Calcitonin impairs the anabolic effect of PTH in young rats and stimulates expression of sclerostin by osteocytes

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ABSTRACT

The therapeutic goal of increasing bone mass by co-treatment of parathyroid hormone (PTH) and an osteoclast inhibitor has been complicated by the undefined contribution of osteoclasts to the anabolic activity of PTH. To determine whether active osteoclasts are required at the time of PTH administration, we administered a low dose of the transient osteoclast inhibitor salmon calcitonin (sCT) to young rats receiving an anabolic PTH regimen. Co-administration of sCT significantly blunted the anabolic effect of PTH as measured by peripheral quantitative computer tomography (pQCT) and histomorphometry in the femur and tibia, respectively. To determine gene targets of sCT, we carried out quantitative real time PCR and microarray analysis of metaphyseal samples 1.5, 4 and 6.5 hours after administration of a single injection of PTH, sCT or PTH+sCT. Known targets of PTH action, IL-6, ephrinB2 and RANKL, were not modified by co-administration with sCT. Surprisingly, at all time points, we noted a significant upregulation of sclerostin mRNA by sCT treatment, as well as down-regulation of two other osteocyte gene products, MEPE and DMP1. Immunohistochemistry confirmed that sCT administration increased the percentage of osteocytes expressing sclerostin, suggesting a mechanism by which sCT reduced the anabolic effect of PTH. Neither mRNA for CT receptor (Calcr) nor labeled CT binding could be detected in sclerostin-enriched cells differentiated from primary calvarial osteoblasts. In contrast, osteocytes freshly isolated from calvariae expressed a high level of Calcr mRNA. Furthermore immunohistochemistry revealed co-localization of CT receptor (CTR) and sclerostin in some osteocytes in calvarial sections. Taken together these data indicate that co-treatment with sCT can blunt the anabolic effect of PTH and this may involve direct stimulation of sclerostin
production by osteocytes. These data directly implicate calcitonin as a negative regulator of bone formation through a previously unsuspected mechanism.

**Keywords:** osteocyte; sclerostin; PTH; calcitonin; osteoclast; coupling
INTRODUCTION

Although continuous administration of PTH and hyperparathyroidism lead to increased bone resorption and reduced bone mass, both clinical and animal studies have established that parathyroid hormone (PTH) given by daily injection is an effective anabolic agent for bone [1, 2]. The mechanism of the anabolic action of PTH has not been clearly defined, but a proper understanding of PTH action has great potential to lead to new therapeutic approaches. Several mechanisms have been proposed to explain the PTH anabolic effect, including direct actions favoring committed osteoblast precursor differentiation [3], inhibition of osteoblast apoptosis [4], and more recently, reduced production of the osteocyte-derived anti-anabolic agent sclerostin [5, 6]. Since PTH influences both bone formation and resorption, and the activities of both osteoclasts and osteoblasts are linked through the normal process of bone remodeling [7-9], another important possibility is that the anabolic effect of PTH is related, directly or indirectly, to osteoclast activity in the bone remodeling process [10].

Rapid morphological changes in osteoclasts consistent with increased activation were observed many years ago in rats injected with PTH [11]. This suggested that the effects of PTH administration are not limited to an enhancement of osteoclast differentiation. Dependence on osteoclasts for PTH anabolic effects was indicated by blunting of the anabolic effect by concomitant bisphosphonate treatment [12], and a lack of anabolic response to PTH in mice deficient in osteoclasts [13]. On the other hand, genetically altered mice with osteoclasts that are present, but not functional, retain an anabolic response to intermittent PTH administration [14], suggesting that an osteoclast contribution to PTH anabolic action may not require bone resorption. In approaching the ways in which PTH might influence remodeling, we considered that
a component of the anabolic action of PTH might depend upon a transient effect to produce an osteoclast-derived activity that contributes to bone formation [10, 15].

In the present work we tested this concept by studying the anabolic action of PTH together with an agent that could prevent an osteoclast response at the time of PTH administration without a prolonged effect on osteoclast activity or bone volume. To do this, we used a low dose of salmon calcitonin (sCT), a peptide hormone that rapidly, transiently, and specifically inhibits osteoclast activity [16, 17]. Co-administration of sCT and PTH did indeed reduce the anabolic effect of PTH. sCT also modified expression of a number of osteocytic gene products, including enhanced expression of the osteocyte-expressed bone inhibitory agent, sclerostin. In this way, we have identified a novel mechanism by which calcitonin might modify bone formation, and provide further evidence for a role for regulated sclerostin production in bone remodeling, which can be a limiting factor in the anabolic action of PTH.

MATERIALS AND METHODS

Materials

Synthetic human PTH(1-34) was purchased from Bachem (Bubendorf Switzerland). Synthetic salmon calcitonin was the kind gift of Dr M Azria, Novartis AG, Basel, Switzerland. Mouse oncostatin M (mOSM) was purchased from R&D Systems (Minneapolis, MN, USA).

Animal experiments
Female Sprague Dawley rats and C57BL/6 mice were purchased from the Animal Resources Centre, Canning Vale, Australia. Animals were housed in a 12 h light and dark cycle with food and water provided *ad libitum*. All animal experiments were approved by the St Vincent’s Health Animal Experimentation Committee. These experiments were carried out in young rats to study these effects in the presence of many osteoclasts and to enable estimation of a sCT dose that ensured a transient effect of the hormone on osteoclasts, distinct from a prolonged effect on bone resorption. To select a sCT dose that would achieve transient osteoclast inhibition, a biological assay of sCT was carried out by measuring the calcium-lowering effect of sCT injected subcutaneously in fasting 45-50g rats, with blood sampling under anaesthesia after 1 hour [18].

To determine whether transient osteoclast inhibition modifies the anabolic response to PTH(1-34), three week-old female Sprague Dawley rats (45-50 gms) were randomly allocated to control, PTH, sCT, or PTH+sCT treatment groups with 8 rats per group. Rats were given daily subcutaneous injections, 5 days a week for 3 weeks, of either vehicle (0.9% saline with 2% heat-inactivated rat serum), 30μg/kg PTH(1-34), 0.5μg/kg sCT, or a combination of PTH(1-34) and sCT (PTH+sCT) at the same doses. The PTH dose of 30μg/kg was aimed at achieving an anabolic effect that could be measured with confidence, but was not the maximum that could be achieved with higher doses and was based on previous experiments in similar aged animals [19]. Rats were weighed daily and doses were adjusted according to weight changes over the 3 week period. Three hours after the last injection, a terminal blood sample was collected by cardiac puncture exsanguination.
To determine acute effects of each treatment on serum biochemistry and gene expression in bone, three week old female Sprague Dawley rats were randomly divided into four groups of 7 and were treated after overnight fast with a single subcutaneous injection of either vehicle, hPTH(1-34), sCT, or a combination of both hPTH(1-34) and sCT as above. Terminal blood samples were collected by cardiac puncture exsanguination under inhalation anaesthesia at 1.5, 4, or 6.5 hours after injection. The femoral distal epiphysis including the growth plate was removed and a subjacent 5 mm band of the metaphyseal primary spongiosa was resected and snap-frozen in liquid nitrogen for RNA preparation as previously described [20]. Tibiae were fixed in 4% paraformaldehyde, decalcified and embedded in paraffin for immunohistochemistry [21].

