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Osteocyte-derived RANKL is a critical mediator of the increased bone resorption caused by dietary calcium deficiency

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Abstract

Parathyroid hormone (PTH) excess stimulates bone resorption. This effect is associated with increased expression of the osteoclastogenic cytokine receptor activator of nuclear factor κ B ligand (RANKL) in bone. However, several different cell types, including bone marrow stromal cells, osteocytes, and T lymphocytes, express both RANKL and the PTH receptor and it is unclear whether RANKL expression by any of these cell types is required for PTH-induced bone loss. Here we have used mice lacking the RANKL gene in osteocytes to determine whether RANKL produced by this cell type is required for the bone loss caused by secondary hyperparathyroidism induced by dietary calcium deficiency in adult mice. Thirty days of dietary calcium deficiency caused bone loss in control mice, but this effect was blunted in mice lacking RANKL in osteocytes. The increase in RANKL expression in bone and the increase in osteoclast number caused by dietary calcium deficiency were also blunted in mice lacking RANKL in osteocytes. These results demonstrate that RANKL produced by osteocytes contributes to the increased bone resorption and the bone loss caused by secondary hyperparathyroidism, strengthening the evidence that osteocytes are an important target cell for hormonal control of bone remodeling.

Keywords

osteocyte; osteoclast; RANKL; parathyroid hormone

INTRODUCTION

The skeleton of terrestrial vertebrates functions as a major reservoir for calcium and phosphorus. Access to this reservoir is accomplished by regulating the rate of bone

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resorption by osteoclasts (1). Low dietary calcium causes a decrease in blood calcium which triggers the release of parathyroid hormone (PTH) from the parathyroid gland (2). PTH restores calcium homeostasis by decreasing calcium excretion from the kidney, by increasing synthesis of 1,25-dihydroxyvitamin D3 in the kidney, which promotes intestinal absorption of calcium, and by stimulating bone resorption to release calcium from the skeleton.

One mechanism by which PTH may increase bone resorption is stimulation of the expression of the osteoclastogenic cytokine receptor activator of nuclear factor kappa-B ligand (RANKL) (3–5). RANKL is essential for osteoclast differentiation, activity, and survival (6–10), and mice and humans deficient in RANKL are osteopetrotic due to a complete lack of osteoclasts (6;11). RANKL exerts its effects on osteoclastogenesis by binding to its receptor RANK on the surface of osteoclast progenitors. Osteoprotegerin (OPG), a soluble decoy receptor, competes with RANK for binding to RANKL and thereby inhibits osteoclastogenesis (12;13). Both PTH and 1,25-dihydroxyvitamin D3 stimulate expression of RANKL in vitro and in vivo (4;14–16). This effect is mediated in part by a distant transcriptional enhancer known as the distal control region (DCR) (4;17). In addition, both hormones suppress expression of OPG (5;18), although the molecular mechanisms are unclear.

Various cell types, including bone marrow stromal cells, osteocytes, chondrocytes, and lymphocytes express RANKL (7;19–21). Recently, we and others have demonstrated that osteocytes are the major source of the RANKL required for osteoclastogenesis in remodeling cancellous bone under physiological conditions in adult mice (14;22). However, whether RANKL produced by osteocytes also contributes to the increased bone resorption caused by high PTH levels is unclear. Nonetheless, abundant evidence suggests that osteocytes are indeed direct targets of PTH action. Specifically, PTH rapidly suppresses expression of sclerostin, which in bone is expressed specifically in osteocytes (23;24). Moreover, transgenic expression of a constitutively active form of the PTH receptor specifically in osteocytes is sufficient to increase RANKL levels, osteoclast number, and bone resorption in mice (25). Conversely, mice lacking the PTH receptor in osteocytes have reduced RANKL expression and an altered calcemic response to a low calcium diet (26;27). Thus, PTH may promote osteoclastogenesis by stimulating RANKL expression in osteocytes. The goal of the studies described here was to determine whether RANKL produced by osteocytes plays a role in PTH-induced bone resorption. To do this, mice lacking the RANKL gene in osteocytes were placed on a calcium deficient diet to induce secondary hyperparathyroidism. These studies show that RANKL produced by osteocytes contributes to the increase in bone resorption and the bone loss caused by secondary hyperparathyroidism and thereby demonstrate that osteocytes are important cellular targets for the hormonal control of bone resorption.

