gp130 in late osteoblasts and osteocytes is required for PTH-induced osteoblast differentiation

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Abstract

Parathyroid hormone (PTH) treatment stimulates osteoblast differentiation and bone formation, and is the only currently approved anabolic therapy for osteoporosis. In cells of the osteoblast lineage, PTH also stimulates the expression of members of the interleukin 6 (IL-6) cytokine superfamily. Although the similarity of gene targets regulated by these cytokines and PTH suggest cooperative action, the dependence of PTH anabolic action on IL-6 cytokine signaling is unknown. To determine whether cytokine signaling in the osteocyte through glycoprotein 130 (gp130), the common IL-6 superfamily receptor subunit, is required for PTH anabolic action, male mice with conditional gp130 deletion in osteocytes (*Dmp1Cre.gp130$^{f/f}$*) and littermate controls (*Dmp1Cre.gp130$^{w/w}$*) were treated with hPTH(1–34) (30 μg/kg 5× per week for 5 weeks). PTH dramatically increased bone formation in *Dmp1Cre.gp130$^{w/w}$* mice, as indicated by elevated osteoblast number, osteoid surface, mineralizing surface, and increased serum N-terminal propeptide of type 1 collagen (P1NP). However, in mice with *Dmp1Cre*-directed deletion of gp130, PTH treatment changed none of these parameters. Impaired PTH anabolic action was associated with a 50% reduction in *Pth1r* mRNA levels in *Dmp1Cre.gp130$^{f/f}$* femora compared with *Dmp1Cre.gp130$^{w/w}$*. Furthermore, lentiviral-*Cre* infection of *gp130$^{f/f}$* primary osteoblasts also lowered *Pth1r* mRNA levels to 16% of that observed in infected C57/BL6 cells. In conclusion, osteocytic gp130 is required to maintain PTH1R expression in the osteoblast lineage, and for the stimulation of osteoblast differentiation that occurs in response to PTH.

Key Words
- glycoprotein-130 (gp130)
- osteoblast
- osteocyte
- osteoclast
- PTH
- PTH1R
- trabecular
- cortical
- bone formation

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Introduction

Intermittent administration of parathyroid hormone (PTH) to animal models and humans (teriparatide (Forteo)) increases bone mass (Reeve *et al.* 1980, Neer *et al.* 2001, Lindsay *et al.* 2007), and is the only approved treatment for osteoporosis capable of inducing bone formation (reviewed in Hodsman *et al.* (2005) and Khosla *et al.* (2008)). However, the mechanisms by which intermittent PTH increases bone mass remain unclear, and identifying downstream targets of this pathway may aid in the design of improved anabolic therapies.

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The effects of PTH on bone mass are likely to be mediated by cells of the osteoblast lineage. This lineage includes committed pre-osteoblasts, matrix-producing osteoblasts, bone lining cells, and matrix-embedded osteocytes. PTH acts directly at each stage of differentiation, as follows. PTH promotes pre-osteoblast differentiation (Dobnig & Turner 1995), inhibits osteoblast apoptosis (Jilka *et al*. 1999), and reactivates quiescent lining cells to become active osteoblasts (Kim *et al*. 2012). PTH also acts directly on osteocytes to reduce their expression of the WNT antagonist sclerostin, an inhibitor of bone formation (Bellido *et al*. 2005, Keller & Kneissel 2005).

PTH also stimulates the expression of receptor activator of NF-kappa-B ligand (RANKL) by early osteoblast lineage cells, thereby promoting osteoclast differentiation (Udagawa *et al*. 1999). However, the stages of osteoblast differentiation most important for the actions of PTH remain controversial, because the expression of RANKL by matrix-embedded osteocytes is also stimulated by PTH (Xiong *et al*. 2011).

PTH also acts on the osteoblast lineage to rapidly promote the transcription of interleukin 6 (IL-6) family cytokines and receptors. These include *Il6* (Greenfield *et al*. 1996), *Il11*, oncostatin M receptor (*Osmr*), leukemia inhibitory factor (*Lif*), and cytokine receptor-like factor 1 (*Crlf1*) (Walker *et al*. 2012). These cytokines all depend on the promiscuous co-receptor glycoprotein 130 (gp130) for signaling (reviewed in Sims & Walsh (2010)), and gp130 expression by the osteoblast lineage is also stimulated by PTH (Romas *et al*. 1996).

Many of the actions and gene targets of IL-6 family cytokines are common to those of PTH. As is the case with PTH, the cytokines IL-6, IL-11, OSM, LIF, and cardiotrophin (CT-1) promote osteoblast differentiation *in vitro* (Walker *et al*. 2008, 2010) and OSM, LIF, and CT-1 stimulate bone formation *in vivo* (Cornish *et al*. 1993, Walker *et al*. 2008, 2010). The family members IL-11, LIF, OSM, CT-1, and CNTF also inhibit osteocytic sclerostin expression (Walker *et al*. 2010, Johnson *et al*. 2014*a*). In addition, IL-6, IL-11, OSM, LIF, and CT-1, stimulate osteoblast lineage expression of RANKL (O'Brien *et al*. 1999, Palmqvist *et al*. 2002, Walker *et al*. 2008) and promote osteoclastogenesis when precursors are co-cultured with osteoblasts *in vitro* (Tamura *et al*. 1993, Richards *et al*. 2000). These similar effects and the upregulation of IL-6 family cytokines in osteoblasts by PTH suggest that this cytokine family may play a role in the actions of PTH on the osteoblast lineage.