To assess local effects of sCT on bone, C57BL/6 mice were divided into 3 groups of 10 and treated with 2 daily 25μl injections of saline, sCT (7.5ng) or mOSM (0.2μg) over the calvariae as previously described [22]. Calvariae were collected 4 hours following the last injection and snap frozen in liquid nitrogen for RNA preparation as previously described [20].

**Biochemical analyses**

Serum calcium was measured by reaction with o-cresolphthalein (Sigma-Aldrich, St. Louis, Missouri, USA). Serum type 1 carboxy-terminal collagen crosslinks (CTX-1) were measured using the RatLaps ELISA (Nordic Bioscience, Denmark) and amino-terminal propeptide of type 1 procollagen (P1NP) was assayed by mass spectrometry [23]. Serum PTH was measured by a rat Intact PTH ELISA Kit (Immutopics, CA, USA).
**Histomorphometry and peripheral quantitative computer tomography (pQCT)**

Tibiae collected after treatment for 3 weeks were dehydrated in acetone and embedded in methylmethacrylate [24]. Undecalcified 5μm longitudinal sections were stained with toluidine blue for histomorphometry of the secondary spongiosa of the proximal tibial metaphysis as previously described [25]. The region measured was a 1 mm long by 1.4 mm wide rectangle commencing 1.9 mm below the growth plate to avoid the primary spongiosa, subcortical bone and trabecular bone newly formed during the 3 week treatment period. Femoral densitometric and geometric parameters were measured by pQCT (Stratec X-CT Research SA+, Version 5.5) as previously described [24].

**RNA Isolation and Real time RT-PCR**

RNA was prepared from individual samples using a QIAGEN RNeasy Lipid Midi Kit after homogenization in Ultraspec-II™ reagent (Biotecx, Houston, TX) with an LS 10-35 Polytron homogenizer (Brinkmann Instruments, Westbury, NY) [26]. cDNA was prepared from RNA using random hexamers (Promega, Australia) and Superscript III (Invitrogen, Australia) according to the manufacturer’s protocol. Primers are listed in Table 1. Real-time quantitative RT-PCR (qPCR) was performed using Sybr green detection with a Stratagene MX3000 (Invitrogen, Australia). The expression levels of all genes are expressed relative to HPRT1 or HMBS. The levels of these housekeeping genes were not altered in the experiments where they were used.

**Immunohistochemistry.**
Changes in sclerostin expression were assessed by immunohistochemistry in paraffin-embedded, decalcified 5µm tibial sections as previously described [22], using goat anti-mouse sclerostin (R&D Systems, Minneapolis, MN). Sclerostin positive osteocytes and unstained osteocytes were counted in cortical bone on both the lateral and medial side of each tibia as shown in Figure 4. The metaphyseal region was measured 1.5mm below the growth plate for 1.9mm, while diaphyseal measurements were taken 5.2mm below the growth plate for 1.9mm.

Dual labeling for sclerostin and calcitonin receptor (CTR) was carried out on sections of calvarial bone from 6 week old C57Bl/6 mice previously described [22]. Sections were dehydrated and non-specific binding blocked as previously described [27], then incubated in the following reagents, and washed with TNT ((0.1M Tris-HCl 0.15M NaCl and 0.05%Tween-20) between each step. A mixture of anti-Sclerostin antibody at 40µg/ml and 20µg/ml CTR antibody (AbD Serotec, Kidlington, UK) in blocking solution overnight, followed by 45 minutes in 1:500 biotinylated rabbit anti rabbit (DAKO, Glostrup, Denmark), 45 minutes of 1:500 streptavidin horseradish peroxidase, 10 minutes in biotin tyramine (1:50) in amplification diluent 10 min (TSA Biotin system kit, Perkin Elmer), then 45 minutes of 1:200 FITC swine anti goat (Invitrogen CA, USA) plus 1:200 avidin-Texas-Red (Vector Lab. CA, USA), then 3 minutes 1:1000 DAPI (4’,6-diamidino-2-phenylindole) (Invitrogen CA, USA). For visualization samples were examined by fluorescence microscopy under 488nm (sclerostin) and 595nm (CTR) excitation filters.

Gene expression profiling by DNA microarray analysis
Samples for mRNA profiling studies were processed by Asuragen (Austin, Texas) according to standard operating procedures. The integrity of total RNA was qualified by Agilent Bioanalyzer 2100 capillary electrophoresis and used for preparation of biotin-labeled targets (cRNA) using a MessageAmp™ II-based protocol (Ambion Inc., Austin, TX). cRNA yields were quantified by UV spectrophotometry and the distribution of transcript sizes was assessed using the Agilent Bioanalyzer 2100 capillary electrophoresis system. Labeled cRNA was fragmented and used for array hybridization and washed according to the standard Affymetrix protocol. In brief, labeled cRNA was resuspended in 5X fragmentation buffer and incubated at 94°C for 35 minutes then stored on ice. The hybridization cocktail and the fragmented cRNA mixture were heated to 99°C for 5 minutes and hybridized to Rat Genome 230_2.0 arrays (Affymetrix) at 45°C for 16 hours in an Affymetrix Model 640 hybridization oven. The microarrays were then automatically washed and stained with streptavidin-phycoerythrin conjugate in an Affymetrix FS450 Fluidics station. Fluorescence intensities were scanned on an Affymetrix GeneChip Scanner 3000 7G. A summary of the image signal data, p-values with significance flags, and gene annotations for every gene interrogated on the array was generated using the Affymetrix Statistical Algorithm MAS 5.0 (GCOS v1.3) algorithm, with all arrays scaled to 1500. The normalized signal data were fitted to an ANOVA model to identify differentially expressed genes between different groups. The false discovery rate (FDR) was estimated using as described by Benjamini et al [28]. Probesets with FDR of 0.2 or smaller were considered as significant and followed up for further analysis and the probesets with absent calls were excluded from all chips in the data set.

Bioinformatic analysis
Differentially expressed probe sets were identified as significant if the comparison had (1) two-independent-sample t-test *p*-values less than 0.05; (2) the median signal for all the chips in a group was greater than 250; (3) the comparisons had an absolute fold change greater than 1.2. Affymetrix probe sets were mapped to genes using proprietary probe set to gene annotations. The differentially expressed genes were mapped to Gene Ontology (GO) categories and counts were computed based on how many genes fell into each category. Statistical significance of enrichment was calculated for each GO term using a hypergeometric distribution statistics with the entire set of annotated genes on the Affymetrix chip serving as a background distribution. Familywise error rates for each group were computed to estimate the probability of FDR due to multiple testing.