MATERIALS AND METHODS

Animal studies

Generation of mice harboring a conditional RANKL allele (RANKL^{f/f}) and mice harboring a transgene consisting of 9.6 kb of the Dmp1 gene 5'-flanking region inserted upstream from

the Cre coding sequence (Dmp1-Cre transgenic mice) has been described previously (14;28). In the present study, experimental animals were obtained by crossing RANKL^{f/f} mice with RANKL^{f/f} mice that were also hemizygous for the Dmp1-Cre transgene, as previously described (14). At the time of the initial cross, RANKL^{f/f} mice, which were derived from embryonic stem cells from 129/Sv mice, had been crossed into the C57BL/6J background for 2 generations. The Dmp1-Cre mice used in the initial cross had been crossed into the C57BL/6J background for more than 10 generations. Thus, the mice used in the experiments described herein have a mixed genetic background derived from 129/Sv and C57BL/6. The offspring were genotyped by PCR using the following primer sequences: Cre-for, 5'-GCGGTCTGGCAGTAAAACTATC-3', Cre-rev, 5'-GTGAAACAGCATTGCTGTCACTT-3', product size 102 bp; RANKL-flox-for, 5'-CTGGGAGCGCAGGTAAATA-3', RANKL-flox-rev, 5'-GCCAATAATTAAATACTGCAGGAAA-3', product size 108 bp (WT) and 251 bp (floxed allele). In order to induce secondary hyperparathyroidism, 5-month-old male or 7-month-old female mice were fed a calcium deficient (0.01% calcium) for 30 days, while control mice were fed a diet containing normal calcium (0.516% calcium). Both diets were obtained from MP Biomedicals (Solon, OH) and contained 2340 IU of vitamin D2 per kg of diet. All animal studies were approved by the Institutional Animal Care and Use Committees of the University of Arkansas for Medical Sciences and the Central Arkansas Veterans Healthcare System.

Bone mineral density (BMD) determination

BMD was determined by dual energy x-ray absorptiometry (DEXA) using a PIXImus densitometer (GELunar Corp, Madison, WI.) and software version 2.0. BMD was measured at three sites. The total body window was defined as the whole body image minus the calvarium, mandible, and teeth. Except for the first few caudal vertebrae, the tail was not included. The spine window was a rectangle depending on animal body length, reaching from just below the skull to the base of the tail. The femoral window captured the right femur. Scan acquisition time was four minutes. The mice were sedated with isoflurane inhalation during scanning to keep the animals motionless for the required four minutes and to facilitate rapid post examination recovery. The animals were monitored by observation of the righting reflex, respiration, and heart rate. Using a proprietary skeletal phantom, the total body BMD was measured over the past 4 years and a mean coefficient of variation of 3.1% was obtained (n = 285).

μCT analysis

The femurs and vertebrae (L4) were dissected, cleaned of soft tissues, fixed in 10% Millonig's formalin, and gradually dehydrated into 100% ethanol. Bones were loaded into 12.3 mm diameter scanning tubes and imaged in a μCT (model μCT40, Scanco Medical, Wayne, PA). The scans were integrated into 3-D voxel images (1024 × 1024 pixel matrices for each individual planar stack) and a Gaussian filter (sigma = 0.8, support = 1) was used to reduce signal noise. A threshold of 200 was applied to all scans at medium resolution (E = 55 kVp, I = 145 μA, integration time = 200ms). Whole vertebrae were scanned with a transverse orientation excluding any bone outside the vertebral body. The cortical bone and the primary spongiosa were manually excluded from the analysis. All trabecular

measurements were made by drawing contours every 10 to 20 slices and voxel counting was used for bone volume per tissue volume and sphere filling distance transformation indices, without pre-assumptions about the bone shape as a rod or plate for trabecular microarchitecture. Cortical thickness, bone volume, and porosity were measured at the femoral mid-diaphysis. Calibration and quality control were performed weekly using five density standards and spatial resolution was verified monthly using a tungsten wire rod. We based beamhardening correction on the calibration records. We made corrections for 200 mg hydroxyapatite for all energies. Over the past 3 years, the coefficient of variation for the fifth density standard (mean five) was $1.28 (781 \pm 10 \text{ SD mg HA/cm}^3)$ and for rod volume was $3.16 (0.0633 \pm 0.002 \text{ SD cm}^3)$.

