilent) with Brilliant II SYBR green QPCR master mix (Agilent Technologies, Santa Clara, CA, USA) or Multiplex SensiMix II Probe kits (Bioline, London, UK) with primers specific to genes of interest, as follows: *Alpl*,[30] *Bglap1*,[30] *Dmp1*,[31] *Mepe*,[32] *Nr4a1*,[29] *Pthlh*,[33] *Pth1r*,[30] *Rgs2*,[29] *Sost*,[32] *Tnfsf11*,[30] and *Tnfrsf11b*.[30] To detect the PTHrP signal peptide and deletion of the nuclear localization sequence (NLS), the following primers were used: *Pthlh* signal peptide: forward 3′-TGGTTCAGCAGTGGAGTGTC-5′, reverse 3′-CAGACACAGCGCGTTTGAG-5′. Primers surrounding NLS: forward 3′-CTCCAAACCTGCTCCCAACA-5′, reverse 3′-GACCGAGTCCTTCGCTTCTT-5′. Gene expression between samples was normalized to hypoxanthine

phosphoribosyltransferase 1 (*Hprt1*) expression. Relative expression was quantified using the comparative threshold cycle (Ct) method ($2^{-(\text{Gene Ct} - \text{Normalizer Ct})}$).

PTHrP biological assay and radioimmunoassay

PTHrP secreted into media was assayed as the cAMP generated in response to treatment of UMR106-01 cells, using PTH(1-34)-induced cAMP response as a standard curve.[34] The same cAMP assay was used to test the response of Ocy454 cells to PTH(1-34), recombinant human PTHrP(1-141),[27] or isoproterenol. Replicate cell cultures in 12-well plates were incubated in cell culture medium with 1mM isobutylmethylxanthine (IBMX) added. After treatment for 12 min with indicated agonists, cAMP was measured by removing medium and adding acidified ethanol, drying, reconstituting in assay buffer, and cAMP assay as described.[27] To test the effect of *Pthlh* knockdown on cAMP generation in Ocy454 cells, vector control or shRNA infected cells were treated with 1mM IBMX for 60 min and assayed for intracellular cAMP.[34] For comparison of cAMP responses between different cells, total protein was quantified from groups of cells treated identically to those in the cAMP assay described above. Cells were washed three times with phosphate buffered saline (PBS), and solubilized with 500 μL of radioimmunoprecipitation assay (RIPA) buffer (1% proteinase inhibitor [PI], 150mM NaCl, 1mM EDTA, 1% triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20mM Tris acetate, pH 8.0). Extracts were centrifuged and protein supernatants assayed using bicinchoninic acid (BCA) protein assay buffer (Pierce). Absorbance was measured at optical density (OD) 562 nm using the Polarstar Optima+ and a bovine serum albumin standard curve.

Amino-terminal PTHrP radioimmunoassay (RIA) was carried out as described, with a sensitivity of 2pM.[35]

Pthlh overexpression gene constructs

Four *Pthlh* constructs were synthesized by Integrated DNA Technologies (IDT) (Coralville, IA, USA): *Pthlh*(-36-139), *Pthlh*(1-139), *Pthlh*(-36-67), *Pthlh*(-36-139ΔNLS) were cloned into plasmid murine stem cell virus (MSCV)-zeo by Xhol1/EcoR1 enzyme digestion and ligation. The *Pthlh*(-36-139ΔNLS) construct omitted the sequence encoding residues 68 to 94. Each construct has a human influenza hemagglutinin (HA) epitope tag at the C terminus (see Supporting Fig. 1). DNA sequencing was undertaken by the

Australian Genome Research Facility to confirm the sequences. Each mutant plasmid was used to transfect Phoenix cells. Viruses produced were used to infect Ocy454 cells and Zeocin was used for selection and establishment of stable lines. Frozen aliquots of cells were thawed and grown first at the permissive temperature (33°C) for 2 to 3 days before transfer to 37°C for differentiation. RNA isolation, qRT-PCR, cAMP assay, and PTHrP RIA of the Ocy454 cells with the constructs was carried out as described above.

Mice

Dmp1-Cre mice (Tg(*Dmp1*-cre)^{1Jqfe}) mice (containing the *Dmp1* 10-kb promoter region) were kindly provided by Lynda Bonewald (University of Kansas, Kansas City, KS, USA),[36] and *Pthlh*-flox (*Pthlh*^{tm1Ack}) mice by Andrew Karaplis (McGill University, Montreal, Canada).[37] Mice hemizygous for *Dmp1-Cre* were crossed with *Pthlh*$^{f/f}$ mice to generate *Dmp1Cre.Pthlh*$^{f/w}$ breeders, which were used to generate PTHrP-deficient (*Dmp1Cre.Pthlh*$^{f/f}$) mice and *Dmp1Cre.Pthlh*$^{w/w}$ littermates, which were used as controls. To purify osteocytes, these mice were crossed with 8 kb-*Dmp1-GFP* (Tg(Dmp1-Topaz)^{1Ikal} mice obtained from Ivo Kalajzic (University of Connecticut Health Science Center, Farmington, CT, USA), via the colony of Hong Zhou (ANZAC Research Institute, Sydney, Australia).[38] All mice were housed at the St Vincent's BioResources Centre, a specific-pathogen-free facility in a 12-hour light and dark cycle and provided food and water *ad libitum*. Observers were blinded to the genotypes of the mice for all procedures and analyses. St. Vincent's Health Animal Ethics Committee approved all animal procedures.

Confirmation of PTHrP knockdown: genomic excision, PCR, and immunohistochemistry

Tissues were collected from various organs including marrow flushed-out femurs and tibias, calvaria, heart, spleen, brain, kidneys, mammary glands, and uterus of 6-week-old female *Dmp1Cre.Pthlh*$^{w/w}$ and *Dmp1Cre.Pthlh*$^{f/f}$ mice. Genomic DNA (gDNA) was extracted using Isolate II genomic DNA kit (Bioline). LoxP locations were determined by sequencing gDNA of *Dmp1Cre.Pthlh*$^{f/f}$ mice using forward 5′-TCCAACAGAACTCGGGAGAC-3′ and reverse 5′-GAGGCTAAGCCAGGAGGATT-3′ primers to span these LoxP sites. PCR of 100 ng of gDNA was performed by using Taq polymerase (Mytaq HS Red; Bioline). PCR products were electrophoresed on 3% agarose gel and visualized by VersaDoc Imaging system (Bio-Rad).

To confirm specific knockdown of *Pthlh* mRNA in osteocytes, *Dmp1Cre.Pthlh*$^{f/f}$ mice were crossed with *Dmp1-GFP* mice. Use of the *Dmp1-GFP* mice allows purification of osteocytes without contamination by osteoblasts[32,39,40] because the 8-kb *Dmp1* promoter used in *Dmp1-GFP* transgenic mice leads to more restricted expression of GFP in osteocytes than using *Dmp1*-Cre, which is also expressed in late osteoblasts. FACS-purified GFP+ osteocytes from 6-week-old (male and female) *Dmp1Cre.Pthlh*$^{f/f}$*.Dmp1-GFP*$^{+}$ and *Dmp1Cre.Pthlh*$^{w/w}$*.Dmp1-GFP*$^{+}$ mice were isolated from marrow-flushed long bones as described.[32] Briefly, bones were processed by seven sequential 15-min digestions in 2 mg/mL dispase (Gibco, Grand Island, NY, USA) and 1 mg/mL collagenase type II (Worthington, Australia). Fractions 2 to 7 were collected, pooled, and resuspended in alpha modified Eagle's medium (α-MEM; Gibco) containing 10% FBS and centrifuged. Pellets were resuspended in FACS buffer before cell sorting. Dead cells and debris were removed based on side scatter (SSC) and forward scatter (FSC) areas, and doublets were excluded based both on SSC width (W) versus SSC height (H) and on FSC-W versus FSC-H. Cells were sorted with excitation 488 nm and 530/30 or 530/40-emission filter for GFP on a BD FACS Influx cell sorter (BD Bioscience). RNA was extracted using Isolate II Micro RNA kit (Bioline, London, UK). cDNA was prepared with a Superscript III kit.

Immunohistochemistry was carried out as described[41,42] on decalcified paraffin-embedded tibial sections from 12-week-old male and female mice using goat anti-sclerostin (1:500; R&D Systems, Minneapolis, MN, USA) or rabbit anti-PTHrP (1:5000, R87, generated against PTHrP(1-34)[43]) antibodies. To quantify positive osteocytes in cortical bone, measurements were made commencing 1110 μm distal to the growth plate, extending for 1110 μm toward the ankle; in trabecular bone, measurements were made in the proximal tibial secondary spongiosa, commencing 370 μm below the growth plate and extending 1110 μm distally using the OsteoMeasure system (Osteometrics Inc., Decatur, GA, USA).

Micro–computed tomography, histomorphometry, and serum biochemistry

Bone and serum samples were collected after a 12-hour fast. Terminal blood samples were collected by cardiac puncture exsanguination and sera kept at –80°C until analysis. Femurs and tibias were fixed overnight in 4% paraformaldehyde at 4°C, then stored in

70% ethanol until further analysis. Femoral morphology and microarchitecture were assessed using the Skyscan 1076 micro–computed tomography (μCT) system (Skyscan, Aartselaar, Belgium) as described.[30] The X-ray source was set at 45 kV and 220 mA. Projections were acquired over a pixel size of 9 μm. For ex vivo scans, femurs were enclosed in a plastic tube filled with 70% ethanol during scanning. Image slices were then reconstructed by Nrecon (Skyscan) with beam-hardening correction of 35%, ring artifact correction of 6, smoothing of 1, and defect pixel masking of 50%. For trabecular and cortical analyses, regions of interest (ROIs) commenced at a distance equal to 7.5% or 30%, respectively, of the total femur length proximal to the distal end of the femur; for each, an ROI of 15% of the total femur length was analyzed. For 6-week-old mice, the lower adaptive threshold limits for trabecular and cortical analysis were equivalent to 0.38 g/mm^3 and 0.65 g/mm^3 calcium hydroxyapatite (CaHA), respectively. For 12-week-old mice, the thresholds used for trabecular and cortical analysis were equivalent to 0.37 g/mm^3 and 0.72 g/mm^3 CaHA, respectively. Cortical bone tissue mineral density was determined in the cortical ROI (Ct.TMD) using the built in TMD function in CTAn (version 1.12.0.0; Bruker, Aartselaar, Belgium); 2 mm CaHA phantoms (Bruker, Aarteslaar, Belgium) were used as calibrators.

Tibias were embedded in methylmethacrylate and sectioned at 5 μm thickness for histomorphometric analysis as described.[44] To determine bone formation rates, calcein was injected intraperitoneally (20 mg/kg) at 10 and 3 days before tissue collection. Sections were stained with Toluidine blue or Xylenol orange, as described.[45] Static and dynamic histomorphometry of trabecular bone surfaces was carried out in the secondary spongiosa, commencing 370 μm below the growth plate and extending 1110 μm distal to the growth plate of the proximal tibia using the OsteoMeasure system (Osteometrics Inc., Decatur, GA, USA).