Hence, in this study we examined the requirement of gp130 signaling in osteocytes for the anabolic action of PTH, using mice with *Dmp1Cre*-directed deletion of gp130 in osteocytes (*Dmp1Cre.gp130*$^{f/f}$; Johnson *et al*. 2014*b*) and mature osteoblasts (Xiong *et al*. 2011, Torreggiani *et al*. 2013). We found that gp130 in these cells is required for PTH to increase osteoblast number and bone forming surfaces, and to maintain PTH1R expression in the osteoblast lineage.

Materials and methods

Mice

All animal procedures were conducted with the approval of the St. Vincent's Health Melbourne Animal Ethics Committee. *Dmp1Cre* mice were obtained from Lynda Bonewald (University of Kansas, Kansas City, MO, USA; Lu *et al*. 2007). Floxed gp130 mice backcrossed onto C57/BL6 were obtained from Rodger McEver (Oklahoma Medical Research Foundation, Oklahoma City, OK, USA; Betz *et al*. 1998). Mice hemizygous for the *Cre* transgene were crossed with the gp130 flox mouse in which the transmembrane domain (exon 15) was flanked by loxP sites, resulting in ablation of intracellular gp130 signaling, as previously reported (Betz *et al*. 1998) and confirmed at the mRNA level in bone (Johnson *et al*. 2014*b*). For all experiments, *Dmp1.Cre+* cousins were used as controls.

Six-week-old male *Dmp1Cre.gp130*$^{w/w}$ or floxed *Dmp1Cre.gp130*$^{f/f}$ mice were injected i.p. with 30 μg/kg human PTH 1–34 (hPTH 1–34) or vehicle, 5 days a week for 5 weeks (n=9/10 per group). This dose and duration of PTH treatment were chosen because it provides a robust increase in lamellar bone formation rate and osteoblast surface in male mice without increasing osteoclastogenesis (Walker *et al*. 2012, Takyar *et al*. 2013, Tonna *et al*. 2014). The mice were also injected with calcein (20 mg/kg) 7 and 2 days before tissue collection. The bones were collected 1 h after the last PTH injection. The mice were fasted for 12 h before anaesthesia with ketamine/xylazine and a final blood sample was collected by cardiac puncture. The blood samples were centrifuged for 10 min at 4000 ***g*** and the serum was collected in a fresh tube and stored at −80 °C until analysis for cross-linked C-terminal telopeptide of type 1 collagen (CTX1), N-terminal propeptide of type 1 collagen (P1NP) (Immunodiagnostic Systems Limited, Boldon, Tyne and Wear, UK), and PTH (Immunotopics, San Clemente, CA, USA) as per manufacturer's instructions. One femur was flushed of marrow and the bone shaft was collected for RNA analyses as described previously (Walker *et al*. 2012). Briefly, bones were homogenized with a LS-10-35 Polytron homogenizer in

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TRI for 4×5 s bursts and stored at −80 °C. RNA from each bone was purified using the RNeasy lipid tissue minikit (Qiagen), according to manufacturer's instructions.

The other femur was analyzed by micro-computed tomography as described previously (Johnson *et al.* 2014*b*) using the SkyScan 1076 System (Bruker-microCT, Kontich, Belgium). The images were acquired using the following settings: 9 μm voxel resolution, 0.5 mm aluminium filter, 48 kV voltage, and 100 μA current, exposure time, rotation 0.5°, frame averaging=1. The images were reconstructed and analyzed using SkyScan Software programs NRecon (version 1.6.3.3), DataViewer (version 1.4.4), and CT Analyser (version 1.12.0.0). Femoral trabecular analysis region of interest (ROI) was determined by identifying the distal end of the femur and calculating 15% of the total femur length toward the femora mid-shaft, where we then analyzed an ROI of 12.6% of the total femur length. The analysis of bone structure was completed using adaptive thresholding (mean of min and max values) in CT Analyser. The thresholds for analysis were determined based on multilevel Otsu thresholding of the entire data set, and were set at 45–255 for trabecular bone. The cortical analyses were performed at 35% above the distal end of the femur toward the femora mid-shaft, also with a 12.6% ROI with the threshold values set at 100–255.

Tibiae were collected for histomorphometric analyses as previously described (Sims *et al.* 2006). Briefly, trabecular histomorphometry was carried out on undecalcified sections in the secondary spongiosa of the proximal tibia, in a region 370 μm below the proximal edge of the hypertrophic zone of the growth plate, extending 1.11 mm in the proximal direction. Periosteal histomorphometry was carried out on the antero-fibular side of the tibia, commencing 1.11 mm below the chondro-osseus junction of the growth plate, and extending 1.11 mm in the proximal direction. The nomenclature is as described previously (Parfitt *et al.* 1987).