RNA purification and gene expression analysis

Calvariae and the 5th lumbar vertebrae were dissected from animals, cleaned of muscle, frozen immediately in liquid nitrogen, and stored at -80°C . Soft tissues were dissected from animals, frozen immediately in liquid nitrogen, and stored at -80°C . The distal and proximal ends of the tibiae were excised and bone marrow cells were removed by centrifugation at 13,000 g for 2 minutes. Total RNA was purified from tissues using Ultraspec reagent (Biotecx Laboratories, Houston, TX), according to the manufacturer's instructions. Three μg of total RNA was reverse-transcribed into cDNA as using the High Capacity Reverse Transcriptase kit from Applied Biosystems (Carlsbad, CA). Taqman quantitative RTPCR was performed using the following Taqman assays from Applied Biosystems: RANKL (Mm0041908_m1); OPG (Mm00435452_m1); Cathepsin K (Mm00484036_m1); tartrate resistant acidphosphatase (TRAP) (Mm00475698_m1); M-CSF (Mm00432688_m1); IL-6 (Mm00446190_m1); and ribosomal protein S2 (for, 5'-CCCAGGATGGCGACGAT-3', rev, 5'-CCGAATGCTGTAATGGCGTAT-3', probe, 5'-FAM-TCCAGAGCAGGATCC-NFQ-3'). Relative mRNA levels were calculated by normalizing to the house-keeping gene ribosomal protein S2 using the Ct method (29).

Genomic DNA isolation from enriched osteocytes

The distal and proximal ends of the femur were removed and bone marrow cells were flushed out completely with PBS. The surfaces of the bone shafts were scraped with a scalpel to remove the periosteum and then cut into a few small pieces. Bone pieces were then digested with 1 ml of Hank's solution containing 1 mM CaCl_2 and 1 mg/ml of collagenase (type I:II, ratio 1:3) (Worthington Biochemical Corporation, Freehold, NJ) in a 12-well-plate. A total of 6 digestions for 15 minutes each were performed at 37°C in a water bath shaker to remove the cells on the bone surface. After the final digestion, bone pieces were decalcified in 14% EDTA for 1 week. Decalcified bone was then digested with proteinase K (0.5 mg/ml in 10 mM Tris, pH 8.0, 100 mM NaCl, 20 mM EDTA, and 1% SDS) at 55°C overnight. Genomic DNA was then isolated by phenol/chloroform extraction and ethanol precipitation. Two custom Taqman assays were obtained from Applied Biosystems for quantifying RANKL gene deletion efficiency: one specific for sequences between the loxP sites (for, 5'-GCCAGTGGACTTACTCAAACCTT-3'; rev, 5'-GGTAGGGTTCAACTGAAGGGTTTA-3'; probe, 5'-FAM-CCTCCTCCTCATGGTTTAGT-NFQ-3') and the other specific for sequences downstream from the 3' loxP site (for, 5'-GGTGCCGTGCATTATCCTAGAC-3'; rev, 5'-

AAGTAATGTGACCCTTGGAGAACTG-3'; probe, 5'-FAM-CTAGCACACGTGCCTGCT-NFQ-3').

Histology

Bones (femurs and L2 vertebrae) were fixed for 24 h in 10% (vol/vol) Millonig's formalin, decalcified in 14% (wt/vol) EDTA for 1 week, embedded in paraffin, and then 5 μ m longitudinal sections were cut. After removal of paraffin and rehydration, the sections were stained for TRAP activity and counterstained with methyl green. Quantitative histomorphometry to determine osteoclast number was performed on the TRAP-stained sections using a computer and digitizer tablet (OsteoMetrics, Decatur, GA) interfaced to a Zeiss Axioscope (Carl Zeiss, Thornwood, NY) attached with a drawing tube. In L2 vertebrae, all cancellous bone was analyzed with the exception of the region within two visual fields of the growth plates and any trabeculae that were in direct contact with cortical bone. In femurs, cancellous bone analysis was performed beginning two visual fields below the growth plate and extended the entire remaining area of cancellous bone in the samples from RANKL^{f/f} mice. Since the cancellous bone of the conditional knockout mice was much more extensive, a region similar in area to the RANKL^{f/f} controls was measured and averaged approximately 3 mm². Histological measurements of the endocortical bone of the femur also began two visual fields below the growth plate and were continued to the diaphysis, with the diaphysis defined as the midway point between the ends of the femur.