*Calcitonin receptor studies*

To determine whether there was an indirect effect of sCT on sclerostin expression induced by a signal from the CT-treated osteoclast. Primary calvarial osteoblasts differentiated to express high levels of sclerostin [22] or UMR106-01 cells were co-cultured with osteoclasts generated from RANKL and M-CSF stimulated cultures of mouse bone marrow as previously described[29]. These co-cultures were treated with sCT (10nM) and SOST mRNA levels assessed after 1 and 4 hours of treatment. Further to this, media was collected from RANKL-derived osteoclasts that had been treated with 10nM sCT for 1 hour, and differentiated primary calvarial osteoblasts or UMR106-01 cells were incubated for 24 hours in this conditioned media diluted 1:1 with 2% fetal bovine serum. Each of these cell preparations was carried out 3 times, in triplicate.
Calvarial osteoblasts were prepared from wild type C57Bl/6 mice and from transgenic mice expressing green fluorescent protein (GFP) driven by the osteocyte-specific dentin matrix protein-1 (DMP1) promoter [30] as previously described [31]. Calvarial cells from each of these sources were differentiated in culture for 21 days, and used to seek specific binding of $^{125}$I-labeled sCT by autoradiography as previously described [32-34]. Positive controls for CT binding included osteoclasts derived by treatment of mouse bone marrow cells with RANKL and M-CSF [33], and UMR 106-06 cells [35]. Calvarial osteoblasts differentiated in this way for 21 days were also treated with 10nM sCT, CGRP, amylin, or PTH for 24 hours, and gene expression was examined by qPCR as above.

**Isolation of osteocytes**

Calvariae were removed from 2-8 day old C57Bl/6, DMP1-GFP transgenic mice or from double transgenic mice (Col2.3GFPCyan / DMP1GFPTopaz) [31]. After removal, calvariae were subject to four sequential, 30 minute digestions [30] in a 15 ml mixture containing 30 mg dispase (Gibco, NY, USA) and 15 mg collagenase type 2 (Worthington, Australia). Cell fractions 1 and 2-4 were collected and resuspended in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) containing 10% FBS and centrifuged. Cells were resuspended in PBS and filtered through a 70μm cell strainer, centrifuged and resuspended in 2% FBS/PBS, and filtered through a 45μm filter. DMP1-GFP positive cells were sorted on a FACS Aria (BD Biosciences) with excitation at 488 nm and 530/30 emission filter for GFP and 695/40 for propidium iodide (PI). Cells were separated using a 100μm nozzle at 20 psi first, live cells were selected based on their granularity, measured by side scatter (SSC) and by size,
measured by forward scatter (FSC). Doublets or cellular aggregates were excluded based on the pulse-width parameter they emitted. The cells were gated first on FSC-width by FSC-height and then gated on SSC-width by SSC-height. Further sorting identified GFP +ve and GFP –ve populations. For cells from DMP1GFPtopaz/pOB-col2.3GFPcyan mice, sorting was performed using a FACS-Vantage BD cell sorter with a 130-μm nozzle at a speed of 3-5k cells/s. Sorting was performed using appropriate lasers to distinguish GFPcyan from GFPtopaz-expressing cells. This double transgenic mouse model allowed identification of four populations a) DMP1GFPtopaz/pOB-col2.3GFPcyan– (termed GFP negative), b) DMP1GFPtopaz+/pOB-Col2.3GFPcyan+ (termed Col2.3cyan+), c) DMP1GFPtopaz+/pOB-Col2.3GFPcyan+ cells and d) DMP1GFPtopaz+/pOB-Col2.3GFPcyan–. Populations that are DMP1GFPtopaz positive and identified as groups C and D were combined into one sample during the cell sorting procedure. These are termed DMP1topaz+ and are concluded to be osteocytes [31].

Expression of the two Calcr transcripts Calcr1a and Calcr1b was determined by RT-PCR using primers designed to distinguish the two transcripts based on amplification product size (Calcr1a, 446; Calcr1b 557)[36] when run on a 2 % TBE agarose gel (Table 1). RT-PCR was performed using High Fidelity Taq Polymerase (Roche) with PCR cycling conditions as follows: 95°C 5 min; followed by 10 cycles 95°C-15 sec, 58°C – 20 sec, 72°C-30 sec; and then 25 cycles 95°C-15 sec, 58°C – 20 sec, 72°C-30 sec+5 sec/cycle; and final extension 72°C for 7 min. RT-PCR for HPRT1 was performed according to the same protocol, with the exception that 20 cycles were performed for the last period of cycling. PCR products were cloned into pGEMT™ T-A cloning vector (Promega) and transformed into Stable3 bacterial cells. Plasmid
DNA was prepared using Qiagen mini-prep kit. Cycle sequencing was performed by Applied Genetic Diagnostics (Department of Pathology, The University of Melbourne).

Statistical analyses

All data are presented as means ± SEM. Significant differences were determined by one-way or two-way ANOVA followed (where significant) by Fisher’s protected least significant difference post hoc test to identify significant pair wise differences. Where data were not normally distributed (for sclerostin positive osteocyte immunohistochemistry measurements), significant differences were identified by the non-parametric Kruskal-Wallis test. For all analyses, \( p<0.05 \) was considered significant. Statistical analyses were carried out using StatView 5.0.1 (SAS Institute, Cary, North Carolina, USA).

RESULTS

Effect of sCT on the anabolic response to PTH

Daily administration of 30μg/kg PTH for 3 weeks was sufficient to double femoral trabecular bone mineral density (Tb.BMD) (Fig.1a) compared to the Tb.BMD of vehicle treated rats. This dose of PTH specifically increased trabecular bone; no alteration in femoral cortical thickness or cortical BMD was detected in any treatment group (data not shown). sCT treatment alone did not alter femoral Tb.BMD (Fig. 1a), cortical thickness or cortical BMD (data not shown), but co-treatment of sCT with PTH prevented the increase in femoral Tb.BMD caused by PTH treatment (Fig. 1a).
This inhibitory effect of sCT on the anabolic effect of PTH was also observed at an alternate site when tibial samples were analyzed by histomorphometry. PTH administration caused a doubling of tibial trabecular bone volume (BV/TV) compared with that of vehicle treated rats (Fig. 1b&c). This was associated with a significant increase in trabecular thickness (Tb.Th) and trabecular number (Tb.N), as well as a significant reduction in trabecular separation (Tb.Sp) (Fig. 1d-f). As in the femur, the low dose of sCT did not cause a significant change in tibial BV/TV, Tb.N or Tb.Th (Fig. 1b-e) but Tb.Sp was significantly increased compared to vehicle (Fig. 1f) suggesting a very small decrease in trabecular bone. Co-treatment of sCT with PTH halved the stimulatory effect of PTH on tibial BV/TV (Fig. 1b&c), as observed in the femur, and fully prevented the increase in Tb.Th associated with PTH treatment (Fig. 1d). The PTH-induced changes in Tb.N and Tb.Sp were not significantly modified by co-administration with sCT (Fig.1e&f).