Serum PTH(1-84) was measured by radioimmunoassay (Immunotopics, San Clemente, CA, USA). Serum procollagen type 1 N-propeptide (P1NP) and cross-linked C-telopeptide of type I collagen (CTX-1) were measured by enzyme immunoassays (IDS, Abacus, Berkeley, CA, USA) according to the manufacturer's instructions.

Three-point bending test and reference point indentation

Mechanical properties were derived from three-point bending tests using an Instron

5565A dual column material testing system and Bluehill 2 software (Instron, Norwood, MA, USA), as described.[46] Briefly, right femurs (the same samples that were studied by μCT) were kept moist in gauze swabs soaked in PBS and positioned horizontally on two supports 6.0 mm apart for loading. Load was applied to the anterior surface of the femoral midshaft at 1 $mm.s^{-1}$ until failure. Load-displacement curves were recorded at a crosshead speed of 1.0 mm/s. Cortical dimensions including anteroposterior, mediolateral, and cortical thickness were measured by μCT at the midshaft and moment of inertia was calculated based on these values. Calculations from the load-displacement, and stress-strain curves were based on the method of Turner and Burr.[47] The yield point was determined from the load deformation curve at the point at which the curve deviated from linear. Local bone material properties were determined on the posterior side of the femoral midshaft by reference point indentation using a BP2 probe assembly apparatus (Biodent Hfc; Active Life Scientific Inc., Santa Barbara, CA, USA) as described.[46]

Purification of PTHrP from Ocy454 conditioned medium and cell lysates

Serum-free medium was collected from Ocy454 cells overexpressing the PTHrP gene constructs. Proteinase inhibitor (Sigma P8340) was added to 50 mL medium, which was then diluted to 150 mL with 0.0375M sodium acetate buffer, pH 4.5. One tenth volume of SP Sepharose Fast Flow gel (GE Healthcare 17-0729-10) pre-equilibrated in starting buffer was mixed with the diluted conditioned medium on a suspension mixer overnight at 4°C. A column was poured using the gel slurry, the column then washed with four bed volumes of starting buffer, followed by 0.15M and 0.3M NaCl washes in 0.0375M sodium acetate. PTHrP bound to SP Sepharose was eluted with 1M NaCl. Eluates were then desalted using Sep Pak C18 cartridges (Waters), lyophilized, and stored at –20°C. Bioactivities of the starting material, and of subsequent eluates were assessed by cAMP stimulation of UMR106.01 cells, using PTH(1-34) as a positive control and to generate a standard curve. The overall recovery of the final product in the Sep-Pak eluate was about 25% of the starting material. The Sep Pak eluates were reconstituted in 300 μL 0.01M acetic acid and stored at –20°C. Fifteen-microliter (15-μL) samples were run on 4-12% Bis-Tris polyacrylamide gels alongside molecular weight markers.

Three-millimeter (3-mm) gel slices were excised and macerated with a scalpel into small pieces, then extracted by 200 μL elution buffer (50mM Tris-HCl, 150mM

NaCl, and 0.1mM EDTA, pH 7.5) overnight with shaking at 4°C. Radioimmunoassay of PTHrP was carried out on each gel slice extract using an N-terminal directed assay with a sensitivity of 2pM, as described.[35] Cell lysates from each of the PTHrP constructs were run on similar gels that were sliced and eluted in the same way and assayed for PTHrP by radioimmunoassay.

Results

Adult *Dmp1Cre.Pthlh*$^{f/f}$ mice have low trabecular bone mass and bone formation

Because *Pthlh* gene nomenclature has changed since the first publication of the *Pthlh*-flox mouse,[48] we confirmed that LoxP sites spanned *Pthlh* exon III (Fig. 1*A*) in tissues expressing Cre under the *Dmp1* promoter. *Dmp1Cre.Pthlh*$^{f/f}$ mice showed *Pthlh* exon III genomic excision in marrow flushed-out long bones (femurs and tibias) and calvariae (Fig. 1*B*). *Dmp1Cre*-directed *Pthlh* recombination was also observed in brain, consistent with previous studies,[49] and mammary glands (Fig. 1*B*). This recombination in mammary glands led us to carry out all subsequent studies in mice bred from mothers hemizygous for the *Pthlh*-loxP gene, with littermate *Dmp1Cre.Pthlh*$^{w/w}$ mice as controls.

<Insert Figure 1>

Because *Pthlh* knockdown has not previously been quantified at either the mRNA or protein level in any bone-targeted *Pthlh*-flox mouse, we sought to quantify the level of deletion at both the mRNA and protein level. *Pthlh* mRNA knockdown in osteocytes was confirmed in GFP+ osteocytes isolated from *Dmp1Cre.Pthlh*$^{f/f}$ mice crossed with the *Dmp1-GFP* reporter mouse to allow purification of osteocytes, without contamination by osteoblasts that may be *Dmp1Cre*-positive.[32,39,40] *Pthlh* mRNA levels were 65% lower than in GFP+ osteocytes from *Dmp1-GFP.Dmp1Cre.Pthlh*$^{w/w}$ mice (Fig. 1*C*).

Pthlh knockdown was also detected in osteocytes at the protein level by immunohistochemistry. In adult *Dmp1Cre.Pthlh*$^{f/f}$ mice, PTHrP was detected at similar levels in growth plate chondrocytes (Fig. 1*D*) and osteoblasts (not shown), but fewer osteocytes stained positive for PTHrP than in age- and sex-matched controls (Fig. 1*E*). We measured PTHrP+ osteocytes on medial and lateral sides of the tibias to account for any possible mechanoregulation of PTHrP. In both regions, female and male *Dmp1Cre.Pthlh*$^{f/f}$ mice had ~30% less PTHrP+ osteocytes than controls (Fig. 1*F*).

Dmp1Cre.Pthlh$^{f/f}$ mice were born at expected Mendelian ratios and displayed no

obvious skeletal abnormalities at birth compared to littermate *Dmp1Cre.Pthlh*$^{w/w}$ controls. They grew normally and weighed the same as control mice (data not shown). No significant differences were detected by in vivo μCT scans in femoral length, width, trabecular, or cortical structure (Fig. 2*A*; Supporting Table 1) at 6 weeks old, indicating normal bone growth and modeling. At 12 weeks, femoral length was also normal (Supporting Table 1), and no changes in growth plate or primary spongiosa were observed (data not shown). At this age, however, trabecular bone volume was 24% lower in male and 51% lower in female *Dmp1Cre.Pthlh*$^{f/f}$ mice, respectively, compared to sex-matched *Dmp1Cre*-positive littermates (Fig. 2*A*, *B*). Trabecular number was reduced to a similar extent in both male and female *Dmp1Cre.Pthlh*$^{f/f}$ mice (Fig. 2*A*), while trabecular separation remained unchanged (Supporting Table 1); in female mice, trabecular thickness was also significantly reduced (Supporting Table 1). In contrast to the changes in trabecular bone mass, there were no significant differences in cortical thickness or size in male or female *Dmp1Cre.Pthlh*$^{f/f}$ mice compared to controls (Supporting Table 1).
<Insert Figure 2>

Histomorphometric analysis revealed that, in female *Dmp1Cre.Pthlh*$^{f/f}$ mice, low trabecular bone mass was associated with low osteoid volume, osteoid thickness, and osteoblast surface compared to littermate controls (Fig. 3*A*), without any significant change in osteoclast surface (Fig. 3*A*) or number (not shown). Dynamic histomorphometry, using dual calcein injection, showed lower double-labeled (mineralizing) surface in female *Dmp1Cre.Pthlh*$^{f/f}$ mice compared to controls, confirming low bone formation in these mice (Fig. 3*B*). Where bone formation was detected, there was no change in mineral appositional rate (Fig. 3*B*). Consistent with lower bone formation, female and male *Dmp1Cre.Pthlh*$^{f/f}$ mice had more sclerostin-positive osteocytes than *Dmp1Cre.Pthlh*$^{w/w}$ controls in their cortical bone. However, in trabecular bone, where the changes in osteoblast and mineralizing surface were detected in females, only males exhibited a significant increase in sclerostin-positive osteocytes (Fig. 3*D*). This might mean that the greater sclerostin expression noted does not cause the low level of bone formation in the absence of PTHrP. Osteocyte density (cells per unit cortical bone area) was not changed in trabecular or cortical bone in male or female *Dmp1Cre.Pthlh*$^{f/f}$ mice.

<Insert Figure 3>

Male *Dmp1Cre.Pthlh*$^{f/f}$ mice, although they also exhibited low trabecular bone mass, did not show significantly lower osteoblast surface or number, nor any significant change in bone formation rate at 12 weeks old (Fig. 3*A*). Whether this is a true sex-difference in the effect of PTHrP deletion in osteocytes is not clear. Both male and female mice have a high level of bone remodeling until 12 weeks of age, when male mice exhibit a decline in osteoblast numbers.[50] Because male controls at this age have osteoblast surface at the lower limit of the range,[46,51] detecting a further reduction may not have been technically possible. The low trabecular bone mass in *Dmp1Cre.Pthlh*$^{f/f}$ mice may also relate to reduced bone formation before 12 weeks of age. Like females, male *Dmp1Cre.Pthlh*$^{f/f}$ mice showed no significant change in osteoclast surface (Fig. 3*A*) or number (data not shown) compared to controls.

Neither male nor female *Dmp1Cre.Pthlh*$^{f/f}$ mice exhibited any significant change in serum P1NP, CTX-1 (Supporting Table 2), or PTH levels (Fig. 3*C*) compared to age- and sex- matched controls.

Dmp1Cre.Pthlh$^{f/f}$ femurs have lower fracture resistance

Although femoral cortical thickness and diameter were not altered, three-point bending tests of cortical bone strength revealed that *Dmp1Cre.Pthlh*$^{f/f}$ femurs were mechanically compromised. Male *Dmp1Cre.Pthlh*$^{f/f}$ femurs exhibited significantly lower ultimate force, ultimate deformation, energy to failure, and displacement at yield (Fig. 4*A*–*E*) compared to controls. Work to fracture, determined from the area under the curve (Fig. 4*A*), was reduced by 29% in male *Dmp1Cre.Pthlh*$^{f/f}$ mice. These structural properties indicate that bone strength and fracture resistance are significantly impaired in male *Dmp1Cre.Pthlh*$^{f/f}$ mice. When mechanical strength testing data were adjusted for bone dimensions, the male femoral cortex had significantly impaired material strength. Strain at yield and strain at fracture were significantly lower than controls (Fig. 4*F*, *G*). Studying cortical bone tissue properties by reference point indentation reinforced these data: male *Dmp1Cre.Pthlh*$^{f/f}$ femurs had lower energy dissipation in response to reference point indentation at the mid-shaft compared to *Dmp1Cre.Pthlh*$^{w/w}$ mice, but total indentation distance was not significantly different (Supporting Table 2). Female *Dmp1Cre.Pthlh*$^{f/f}$ mice showed similar magnitude reductions in ultimate force, energy to

failure, and ultimate strain compared to controls as male mice (Fig. 4*B*, *D*, *E*, *F*). There was no significant interaction between genotype and sex effects in three-point bending data, suggesting that although sample numbers were insufficient to detect a significant change in bone strength in female *Dmp1Cre.Pthlh*$^{f/f}$ femurs, female mice may share the defect in cortical strength observed in the males. No difference in cortical tissue mineral density was detected at the femoral midshaft in either male or female bones (Supporting Table 2).