Immunoblot

Protein extraction from cortical bone shafts was performed as previously described (30). Briefly, protein was extracted from tibia shafts by freezing them in liquid nitrogen followed by pulverization in liquid nitrogen. Tibia shafts from two mice of the same genotype and same diet were pooled. The pulverized bone powder was then incubated in SDS-PAGE loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 6% glycerol, 1% β -mercaptoethanol, and 0.004% bromophenol blue) for 10 min at 100°C. Proteins were resolved in SDS-polyacrylamide gels and electroblotted onto polyvinylidenedifluoride membranes. Membranes were subsequently blocked with 5% nonfat dry milk in TBS with 0.1% Tween-20 and then incubated with primary antibodies and secondary antibodies. The following antibodies were used: anti-RANKL antibody EPR4999 (Abcam, Cambridge, MA) and anti-actin antibody SC81178 (Santa Cruz Biotechnology, Dallas, TX). Blots were developed using enhanced chemiluminescence and the intensity of the bands was quantified using a ChemDoc XRS-plus system (Bio-Rad, Hercules, CA).

Biomechanical Testing

Three-point bending of the femur was performed at 37 ± 0.5 °C using a miniature bending apparatus with the posterior femoral surface lying on lower supports (7 mm apart) and the left support immediately proximal to the distal condyles. Load was applied to the anterior femoral surface by an actuator midway between the two supports moving at a constant rate of 3 mm/min to produce a physiological in vivo strain rate of 1% for the average murine femur. The external measurements (length, width and thickness) of the femora were recorded with a digital caliper. Measurements of the internal marrow cavity (greater and lesser diameters) were obtained with a hand-held microscope at $\times 100$ magnification using a

calibrated linear reticule eyepiece (Klarmann Rulings, Manchester, NH). Maximum load (N) and displacement (mm) were recorded. The mechanical properties were normalized for bone size and ultimate strength or stress (N/mm²; in megapascals or MPa) was calculated. Standard precision steel piano wire with stiffness in the same range as murine femoral bone was evaluated before each set of determinations for quality control (31).

Blood chemistry

Blood was collected by tail bleeding into heparinized tubes and was centrifuged at 1500 g for 10 minutes to separate plasma from blood cells. Circulating PTH was measured using a mouse intact PTH ELISA kit from Immutopics (San Clemente, CA) according to the manual provided by the manufacturer. Soluble RANKL in blood plasma was measured using a Quantikine mouse RANKL kit from R&D Systems (Minneapolis, MN) according to the manual provided by the manufacturer. Carboxy-terminal cross-linked telopeptide of type I collagen (CTX) was measured using the RatLaps EIA CTX kit from Immunodiagnostic Systems Inc (Scottsdale, AZ). Plasma calcium was measured using the StanbioTotal Calcium LiquiColor kit from Stanbio Laboratory (Boerne, TX) and phosphorous was measured using a QuantiChrom phosphorous assay kit from BioAssay Systems (Hayward, CA), both according to the manufacturer's instructions.

Statistics

Two-way ANOVA was used to detect statistically significant treatment effects, after determining that the data were normally distributed and exhibited equivalent variances. In some cases, log (Fig. 5D and supplemental Fig. 5D–E) or reciprocal (Fig. 5A and supplemental Fig. 5C) transformations were used to obtain normally-distributed data and equal variance. A Tukey correction was used for multiple comparisons. In cases where normalization was not possible (Fig. 1A and Fig. 2A), non-parametric two-way ANOVA on the ranks was used. For experiments involving comparison of only two groups, Student's t-test was used. P-values less than 0.05 were considered as significant. Results in all graphs represent the mean $\pm$ s.d..

RESULTS

Deletion of RANKL from osteocytes attenuates bone loss caused by dietary calcium deficiency

To determine whether RANKL expression in osteocytes is required for the bone loss caused by secondary hyperparathyroidism, we generated a cohort of animals in which the RANKL gene was deleted from osteocytes. This was accomplished by crossing RANKL^{f/f} mice, which contain a conditional RANKL allele, with Dmp1-Cre transgenic mice (28), which express the Cre recombinase predominantly in osteocytes. We have used such mice previously to show that osteocyte-derived RANKL is essential for osteoclastogenesis during bone remodeling and is required for cortical bone loss induced by mechanical unloading (14). Quantitative analysis of the region of DNA flanked by loxP sites in Dmp1-Cre;RANKL^{f/f} mice, hereafter referred to as RANKL^{Ot} mice, confirmed that the Dmp1-Cre transgene caused deletion of this region in osteocyte-enriched cortical bone but not in spleen (supplemental Fig. 1). Five-month-old male RANKL^{Ot} mice and their control