Histomorphometric indices of bone formation measured in the tibia, including osteoblast surface, osteoid surface, osteoid thickness and osteoid volume were all significantly increased by PTH treatment (Table 2). Although sCT clearly blocked the increase in BV/TV, Tb.Th and Tb.BMD caused by PTH treatment over the 3 week treatment period (Fig. 1), osteoblast and osteoid extent were not further modified by co-administration with sCT (Table 2). These indices were also increased by sCT treatment which probably relates to regular intermittent increases in PTH following the calcium-lowering effect of sCT (Figure 2a,b). There was no effect of any treatment on osteoclast surface or osteoclast number (Table 2). Dynamic histomorphometric indices were also measured, but possibly due to the high level of resorption in these young animals, no statistically significant changes in mineral apposition rate (data not shown), mineralizing surface (data not shown) or bone
formation rate (Table 2) were detected in any treatment group. Serum CTX-1 and osteocalcin levels, analysed 24 hours after the final of three weeks of treatment, were not significantly altered in any of the treatment groups (data not shown).

**Acute effects of sCT and PTH on serum biochemistry.**

Treatment with a single injection of 30 μg/kg PTH did not alter serum calcium levels significantly at 1, 4.5 or 6.5 hours after administration (Fig. 2b). Serum CTX-1 and P1NP levels were significantly increased 4 hours after PTH injection, consistent with a transient increase in bone resorption and bone formation, respectively (Fig. 2c&d).

Consistent with the sCT biological assay data, administration of a single injection of sCT rapidly reduced serum calcium levels by 1.5 hours post injection (Fig. 2a). This effect was transient, and was associated with an increase in circulating PTH (Fig. 2b); serum calcium levels returned to control levels by 6.5 hours after the injection (Fig. 2a). Serum CTX-1 (Fig. 2c) was also significantly reduced by sCT at 1.5 and 4 hours post injection, confirming a transient inhibition of osteoclast activity. The bone formation marker serum P1NP was not significantly modified at any time point by sCT (Fig. 2d).

The effects of co-treatment of sCT with PTH were similar to those of sCT administration alone, including a significant reduction in serum calcium for 4 hours post injection (Fig. 2a), a transient increase in circulating PTH (Fig. 2b) and a transient reduction in CTX-1, although the timing of these changes was slightly altered compared to sCT alone (Fig. 2c). Serum P1NP remained unaffected (Fig. 2d). This indicates that sCT co-treatment abolished the transient increase in markers for
bone resorption (CTX-1) and bone formation (P1NP) induced by a single injection of PTH, without modifying P1NP by itself.

Effect of sCT on regulation of PTH-induced genes

In a search for osteoclast-derived signals that may be modified by sCT co-administration with PTH, we examined gene expression in metaphyseal bone and marrow of 3-week-old female rats at 1.5, 4 and 6.5 hours after treatment in each group. A number of candidate genes were selected as positive controls to establish that known PTH-regulated genes were altered. RANKL, IL-6 and ephrinB2 mRNA levels were significantly upregulated as expected, but were not modified by simultaneous sCT treatment, nor were they significantly altered by sCT alone (Fig.3a-c). The mRNA levels for the osteocyte–derived bone formation inhibitor sclerostin were slightly decreased by PTH treatment at 1.5 hours (Fig.3d). A higher dose of PTH (80µg/kg) has been shown to lower sclerostin mRNA in rat bone at 6 hrs [6]. Surprisingly, treatment with sCT alone increased mRNA levels for sclerostin at 1.5, 4, and 6.5 hours (1.9, 2.2, and 2.6 fold, respectively; Fig.3d). When sCT was administered together with PTH the level of sclerostin mRNA was increased at 4 hrs but was not distinguishable from control levels at the 1.5 and 6.5 hr time points (Fig 3d). Treatment with sCT also reduced mRNA for the osteocyte product DMP1 by -1.9 fold, while sCT co-treatment with PTH resulted in a reduction of -3.6 fold. MEPE expression was reduced -1.4 and -1.3 fold in sCT alone and sCT co-treated with PTH, respectively (Fig. 3e&f).
Microarray gene profiling

In view of the surprising finding of upregulated sclerostin in response to sCT, we undertook cDNA microarray analysis of the rat bone mRNA after each treatment described above. Microarray analysis revealed a significant number of differentially expressed genes ≥1.2 fold upon treatment with either hPTH(1-34) alone, sCT alone, or co-administration of sCT with hPTH(1-34), with a number of genes overlapping different treatment groups, as indicated in Fig 3g. Microarray analysis confirmed the changes observed in levels of PTH-regulated genes identified by qPCR, as well as CREM. Microarray analysis of candidate osteoclast-derived coupling factors including cardiotrophin-1 [24] and sphingosine-1 kinase [37, 38], did not reveal any difference between PTH and PTH+sCT treated groups (Fig 3h). Microarray analysis confirmed that in addition to sclerostin, mRNA levels of the osteocytic markers DMP1 and MEPE were significantly down-regulated following administration of sCT at 4 hours (Fig. 3h). Calcitonin receptor mRNA levels were slightly elevated by both PTH and sCT at 4 hours (Fig 3h). Co-administration of PTH did not affect the sCT-induced reduction in DMP1 and MEPE (Fig. 3g).

Having shown that systemic administration of sCT treatment in the young rat resulted in increased sclerostin mRNA in long bones, we examined responses in mouse calvariae, since PTH [6] and oncostatin M [22] treatment both rapidly reduced sclerostin production when administered in this manner. This model might be free of influence of weight-bearing, since sclerostin +ve osteocytes were found to be evenly distributed across the calvarial span [22]. The amount of mRNA for sclerostin was decreased by 80% with mOSM treatment, consistent with our previous data [22], and
was increased 2-fold by sCT treatment (Fig 3i) confirming this effect of sCT on sclerostin expression.