<Insert Figure 4>

Pthlh knockdown in Ocy454 cells modifies osteocytic differentiation

We next investigated whether endogenously produced PTHrP modified osteocyte gene expression in vitro. *Pthlh* shRNA in the Ocy454 osteocyte cell line reduced their endogenous PTHrP production. This was confirmed at the mRNA level in three different culture conditions: on plasticware plates (2D), on collagen I-coated plates (2D), and in 3D Alvetex scaffolds (Fig. 5*A*). In 3D scaffolds *Pthlh* mRNA levels were reduced by 72%, whereas in plastic and collagen-coated plates this was ~58% (Fig. 5*A*).

<Insert Figure 5>

When Ocy454 cells are transferred to semipermissive temperatures they differentiate and increase osteocytic marker gene expression, including *Dmp1*, *Mepe*, and *Sost*,[28,52,53] thereby replicating osteocyte gene expression patterns in vivo as they become embedded in the mineralizing osteoid matrix. *Pthlh* mRNA levels declined slightly during Ocy454 cell differentiation, and were significantly lower in shRNA-treated cells at all time points, confirming stable knockdown (Fig. 5*B*); similar results were obtained in 2D cultures (data not shown). *Pth1r* mRNA levels increased in both knockdown and control Ocy454 cells during differentiation (Fig. 5*C*). *Pthlh* knockdown cells showed higher *Pth1r* mRNA levels compared to vector controls at the start of differentiation, when *Pth1r* mRNA levels were low in control cells, and only at that time point (Fig. 5*C*).

Pthlh knockdown was associated with an early increase in all three osteocyte markers: *Sost* and *Dmp1* mRNA levels were approximately threefold greater than control at day 7 and 14 of differentiation, respectively (Fig. 5*D, E*), whereas *Mepe* mRNA was twofold greater in knockdown compared to control at day 14 of differentiation (Fig. 5*F*).

Knockdown of *Pthlh* also resulted in lower mRNA levels of the osteoblast markers alkaline phosphatase (*Alpl*) and osteocalcin (*Bglap1*) at both day 7 and 14 (Fig. 5*G*, *H*). *Tnfsf11* (RANKL) mRNA was lower at all time points, and *Tnfrsf11b* (OPG) was lower at day 7 of differentiation (Fig. 5*I*, *J*). These changes suggest osteocyte-derived PTHrP acts in an autocrine/paracrine manner to regulate osteocytic and osteoblastic genes involved in bone remodeling and mineralization.

To confirm PTHrP's autocrine/paracrine effect in Ocy454 cells, we assessed cAMP levels (with maximal phosphodiesterase inhibition) without adding exogenous PTHrP. In this way cAMP is generated only by autocrine receptor-linked adenylyl cyclase activation by cell-derived ligand(s).[29] Although *Pthlh* knockdown cells had higher *Pth1r* mRNA levels than vector control (Fig. 5*C*) at day 0, their cAMP generation was 65% lower than vector control (Fig. 5*K*). This is consistent with loss of PTHrP action as an autocrine/paracrine factor in Ocy454 cells. By day 14 of differentiation, *Pthlh* mRNA had decreased in vector control (Fig. 5*B*), functional PTHR1 was greater in both vector control and *Pthlh* knockdown cells (Fig. 5*C*), and the difference in cAMP generation was no longer apparent.

Ocy454 cells respond to exogenous full-length recombinant PTHrP

How full-length exogenous PTHrP affects osteocytes is not known. PTHrP(1-141) and shorter forms containing the N-terminus activate adenylyl cyclase in target cells on an equipotent molar basis with PTH(1-34) and PTHrP(1-34),[15,27] so we used the same doses to test effects of full-length PTHrP compared to N-terminal PTH on Ocy454 cells. Although *Pth1r* levels were low in undifferentiated Ocy454 cells at day 0 (Fig. 5*A*), a small increase in cAMP production was elicited by treating with PTH(1-34) or recombinant human PTHrP(1-141) (hPTHrP(1-141)) (Fig. 6*A*). At day 7 and 14 of differentiation, exogenous treatment with PTH(1-34) and hPTHrP(1-141) increased cAMP levels more than 100-fold (Fig. 6*A*), confirming increased PTHR1 expression with Ocy454 cell differentiation. Treatment at day 14 with PTH(1-34) or hPTHrP(1-141) for 6 hours significantly reduced *Sost* mRNA levels by 64% and 80%, respectively (Fig. 6*B*). PTH(1-34) and hPTHrP(1-141) treatment also decreased *Dmp1* and *Mepe* levels in differentiated cells (Fig. 6*C*, *D*), while *Tnfsf11* (RANKL) levels were increased more than 100-fold (Fig. 6*E*) and *Tnfrsf11b* (OPG) levels were significantly reduced (Fig. 6*F*).

<Insert Figure 6>

Secreted PTHrP overexpression in Ocy454 cells activates cAMP and regulates known cAMP targets

Because the gene expression data from the *Pthlh* knockdown cells indicated that PTHrP was produced by and acted upon osteocytes, we established an approach that could be used to determine whether this was mediated through the PTHR1 by osteocyte-secreted PTHrP, or whether PTHrP action through its NLS or C-terminus may be involved. We prepared Ocy454 cells expressing murine *Pthlh* gene constructs engineered to express secreted full-length PTHrP (*Pthlh*(-36-139)), PTHrP lacking the leader sequence required for secretion (*Pthlh*(1-139)), PTHrP lacking the nuclear localization sequence (*Pthlh*(-36-139ΔNLS)), or PTHrP lacking the C-terminus (*Pthlh*(-36-67)) (see Supporting Fig. 1 for construct design). Using primers targeting regions common to the four constructs (Fig. 7*A*, *E*), we confirmed that all constructs produced *Pthlh* mRNA at levels higher than vector control (Fig. 7*E*). Primers directed to the leader sequence (Fig. 7*B*) confirmed leader sequence transcription in cells expressing all constructs, except the one designed to lack this sequence, *Pthlh*(1-139). Primers designed to bind surrounding the NLS generated amplicons in cells expressing all constructs except *Pthlh*(-36-67), which lacks this region entirely (Fig. 7*C*). In the cells expressing *Pthlh*(-36-139ΔNLS) an amplicon was detected, but at a lower temperature, reflecting its lower GC content (Fig. 7*C*, *D*).

<Insert Figure 7>

We measured PTHrP protein biological activity in conditioned media from the cells by assaying cAMP response in UMR106-01 cells (Fig. 7*F*) and by RIA for PTHrP protein (Fig. 7*G*). All three constructs containing the leader sequence/secretion signal generated conditioned media containing PTHrP protein with substantial biological activity. Activity level in their media was equivalent to approximately 150nM hPTH(1-34), used as standard in the cAMP response assay. No significant activity nor any PTHrP protein greater than vector control was detected either by bioassay or RIA in media from those cells transduced with the construct for *Pthlh*(1-139), which contains no secretory mechanism (Fig. 7*F*, *G*).

All osteocyte-secreted PTHrP isoforms regulated target genes known to be regulated through PTHR1/cAMP in Ocy454 cells. *Pth1r* mRNA was significantly less

from day 8 in cells transfected with all secreted PTHrP forms compared to MSCV control (Fig. 8*A*), consistent with receptor downregulation through N-terminal action. *Pth1r* was not modified by the non-secreted form, confirming that it did not act through the PTHR1. *Tnfsf11* mRNA (Fig. 8*B*) was increased and *Tnfrsf11b* decreased (Fig. 8*C*) by all secreted constructs, but not by non-secreted *Pthlh*(1-139). At day 14, *Bglap1* and the cAMP response element-binding protein (CREB) targets *Nr4a1* and *Rgs2* were increased by all three constructs that secreted active PTHrP, but not by *Pthlh*(1-139) (Fig. 8*D–F*). *Dmp1* was also downregulated by all three secreted PTHrP forms, but not by unsecreted *Pthlh*(1-139) at day 14 (*Dmp1:Hprt1* MSCV = 0.056 ± 0.024, *Pthlh*(-36-139) = 0.006 ± 0.001, *Pthlh*(-36-139ΔNLS) = 0.001 ± 0.0001, *Pthlh*(-36-67) = 0.006 ± 0.002, *Pthlh*(1-139) = 0.047 ± 0.011; mean ± SE of three replicate experiments). This confirmed that PTHrP produced by osteocytes activates PTHR1/cAMP action in the same cells.

<Insert Figure 8>

Ocy454 cells secrete full-length PTHrP

The form of PTHrP secreted by mesenchymal cells has never been identified, but neuroendocrine cells have been shown to process PTHrP to cleaved daughter peptide forms prior to secretion.[54,55] We therefore sought to determine the molecular form of PTHrP secreted by osteocytes. Because extracting sufficient protein from osteocytes purified from bone would not be feasible,[32] we used overexpression of the murine *Pthlh* gene in the osteocyte cell line Ocy454[28] to characterize the PTHrP that they secrete. The biological assay measures PTHrP species containing intact N-terminal protein (at least 34 residues), with a sensitivity of 0.01nM to 0.1nM.[29] This showed PTHrP activity was retained in conditioned medium in cation exchange chromatography eluates (Fig. 9*A*); a similar pattern of biological activity elution was obtained with all three secreted gene products. After cation exchange chromatography, active fractions were desalted and concentrated by Sep-Pak and subjected to polyacrylamide gel electrophoresis. Extracted gel slices (3 mm) were assayed for N-terminal PTHrP by RIA with a 2pM sensitivity of detection[35]; all forms of PTHrP that contain PTHrP(1-34) are detected equally on a molar basis using this RIA. *Pthlh*(-36-139) cells yielded culture medium protein of approximately 17 to 20 kDa, consistent with the size of full-length PTHrP, with no evidence of any lower molecular weight material that is active on the PTHR1 (Fig. 9*B*).

The constructs for *Pthlh*(-36-139ΔNLS) and *Pthlh*(-36-67) yielded peaks on electrophoresis that were not resolved from each other, but were, at 14 kDa, significantly lower in molecular weight than the *Pthlh*(-36-139) peak (Fig. 9*B*). Synthetic PTHrP(1-36) was subjected to electrophoresis also, and appeared at a much lower molecular weight, as expected (Fig. 9*B*).

<Insert Figure 9>

When cell lysates were examined, the construct for *Pthlh*(-36-139) also yielded a peak at 17 kDa (Fig. 9*C*) that was coincident with the peak from cells containing the non-secreted *Pthlh*(1-139) construct. The lysate peaks for *Pthlh*(-36-139ΔNLS) and *Pthlh*(-36-67) were at a lower molecular weight. These findings indicate that secreted PTHrP from the Ocy454 cells is full length PTHrP and is not processed within the cells to any lower molecular weight forms that contain the N-terminal region.