littermates, RANKf/f mice, were fed a calcium-deficient diet or a control diet for 30 days. Dietary calcium deficiency caused a significant loss of spinal and femoral BMD in both genotypes, but whole body BMD was significantly decreased only in control littermates (Fig. 1A). Moreover, even though the differences were not statistically significant, the degree of bone loss appeared lower in the conditional knockout mice in the spine and femur. Since animal numbers were small in this experiment with male mice, we performed a second experiment in female mice that were slightly older (7 months old) using a larger number of animals per group. In this second experiment, the bone loss caused by dietary calcium deficiency was blunted in RANKL^{Ot} at all skeletal sites measured (Fig. 1B). Similar results were obtained when comparing beginning and final group means rather than percent change with the exception that this approach could not detect bone loss in the femur of either genotype (supplemental Fig. 2). The different response of RANKL^{Ot} mice was not due to a difference in body weight (supplemental Fig. 1).

Microcomputed tomography (μ CT) analysis revealed that dietary calcium deficiency reduced cortical thickness in the femur of RANKLf/f mice (Fig. 2A, Fig. 2D, and supplemental Fig. 3). However, this cortical bone loss did not occur in RANKL^{Ot} mice (Fig. 2A, Fig. 2D, and supplemental Fig. 3). Similar differences were reflected in the changes in cortical bone volume (supplemental Fig. 3). In addition, dietary calcium deficiency increased cortical porosity in the femur of RANKLf/f mice but not RANKL^{Ot} mice (Fig. 2B and Fig. 2E). Consistent with these bone mass changes, bone strength in the femurs of RANKLf/f mice on the calcium-deficient diet was lower than RANKL^{Ot} mice on the same diet (Fig. 2F). Taken together, these results demonstrate that loss of RANKL in osteocytes attenuates the loss of cortical bone caused by dietary calcium deficiency.

In agreement with our earlier work (14), deletion of the RANKL gene from osteocytes caused a large increase in cancellous bone volume in both the spine and the femur in the animals of the current study (supplemental Fig. 4). However, dietary calcium deficiency did not cause a significant change of vertebral or femoral cancellous bone volume or architecture in either RANKLf/f or RANKL^{Ot} mice (supplemental Fig. 4). Matrix mineralization of vertebral cancellous bone was slightly decreased by dietary calcium deficiency in female RANKLf/f mice, consistent with increased bone remodeling, but this did not occur in RANKL^{Ot} mice (supplemental Fig. 4). We have shown previously that 30 days of dietary calcium-deficiency causes loss of cancellous bone volume in adult C57BL/6 mice (32). Therefore, the failure to observe significant loss of cancellous bone volume in the present study is most likely due to the mixed genetic background (C57BL/6 and 129/Sv) of the mice used here, which resulted in higher intra-group variance than in pure C57BL/6 mice.

Osteocyte RANKL contributes to endocortical bone resorption caused by secondary hyperparathyroidism

Secondary hyperparathyroidism caused by dietary calcium deficiency stimulates RANKL gene expression in bone (4;32). Therefore, we next measured RANKL mRNA levels in different skeletal sites to determine what portion, if any, of the change in RANKL expression can be accounted for by increased production in osteocytes. Dietary calcium

deficiency stimulated RANKL mRNA abundance in tibial cortical bone, intact vertebra, and calvaria of RANKL^{f/f} mice (Fig. 3A). However, this stimulation did not occur in RANKL^{O_t} mice (Fig. 3A). RANKL mRNA levels in the spleen, bone marrow, or kidney of either genotype did not change with dietary calcium deficiency (Fig. 3A). Consistent with the mRNA results, RANKL protein levels, as measured by immunoblot of tibial cortical bone extract, were elevated by dietary calcium deficiency in the control mice but not in the conditional knockout mice (Fig. 3B). In contrast, soluble RANKL levels in the circulation were unchanged by dietary calcium deficiency in mice of either genotype, as were OPG and M-CSF mRNA levels in bone (Supplemental Figs. 5 and 6). Earlier studies had suggested that IL-6 may mediate some of the osteoclastogenic actions of PTH (33). However, IL-6 mRNA was similarly suppressed by dietary calcium deficiency in the cortical bone of both genotypes and unchanged in whole L5 vertebra (supplemental Fig. 6).