Sclerostin detection by immunohistochemistry

To establish whether the increase in sclerostin mRNA led to an increase in sclerostin protein levels in osteocytes, immunohistochemistry was performed on individual tibiae from 3-week-old female rats given a single treatment with sCT, PTH or the two combined. Since sclerostin expression is modified by mechanical strain [39], sclerostin-positive osteocytes as a percentage of the total number of osteocytes were counted in 4 regions (Fig 4b), to take into account possible effects of differing rates of bone turnover, loading and density. No significant variation in sclerostin staining was detected between the regions, but there was a significantly increased proportion of sclerostin-positive osteocytes in all regions when measured 4 hours after a single sCT injection (Fig 4a&c). This was observed both in the absence and presence of PTH. In contrast, a single 30µg/kg injection of PTH did not significantly alter the proportion of sclerostin positive osteocytes in any region measured (Fig 4a&c). In the medial tibiae, a significant increase in the percentage of sclerostin-positive osteocytes was also observed at 1.5 hours (data not shown).

Lack of CTR in cultured osteocyte-like cells

The rapid regulation of sclerostin, MEPE and DMP1 by sCT raised the possibility of either a direct effect of sCT on osteocytes, or a signal from the CT-treated osteoclast that influences the osteocyte. Differentiation of calvarial osteoblasts from normal or
DMP1-GFP mice for 21 days [30, 31] resulted in several hundred-fold increases in mRNA levels of the osteocyte markers sclerostin, MEPE and DMP1 with levels reaching Ct values of 28, 26, and 26, respectively. When osteoclasts were added to these cultures, sclerostin expression was not modified by either sCT treatment or exposure to conditioned medium from osteoclasts treated with sCT (data not shown). Similarly, UMR106-01 cells (which express high levels of sclerostin [22]) were co-cultured with osteoclasts and showed no sclerostin regulation in response to sCT (data not shown). As we could find no evidence for communication from the sCT-treated osteoclast to the osteocytes, we assessed Calcr mRNA levels and sCT response in primary calvarial osteoblasts differentiated in culture as above. Calcr mRNA was not detectable (Ct>40) in repeated experiments with these cells. Receptor autoradiography with iodinated sCT (Fig.5a) showed accumulation of silver grains over cultured osteoclasts, reflecting a high level of binding due to the large number of CTR (> 1 million per cell) in those cells [32]. Very much weaker specific binding was observed in the UMR106-06 osteosarcoma cell-line (Fig.5c); these cells have been express to express much lower levels of CTR (5-10,000 per cell) than osteoclasts [35]. Specificity of radiolabelled calcitonin binding in both osteoclasts and UMR106.06 cells was confirmed by competition with unlabelled sCT, which abolished the autoradiographic signal (Fig. 5b, d). In contrast, no specific silver granules were observed over GFP positive cells from DMP1-GFP mice, where only osteocytes express GFP (Fig.5e-g) or wild-type osteoblasts differentiated until high levels of osteocyte markers were expressed (data not shown). Furthermore, while these highly differentiated cells responded to PTH treatment with a rapid and substantial decrease in expression of mRNA for sclerostin, sclerostin mRNA expression was not
significantly modified by treatment with sCT, or the other ligands for CTR, amylin and calcitonin gene-related peptide (CGRP) (Fig.5h).

Primary calvarial osteocytes possess calcitonin receptor

Although sCT binding and Calcr mRNA were not detectable in calvarial osteoblasts differentiated in culture to a stage of enrichment in sclerostin and DMP1 expression, recent work identified mRNA for Calcr in calvarial osteocytes that were freshly isolated and separated by FACS sorting and subjected to microarray [31]. In light of this finding, we went on to study osteocytes freshly isolated from bone, since the generation of osteocytes by differentiation of precursor cells in culture might result in the loss of Calcr expression.

Osteocytes were isolated from calvariae of wild type mice and two transgenic mouse lines expressing GFP under the control of the DMP1 promoter: DMP1-GFP and pOBCol2.3GFPCyan / DMP1GFPTopaz mice following sequential collagenase digestion [31]. Freshly isolated unsorted osteocytes from WT mice expressed high levels of CTR mRNA (Ct = 26.5). Osteocytes isolated from pOBCol2.3GFPCyan / DMP1GFPTopaz mice sorted by FACs revealed mRNA levels of Calcr in DMP1GFPTopaz cells greater than that of osteoclasts derived from RAW264.7 cells (Fig 6a). Freshly isolated osteocytes from DMP1-GFP transgenic mice sorted by FACS for GFP displayed higher levels of both sclerostin and DMP1 mRNA (Fig.6b&c) than GFP negative cells. GFP-positive cells also displayed very high levels of Calcr mRNA (Ct =23.5) compared to GFP negative cells (Ct =27) (Fig.6d). Freshly isolated osteocytic cells from WT calvaria incubated with PTH did not, however, display a cAMP elevation response (data not shown), suggesting the
collagenase/dispase digestion process results in receptor destruction. Likely for the same reason, CTR could not be detected either by immunostaining or receptor autoradiography in the cells isolated with prolonged enzymatic digestion (not shown). In order to establish how long production of mRNA for Calcr might persist in monolayer culture, freshly isolated 2-hr digests of calvarial cells were cultured for 1, 2, 3 or 7 days and assessed by qPCR. Production of mRNA for Calcr was almost completely lost within 24 hrs, as were production of the osteocyte markers SOST and DMP1 (Fig 6e).

There are two isoforms of the CTR formed by alternative splicing in the mouse, C1a and C1b. These are identical but for a 37-amino-acid insert in the second extracellular domain in C1b [40, 41]. RT-PCR detected only the C1a isoform in freshly prepared osteocytes from WT and DMP1-GFP mice (Fig. 6f), as also observed with mRNA from osteoclasts, as shown previously [40, 42]. UMR106-06 cells, shown to possess CTR [35], express predominantly C1a mRNA. Calcr was not detected in UMR106-01 cells or in calvarial digests cultured for 7 days. The brain Calcr consisted of both C1a and C1b, but predominantly the latter as previously shown [41, 42]. Sequencing of the RT-PCR product confirmed that the isoform expressed in wild type and DMP1-GFP positive osteocytes was C1a.

Localization of CTR in osteocytes by immunohistochemistry.

To determine CTR protein expression in calvarial osteocytes in situ, we carried out sclerostin and CTR immunohistochemical colocalization in calvarial sections of 6 week old mice (Figure 7). The antibody for CTR was directed to the intracellular region of the protein [43]. CTR was detected in the perinuclear region of a
subpopulation of osteocytes. No staining was observed in osteocyte canaliculi. Notably, while some osteocytes were positive for both sclerostin and CTR, there were subpopulations that were positive for only sclerostin, or only CTR. No distinct distribution of CTR positive osteocytes was noted.