Discussion

We have established that osteocytes release PTHrP, which maintains normal adult trabecular bone mass and maintains cortical resistance to fracture. Further, using cultured cells, we have shown that PTHrP secreted by osteocytes is full length and acts in an autocrine/paracrine manner to regulate osteocyte gene expression.

This is the first report detailing the nature of PTHrP secreted by osteoblast lineage cells. We report release of full-length PTHrP from osteocytes, with no evidence for release of any shorter peptides capable of action upon PTHR1. We previously detected PTHrP protein in osteoblasts and osteocytes during endochondral ossification and intramembranous bone formation.[8] The nature of osteocyte-derived PTHrP could not be determined in the earlier study, and until now has not been known. Previous PTHrP secretion studies analyzed neuroendocrine cells that use the regulated secretory pathway to package, process, and secrete truncated daughter peptides[54,55] including PTHrP(1-36), PTHrP(38-84), either free or amidated carboxy-terminus, and PTHrP(107-138).[56–58] Our new findings suggest osteocytes use the constitutive secretory pathway for PTHrP. A similar conclusion was reached in a recent study of FGF23 secretion by IDG-SW3 osteocytes.[59] As we observed for PTHrP, FGF23 used the constitutive secretory pathway, with no evidence for using the regulated secretory pathway.[59] PTHrP biological activity through PTHR1 is destroyed by kexin-2 cleavage carboxyl to

$Arg_{19}Arg_{20}Arg_{21}$.[60] Because our PTHrP bioassay detects its N-terminal biological activity, our new findings do not exclude generation of short PTHrP fragments lacking biological activity through PTHR1 by osteocytes. However, the PTHrP form secreted by osteocytes that acts on the PTHR1 is full-length PTHrP, with no PTH1R-active truncated forms. Our data suggest that osteocytes do not use the regulated secretory pathway for PTHrP production, although this mechanism can be used in neuroendocrine cells. This difference between these two cell types indicates tissue- and cell-specific PTHrP processing.

The trabecular phenotype of low bone mass with low osteoblast numbers and mineralizing surface in *Dmp1Cre.Pthlh*$^{f/f}$ mice has some similarity with a previously described model targeting PTHrP earlier in the osteoblast lineage, the *Col1(2.3kb)Pthlh*$^{f/f}$ mouse,[9] but also shows some differences. First, the low osteoblast numbers in both models indicates production of PTHrP by cells in late stages of osteoblast/osteocyte differentiation (targeted by both models) is required to support osteoblast differentiation and activity. Osteocyte-secreted PTHrP may promote bone formation in a manner similar to osteocyte-secreted sclerostin.[61] Secreted full-length PTHrP transported through the lacunar canalicular network would act upon osteocytes, osteoblasts, and other cells on or near the bone surface (illustrated schematically in Fig. 10).

<Insert Figure 10>

Sclerostin suppresses bone formation, and can be inhibited by PTHR1-mediated activation of cAMP.[62] Because treatment with either exogenous recombinant PTHrP(1-141) or PTH(1-34) reduced sclerostin in Ocy454 cells, and had equivalent effects on other target genes, some bone-forming actions of PTHrP are likely to be mediated through PTHR1/cAMP. The downregulation of sclerostin by PTHrP was reflected in increased sclerostin expression in *Pthlh*-deficient Ocy454 osteocytes and by the greater numbers of sclerostin-positive osteocytes in cortical bone of *Dmp1Cre.Pthlh*$^{f/f}$ mice. However, in female *Dmp1Cre.Pthlh*$^{f/f}$ mice, where osteoblast numbers and mineralizing surface were low, there was no significant reduction in the number of sclerostin-positive osteocytes, suggesting that this may not be the primary cause of the reduction in trabecular bone formation in the absence of PTHrP.

The *Dmp1Cre.Pthlh*$^{f/f}$ mouse lacked two specific features of the

Col1(2.3kb)Cre.Pthlh$^{f/f}$ mouse: (i) a low bone mass phenotype in younger animals (6 weeks old), and (ii) low osteoclast numbers. These differences suggest PTHrP production by osteocytes is not required for: (i) trabecular bone development in the juvenile skeleton, nor (ii) support of osteoclastogenesis. The osteocyte has been proposed as an essential source of RANKL for normal osteoclast formation.[63,64] Although RANKL transcription is stimulated by PTH action through PTHR1/cAMP[65] and both exogenous PTHrP treatment and secreted PTHrP overexpression increased RANKL mRNA (*Tnfsf11*) in Ocy454 cells, *Dmp1Cre.Pthlh*$^{f/f}$ mice showed no reduction in osteoclastogenesis compared to controls. This contrast with the *Col1(2.3kb).Pthlh*$^{f/f}$ mice suggests PTHrP expressed at earlier stages of osteoblast differentiation is required for physiological osteoclastogenesis, and although osteocyte-derived PTHrP may stimulate RANKL production, this source of PTHrP is not required for normal osteoclastogenesis.

How might PTHrP production by early, but not late, stages of the osteoblast lineage regulate osteoclastogenesis? Perhaps osteocyte-derived full-length PTHrP is not transported to RANKL-producing cells located close to osteoclast precursors because of its size and the constraints of the lacunocanalicular network.[66] Another possibility is that endogenous PTHrP stimulates RANKL production in vivo, but either (i) there is insufficient contact between RANKL-producing osteocytes and osteoclast precursors; (ii) RANKL is expressed only in newly embedded osteocytes,[8] and there are insufficient of them targeted by our genetic deletion model; or (iii) RANKL expression by osteocytes, even when stimulated by PTHrP, is insufficient to support osteoclastogenesis. Consistent with the latter suggestion, purified osteocytes do not fully support osteoclastogenesis, even with direct contact in vitro.[32] We conclude that RANKL induction in osteocytes by PTHrP is not required physiologically, even though in vitro effects are evident. This does not exclude the possibility that osteocyte-derived PTHrP might contribute to pathological osteoclastogenesis, such as in bone metastasis.[67]

The normal osteoclast numbers in *Dmp1Cre.Pthlh*$^{f/f}$ mice also contrasts with two previous models where PTHR1, the receptor used by both PTHrP and PTH, was deleted in *Dmp1Cre*-expressing cells. Those models used either the same *Dmp1Cre* as ours (with 10 kb of the promoter)[25] or an alternative *Dmp1Cre* (with 8 kb of the promoter).[26] Although the analyses cannot be directly compared because of methodological

differences, both PTHR1-deficient mice exhibited high trabecular bone mass and low levels of osteoclast formation: femoral trabecular bone had fewer osteoclasts in the *Dmp1Cre(10kb).Pth1r* model,[25] and the *Dmp1Cre(8kb).Pth1r* model showed a mild (though inconsistent) reduction in CTX1, a systemic bone resorption marker.[26] In the *Dmp1Cre(8kb).Pth1r* model, high endogenous PTH levels, induced by a low calcium diet, robustly stimulated osteoclastogenesis.[26] This latter observation supports a model in which RANKL induction by endogenous PTH is mediated by effects on cells earlier in the lineage, and not osteocytes. Our work suggests the phenotype of low bone resorption in both *Dmp1Cre.Pth1r* models is not explained by loss of endogenous osteocyte-derived PTHrP.

Although PTH actions through the PTHR1 in osteocytes have been noted previously,[62,68] this work contains the first description of the action of full-length PTHrP on osteocytic gene expression. Overexpression of all secreted forms of PTHrP, and treatment with exogenous full-length PTHrP reduced *Pth1r* levels, increased *Tnfsf11* (RANKL), and decreased *Tnfrsf11b* (OPG). Overexpression of secreted forms also increased two cAMP/CREB/PKA–responsive genes *Nr4a1* and *Rgs2*, consistent with action through the PTHR1/cAMP pathway. No changes in any of these genes were observed in cells overexpressing the non-secreted form of PTHrP (*Pthlh*(1-139)), confirming that intracellular PTHrP does not induce cAMP signaling.

High trabecular bone mass in mice with *Dmp1Cre*-targeted deletion of PTHR1 contrasts with the low trabecular bone mass of both cell-specific models of PTHrP deletion (our *Dmp1Cre.Pthlh*$^{f/f}$, and the prior *Col1(2.3kb).Pthlh*$^{f/f}$ mouse). This suggests that, in addition to PTHR1-mediated activities of PTHrP, some osteocyte-derived PTHrP actions required to maintain normal bone formation may be independent of PTHR1. PTHrP signaling pathways independent of PTHR1 have been reported previously: PTHrP residues 34-139 have non–receptor-mediated intracrine actions, and can translocate to the nucleus.[1,34,69–72] Intracrine actions include anti-apoptotic action in chondrocytes[73] and breast cancer cells.[74] Non–receptor-mediated PTHrP actions can contrast with receptor-mediated actions, as exemplified by its mitogenic action in mesangial cells and smooth muscle cells, using signaling through the nucleus, but anti-apoptotic action in the same cells mediated by PTHR1.[7,75] In the MC3T3-E1 osteoblast cell line, although secreted

PTHrP increased cell growth, viability, osteocalcin, and alkaline phosphatase mRNA levels, the latter effects were inhibited in PTHrP mutants lacking the nuclear localizing sequence or residue 107[76]; osteocytes may also exhibit such intracrine activity. The actions of these regions of PTHrP in bone remain poorly defined. Low bone mass, retarded growth, and early senescence[77] have been reported in knock-in mice expressing PTHrP lacking both its NLS and C-terminal regions, but retaining the PTHR1-activating N-terminus. In another knock-in mouse expressing PTHrP(1-66) there were major impairments of skeletal development, hemopoiesis, and survival.[78] These models indicate that domains of PTHrP other than the amino-terminal are physiologically important, although their extreme defects in bone growth and development make it difficult to discern their exact roles in the mature adult skeleton.