Lenti-Cre viral infection

Calvarial osteoblasts were collected from C57/BL6 WT and *gp130*$^{f/f}$ neonates by digesting calvaria in 1:2 collagenase II/dispase solution at 37 °C on a shaker (1×5 min 4× 10 min digestions). The cells were resuspended in culture media (alpha-MEM+10% fetal bovine serum), and allowed to adhere overnight before being frozen and stored in liquid nitrogen. When required, isolated cells were thawed and expanded in culture and infected with a GFP-tagged lenti-Cre virus synthesized as described previously (Tonna *et al.* 2014) for 24 h with polybrene in the maintenance media. Following infection, media was changed and cells were evaluated for GFP expression by microscopy; >30–60% transfection efficiency was observed ($n=3$ independent experiments). The cells were expanded in culture for 2–3 weeks in alpha-MEM+10% fetal bovine serum, and GFP positive cells (fluorescence driven by *Cre* transgene expression) were sorted on a FACS Aria (BD Biosciences, San Jose, CA, USA) for GFP. The GFP+ cells were harvested for RNA in trizol (Life Technologies) and separated and precipitated using chloroform and isopropanol. Extracted RNA was treated with DNase using Ambion TURBO DNA-free Kit (Life Technologies) and quantified on a NanoDrop ND1000 Spectrophotemeter (Thermo Scientific, Wilmington, DE, USA).

Semi-quantitative real-time PCR

cDNA synthesis from 50 to 100 ng DNase-treated RNA from each femur or cell culture preparation was carried out using AffinityScript (Agilent Technologies, Santa Clara, CA, USA) as per the manufacturer's instructions. The stock cDNA was diluted to a concentration of 5 ng/μl and semi-quantitative real-time PCR (qPCR) was performed on 12.5 ng cDNA in a reaction volume of 10 μl using in-house master mix of 10× AmpliTaq Gold with SYBR Green nucleic acid gel stain (Life Technologies). *Dkk1* primers were designed using NCBI Primer Blast: forward, GAGGGGAAATTGAGGAAAGC and reverse, ACGGAGCCTTCTTGTCCTTT. Other primers were as previously described for *Pth1r*, hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), *Sost*, *Tnfsf11*, *Il6* (Allan *et al.* 2008), β-2 microglobulin (*B2m*) (McGregor *et al.* 2010), and hydroxymethylbilane synthase (*Hmbs*) (Johnson *et al.* 2014*b*).

The samples were dispensed onto optically clear 96-well plates (Thermo Scientific) and run on a Stratagene Mx3000P (Agilent Technologies). The cycling conditions were 95 °C for 10 min (95 °C for 30 s, 58 °C for 1 min, and 72 °C for 30 s)×40 cycles, followed by a dissociation step (95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s). The post-run samples were analyzed using MxPro (Agilent Technologies, Santa Clara, CA, USA) and reported using linear ΔCT values normalized to the geometric mean of the two housekeeping genes (HKG) *Hprt1* and *Hmbs* or to *B2m* as indicated.

Statistical analysis

All graphs are presented as the mean/genotype+S.E.M.. $N=5$–10 animals/group as indicated in the figure legend. For *in vitro* experiments, data shown is the average of three

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independent biological replicates. Statistical significance was considered when $P<0.05$. Differences between groups were analyzed by two-way ANOVA and *post hoc* Šidak multiple comparison test. Skewed variables were transformed using the natural logarithm before statistical analyses. For the lenti-viral *Cre*-infected primary calvarial osteoblasts, Student's *t*-test was used to assess significance. Statistical analyses were performed using GraphPad Prism version 6.0c for Mac OS X (GraphPad Software, La Jolla, CA, USA).

Results

Dmp1Cre.gp130$^{f/f}$ mice show no increase in the number of trabecular osteoblasts in response to PTH

PTH treatment at 30 μg/kg per day significantly increased osteoblast number/bone perimeter (NOb/BPm) on trabecular bone in *Dmp1Cre.gp130$^{w/w}$* mice by 76% (Fig. 1A). Osteoblast surface/bone surface (ObS/BS; Fig. 1B) and osteoid surface/bone surface (OS/BS; Fig. 1C) were also elevated by PTH treatment to similar extents. We detected no significant changes in osteoid thickness in *Dmp1Cre.gp130$^{w/w}$* mice after PTH treatment (Fig. 1D).

In contrast to *Dmp1Cre.gp130$^{w/w}$* mice, PTH treatment did not increase osteoblast or osteoid-derived parameters in age- and sex-matched *Dmp1Cre.gp130$^{f/f}$* mice (Fig. 1A, B, C and D). Two-way ANOVA revealed that the effects of PTH treatment on both NOb/BPm and ObS/BS were significantly reduced in the *Dmp1Cre.gp130$^{f/f}$* mice compared with *Dmp1Cre.gp130$^{w/w}$* controls (interaction $P=0.039$ and $P=0.043$ respectively). This indicates that the effect of PTH on osteoblast differentiation is dependent on gp130 expression in osteocytes.

In line with the effects on osteoblast numbers, bone forming surfaces, indicated by incorporation of calcein labels, including both double-labeled surface (dLS/BS) (Fig. 1E) and single-labeled surface ($P\leq0.05$, not shown) were significantly greater in PTH-treated *Dmp1Cre.gp130$^{w/w}$* mice compared with controls. Again, this was not observed in *Dmp1Cre.gp130$^{f/f}$* mice. Mineral apposition rate (MAR) was significantly greater in both *Dmp1Cre.gp130$^{f/f}$* and *Dmp1Cre.gp130$^{w/w}$* mice treated with PTH compared with their vehicle-treated controls (Fig. 1F), indicating that an increase in mineralization rate in response to PTH is retained on those surfaces on which bone formation occurs in *Dmp1Cre.gp130$^{f/f}$* mice.