DISCUSSION

This study began with the aim of using sCT as a transient inhibitor of osteoclast activation, with the hypothesis that its effect could impair the ability of osteoclasts to contribute to the anabolic response to PTH. Concomitant treatment with PTH and sCT significantly attenuated the anabolic response of trabecular bone to PTH in two sites, assessed by quantitative pQCT and by histomorphometry. The PTH effect on tibial BV/TV and Tb.Th, and femoral Tb.BMD was greatly diminished by sCT co-treatment despite the fact that osteoblast and osteoid surfaces were unchanged. This suggested that the greater level of osteoblast activity induced by PTH over the treatment period was impaired by sCT. This was supported by serum biochemistry, where a PTH-induced transient increase in P1NP was abrogated by co-treatment with sCT. Although osteoblast and osteoid surfaces were increased in the PTH+sCT treated rats (and, indeed the sCT treated rats) to the same levels as observed in PTH-treated rats, this was not sufficient to increase BV/TV or Tb.BMD. This observation is very similar to those made by Kramer et al in a SOST overexpressing mouse line [44]. Together, these studies indicate that an increase in osteoblast differentiation, without a sufficient reduction in sclerostin expression is insufficient for an increase in trabecular bone volume to occur[45].
The blunting of PTH anabolic action by co-administration with sCT seemed to accord with the hypothesis that active osteoclasts are required for the anabolic effect of PTH. While no clear CT-regulated factor that was both osteoclast-derived and a known inhibitor of osteoblast activity was revealed by microarray or PCR analysis we cannot exclude this possibility. However, the striking and surprising finding, that both systemic and local injections of calcitonin induced a rapid increase in mRNA and protein levels of the osteocyte-derived osteoblast inhibitor sclerostin points to an alternative mechanism. This could, at least in part, explain the sCT inhibition of the PTH effect and suggests a mechanism by which endogenous CT itself may regulate bone formation.

The *sost* gene product, sclerostin, is a powerful inhibitor of bone formation that inhibits Wnt signaling through binding to the Wnt co–receptors, LRP5 and LRP6 [46]. Since the discovery of sclerostin there have been extensive analyses of its localization by immunohistochemistry indicating sclerostin is produced in bone only by osteocytes [47-49]. There has been a single report that sclerostin co-localises with MMP-9, raising the possibility that osteoclasts may produce sclerostin [50]. However, using immunohistochemistry in rat and mouse bone we could not detect sclerostin staining in osteoclasts (data not shown). Production of sclerostin by osteocytes is rapidly decreased by treatment with PTH [5, 6], cytokines including oncostatin M, leukemia inhibitory factor and cardiotrophin-1 [22, 27], and by mechanical loading [39], and this reduction has been proposed to explain, at least in part, the increased bone formation resulting from those treatments.
Rapid reductions in sclerostin production could signal to limit the filling of remodeling spaces by osteoblasts, in addition to keeping lining cells in a quiescent state on non-remodeling bone surfaces [49]. To date, this is the only report of rapidly enhanced sclerostin expression. A more delayed stimulation has been reported for the family of bone morphogenetic proteins (BMPs) which, after 72 hours of treatment, induced sclerostin mRNA expression by isolated human osteoblasts and mesenchymal stem cells, suggesting stimulation of osteocytic differentiation [51]. The cytokine TWEAK, also increased sclerostin mRNA levels in human bone-derived cells after 72 hours [52].

We may have inadvertently used an alterative method to demonstrate the importance of sclerostin in the PTH anabolic effect without the complication of the basal phenotype of genetically manipulated animals. We are not able to conclude that the enhancement in sclerostin alone is sufficient to explain the reduced PTH anabolic effect seen in the presence of sCT, but the rapid change in sclerostin production is compatible with the notion that regulation of sclerostin production contributes to the PTH anabolic effect. This has been recently confirmed by reports of impaired PTH anabolic activity in mice genetically engineered to express constitutively higher or lower levels of sclerostin [53].

The rapid effects of sCT on osteocytic gene expression were not restricted to sclerostin. mRNA levels for the osteocyte products DMP1 and MEPE were both reduced by sCT treatment, regardless of the presence of PTH. The significance of these effects remains to be determined, but might imply a more general mechanism
whereby CT may indirectly affect osteoblastic mineralization through the mediation of osteocytes. Reduced sclerostin production with skeletal loading [39] has been associated with increased production of DMP1 and MEPE in the same conditions [54]; sCT also induced contrary regulation of these osteocyte products, but in the opposite direction to mechanical loading. This was not observed, nor has this been reported for PTH treatment.

The physiological role of CT has been long argued. It is firmly established that CT acts directly on osteoclasts, and receptor autoradiography established osteoclasts as the only discernible bone cell target [32]. Although the best understood action of CT in bone is as an antiresorptive agent, there are reports of actions of very high doses of CT on osteoblast-like cells in vitro [55, 56]. CTR has not been detected by high affinity specific binding studies, receptor autoradiography with competitive inhibition, or by immunostaining nor has cDNA been detected in osteoblastic cells [32-34], with the exception of the osteosarcoma-derived UMR106-06 cell line[35]. A low level of sCT binding has been reported in MLO-Y4 cells [57], a cell-line that shares some characteristics of osteocytes. Since sCT is able to bind at low affinity to the calcitonin-like receptor, which is expressed at high levels in osteoblasts [58], inhibition of apoptosis by 5 ng/ml sCT in undifferentiated calvarial cells [57], may be explained by effects through low-affinity binding to the CGRP receptor, which is a dimer of the calcitonin-like receptor and RAMP1, as discussed in a recent review [58].
Since modulation of sclerostin expression by sCT was not observed in cultured sclerostin-producing osteoblasts in the presence of osteoclasts, we revisited the question of whether CTR is expressed in osteocytes using techniques not previously available. Freshly isolated cells identified as osteocytic in nature through DMP1 promoter-directed transgenic expression of GFP [30] expressed substantial levels of Calcr mRNA, and CTR was detected in osteocytes by immunostaining of calvarial sections. CTR staining in a subset of osteocytes is consistent with a 1981 report [59] using an immunohistochemical technique to identify CT binding to osteoclasts and some osteocytes. This work was inconsistent with a contemporary report that failed to detect intravenously administered radiolabelled sCT in osteocytes [60]. This difference could reflect not only different levels of CTR expression by the two cell types, but also differing sensitivities of the methods used.

Although osteocytes freshly isolated from calvarial bone expressed abundant Calcr mRNA, CTR protein could not be detected. It is likely that this is due to the prolonged (2.5 hour) enzymatic digestion step required to extract these cells. Consistent with the destruction of surface receptors, no cAMP response to PTH was detected in these cells. For this reason it has not been possible to study the CT signaling pathway in these cells. Decreased sclerostin production induced by PTH is cAMP-dependent and can be reproduced with forskolin, an adenylyl cyclase stimulator [6], although it is not clear that all this signaling occurs through the cAMP/PKA/CREB pathway [61]. Since CT has the opposite effect on sclerostin levels, the effect of CT is unlikely to be cAMP-dependent, and while both CTR isoforms signal through cAMP, they do so also through calcium-dependent pathways.
This highlights the need for more detailed study of signaling mechanisms in the control of sclerostin production.