We next measured osteoclast number and the percentage of bone surface covered by osteoclasts in the cancellous bone and endocortical surface of the femur. Deletion of RANKL from osteocytes reduced osteoclast number and perimeter in cancellous bone by approximately 70% (Fig. 4A and C), similar to what has been reported previously (14;22). In contrast, osteoclast number and perimeter were reduced by only 28% at the endocortical surface when comparing RANKL^{O_t} and RANKL^{f/f} mice on the control diet (Fig. 4B and D). Thus, deletion of RANKL from osteocytes had a greater impact on osteoclast formation in cancellous bone compared to endocortical bone. Despite this difference, dietary calcium deficiency increased osteoclast number and perimeter in both cancellous and cortical bone of RANKL^{f/f} mice but these increases were either abolished (cancellous bone) or significantly blunted (endocortical bone) in mice lacking RANKL in osteocytes (Fig. 4A–D). Similar results were obtained in the cancellous bone of the spine (supplemental Fig. 7). Consistent with the histological results, the increase in osteoclast-specific gene expression caused by dietary calcium deficiency was also blunted in RANKL^{O_t} mice compared with RANKL^{f/f} littermates (supplemental figure 6). Likewise, levels of the resorption marker CTX were elevated by dietary calcium deficiency in control but not conditional knockout mice (Fig. 5A). Taken together, these results demonstrate that production of RANKL by osteocytes increases with dietary calcium deficiency and that this contributes to the increased bone resorption.

Since the bone loss caused by dietary calcium deficiency was blunted in mice lacking RANKL in osteocytes, it is possible that less calcium was released from the skeleton and that calcium homeostasis was not maintained. However, despite losing less bone mass, blood calcium and phosphorous levels were normal in RANKL^{O_t} mice fed the calcium deficient diet (Fig. 5B–C). Circulating PTH levels were significantly increased by dietary calcium deficiency in RANKL^{O_t} mice but not control littermates, suggesting the possibility of a greater degree of hyperparathyroidism in RANKL^{O_t} mice (Fig. 5D). However, Cyp27b1 mRNA in the kidney and Sost mRNA in bone were increased by dietary calcium deficiency to similar extents in RANKL^{f/f} and RANKL^{O_t} mice, which is consistent with a comparable increase in PTH in both genotypes (Fig. 5E).

DISCUSSION

RANKL plays important roles in bone growth and maintenance, lymphocyte differentiation, lymphnode and mammary gland development, and thermoregulation (34). To accomplish these diverse functions, RANKL is expressed in a variety of cell types and many of these, including bone marrow stromal cells, lymphocytes, hypertrophic chondrocytes, and osteocytes, support osteoclast formation in vitro (35–38). However, despite such broad expression, only RANKL production by osteocytes is essential for osteoclast formation in remodeling cancellous bone in mice (14;22;39). In the present study, we demonstrate that RANKL produced by osteocytes is also required for the increase in cortical bone resorption and subsequent bone loss caused by secondary hyperparathyroidism.

The results presented here reveal that high PTH levels induce bone resorption in part by stimulating osteocytes to produce more RANKL, thereby increasing osteoclast number and function. Continuous infusion of PTH in mice has been shown to increase production of TNF α by T lymphocytes, which in turn promoted RANKL expression in bone marrow stromal cells, which was associated with cortical bone loss (40). In a follow-up study, deletion of the PTH receptor from T lymphocytes blunted the bone loss caused by continuous PTH infusion (41). Since T lymphocytes play a role in the bone loss caused by primary hyperparathyroidism, it is possible that they could also contribute to bone loss caused by secondary hyperparathyroidism. Moreover, since T lymphocytes express RANKL (6), RANKL produced by these cells may also contribute to PTH-induced bone loss. However, the results of the present study demonstrate that RANKL produced by any cells other than osteocytes is not sufficient to cause cortical bone loss in response to elevated PTH in the absence of osteocyte RANKL.

The blunting of the bone loss in RANKL^{Ot} mice could be caused by the very low rate of bone remodeling resulting from this genetic change (14;22). Specifically, other factors controlled by PTH that can change osteoclast number, such as OPG, IL-6, and M-CSF, would be less effective if osteoclast number was already very low due to low levels of RANKL. Consistent with this idea, we observed an increase in osteoclast number in the cortical bone of RANKL^{Ot} mice on the calcium-deficient diet, perhaps due to changes in these other PTH target genes. Nonetheless, this increase was not sufficient to cause measurable bone loss at this site.