PTH-treated *Dmp1Cre.gp130$^{w/w}$* mice had significantly higher serum P1NP levels than *Dmp1Cre.gp130$^{w/w}$* untreated controls. In contrast, in *Dmp1Cre.gp130$^{f/f}$* mice there was no significant effect of PTH on P1NP levels

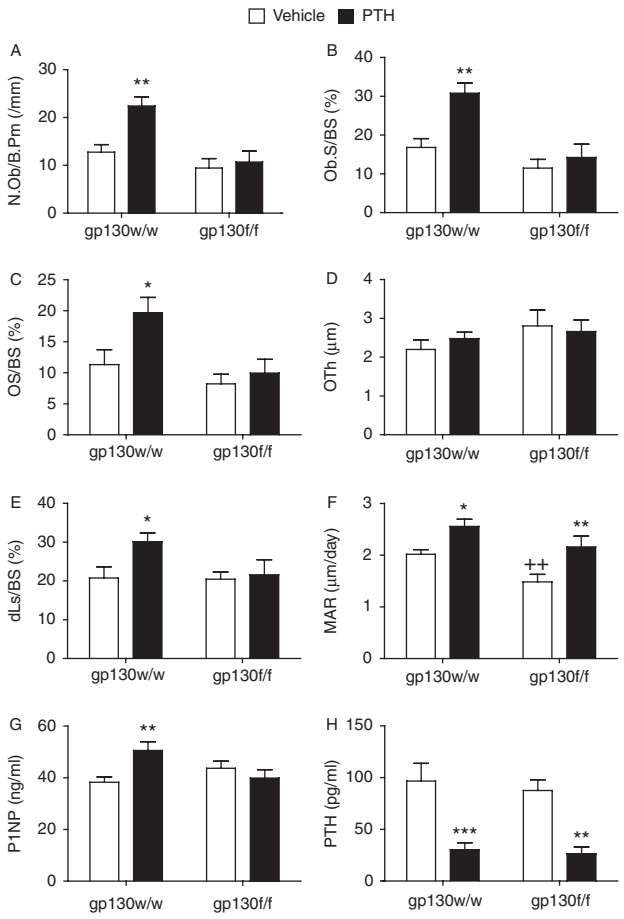

Figure 1
Osteocytic gp130 is required for PTH to increase osteoblast numbers and bone formation in trabecular bone. Male mice were treated with hPTH (1–34) at 30 μg/kg per day for 5 weeks. (A) Numbers of osteoblasts/bone perimeter (N.Ob/B.Pm), (B) osteoblast surface/bone surface (Ob.S/BS), (C) osteoid surface/bone surface (OS/BS), (D) osteoid thickness (OTh), (E) double calcein-labeled surface (dLS/BS), and (F) mineral apposition rate (MAR) from trabecular bone in the proximal tibial secondary spongiosa in *Dmp1Cre.gp130$^{w/w}$* (gp130 w/w) and *Dmp1Cre.gp130$^{f/f}$* (gp130 f/f) mice. (G) The serum levels of procollagen type 1 amino-terminal propeptide (P1NP) and (H) endogenous murine PTH measured at the end of the treatment protocol are also shown. Scale bars are mean+S.E.M., $n=8$–10 per group. *$P\leq0.05$, **$P\leq0.01$ and ***$P\leq0.001$, PTH-treated compared with genotype-matched vehicle-treated mice; $^{++}P<0.01$ compared to vehicle-treated gp130 w/w mice.

compared with vehicle-treated *Dmp1Cre.gp130$^{f/f}$* mice (Fig. 1G); interaction P value=0.009 by two-way ANOVA. These results are consistent with the histomorphometry data and confirm that at a systemic level, the effect of PTH on bone formation is blunted in *Dmp1Cre.gp130$^{f/f}$* mice.

In both *Dmp1Cre.gp130$^{f/f}$* and *Dmp1Cre.gp130$^{w/w}$* mice, intermittent human PTH treatment led to reduced production of endogenous circulating murine PTH levels (Fig. 1H), demonstrating that negative feedback at the parathyroid gland induced by exogenous PTH administration was maintained in both groups of mice.

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Table 1 Effects of PTH on trabecular and cortical bone in femora from *Dmp1Cre.gp130*$^{w/w}$ and *Dmp1Cre.gp130*$^{w/w}$ mice.

	Dmp1Cre.gp130w/w		***Dmp1Cre.gp130f/f***	
	Vehicle ($n=9$)	PTH ($n=10$)	Vehicle ($n=9$)	PTH ($n=9$)
BV/TV (%)	24.86±0.42	21.54±1.32	17.70±1.55^{+++}	20.61±0.96
Tb.Th (μm)	57.61±1.80	56.49±2.22	57.92±3.01	57.42±3.83
Tb.N (/mm)	4.35±0.15	3.80±0.16	3.03±0.15^{+++}	3.64±0.18
Tb.Sp (μm)	122.71±2.96	144.39±14.04	171.05±15.74^{+++}	158.98±10.37
Ct.Ar (mm^2)	0.60±0.02	0.65±0.02	0.63±0.02	0.67±0.03

Fixed nondemineralized femora from vehicle- or PTH-treated mice were analyzed by μCT. Effect of gp130$^{f/f}$ transgene: $^{+++}P<0.001$ vs *Dmp1Cre.gp130*$^{w/w}$ (two-way ANOVA with Šidak multiple comparisons test). BV/TV, bone volume per total volume of the region of interest; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; Ct.Ar, cortical area.

Although this dose of PTH significantly increased all markers of bone formation in *Dmp1Cre.gp130*$^{w/w}$ mice, we did not detect a significant increase in trabecular bone mass by micro-computed tomography with this short-time course of low-dose treatment (Table 1). The low trabecular bone mass of these mice, previously reported (Johnson *et al.* 2014*b*), was confirmed.