Cortical bone strength was impaired in male and female *Dmp1Cre.Pthlh*$^{f/f}$ mice. Because this was not associated with any change in bone size or shape, there is likely to be a material defect in the cortical bone matrix. The inhibition of *Dmp1* and *Mepe* mRNA levels by PTHrP Ocy454 cells, and the opposite regulation of these genes with PTHrP knockdown suggests an effect of PTHrP on mineralization via osteocytes; whether it is due to osteocytes changing their surrounding matrix, or sending signals to osteoblasts and thereby changing their activity is not yet known. Osteocytic PTHrP may also control collagen orientation, content, or crosslinking by an influence on bone-forming osteoblasts, or may modify other aspects of hydroxyapatite crystal composition. All these ideas require further investigation. We suggest PTHrP affects cortical strength through non–receptor-mediated signaling because intermittent PTH administration, which would also act through PTHR1, did not change bone matrix composition or material strength in mice apart from its ability to increase bone mass.[79]

Our discovery of full-length PTHrP production and secretion by osteocytes provides a way to distinguish the skeletal effects of locally-produced PTHrP from circulating PTH. Although both act on PTHR1, they differ in their roles in skeletal physiology and pathology. This first became clear when mice null for *Pthlh* showed short limb dwarfism characterized by premature chondrocyte differentiation,[3,48] which led to perinatal death due to abnormal rib cage development and respiratory failure. Conversely, murine genetic studies revealed that PTH promotes bone modeling in the fetus and

newborn, but functions physiologically in the adult skeleton to regulate calcium homeostasis rather than bone remodeling per se.[11,80] How can PTHrP and PTH have such different actions when both act on the same receptor? There are two possibilities, and the finding that osteocytes secrete full-length PTHrP is consistent with both. First, PTHrP production is tightly regulated within the bone microenvironment and brought into play where remodeling is required.[13] In contrast, pharmacologically delivered PTH is distributed widely via the bloodstream and would rapidly stimulate PTHR1-mediated responses at all bone remodeling sites at once. Second, although PTH and the PTHrP N-terminus both act through PTHR1, PTHrP has additional non–receptor-mediated actions mediated by the rest of the molecule. Our data suggest that osteocytes and osteoblasts are exposed physiologically not to N-terminal PTHrP, but to full-length PTHrP. This underlies the importance of specifying the nature of locally generated PTHrP. The clinical trials undertaken of PTHrP(1-36) therapy for osteoporosis[81] were predicated on the view that PTHrP(1-36) is a specific locally secreted form of PTHrP. Our data suggest that this is not the case. Although slight differences in conformation of PTHR1 binding have been reported between PTHrP(1-36) (or the modified protein abaloparatide) and PTH(1-34),[16,82,83] these are unlikely to reflect physiological differences within the bone microenvironment.

Although PTHrP levels were reduced at both the protein and mRNA level in osteocytes in this model, we did not observe full deletion of PTHrP. Other studies using the same *Dmp1Cre* do not show full knockdown of target genes at the mRNA level in homogenized marrow-flushed bone, but this contains multiple cell types that also express the target genes.[46,50,84,85] Because PTHrP is expressed in many cells we made use of FACS-purification to specifically purify osteocytes, but the level of knockdown at the mRNA level was only 65%. This is similar to the level of knockdown of mRNA in the only other study to quantify PTHrP knockdown with this PTHrP-loxP mouse, in aortic smooth muscle.[86] No other study using the PTHrP-flox mouse has quantified deletion at the protein level, so we carried out immunohistochemistry and point counting of PTHrP-positive osteocytes. A similar approach has been used by another investigator assessing osteocytic knockdown of IGF1 using the same Dmp1Cre; a reduction in IGF1-positive cells of a little over 50% was achieved.[87] As those authors observed with IGF1, our

point counting of PTHrP-positive osteocytes did not show complete deletion of the gene of interest. We cannot exclude the possibility that positive immunostaining of osteocytes reflects transport of PTHrP from non-osteocytic/non–*Dmp1Cre*-positive cells to lacunae (and perhaps even bound to PTH1R, and internalized). Given the incomplete knockdown at the mRNA level, we suggest this mouse may more accurately reflect a knockdown of PTHrP rather than a knockout. If PTHrP had been knocked out fully, a more severe phenotype may have been observed.

In conclusion, osteocyte production of PTHrP is required for normal trabecular bone mass in the adult murine skeleton, and for production of strong cortical bone. We propose two necessary mechanisms of action for osteocyte-derived PTHrP. First, it is secreted as a full-length form which is transported through the lacunocanalicular network to influence nearby osteocytes, osteoblasts, and their precursors through PTHR1/cAMP signaling; this PTHR1-mediated action is mimicked by therapeutic PTH (Fig. 10). Second, endogenous PTHrP has cytoplasmic or nuclear intracrine actions on the osteocyte network, independent of PTHR1, that also support bone formation and promote strong matrix production.

Disclosures

All authors state that they have no conflicts of interest.

Acknowledgments

This work was partially supported by National Health and Medical Research Council (Australia) Project Grants 1042129 and 1081242. NAS is supported by a National Health and Medical Research Council (Australia) Senior Research Fellowship. St. Vincent's Institute is supported by the Victorian Government's Operational Infrastructure Support Programme. The authors thank the staff of the St. Vincent's Health Bioresources Centre for excellent animal care and assistance, and Carl R Walkley and Emma C Walker for advice on detecting PTHrP recombination.

Authors' roles: NA, PWMH, BC-I, JHG, IJP, ARB, conducted experiments and acquired data. NA, PWMH, BC-I, JHG, IJP, TJM, and NAS analyzed data. NAS and TJM designed the experiments. NA, PWMH, MRF, TJM, and NAS interpreted data. PDP provided the Ocy454 cell line. NA, NAS, and TJM wrote the manuscript. All authors edited the manuscript and approved the final version.

References

1. McCauley LK, Martin TJ. Twenty-five years of PTHrP progress: from cancer hormone to multifunctional cytokine. J Bone Miner Res. 2012;27(6):1231–9.

2. Suva LJ, Winslow GA, Wettenhall RE, et al. A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. Science. 1987;237(4817):893–6.

3. Amizuka N, Warshawsky H, Henderson JE, Goltzman D, Karaplis AC. Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. J Cell Biol. 1994;126(6):1611–23.

4. Powell GJ, Southby J, Danks JA, et al. Localization of parathyroid hormone-related protein in breast cancer metastases: increased incidence in bone compared with other sites. Cancer Res. 1991;51(11):3059–61.

5. Simmonds CS, Kovacs CS. Role of parathyroid hormone (PTH) and PTH-related protein (PTHrP) in regulating mineral homeostasis during fetal development. Crit Rev Eukaryot Gene Expr. 2010;20(3):235–73.

6. Wysolmerski JJ, McCaughern-Carucci JF, Daifotis AG, Broadus AE, Philbrick WM. Overexpression of parathyroid hormone-related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. Development. 1995;121(11):3539–47.

7. Massfelder T, Dann P, Wu TL, Vasavada R, Helwig J-J, Stewart AF. Opposing mitogenic and anti-mitogenic actions of parathyroid hormone-related protein in vascular smooth muscle cells: a critical role for nuclear targeting. Proc Natl Acad Sci U S A. 1997;94(25):13630–5.

8. Kartsogiannis V, Moseley J, McKelvie B, et al. Temporal expression of PTHrP during endochondral bone formation in mouse and intramembranous bone formation in an in vivo rabbit model. Bone. 1997;21(5):385–92.

9. Miao D, He B, Jiang Y, et al. Osteoblast-derived PTHrP is a potent endogenous bone anabolic agent that modifies the therapeutic efficacy of administered PTH 1-34. J Clin Invest. 2005;115(9):2402–11.

10. Martin TJ. Osteoblast-derived PTHrP is a physiological regulator of bone formation. J Clin Invest. 2005;115(9):2322–4.

11. Miao D, He B, Karaplis AC, Goltzman D. Parathyroid hormone is essential for normal fetal bone formation. J Clin Invest. 2002;109(9):1173–82.
12. Miao D, Li J, Xue Y, Su H, Karaplis AC, Goltzman D. Parathyroid hormone-related peptide is required for increased trabecular bone volume in parathyroid hormone-null mice. Endocrinology. 2004;145(8):3554–62.
13. Martin TJ, Sims NA. Integrating endocrine and paracrine influences on bone: lessons from parathyroid hormone and parathyroid hormone-related protein. In: Thakker RW, Whyte MP, Eisman JA, Igarashi T, editors. Genetics of bone biology and skeletal disease. New York: Academic Press; 2013.
14. Martin TJ. Parathyroid hormone-related protein, its regulation of cartilage and bone development, and role in treating bone diseases. Physiol Rev. 2016;96(3):831–71.
15. Kemp B, Moseley J, Rodda C, et al. Parathyroid hormone-related protein of malignancy: active synthetic fragments. Science. 1987;238(4833):1568–70.
16. Hattersley G, Dean T, Corbin BA, Bahar H, Gardella TJ. Binding selectivity of abaloparatide for PTH-type-1-receptor conformations and effects on downstream signaling. Endocrinology. 2016;157(1):141–9.
17. Martin TJ, Seeman E. Abaloparatide is an anabolic, but does it spare resorption? J Bone Miner Res. 2017;32(1):11–6.
18. Sims NA, Martin TJ. Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit. Bonekey Rep. 2014;3:Article number 481.
19. Sims NA, Quinn JM, Martin TJ. Coupling between immune and bone cells.. In: Lorenzo JA, Choi Y, Horowitz MC, Takayanagi H, editors. Osteoimmunology: interactions of the immune and skeletal systems. 2nd ed. London: Academic Press; 2015. p. 169–85.
20. Bonewald LF. The role of the osteocyte in bone and nonbone disease. Endocrinol Metab Clin North Am. 2017;46(1):1–18.
21. Buenzli PR, Sims NA. Quantifying the osteocyte network in the human skeleton. Bone. 2015;75:144–50.
22. Feng JQ, Ward LM, Liu S, et al. Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. Nat Genet. 2006;38(11):1310–5.

23. Tatsumi S, Ishii K, Amizuka N, et al. Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. Cell Metab. 2007;5(6):464–75.
24. van Bezooijen RL, ten Dijke P, Papapoulos SE, Lowik CW. SOST/sclerostin, an osteocyte-derived negative regulator of bone formation. Cytokine Growth Factor Rev. 2005;16(3):319–27.
25. Saini V, Marengi DA, Barry KJ, et al. Parathyroid hormone (PTH)/PTH-related peptide type 1 receptor (PPR) signaling in osteocytes regulates anabolic and catabolic skeletal responses to PTH. J Biol Chem. 2013;288(28):20122–34.
26. Delgado-Calle J, Tu X, Pacheco-Costa R, et al. Control of bone anabolism in response to mechanical loading and PTH by distinct mechanisms downstream of the PTH receptor. J Bone Miner Res. 2017;32(3):522–35.
27. Hammonds RG Jr, McKay P, Winslow GA, et al. Purification and characterization of recombinant human parathyroid hormone-related protein. J Biol Chem. 1989;264(25):14806–11.
28. Spatz JM, Wein MN, Gooi JH, et al. The Wnt inhibitor sclerostin is up-regulated by mechanical unloading in osteocytes in vitro. J Biol Chem. 2015;290(27):16744–58.
29. Walia MK, Ho PM, Taylor S, et al. Activation of PTHrP-cAMP-CREB1 signaling following p53 loss is essential for osteosarcoma initiation and maintenance. eLife. 2016;5:e13446.
30. Takyar FM, Tonna S, Ho PW, et al. EphrinB2/EphB4 inhibition in the osteoblast lineage modifies the anabolic response to parathyroid hormone. J Bone Miner Res. 2013;28(4):912–25.
31. Allan EH, Hausler KD, Wei T, et al. EphrinB2 regulation by PTH and PTHrP revealed by molecular profiling in differentiating osteoblasts. J Bone Miner Res. 2008;23(8):1170–81.
32. Chia LY, Walsh NC, Martin TJ, Sims NA. Isolation and gene expression of haematopoietic-cell-free preparations of highly purified murine osteocytes. Bone. 2015;72:34–42.
33. Takarada T, Kodama A, Hotta S, et al. Clock genes influence gene expression in growth plate and endochondral ossification in mice. J Biol Chem. 2012;287(43):36081–95.