It will be important also to investigate why osteocytic cells generated or maintained in culture do not express CTR mRNA or protein, when they clearly do in vivo. This may relate to required interaction of the cells with components of the bone matrix, as suggested by super-induction of Calcr in osteoclasts grown on bone [65]. It is also possible that regulatory mechanisms including neural innervation may support CTR expression in osteocytes in vivo. It might be relevant to note that in osteocyte/osteoblast gene array comparisons [31, 66], the osteocyte population was found to express a large number of genes associated with the neuronal phenotype. It is not clear whether the rapid reduction in mRNA levels of Calcr, SOST and DMP1 when freshly isolated osteocytes are cultured relates to a loss of the osteocytic phenotype, or to poor survival of the osteocytic cells in culture.

In a review one of the co-discoverers of CT [67] argued that it is not involved in calcium homeostasis or in any other important physiological function, except possibly in protection of the skeleton under calcium stress. Some more recent work has raised interesting possibilities, however. Ablation of the CT/CGRP gene resulted in mice (Calca−/−) that were much less able than wild-type mice to overcome hypercalcemia induced by a calcium load [68], and lost excessive bone during lactation [69]. The great surprise with these mice however, was that they had increased bone mass, with histomorphometry showing increased bone formation [68], suggesting that CT might inhibit bone formation during normal bone remodeling. It was possible that the dual
ablation of CT and CGRPα might explain it, but the increased bone formation phenotype was not found in mice deficient for CGRPα alone [70], leaving calcitonin deficiency likely responsible for the increased bone formation in Calca−/− mice. Although interpreting the findings from the Calca−/− mouse model was complicated by the mixed genetic background of the mouse model, CTR+/− mice also showed increased bone formation [71]. Embryo lethality of CTR−/− mice led these investigators to develop a further global CTR knockout mouse, with deletion of greater than 94% of CTR, yielding mice with a mild increase in bone formation rate [72]. Recent findings reported in abstract [73] show that in a new CTR null mouse strain, knockout animals are viable and again demonstrate increased bone formation rate, with gene dose-dependence in the heterozygotes. These observations indicate an inhibitory effect of calcitonin on bone formation, as in the other genetic studies [68, 71, 72, 74]. The increased sclerostin expression induced by sCT that we report here in young mice and rats provides a means to achieve this effect. Strikingly though, as mice lacking CT and CGRPα aged, they developed high turnover bone loss, consistent with a role for CT as an inhibitor of bone remodeling [73, 75]. Taken together with the pharmacological effects that we describe here in young animals, this might imply that the physiological roles of CT differ between younger and old animals. This question will be addressed in further work.

This study demonstrated that co-administration of sCT with PTH significantly attenuated the anabolic action of PTH. qPCR and DNA microarray analysis revealed a large number of significantly altered genes, but none of them were known as candidate coupling factors. It has been reported that IL-6 [25], ephrinB2 [76],
cardiotrophin-1 [27], and sphingosine-1 kinase [37, 38] are potential coupling factors, however calcitonin co-administration did not modify mRNA levels of any of these genes (Fig 3g). Increased sclerostin production in response to CT treatment reported here provides a possible explanation for the inhibition by CT of the PTH anabolic effect, but cannot exclude an osteoclast contribution. The present work provides further evidence that local regulation of sclerostin production by osteocytes can influence the amount of bone formed, and the CT effect described here might provide a mechanism to explain the findings in genetically manipulated mice that suggest CT as a physiological inhibitor of bone formation, at least in young animals.

Acknowledgements

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Figure Legends

Fig. 1. Effects of salmon calcitonin (sCT) on the anabolic effect of parathyroid hormone (PTH). a-f: Femoral trabecular BMD (Tb.BMD), tibial trabecular bone volume (BV/TV), trabecular thickness (Tb.Th.) and trabecular number (Tb.N.) were all significantly greater, and trabecular separation (Tb.Sp) was lower after 3 weeks of
PTH treatment at 30µg/kg compared to vehicle treated rats. The PTH effect was inhibited by co-administration with 0.5µg/kg sCT (PTH+sCT); sCT alone influenced only Tb.Sp. c: Representative Von Kossa stained tibia; boxes denote measurement region. Data are mean ± SEM (n=7 per group). \(^a\)p<0.05, \(^aa\)p<0.01, \(^aaa\)p<0.001, compared with vehicle-treated animals. \(^b\)p<0.05 compared to PTH-treated animals.

**Fig. 2. Effects of sCT and PTH on serum biochemistry.** Serum levels of (a) intact (iPTH), (b) calcium, (c) carboxy-terminal collagen crosslinks (CTX-1) and (d) amino-terminal propeptide of type 1 procollagen (P1NP) at 1.5, 4, & 6.5 hours following a single injection of PTH (30µg/kg), sCT (0.5µg/kg) or PTH+sCT co-administration in 3 week old female Sprague-Dawley rats. Vehicle (●), PTH (△), sCT (○), PTH+CT (▲). Data are mean ± SEM (n=6 per group). \(^a\)p<0.05, \(^aa\)p<0.01, \(^aaa\)p<0.001, compared with vehicle-treated animals at the same time point. \(^b\)p<0.05 compared to PTH-treated animals at the same time point.

**Fig. 3. Microarray analysis of genes regulated by PTH and CT and qPCR validation of osteocytic genes.** mRNA levels of PTH-regulated genes (a) RANKL, (b) IL-6, (c) Ephrin B2, (d) sclerostin, (e) DMP1 and (f) MEPE, determined by qPCR of metaphyseal specimens collected 1.5, 4 and 6.5 hours after a single injection of vehicle (black), 30µg/kg PTH (white), 0.5µg/kg sCT (vertical stripes), or both PTH and sCT (horizontal stripes). Data are mean levels of the genes of interest relative to HPRT1 ± SEM (n=6 per group). g: Venn diagrams showing number of genes regulated by PTH, sCT and PTH+CT compared to vehicle at 1.5 and 4 hours after a single injection of each treatment. h: Fold change in mRNA levels detected by microarray for osteocytic genes, PTH-regulated genes, and osteoclast-derived coupling factors; TNFSF11=RANKL, EFNB2 = Ephrin B2, CT-1 = Cardiotrophin-1 and SPHK1 = Sphingosine kinase 1. NSD, no significant difference. i: mRNA levels
of sclerostin in calvariae of mice 4 hours after 2 daily calvarial injections of sCT (7.5ng) or mOSM (0.2μg); n=10 mice per group. \textsuperscript{a}p<0.05, \textsuperscript{aa}p<0.01, \textsuperscript{aaa}p<0.001 compared with vehicle treated specimens at the same time point; \textsuperscript{bb}p<0.01, \textsuperscript{bbb}p<0.001 compared with PTH-treated specimens at the same time point.