No effect of intermittent PTH treatment on bone resorption

This protocol of intermittent PTH treatment did not significantly change osteoclast number/bone perimeter (NOc/BPm) (Fig. 2A), osteoclast number/osteoclast perimeter (Fig. 2B), osteoclast surface/bone surface (OcS/BS) (Fig. 2C), or serum levels of cross-linked CTX1 (Fig. 2D) in either *Dmp1Cre.gp130*$^{f/f}$ or *Dmp1Cre.gp130*$^{w/w}$ mice. This confirms our previous observations using similar protocols over 4 weeks of treatment (Walker *et al.* 2012, Takyar *et al.* 2013, Tonna *et al.* 2014).

Effects of PTH on cortical bone

Periosteal MAR (Fig. 3A), periosteal mineralising surface (Fig. 3B) and periosteal perimeter (Fig. 3C) were all significantly greater in PTH-treated *Dmp1Cre.gp130*$^{w/w}$ mice compared with untreated mice. None of these parameters were significantly increased by PTH treatment in *Dmp1Cre.gp130*$^{f/f}$ mice compared with genotype-matched vehicle controls (Fig. 3A–C), indicating that periosteal growth in response to PTH may also be impaired in the absence of osteocytic gp130.

Normal response of osteoclastic genes, but lack of inhibition of WNT signaling inhibitors by PTH treatment in *Dmp1Cre.gp130*$^{f/f}$ mice

RANKL (gene name *Tnfsf11*) and IL-6 (*Il6*) are both potent stimuli of osteoclast formation, and PTH increases their expression in cells of the osteoblast lineage (Greenfield *et al.* 1995, Udagawa *et al.* 1999). Indeed, in marrow-flushed femoral samples collected 1 h after the last of these 5 weeks of injections, mRNA levels of *Tnfsf11* and *Il6* were significantly higher in both genotypes after PTH treatment (Fig. 4A and B); this increase was not significantly affected by the genotype (two-way ANOVA interaction P values = 0.365 and 0.314 respectively). This indicated that among cells in the flushed femora, which would include osteoblasts at different stages of differentiation as well as osteocytes, are some cells that retain normal responses of these genes to PTH.

Wingless (WNT)-signaling is important for osteoblast differentiation and bone formation, and PTH has been shown to stimulate WNT signaling by suppressing Dickopf1 (*Dkk1*) and sclerostin (*Sost*) expression in the

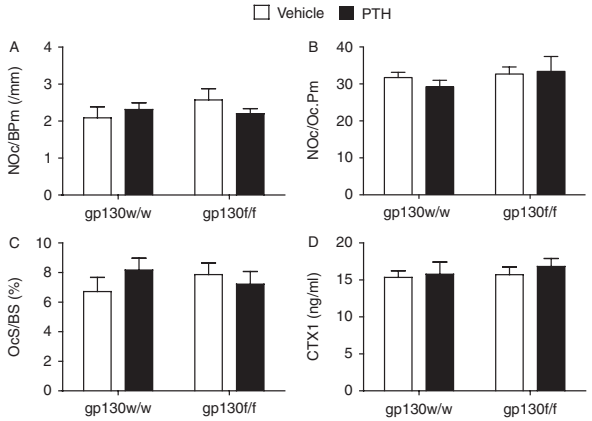

Figure 2
No effect of intermittent PTH on bone resorption. Male mice were treated with hPTH (1–34) at 30 μg/kg per day for 5 weeks. (A) Numbers of osteoclasts per unit bone perimeter (NOc/BPm), (B) numbers of osteoclasts per unit osteoclast perimeter (NOc/OcPm), (C) osteoclast surface per unit bone surface (OcS/BS) measured in the proximal tibial secondary spongiosa, and (D) serum levels of cross-linked C-terminal telopeptide of type 1 collagen (CTX1) in PTH and vehicle-treated *Dmp1Cre.gp130*$^{w/w}$ (gp130 w/w) and *Dmp1Cre.gp130*$^{f/f}$ (gp130 f/f) mice. Scale bars are mean+S.E.M., $n=8$–10 per group.

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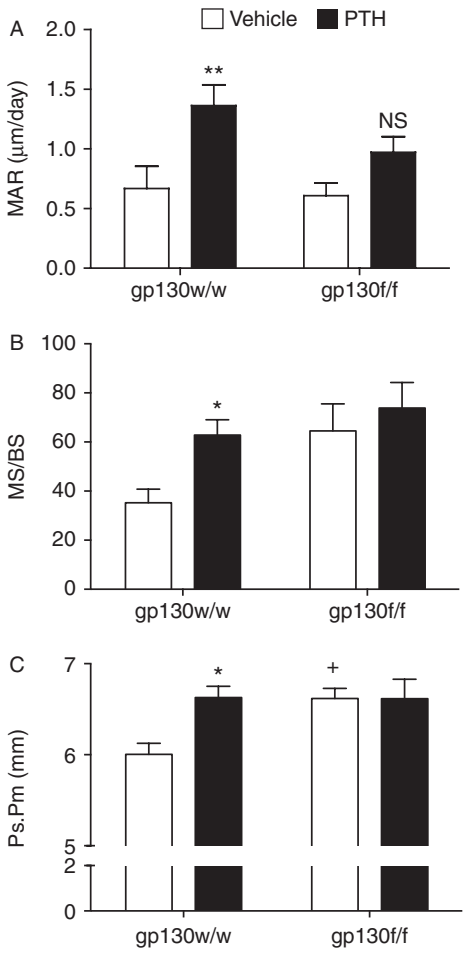