34. Ho PW, Goradia A, Russell MR, et al. Knockdown of PTHR1 in osteosarcoma cells decreases invasion and growth and increases tumor differentiation in vivo. Oncogene. 2015;34(22):2922–33.
35. Grill V, Ho P, Body JJ, et al. Parathyroid hormone-related protein: elevated levels in both humoral hypercalcemia of malignancy and hypercalcemia complicating metastatic breast cancer. J Clin Endocrinol Metab. 1991;73(6):1309–15.
36. Lu Y, Xie Y, Zhang S, Dusevich V, Bonewald LF, Feng JQ. DMP1-targeted Cre expression in odontoblasts and osteocytes. J Dent Res. 2007;86(4):320–5.
37. He B, Deckelbaum RA, Miao D, et al. Tissue-specific targeting of the pthrp gene: the generation of mice with floxed alleles. Endocrinology. 2001;142(5):2070–7.
38. Kalajzic I, Braut A, Guo D, et al. Dentin matrix protein 1 expression during osteoblastic differentiation, generation of an osteocyte GFP-transgene. Bone. 2004;35(1):74–82.
39. Paic F, Igwe JC, Nori R, et al. Identification of differentially expressed genes between osteoblasts and osteocytes. Bone. 2009;45(4):682–92.
40. Halleux C, Kramer I, Allard C, Kneissel M. Isolation of mouse osteocytes using cell fractionation for gene expression analysis. Methods Mol Biol. 2012;816:55–66.
41. McGregor NE, Poulton IJ, Walker EC, et al. Ciliary neurotrophic factor inhibits bone formation and plays a sex-specific role in bone growth and remodeling. Calcif Tissue Int. 2010;86(3):261–70.
42. Walker EC, McGregor NE, Poulton IJ, et al. Oncostatin M promotes bone formation independently of resorption when signaling through leukemia inhibitory factor receptor in mice. J Clin Invest. 2010;120(2):582–92.
43. Lam MH, Thomas RJ, Loveland KL, et al. Nuclear transport of parathyroid hormone (PTH)-related protein is dependent on microtubules. Mol Endocrinol. 2002;16(2):390–401.
44. Sims NA, Clement-Lacroix P, Da Ponte F, et al. Bone homeostasis in growth hormone receptor-null mice is restored by IGF-I but independent of Stat5. J Clin Invest. 2000;106(9):1095–103.
45. Sims NA, Jenkins BJ, Nakamura A, et al. Interleukin-11 receptor signaling is required for normal bone remodeling. J Bone Miner Res. 2005;20(7):1093–102.

46. Johnson RW, Brennan HJ, Vrahnas C, et al. The primary function of gp130 signaling in osteoblasts is to maintain bone formation and strength, rather than promote osteoclast formation. J Bone Miner Res. 2014;29(6):1492–505.
47. Turner CH, Burr DB. Basic biomechanical measurements of bone: a tutorial. Bone. 1993;14(4):595–608.
48. Karaplis AC, Luz A, Glowacki J, et al. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. Genes Dev. 1994;8(3):277–89.
49. Kalajzic I, Matthews BG, Torreggiani E, Harris MA, Divieti Pajevic P, Harris SE. In vitro and in vivo approaches to study osteocyte biology. Bone. 2013;54(2):296–306.
50. Cho DC, Brennan HJ, Johnson RW, et al. Androgen action promotes bone corticalization by inhibiting SOCS3-dependent interleukin-6 signalling. Nat Commun. Forthcoming.
51. Li X, Ominsky MS, Niu QT, et al. Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. J Bone Miner Res. 2008;23(6):860–9.
52. Wein MN, Spatz J, Nishimori S, et al. HDAC5 controls MEF2C-driven sclerostin expression in osteocytes. J Bone Miner Res. 2015;30(3):400–11.
53. Wein MN, Liang Y, Goransson O, et al. SIKs control osteocyte responses to parathyroid hormone. Nat Commun. 2016;7:13176.
54. Burgess TL, Kelly RB. Constitutive and regulated secretion of proteins. Annu Rev Cell Biol. 1987;3:243–93.
55. Philbrick WM, Wysolmerski JJ, Galbraith S, et al. Defining the roles of parathyroid hormone-related protein in normal physiology. Physiol Rev. 1996;76(1):127–73.
56. Orloff JJ, Ganz MB, Nathanson MH, et al. A midregion parathyroid hormone-related peptide mobilizes cytosolic calcium and stimulates formation of inositol trisphosphate in a squamous carcinoma cell line. Endocrinology. 1996;137(12):5376–85.
57. Soifer NE, Dee KE, Insogna KL, et al. Parathyroid hormone-related protein. Evidence for secretion of a novel mid-region fragment by three different cell types. J Biol Chem. 1992;267(25):18236–43.
58. Yang KH, dePapp AE, Soifer NE, et al. Parathyroid hormone-related protein:

evidence for isoform- and tissue-specific posttranslational processing. Biochemistry. 1994;33(23):7460–9.
59. Yamamoto H, Ramos-Molina B, Lick AN, et al. Posttranslational processing of FGF23 in osteocytes during the osteoblast to osteocyte transition. Bone. 2016;84:120–30.
60. Diefenbach-Jagger H, Brenner C, Kemp BE, et al. Arg21 is the preferred kexin cleavage site in parathyroid-hormone-related protein. Eur J Bioch. 1995;229(1):91–8.
61. Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. Nat Med. 2013;19(2):179–92.
62. Keller H, Kneissel M. SOST is a target gene for PTH in bone. Bone. 2005;37(2):148–58.
63. Nakashima T, Hayashi M, Fukunaga T, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. Nat Med. 2011;17(10):1231–4.
64. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. Nat Med. 2011;17(10):1235–41.
65. Fu Q, Jilka RL, Manolagas SC, O'Brien CA. Parathyroid hormone stimulates receptor activator of NFkappa B ligand and inhibits osteoprotegerin expression via protein kinase A activation of cAMP-response element-binding protein. J Biol Chem. 2002;277(50):48868–75.
66. Schaffler MB, Cheung WY, Majeska R, Kennedy O. Osteocytes: master orchestrators of bone. Calcif Tissue Int. 2014;94(1):5–24.
67. Thomas RJ, Guise TA, Yin JJ, et al. Breast cancer cells interact with osteoblasts to support osteoclast formation. Endocrinology. 1999;140(10):4451–8.
68. Bellido T, Ali AA, Gubrij I, et al. Chronic elevation of parathyroid hormone in mice reduces expression of sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis. Endocrinology. 2005;146(11):4577–83.
69. Jans DA, Thomas RJ, Gillespie MT. Parathyroid hormone-related protein (PTHrP): a nucleocytoplasmic shuttling protein with distinct paracrine and intracrine roles. Vitam Horm. 2003;66:345–84.
70. Lam MH, Briggs LJ, Hu W, Martin TJ, Gillespie MT, Jans DA. Importin beta recognizes parathyroid hormone-related protein with high affinity and mediates its nuclear import in the absence of importin alpha. J Biol Chem. 1999;274(11):7391–8.

71. Lam MH, House CM, Tiganis T, et al. Phosphorylation at the cyclin-dependent kinases site (Thr85) of parathyroid hormone-related protein negatively regulates its nuclear localization. J Biol Chem. 1999;274(26):18559–66.
72. Lam MH, Thomas RJ, Martin TJ, Gillespie MT, Jans DA. Nuclear and nucleolar localization of parathyroid hormone-related protein. Immunol Cell Biol. 2000;78(4):395–402.
73. Henderson JE, Amizuka N, Warshawsky H, et al. Nucleolar localization of parathyroid hormone-related peptide enhances survival of chondrocytes under conditions that promote apoptotic cell death. Mol Cell Biol. 1995;15(8):4064–75.
74. Kim W, Takyar FM, Swan K, et al. Calcium-sensing receptor promotes breast cancer by stimulating intracrine actions of parathyroid hormone-related protein. Cancer Res. 2016;76(18):5348–60.
75. Hochane M, Raison D, Coquard C, et al. Parathyroid hormone-related protein is a mitogenic and a survival factor of mesangial cells from male mice: role of intracrine and paracrine pathways. Endocrinology. 2013;154(2):853–64.
76. Garcia-Martin A, Ardura JA, Maycas M, et al. Functional roles of the nuclear localization signal of parathyroid hormone-related protein (PTHrP) in osteoblastic cells. Mol Endocrinol. 2014;28(6):925–34.
77. Miao D, Su H, He B, et al. Severe growth retardation and early lethality in mice lacking the nuclear localization sequence and C-terminus of PTH-related protein. Proc Natl Acad Sci U S A. 2008;105(51):20309–14.
78. Toribio RE, Brown HA, Novince CM, et al. The midregion, nuclear localization sequence, and C terminus of PTHrP regulate skeletal development, hematopoiesis, and survival in mice. FASEB J. 2010;24(6):1947–57.
79. Vrahnas C, Pearson TA, Brunt AR, et al. Anabolic action of parathyroid hormone (PTH) does not compromise bone matrix mineral composition or maturation. Bone. 2016;93:146–54.
80. Miao D, He B, Lanske B, et al. Skeletal abnormalities in Pth-null mice are influenced by dietary calcium. Endocrinology. 2004;145(4):2046–53.
81. Horwitz MJ, Tedesco MB, Garcia-Ocana A, et al. Parathyroid hormone-related protein for the treatment of postmenopausal osteoporosis: defining the maximal tolerable

dose. J Clin Endocrinol Metab. 2010;95(3):1279–87.
82. Dean T, Vilardaga JP, Potts JT Jr, Gardella TJ. Altered selectivity of parathyroid hormone (PTH) and PTH-related protein (PTHrP) for distinct conformations of the PTH/PTHrP receptor. Mol Endocrinol. 2008;22(1):156–66.
83. Okazaki M, Ferrandon S, Vilardaga JP, Bouxsein ML, Potts JT Jr, Gardella TJ. Prolonged signaling at the parathyroid hormone receptor by peptide ligands targeted to a specific receptor conformation. Proc Natl Acad Sci U S A. 2008;105(43):16525–30.
84. Fulzele K, Krause DS, Panaroni C, et al. Myelopoiesis is regulated by osteocytes through Gsalpha-dependent signaling. Blood. 2013;121(6):930–9.
85. Windahl SH, Saxon L, Borjesson AE, et al. Estrogen receptor-alpha is required for the osteogenic response to mechanical loading in a ligand-independent manner involving its activation function 1 but not 2. J Bone Miner Res. 2013;28(2):291–301.
86. Raison D, Coquard C, Hochane M, et al. Knockdown of parathyroid hormone related protein in smooth muscle cells alters renal hemodynamics but not blood pressure. Am J Physiol Renal Physiol. 2013;305(3):F333–42.
87. Sheng MH, Zhou XD, Bonewald LF, Baylink DJ, Lau KH. Disruption of the insulin-like growth factor-1 gene in osteocytes impairs developmental bone growth in mice. Bone. 2013;52(1):133–44.