The blunting of bone loss in mice lacking RANKL in osteocytes did not result in hypocalcemia, perhaps because of increased reabsorption of calcium by the kidney. We measured calcium in urine that was collected at a single time-point, corrected for creatinine concentration, in all four groups but we were unable to detect any differences (data not shown). Nonetheless, a greater efficiency of calcium reabsorption, undetectable by our measurements, could account for the maintenance of serum calcium levels in the conditional knockout mice fed the calcium deficient diet. In addition, it is possible that an increase in 1,25-dihydroxyvitamin D3 contributed to calcium homeostasis by inhibiting bone mineralization via direct actions on osteocytes (42). However, material density of the bone matrix, as measured by μ CT, was not altered by the calcium deficient diet in RANKL^{Ot} mice. Thus reduced mineralization of bone does not appear to have contributed to the

maintenance of serum calcium. Another possibility is that the amount of bone resorption induced by secondary hyperparathyroidism is in excess of what is needed to maintain serum calcium levels. If this were the case, then the small amount of bone resorption that still occurred in RANKL^{Ot} mice on the low calcium diet, together with effects on the kidney, may have contributed to maintenance of calcium homeostasis. In contrast to the deletion of RANKL, deletion of the PTH receptor from osteocytes did result in mild hypocalcemia during dietary calcium deficiency (27), consistent with the idea that PTH regulation of genes other than RANKL contributes to the control of calcium mobilization.

Deletion of the RANKL gene from osteocytes reduced osteoclast number in cancellous bone to a much greater extent than at the endocortical surface (70% versus 28%, respectively). This coincided with a relatively greater increase in cancellous bone volume than cortical thickness in the conditional knockout mice compared with control littermates, under basal conditions. Yet, despite the difference in impact on basal osteoclast number, deletion of RANKL from osteocytes blunted the PTH-induced increase in osteoclast number in both compartments and was able to block the loss of cortical bone. These results suggest that the role of osteocyte-derived RANKL in endocortical bone resorption becomes relatively more important when PTH levels are increased, compared to the conditions that exist during normal growth and remodeling.

We did not observe a significant reduction in RANKL mRNA in bones from the conditional knockout mice. In our previous study (14), we concluded that osteocytes are the major source of the RANKL involved in osteoclast formation in cancellous bone. This is not the same as concluding that osteocytes are the major source of RANKL in bone. The picture that is emerging is that several cell types produce RANKL, each for different purposes. For example, hypertrophic chondrocytes produce abundant RANKL for resorption of calcified cartilage during bone growth (43). In addition, B lymphocytes produce RANKL for B cell differentiation, but this does not contribute to bone remodeling, at least under physiological conditions (39). It is quite possible that the amount of RANKL produced by chondrocytes and lymphocytes exceeds that produced by osteocytes so that deletion of the gene in osteocytes is difficult to detect when measuring RANKL mRNA in whole bones. In contrast, the reduction in RANKL is easily detectable in cortical bone that has been enriched in osteocytes by removing the surface cells with collagenase or by scraping (14). We did not remove the surface cells from the tibia shafts used in the current study since we wanted to also measure osteoclast-specific gene expression in these same samples. Despite the inability to detect a reduction in RANKL mRNA in whole bones when deleting the gene with Dmp1-Cre, it is clear that RANKL produced by these cells is essential for osteoclast formation in cancellous bone, as shown here and in previous studies (14;22).

The Dmp1-Cre transgene used in our study is active in mature osteoblasts as well as osteocytes (14;44). Therefore, it is likely that the Dmp1-Cre transgene also deleted the RANKL gene from mature osteoblasts in RANKL^{Ot} mice. We have shown previously that administration of exogenous PTH to mice lacking mature osteoblasts stimulates RANKL expression in whole tibia and cortical bone to the same extent as mice with osteoblasts (14). In contrast, the PTH-induced increase in RANKL expression was severely blunted in mice lacking RANKL in Dmp1-Cre expressing cells, indicating that osteocytes but not mature

osteoblasts are the major source of RANKL induced by PTH. Based on these earlier findings, we conclude that loss of the RANKL gene in osteocytes best accounts for the results of the present study.