Figure 3
PTH effects on cortical bone are impaired in *Dmp1Cre.gp130*$^{f/f}$ mice. Male mice were treated with hPTH (1–34) at 30µg/kg per day for 5 weeks. (A) Tibial periosteal MAR (Ps.MAR), and (B) mineralizing surface per unit bone surface (MS/BS) in the tibial diaphysis, and (C) periosteal perimeter (Ps.Pm) of the femoral diaphysis in PTH and vehicle-treated *Dmp1Cre.gp130*$^{w/w}$ (gp130 w/w) and *Dmp1Cre.gp130*$^{f/f}$ (gp130 f/f) mice. Scale bars are mean+S.E.M., $n=8$–10 per group. $^{**}P \leq 0.01$, NS, $P > 0.05$ (not statistically significant) in PTH-treated compared with genotype-matched vehicle-treated mice. $^{+}$, $P < 0.05$, vehicle-treated *Dmp1Cre.gp130*$^{f/f}$ compared with vehicle-treated *Dmp1Cre.gp130*$^{w/w}$.

osteoblast lineage (Keller & Kneissel 2005, Yao *et al*. 2011). For this reason, we quantified mRNA levels of *Dkk1* and *Sost* in flushed femurs. As expected, *Dkk1* mRNA levels were significantly lower in PTH-treated *Dmp1Cre.gp130*$^{w/w}$ femurs compared with untreated mice. However, *Dkk1* was not lower in femurs from PTH-treated *Dmp1Cre.gp130*$^{f/f}$ mice compared with controls (Fig. 4C). *Sost* mRNA levels were slightly, but not significantly, lowered in response to PTH in *Dmp1Cre.gp130*$^{w/w}$ femora. *Dmp1Cre.gp130*$^{f/f}$ femora showed a lower level of *Sost* mRNA compared with vehicle-treated *Dmp1Cre.gp130*$^{w/w}$ controls; with PTH treatment these mice showed a significant increase in *Sost* mRNA levels (Fig. 4D). These differences in the effects of PTH treatment on gene expression were significant by two-way ANOVA for both *Dkk1* (interaction $P=0.01$) and *Sost* (interaction $P=0.003$). Thus, PTH treatment does not decrease WNT antagonist expression in *Dmp1Cre.gp130*$^{f/f}$ mice, implying that gp130 signaling in osteocytes is important for the PTH effect on WNT signaling inhibitors.

Pth1r expression is reduced in *DMP1Cre.gp130*$^{f/f}$ mice and gp130-deficient osteoblasts

Since many effects of PTH were blocked in *Dmp1Cre.gp130*$^{f/f}$ mice, we quantified *Pth1r* mRNA levels in flushed femurs from untreated 12-week-old *Dmp1Cre.gp130*$^{f/f}$ and *Dmp1Cre.gp130*$^{w/w}$ mice. Surprisingly, *Pth1r* mRNA expression was 47% lower in *Dmp1Cre.gp130*$^{f/f}$ compared with *Dmp1Cre.gp130*$^{w/w}$ femurs ($P=0.03$; Fig. 5A).

These findings were supported by *in vitro* data, where C57/BL6 and *gp130*$^{f/f}$ calvarial osteoblasts were infected with lentiviral *Cre*-recombinase. In *Cre*-infected *gp130*$^{f/f}$ osteoblasts, *gp130* was significantly lowered by 52%, and *Pth1r* mRNA was 84% lower than in infected C57/BL6 cells

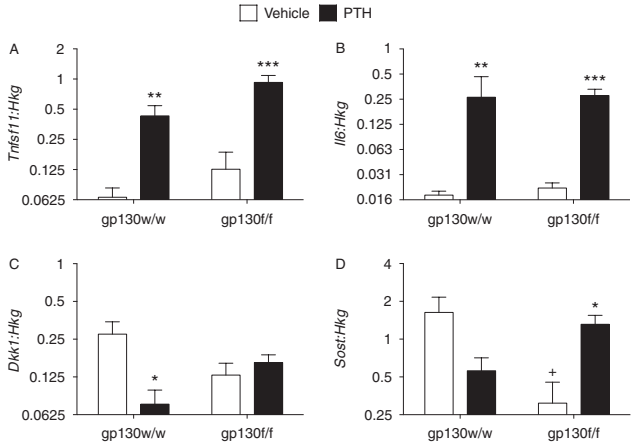

Figure 4
PTH effects on WNT-inhibitor, but not osteoclastogenic, mRNA levels are impaired in *DMP1Cre.gp130*$^{f/f}$ mice. RNA was isolated from femurs flushed of bone marrow and expression of PTH target genes was examined by relative quantitative PCR. *Tnfs11* mRNA (A), *Il6* mRNA (B), *Dkk1* mRNA (C) and *Sost* mRNA (D) in *Dmp1Cre.gp130*$^{w/w}$ and *Dmp1Cre.gp130*$^{f/f}$ mice treated for 5 weeks with PTH, collected 1 h after the final injection. All values are shown relative to housekeeping (*Hkg*) – the geometric mean of hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) and hydroxymethylbilane synthase (*Hmbs*). Scale bars are mean+S.E.M., $n=5$–8 bones per group, with mRNA prepared and analyzed separately. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$, PTH-treated compared with genotype-matched vehicle-treated mice; $^{+}P < 0.05$, vehicle-treated *Dmp1Cre.gp130*$^{f/f}$ compared with vehicle-treated *Dmp1Cre.gp130*$^{w/w}$.