Figure captions

Fig. **1.** Conditional knockdown of PTHrP in osteocytes in *Dmp1Cre.Pthlh*$^{f/f}$ mice. (*A*) Diagram of murine *Pthlh* gene, LoxP locations determined by sequencing, and recombination product obtained by Cre recombinase activity. Specific primers were designed spanning LoxP sites. (*B*) Genomic DNA was extracted from tissues of 6-week-old female *Dmp1Cre.Pthlh*$^{f/f}$ (f/f) and *Dmp1Cre.Pthlh*$^{w/w}$ (w/w) mice. *Pthlh* genomic excision was assessed using PCR. (*C*) RNA was extracted from FACS-sorted GFP+ cells isolated from f/f and w/w long bones. *Pthlh* transcript level was measured by qRT-PCR. Data shown as mean ± SD of two repeats, each with 3 to 6 mice/group. (*D–F*) Immunostaining for PTHrP. Growth plate (*D*) and diaphysis (*E*) of 12-week-old male and female tibias are shown. Positive (black arrowhead) and negative (white arrowhead) osteocytes were counted in tibial cortical bone on both the medial and lateral sides (n = 7 to 10 mice/group), $^{**}p < 0.01$ versus w/w, by two-way ANOVA with Sidak's post hoc test (*F*). Data shown as mean ± SE. Scale bar = 50 μm.

Fig. **2.** Trabecular bone mass in 6-week-old and 12-week-old *Dmp1Cre.Pthlh*$^{f/f}$ mice. (*A*) Analysis of distal femoral primary spongiosa of male and female Dmp1Cre.Pthlhf/f (f/f) mice measured by μCT. Trabecular bone volume (BV/TV) and trabecular number are shown as mean (dot), median (line), and range; n = 6 to 11/group. $^{*}p < 0.05$ compared to sex- and age-matched *Dmp1Cre.Pthlh*$^{w/w}$ (w/w) littermates by two-way ANOVA and Student's t test. (*B*) Representative μCT images of trabecular bone in the distal femoral primary spongiosa of 12-week-old mice.

Fig. **3.** Histomorphometry of 12-week-old *Dmp1Cre.Pthlh*$^{f/f}$ (f/f) tibias. (*A*) Osteoid volume (Osteoid volume/BV), osteoid surface (Osteoid surface/BS), osteoblast surface (Ob.S/BS), and osteoclast surface (Oc.S/BS) were measured in the distal tibial metaphyseal secondary spongiosa. (*B*) Double-labeled mineralizing surface (dL.S/BS), bone formation rate (BFR/BS), and mineral apposition rate (MAR) were measured by dynamic histomorphometry. Data shown as mean (dot), median (line), and range, n = 6 to 11/group. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to sex- and age-matched Dmp1Cre.Pthlh$^{w/w}$ (w/w) littermates by two-way ANOVA with Sidak's post hoc test. (*C*) Serum PTH levels of 12-week-old male and female *Dmp1Cre.Pthlh*$^{w/w}$ (w/w) and *Dmp1Cre.Pthlh*$^{f/f}$ (f/f) mice (mean ± SE), n = 10 to 12/group. (*D*) Tibias of 12-week-old male and female mice were paraffin-embedded and stained with anti-sclerostin specific antibody. Sclerostin-positive (+ve; black arrowhead) osteocytes (Ocys), and sclerostin-negative osteocytes (white arrowhead) were counted in both cortical and trabecular bone. Data shown as mean ± SE, n = 5 to 9 mice/group. $^{*}p < 0.05$ versus w/w by Student's t test. Scale bar = 50 μm.

Fig. **4.** Mechanical properties of 12-week-old *Dmp1Cre.Pthlh*$^{f/f}$ femurs measured by three-point bending test. (*A*) Average load-displacement curves of male *Dmp1Cre.Pthlh*$^{f/f}$ (f/f) and *Dmp1Cre.Pthlh*$^{w/w}$ (w/w) bones. Ultimate force (*B*), ultimate deformation (*C*), yield displacement (*D*), and energy to failure (*E*) were measured in both males and females. Tissue-level parameters, ultimate (*F*) and yield (*G*) strain, were calculated for each sample based on its anteroposterior and mediolateral dimensions. Data shown as mean (dot), median (line), and range, $n = 8$ to 10/group; p values for all group-wise comparisons are reported on each graph. *$p < 0.05$ and **$p < 0.01$ compared to sex-matched *Dmp1Cre.Pthlh*$^{w/w}$ littermates by two-way ANOVA and Fisher's PLSD test. PLSD = projected least significant difference.

Fig. **5.** Effect of PTHrP knockdown in Ocy454 cells. (*A*) Ocy454 cells were treated with PTHrP shRNA. Selected cells were grown in three culture conditions: 3D scaffolds, plastic plates (2D), and collagen-I coated plates (2D). *Pthlh* mRNA levels were measured by qRT-PCR after 3 days. Data shown as mean ± SE of 2 to 5 independent repeats, each with 2 to 3 samples per group. (*B–J*) mRNA levels of *Pthlh* (*B*), *Pth1r* (*C*), *Sost* (*D*), *Dmp1* (*E*), *Mepe* (*F*), alkaline phosphatase (*Alpl*, *G*), osteocalcin (*Bglap1*, *H*), RANKL (*Tnfsf11*, *I*), and osteoprotegerin (*Tnfrsf11b*, *J*) measured by qRT-PCR in *Pthlh* knockdown and vector control Ocy454 cells differentiated in 3D scaffolds at days 0, 7, and 14 of differentiation. Data shown as mean ± SD, $n = 3$ replicates; representative of 3 independent experiments. (*K*) cAMP levels produced in response to endogenous PTHrP in *Pthlh* knockdown and vector control Ocy454 cells, both undifferentiated (day 0) and grown on plastic plates for 14 days; levels measured after a 60-min incubation with maximal concentration (1mM) of phosphodiesterase inhibitor, IBMX. Data shown as mean ± SD, $n = 5$ to 6. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ compared to vector control by Student's t test. IBMX = isobutylmethylxanthine; ND = not detected.

Fig. **6.** Responses to exogenous treatment with PTH and PTHrP by Ocy454 cells. (*A*) cAMP levels were measured at day 0, 7, and 14 of differentiation in Ocy454 cells treated with hPTH(1-34) or hPTHrP(1-141) for 12 min after IBMX preincubation. Data shown as mean ± SD, $n = 3$ replicates, representative of 3 independent experiments. (*B–E*) Ocy454 cells were differentiated on plastic plates for 14 days. Differentiated cells were treated with hPTH(1-34) or hPTHrP(1-141) for 6 hours; mRNA levels for sclerostin (*Sost*, *B*), *Dmp1* (*C*), *Mepe* (*D*), RANKL (*Tnfsf11*, *E*), and OPG (*Tnfrsf11b*, *F*) were measured by qRT-PCR. Data shown as mean ± SD, $n = 5$ to 6 replicates; representative of 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ compared to untreated control by Student's t test.

Fig. **7.** Development and confirmation of *Pthlh* overexpressing constructs in Ocy454 cells. (*A*) Construct design, and locations of region-specific primers to the signal sequence (gray), mid-region (black outline), and surrounding the NLS (black). (*B*) qRT-PCR for the leader sequence. (*C*) qRT-PCR surrounding the NLS region. (*D*) Dissociation curves showing annealing temperature differences of the PCR products generated by *Pthlh*(-36-139ΔNLS) versus *Pthlh*(1-139) and *Pthlh*(-36-139). (*E*) qRT-PCR for the mid-region in Ocy454 cells expressing each *Pthlh* construct or MSCV vector control from 0 to 14 days of differentiation. (*F*,*G*) Analysis of conditioned media from transfected cells, as defined in *A*, assayed by measuring cAMP response to conditioned media treatment of UMR106-01 cells (*F*) and PTHrP RIA (*G*). Data shown as mean + SD; $n = 3$ replicates of 3 independent experiments. NLS = nuclear localization sequence.

Fig. **8.** Regulation of known PTHR1-responsive genes by osteocyte-secreted PTHrP. mRNA levels of *Pth1r* (*A*), RANKL (*Tnfsf11*, *B*), osteoprotegerin (*Tnfrsf11b*, *C*), osteocalcin (*Bglap1*, *D*), *Nr4a1* (*E*), and *Rgs2* (*F*) in Ocy454 cells containing *Pthlh* overexpression vectors (described in Fig. 7) in comparison to cells infected with vector control (MSCV) at days 0 to 14 of differentiation. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ versus MSCV control cells by two-way ANOVA with Sidak's post hoc test. Data shown as mean + SE of 3 replicates of 3 independent experiments.

Fig. **9.** Identification of the form of PTHrP secreted by Ocy454 cells. (*A*) Example of biological activity of starting material (*Pthlh*(-36-139)) and all fractions retrieved from the column, assayed in UMR106-01 cells against a standard curve of cAMP activity induced by PTH(1-34). The same pattern of elution of activity was obtained for all other constructs. (*B*, *C*) N-terminal PTHrP RIA of extracts of 3-mm sequential slices of polyacrylamide gel electrophoresis of (*B*) Sep-Pak eluates, reflecting secreted PTHrP, and (*C*) cell lysates, indicating cellular PTHrP content of cells overexpressing constructs described in Fig. 7.

Fig. **10.** Model of osteocytic PTHrP action. (A) PTHrP is produced by osteocytes, and is secreted in its full-length form. It can be transported through the lacunocanalicular network to influence nearby osteoblasts on the cell surface and their precursors through PTHR1/cAMP signaling to promote bone formation, in the same manner as therapeutic PTH. In addition, PTHrP secreted by osteocytes can act in an autocrine (B) and paracrine (C) manner in osteocytes through PTHR1-cAMP signaling thereby modifying osteocytic gene expression, including suppressing sclerostin production, which promotes bone formation by osteoblasts. RANKL production is also promoted, though this role of PTHrP is

redundant in normal physiology. (D) PTHrP also has non–receptor-mediated intracrine actions, which may be cytoplasmic or nuclear, that are required for normal levels of bone formation; the mechanism by which they promote bone formation is not yet defined.

Supporting Table 1: Additional trabecular and cortical measurements of femora from *Dmp1Cre.Pthlh*$^{w/w}$ *and Dmp1Cre.Pthlh*$^{f/f}$ littermates at 6 and 12 weeks of age by microcomputed tomography. Values are mean ± SEM. n=6-12/group; *, p<0.05 vs sex-matched w/w.