**Fig 4. Increased proportion of sclerostin-positive osteocytes in calcitonin treated rats.** \textbf{a}: Representative images showing immunolocalisation of sclerostin in paraffin embedded tibial sections, showing osteocytes in cortical bone from vehicle, PTH, sCT and PTH+sCT treated rats, scale bar = 20μm. \textbf{b}: Representative image of tibia highlighting metaphyseal (1&2), diaphyseal (3&4), lateral (1&3) and medial (2&4) regions measured. \textbf{c}: Percentage of sclerostin positive osteocytes from each region 4 hours after treatment. Data are mean ± SEM (n=6). \textsuperscript{a}p<0.05, \textsuperscript{aa}p<0.01, \textsuperscript{aaa}p<0.001, compared with vehicle treated animals, \textsuperscript{b}p<0.05 compared with PTH treated animals.

**Fig 5. Autoradiographic detection of cellular binding of \textsuperscript{125}I-sCT** \textbf{a}: \textsuperscript{125}I-sCT binding (silver granules) clustered on \textit{in vitro}-generated murine osteoclasts. \textbf{b}: Non-specific binding of \textsuperscript{125}I-sCT to osteoclasts. \textbf{c}: Low level of \textsuperscript{125}I-sCT binding (c) and non-specific binding (d) on confluent UMR106.06 cells. \textbf{e-g}: Mineralized nodule of primary DMP1-GFP osteoblasts showing GFP expression (e) and lack of \textsuperscript{125}I-sCT binding in two focal planes (f,g). Arrows indicate mineralized nodule location. Scale Bar = 50μm. \textbf{h}: The effect of 24 hours treatment with 10nM PTH, sCT, amylin, or calcitonin-gene-related peptide (CGRP) on sclerostin mRNA levels in calvarial osteoblasts differentiated for 21 days. \textsuperscript{aaa}p<0.001, compared with vehicle treated cells.
**Fig. 6. Calcr expression in isolated calvarial osteoblasts and osteocytes**

a: *Calcr* mRNA levels in M-CSF & RANKL differentiated RAW cells (OCL) and freshly isolated calvarial cells from Col2.3cyan/DMP1topaz mice that were FACS sorted and were negative for both forms of GFP (negative), Col2.3cyan+/DMP1topaz– osteoblasts (cyan) and DMP1topaz+ osteocytes (topaz). b-d: mRNA levels of sclerostin, *Calcr*, or DMP1 relative to HMBS in freshly isolated calvarial cells from DMP1-GFP mice. Populations are from fraction 1 or fractions 2-4 unsorted and sorted into GFP positive osteocytes (GFP+) or GFP negative cells (GFP-). Data are mean ± SEM from three independent experiments performed in triplicate. *p<0.05*, compared with fraction 2-4 GFP+ve cells. e: Calcr, SOST and DMP1 mRNA levels decrease rapidly when cultured for 1-7 days in normal osteoblast differentiation media. f: Calcr PCR products separated by gel electrophoresis show no expression of Calcr in UMR106-01, expression of both C1a and C1b isoforms in brain, C1b in bone marrow-macrophage-derived osteoclasts (OCL) and freshly isolated osteocytes (OCY) from wild type mice (WT) and FACS sorted OCY from DMP1-GFP mice (GFP+ OCY). Calcr mRNA was not detected in WT OCY cultured for 7 days. UMR106-06 cells expressed largely C1a, but also a small amount of C1b. HPRT1 product is shown as a loading control.

**Fig. 7. Immunohistochemical detection of calcitonin receptor in sections of mouse calvaria.**
a: Sclerostin expression, b: calcitonin receptor (CTR) and c: 4’,6-diamidino-2-phenylindole (DAPI) d: Merged image. Arrows indicate a population of osteocytes expressing both sclerostin and CTR. Magnification x60. Scale Bar = 20µm
Table 1: Primer sequences for PCR analysis: sequences detect both mouse and rat transcripts, except where noted.

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<th>Forward Sequence (5' to 3')</th>
<th>Reverse Sequence (5' to 3')</th>
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Table 2: Osteoblast surface (ObS/BS), Osteoid volume (OV/BV), osteoid thickness (OTh), osteoclast surface (OcS/BS) and bone formation rate (BFR/BS) in rats treated with vehicle PTH, CT or PTH+CT for 3 weeks. Data are mean ± SEM (n=7 per group). \(^a\)p<0.05, \(^{aa}\)p<0.01, \(^{aaa}\)p<0.001, compared with vehicle-treated animals. No significant difference between CT and PTH+CT groups was observed.

<table>
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<tr>
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<th>ObS/BS (%)</th>
<th>OS/BS (%)</th>
<th>OV/BV (%)</th>
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<th>OcS/BS (%)</th>
<th>BFR/BS (μm³/μm²/d)</th>
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<td>VEHICLE</td>
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<td>PTH+CT</td>
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References:


Figure(s)
**Osteocyte Genes**

- **DMP1**: NSD, NSD, NSD
- **MEPE**: -1.2, NSD, -1.2
- **Sclerostin**: -1.8, 2.4, -1.8

**PTH Regulated Genes**

- **CREM**: 6.7, 1.8, 9.6
- **TNFSF11**: 2.9, 1.2, 3.2
- **EFNB2**: 8.1, 1.4, 9.8
- **IL6**: 12.6, 1.4, 21.2

**Coupling Factors**

- **CT-1**: NSD, NSD, NSD
- **SPHK1**: 1.9, 1.2, 1.9
- **CALCR**: NSD, NSD, NSD

**Sclerostin:HMBS**

- **Vehicle**: 0
- **sCT**: 5
- **mOSM**: 1

**Gene Expression**

- **RANKL:HPRT1**: 1.5H, 4H, 6.5H
- **IL-6:HPRT1**: 1.5H, 4H, 6.5H
- **EphrinB2:HPRT1**: 1.5H, 4H, 6.5H
- **Sclerostin:HPRT1**: 1.5H, 4H, 6.5H
- **DMP1:HPRT1**: 1.5H, 4H, 6.5H
- **MEPE:HPRT1**: 1.5H, 4H, 6.5H

**Significance Levels**

- a
- b
- c
- d
- e
- f
- g

**Gene Expression Levels**

- **PTH**: 876, 567, 464
- **PTH+CT**: 537, 564, 761
- **1.5H**: 41, 113, 72
- **4H**: 630, 72, 411

**Vehicle**

- sCT: 630
- mOSM: 155
Author/s:
Gooi, JH; Pompolo, S; Karsdal, MA; Kulkarni, NH; Kalajzic, I; McAhren, SHM; Han, B; Onyia, JE; Ho, PWM; Gillespie, MT; Walsh, NC; Chia, LY; Quinn, JMW; Martin, TJ; Sims, NA

Title:
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