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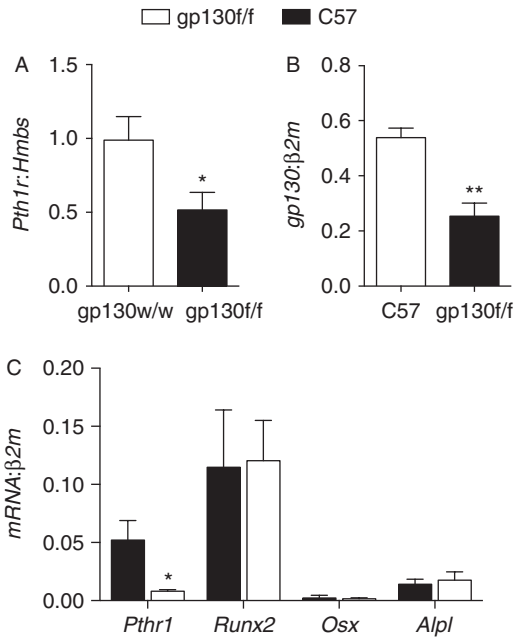

Figure 5
PTH1R expression is reduced in *DMP1Cre.gp130$^{f/f}$* mice and gp130 deficient cultured osteoblasts. (A) *Pth1r* mRNA quantified by qPCR in femurs flushed of bone marrow obtained from untreated 12-week-old *Dmp1Cre.gp130$^{w/w}$* and *Dmp1Cre.gp130$^{f/f}$* mice, normalized to *Hmbs*; $n=8$ samples per group. (B) *gp130* (*Il6st*) and (C) *Pth1r*, *Runx2*, *Osx* and *Alpl* mRNA levels in primary calvarial osteoblasts obtained from *gp130$^{f/f}$* or C57/BL6 WT neonates infected with lentiviral *Cre*-recombinase; levels are shown normalized to beta-2-microglobulin (*B2m*) ($n=3$ biological replicates). *$P\leq 0.05$; **$P\leq 0.01$, vs gp130 w/w or C57/BL6.

(Fig. 5B and C). The mRNA levels of *Runx2*, *Osx*, and *Alpl* were not significantly altered by *Cre*-infection of *gp130$^{f/f}$* osteoblasts (Fig. 5C), consistent with previously published mRNA levels of these genes in the femora of *Dmp1Cre.gp130$^{f/f}$* mice (Johnson *et al.* 2014*b*). This suggests that the cells of the osteoblast lineage require signals mediated by gp130 to maintain PTH1R expression, and that a lack of PTH1R in *Dmp1Cre* expressing cells is responsible for the reduced response to anabolic PTH treatment.

Discussion

This work demonstrates that PTH-induced osteoblast differentiation is dependent on gp130 expression in mature osteoblast lineage cells. gp130 is needed to maintain *Pth1r* expression in osteoblasts, and is required for PTH to suppress the WNT-antagonists *Dkk1* and *Sost*. In contrast, gp130 expression by osteocytes is not required for PTH to stimulate mRNA levels of the pro-osteoclastogenic factors RANKL (*Tnfsf11*) and *Il6* in bone.

The stimulatory effect of PTH on trabecular osteoblast numbers and mineralizing surface was completely ablated in *Dmp1Cre.gp130$^{f/f}$* mice. This may, at least partly, be explained by the lack of a reduction in both WNT signaling inhibitors *Sost* and *Dkk1* in response to PTH. WNT signaling stimulates osteoblast differentiation, and it has been postulated that this is one pathway through which PTH stimulates bone formation (Kulkarni *et al.* 2005), a hypothesis supported by impaired PTH responses in mice overexpressing sclerostin or *Dkk1* (Guo *et al.* 2010, Kramer *et al.* 2010). PTH directly inhibits *Sost* via cAMP–PKA signaling (Keller & Kneissel 2005). The IL-6 family cytokines also rapidly inhibit *Sost*, although the mechanism remains unknown (Walker *et al.* 2010). Whether the reduction in the effect of PTH on WNT signaling is entirely due to the reduced PTH1R expression or results from some dependence on gp130 cytokines by this same pathway in osteoblasts and osteocytes remains unclear.

In contrast to the effect on WNT-antagonists, both *Dmp1Cre.gp130$^{w/w}$* and *Dmp1Cre.gp130$^{f/f}$* mice demonstrated increased femoral *Tnfsf11* and *Il6* mRNA levels in response to PTH. Despite these increases in both genotypes, osteoclast numbers were unchanged, as we have previously reported with this low dose of intermittent PTH treatment (Takyar *et al.* 2013, Tonna *et al.* 2014), likely because the inductions of *Tnfsf11* and *Il6* are transient (Ma *et al.* 2001, Walker *et al.* 2012). IL-6 and RANKL are expressed by a wide range of cells in the bone, including osteoblast lineage cells as well as osteocytes (Lee & Lorenzo 1999, Dai *et al.* 2006, Nakashima *et al.* 2011, Xiong *et al.* 2011), and cells within the bone marrow, including T-cells (Horwood *et al.* 1999, Hirano *et al.* 1986) and, in the case of IL-6, macrophages (Tosato *et al.* 1988). Although PTH has recently been suggested to directly promote RANKL expression in osteocytes (Xiong *et al.* 2011), our findings suggest that the major cellular targets that produce these pro-osteoclastogenic factors in response to PTH are not osteocytes. Notably, although PTH was unable to increase osteoblast numbers or mineralizing surface in the *Dmp1Cre.gp130$^{f/f}$* mice, on those surfaces where double calcein labels were incorporated into the bone matrix, the distance between them (MAR) was significantly greater in PTH-treated mice, regardless of genotype. This suggests that bone-forming osteoblasts in *Dmp1Cre.gp130$^{f/f}$* mice retain sufficient PTHR expression to respond to PTH with increased matrix production. Since marrow was flushed from the femora, and *Pth1r* levels were dramatically reduced in undifferentiated cultured *Cre*-expressing cells, we suggest that the