	6 week old				12 week old			
	Male		Female		Male		Female	
	w/w	f/f	w/w	f/f	w/w	f/f	w/w	f/f
Length (mm)	12.9 ± 0.2	12.7 ± 0.1	12.1 ± 0.1	12.0 ± 0.2	13.8 ± 0.1	13.6 ± 0.2	13.6 ± 0.1	13.6 ± 0.1
Trabecular separation (μm)	280.4 ± 17.3	321.0 ± 10.8	585.0 ± 29.0	652.7 ± 59.2	280.0 ± 16.3	309.8 ± 21.8	545.5 ± 75.2	590.8 ± 54.7
Trabecular thickness (μm)	66.2 ± 1.0	69.2 ± 0.9	60.3 ± 0.8	57.8 ± 3.3	58.8 ± 1.1	58.6 ± 1.5	53.8 ± 1.1	50.9 ± 1.1*
Cortical thickness (μm)	190.8 ± 6.2	191.7 ± 3.9	151.0 ± 4.7	148.0 ± 6.9	213.5 ± 4.7	206.5 ± 5.5	209.5 ± 4.7	202.6 ± 3.4
Cortical area (mm^2)	0.91 ± 0.04	0.86 ± 0.03	0.58 ± 0.01	0.59 ± 0.03	1.01 ± 0.04	0.96 ± 0.04	0.83 ± 0.02	0.82 ± 0.02
Marrow area (mm^2)	1.40 ± 0.05	1.35 ± 0.06	1.10 ± 0.05	1.22 ± 0.06	1.34 ± 0.05	1.37 ± 0.07	0.96 ± 0.05	1.02 ± 0.05

Endocortical perimeter (mm)	3.82 ± 0.10	3.56 ± 0.09	2.88 ± 0.16	3.12 ± 0.05	3.96 ± 0.18	3.70 ± 0.13	3.00 ± 0.06	3.07 ± 0.04
Periosteal perimeter (mm)	9.14 ± 0.78	8.35 ± 0.19	7.27 ± 0.28	7.36 ± 0.19	8.33 ± 0.18	8.28 ± 0.23	7.03 ± 0.13	7.12 ± 0.15

Supporting Table 2: Additional data including micro-CT, femoral 3 point bending test data, reference point indentation, serum biochemistry, and cortical bone mineral density (Ct.TMD) of 12 week old male and female *Dmp1Cre.Pthlh*$^{w/w}$ *and Dmp1Cre.Pthlh*$^{f/f}$ mice at 12 weeks of age. Values are mean ± SEM. n=8-10/group; *, $p<0.05$ vs sex-matched w/w.

	Male		**Female**	
	w/w	f/f	w/w	f/f
Moment of inertia (mm^4)	0.96 ± 0.12	0.93 ± 0.12	0.48 ± 0.03	0.46 ± 0.04
Ultimate stress (MPa)	19.60 ± 1.86	17.67 ± 2.28	25.31 ± 2.12	23.35 ± 3.59
Yield point load (N)	13.44 ± 0.85	11.77 ± 0.78	11.75 ± 0.55	10.39 ± 0.89
Yield point stress (MPa)	15.62 ± 1.36	14.48 ± 1.80	22.38 ± 1.67	21.48 ± 2.80
Stiffness (N/mm)	117.51 ± 8.02	129.63 ± 7.00	110.14 ± 5.79	105.89 ± 5.65
Elastic modulus (MPa)	614.3 ± 65.1	716.5 ± 86.4	1044.9 ± 76.9	1075.7 ± 108.0
Postyield displacement (µm)	89.76 ± 11.53	74.75 ± 10.19	69.91 ± 9.90	56.28 ± 9.69
Toughness (J/mm^3)	0.56 ± 0.07	0.42 ± 0.06	0.58 ± 0.09	0.47 ± 0.10
Total indentation distance (µm)	26.90 ± 0.72	26.15 ± 0.90	26.16 ± 0.45	25.50 ± 0.43
Average loading slope (N/µm)	0.231 ± 0.007	0.247 ± 0.008	0.237 ± 0.003	0.260 ± 0.010*
Average unloading slope (N/µm)	0.292 ± 0.009	0.304 ± 0.011	0.294 ± 0.003	0.318 ± 0.015
Average energy dissipated (µJ)	2.77 ± 0.13	2.44 ± 0.09*	2.55 ± 0.11	2.38 ± 0.10
Serum P1NP (ng/ml)	30.7 ± 2.8	32.0 ± 3.3	23.7 ± 3.7	25.7 ± 5.2
Serum CTX-1 (ng/ml)	34.6 ± 4.5	26.9 ± 4.2	30.5 ± 2.1	28.8 ± 4.2
Femoral Ct.TMD (mg/cm^3)	1.328 ± 0.005	1.336 ± 0.005	1.353 ± 0.006	1.351 ± 0.004
Trabecular osteocyte density ($/mm^2$)	1358 ± 173	1309 ± 143	1970 ± 194	1692 ± 81
Cortical osteocyte density ($/mm^2$)	1645 ± 246	1371 ± 91	1108 ± 94	1165 ± 151

Supporting Fig. 1. CLUSTAL alignment of *Pthlh* overexpression constructs. Leader sequence highlighted in grey, nuclear localization sequence highlighted in black, HA tag in grey typeface. * denotes homology in all constructs. Numbers above the DNA code indicate amino acid numbers of the translated product.

```
                  -36
Pthlh(-36-139)        ATGCTGCGGAGGCTGGTTCAGCAGTGGAGTGTCCTGGTATTCCTGCTCAGCTACTCCGTG 60
Pthlh(1-139)          ------------------------------------------------------------ 0
Pthlh(-36-67)         ATGCTGCGGAGGCTGGTTCAGCAGTGGAGTGTCCTGGTATTCCTGCTCAGCTACTCCGTG 60
Pthlh(-36-139ΔNLS)    ATGCTGCGGAGGCTGGTTCAGCAGTGGAGTGTCCTGGTATTCCTGCTCAGCTACTCCGTG 60

Pthlh(-36-139)        CCCTCCCGCGGGCGTTCGGTGGAGGGGCTTGGCCGCAGGCTCAAACGCGCTGTGTCTGAA 120
Pthlh(1-139)          ------------------------------------------------------------ 0
Pthlh(-36-67)         CCCTCCCGCGGGCGTTCGGTGGAGGGGCTTGGCCGCAGGCTCAAACGCGCTGTGTCTGAA 120
Pthlh(-36-139ΔNLS)    CCCTCCCGCGGGCGTTCGGTGGAGGGGCTTGGCCGCAGGCTCAAACGCGCTGTGTCTGAA 120

                                      1
Pthlh(-36-139)        CATCAGCTACTGCATGACAAGGGCAAGTCCATCCAAGACTTGCGCCGCCGTTTCTTCCTC 180
Pthlh(1-139)          ------------------------GTCCATCCAAGACTTGCGCCGCCGTTTCTTCCTC 34
Pthlh(-36-67)         CATCAGCTACTGCATGACAAGGGCAAGTCCATCCAAGACTTGCGCCGCCGTTTCTTCCTC 180
Pthlh(-36-139ΔNLS)    CATCAGCTACTGCATGACAAGGGCAAGTCCATCCAAGACTTGCGCCGCCGTTTCTTCCTC 180
                                      **********************************

Pthlh(-36-139)        CACCATCTGATCGCGGAGATCCACACAGCCGAAATCAGAGCTACCTCGGAGGTGTCCCCC 240
Pthlh(1-139)          CACCATCTGATCGCGGAGATCCACACAGCCGAAATCAGAGCTACCTCGGAGGTGTCCCCC 94
Pthlh(-36-67)         CACCATCTGATCGCGGAGATCCACACAGCCGAAATCAGAGCTACCTCGGAGGTGTCCCCC 240
Pthlh(-36-139ΔNLS)    CACCATCTGATCGCGGAGATCCACACAGCCGAAATCAGAGCTACCTCGGAGGTGTCCCCC 240
                      ************************************************************

Pthlh(-36-139)        AACTCCAAACCTGCTCCCAACACCAAAAACCACCCCGTGCGGTTTGGGTCAGACGATGAG 300
Pthlh(1-139)          AACTCCAAACCTGCTCCCAACACCAAAAACCACCCCGTGCGGTTTGGGTCAGACGATGAG 154
Pthlh(-36-67)         AACTCCAAACCTGCTCCCAACACCAAAAACCACCCCGTGCGGTTTGGGTCAGACGATGAG 300
Pthlh(-36-139ΔNLS)    AACTCCAAACCTGCTCCCAACACCAAAAACCACCCCGTGCGGTTTGGGTCAGACGATGAG 300
                      ************************************************************

                            67
Pthlh(-36-139)        GGCAGATACCTAACTCAGGAAACCAACAAGGTGGAGACGTACAAAGAACAGCCACTCAAG 360
Pthlh(1-139)          GGCAGATACCTAACTCAGGAAACCAACAAGGTGGAGACGTACAAAGAACAGCCACTCAAG 214
Pthlh(-36-67)         GGCAGATAC--------------------------------------------------- 333
Pthlh(-36-139ΔNLS)    GGCAGATAC--------------------------------------------------- 31
                      *********

                                                95
Pthlh(-36-139)        ACACCCGGGAAGAAGAAGAAAGGCAAGCCTGGGAAACGCAGAGAACAGGAGAAAAAGAAG 420
Pthlh(1-139)          ACACCCGGGAAGAAGAAGAAAGGCAAGCCTGGGAAACGCAGAGAACAGGAGAAAAAGAAG 274
Pthlh(-36-67)         ------------------------------------------------------------ 333
Pthlh(-36-139ΔNLS)    ---------------------------------AAACGCAGAGAACAGGAGAAAAAGAAG 336

Pthlh(-36-139)        CGAAGGACTCGGTCTGCCTGGCCAAGCACAGCTGCGAGTGGCCTGCTTGAGGACCCCCTG 480
Pthlh(1-139)          CGAAGGACTCGGTCTGCCTGGCCAAGCACAGCTGCGAGTGGCCTGCTTGAGGACCCCCTG 334
Pthlh(-36-67)         ------------------------------------------------------------ 333
Pthlh(-36-139ΔNLS)    CGAAGGACTCGGTCTGCCTGGCCAAGCACAGCTGCGAGTGGCCTGCTTGAGGACCCCCTG  396

                                                                    139  HA tag
Pthlh(-36-139)        CCCCACACCTCCAGGCCCTCGCTGGAGCCCAGCTTAAGGACGCATTACCCATACGATGTT 540
Pthlh(1-139)          CCCCACACCTCCAGGCCCTCGCTGGAGCCCAGCTTAAGGACGCATTACCCATACGATGTT 394
Pthlh(-36-67)         ---------------------------------------------CCATACGATGTT 333
Pthlh(-36-139ΔNLS)    CCCCACACCTCCAGGCCCTCGCTGGAGCCCAGCTTAAGGACGCATTACCCATACGATGTT 456
                                                                    ************

Pthlh(-36-139)        CCAGATTACGCT    552
Pthlh(1-139)          CCAGATTACGCT    406
Pthlh(-36-67)         CCAGATTACGCT    333
Pthlh(-36-139ΔNLS)    CCAGATTACGCT    468
                      ************
```