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key PTH-responsive cells producing RANKL and IL-6 in this model are less differentiated osteoblasts, not expressing DMP1Cre, on the bone surface.

Pth1r mRNA was lower in cortical bone of *Dmp1Cre.gp130*$^{f/f}$ mice compared with littermate controls, an effect that was reproduced when gp130 was deleted in cultured primary calvarial osteoblasts. There are two ways to understand this: firstly, as osteoblast differentiation is impaired in the *Dmp1Cre.gp130*$^{f/f}$ mice (Johnson *et al*. 2014*b*) and PTH1R expression in the osteoblast lineage is higher in more mature osteoblasts (Allan *et al*. 2003, 2008, Balic *et al*. 2010), there may be fewer mature PTH1R-expressing osteoblasts present within the bone of these mice. Another interpretation is that gp130 is needed to maintain the expression of PTH1R in the osteoblast lineage. This latter hypothesis is supported by our *in vitro* data, as we observed that a reduction in gp130 by about 50% in calvarial osteoblasts cultured *in vitro* reduced *Pth1r* mRNA levels by nearly 80%. This further suggests that, as well as maintaining PTH1R levels in the osteocyte, gp130 may maintain PTH1R expression throughout the osteoblast lineage.

Although *Pth1r* levels were low in the femora of *Dmp1Cre.gp130*$^{f/f}$ mice, their phenotype is strikingly different to mice with a conditional deletion of *Pth1r* in osteocytes (Ocy-PPRKO), generated using the same *Dmp1Cre* (Saini *et al*. 2013). Ocy-PPRKO mice showed a greater trabecular bone mass than controls, with no significant alteration in osteoblast numbers, indicating that the underlying cause of bone fragility in the *Dmp1Cre.gp130*$^{f/f}$ mice is not simply low PTH1R expression in the osteocyte. As observed in *Dmp1Cre.gp130*$^{f/f}$ mice, Ocy-PPRKO mice failed to reduce *Sost* in response to PTH treatment. However, in direct contrast to *Dmp1Cre.gp130*$^{f/f}$ mice, Ocy-PPRKO mice lacked a *Tnfsf11* in response to PTH. This suggests that the *Dkk1/Sost* and *Tnfsf11/Il6st* responses to PTH occur in different cell populations, and it is only the former that is affected by *Dmp1Cre*-mediated gp130 deletion. Alternatively, the *Dkk1/Sost* induction may require a higher level of PTH1R expression than the *Tnfsf11/Il6st* response; the low level of PTH1R expression in the *Dmp1Cre.gp130*$^{f/f}$ mice may be sufficient for the latter.

In addition to mediating the response of osteoblasts to exogenous PTH treatment, PTH1R also acts as a receptor for PTH-related protein (PTHrP). Although first identified as the mediator of humoral hypercalcemia of malignancy (Suva *et al*. 1987), PTHrP is also produced by the osteoblast lineage (Kartsogiannis *et al*. 1997). This local PTHrP production is essential for normal osteoblast differentiation, as indicated by studies of an osteoblast-lineage PTHrP-null mice (Miao *et al*. 2005). This suggests that basal defects in osteoblast differentiation in our model lacking gp130 in osteocytes may relate specifically to a lack of PTHrP signal. Notably, and in direct contrast to our model, the osteoblast-lineage knockout of PTHrP also exhibited a significant impairment in osteoclastogenesis (Miao *et al*. 2005), a finding that may relate to the difference in the gene-driving expression of the *Cre*-recombinase. The *Pthrp*$^{f/f}$ deletion was driven by the *Col2.3Cre*, which would delete expression in osteocytes, but also in less mature osteoblasts than the *Dmp1Cre* that we have used. Again, this suggests that the PTH-induced expression of RANKL is likely to occur in less mature osteoblasts.

In conclusion, in addition to the recently described role of osteocytic gp130 in maintaining bone formation and strength (Johnson *et al*. 2014*b*), the current study has revealed a new role for gp130 in the osteoblast lineage in bone: it is needed to maintain PTH1R expression and to increase osteoblast numbers in response to anabolic PTH treatment.

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

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Author contributions
N A S and T S designed the study; T S, R W J, N E M, I J P, and P W M H conducted the study and made data collection; T S, R W J, N E M, I J P, P W M H, and N A S analyzed data; T S, R W J, N E M, I J P, P W M H, T J M, and N A S interpreted data; T S and N A S drafted manuscript; T S, N A S, R W J, and T J M revised the manuscript content. All authors approved the final version of manuscript. N A S takes responsibility for the integrity of the data analysis.

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