We have shown previously that deletion of the DCR transcriptional enhancer of the RANKL gene blunted the increase in RANKL mRNA and bone loss caused by secondary hyperparathyroidism in growing mice (32). In contrast, loss of the DCR had no impact on bone loss caused by dietary calcium deficiency in adult mice, even though the increase in RANKL production in bone was blunted. At first glance, the latter finding does not appear to agree with the results of the current study showing that stimulation of osteocyte RANKL production by dietary calcium deficiency contributes to bone loss. However, it is important to point out that the changes in RANKL expression and osteoclast number in DCR knockout mice are quite different than those in RANKL^{Ot} mice. Specifically, global deletion of the DCR modestly reduces RANKL expression in a variety of cell types and reduces cancellous osteoclast number by approximately 25%, whereas deletion of RANKL specifically from osteocytes and osteoblasts reduces cancellous osteoclast number by approximately 70%. Thus, the overall impact on bone resorption is much greater in RANKL^{Ot} mice compared with DCR-null mice. Therefore, the ability of PTH to increase osteoclast formation by acting on factors other than RANKL, such as OPG, IL-6, or MCSF, would be expected to be greater in DCR-null mice compared with RANKL^{Ot} mice. In other words, changes in factors such as OPG cannot increase osteoclast formation in situations in which RANKL is very low or absent but can do so if sufficient amounts of RANKL are present.

As with most forms of hyperparathyroidism, dietary calcium-deficiency elevates circulating levels of 1,25-dihydroxyvitamin D3 as well as PTH (45). Since the RANKL gene responds to both of these hormonal changes (46), the question then arises as to the relative contribution of each in driving the bone resorption caused by dietary calcium deficiency. Consistent with an important role for PTH, deletion of the PTH receptor from osteocytes prevents the increased bone resorption caused by continuous PTH infusion (26). In contrast, elevated 1,25-dihydroxyvitamin D3 levels can induce bone resorption in the absence of VDR in osteocytes, suggesting that changes in osteocyte RANKL are not involved in the stimulation of bone resorption by 1,25-dihydroxyvitamin D3 (42). These findings, taken together with the results of the current study, indicate that secondary hyperparathyroidism induces osteoclast formation primarily via PTH action on osteocytes.

In conclusion, the results presented here add to a growing body of evidence that osteocytes are an important target cell for the hormonal control of bone remodeling (47). It remains unclear, however, whether all osteocytes respond similarly to hormonal changes or whether additional signals, such as those generated by mechanical loading, modulate hormone-induced changes (48). Consistent with the latter possibility, osteocyte apoptosis induced by estrogen-deficiency occurs in specific regions of cortical bone (49). Future studies aiming to identify the population of osteocytes producing RANKL in response to various hormonal changes, as well as studies examining the integration of hormonal and mechanical signals in osteocytes, will be required to address these questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Parfitt, AM. Modeling and Remodeling: How Bone Cells Work Together. In: Feldman, D.; Pike, JW.; Glorieux, FH., editors. Vitamin D. 2nd Edition ed.. San Diego: Academic Press Inc.; 2005. p. 497-513.
2. Brown, EM.; Juppner, H. Parathyroid Hormone: Synthesis, Secretion, and Action. In: Favus, MJ., editor. Primer on the metabolic bone diseases and disorders of mineral metabolism. Sixth Edition ed.. Washington, D.C.: American Society for Bone and Mineral Research; 2006. p. 90-99.
3. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio, Udagawa N, Takahashi N, Suda T. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci U S A. 1998; 95:3597-3602. [PubMed: 9520411]
4. Fu Q, Manolagas SC, O'Brien CA. Parathyroid Hormone Controls Receptor Activator of NF- κ B Ligand Gene Expression via a Distant Transcriptional Enhancer. Mol Cell Biol. 2006; 26:6453-6468. [PubMed: 16914731]
5. Fu Q, Jilka RL, Manolagas SC, O'Brien CA. Parathyroid hormone stimulates receptor activator of NF κ B ligand and inhibits osteoprotegerin expression via protein kinase A activation of cAMP-response element-binding protein. J Biol Chem. 2002; 277:48868-48875. [PubMed: 12364326]
6. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira dSA, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM. OPG is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. Nature. 1999; 397:315-323. [PubMed: 9950424]
7. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi, Guo J, Delaney J, Boyle WJ. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell. 1998; 93:165-176. [PubMed: 9568710]
8. Glantschnig H, Fisher JE, Wesolowski G, Rodan GA, Reszka AA. M-CSF, TNF α and RANK ligand promote osteoclast survival by signaling through mTOR/S6 kinase. Cell Death Differ. 2003; 10:1165-1177. [PubMed: 14502240]
9. Lacey DL, Tan HL, Lu J, Kaufman S, Van G, Qiu W, Rattan A, Scully S, Fletcher F, Juan T, Kelley M, Burgess TL, Boyle WJ, Polverino AJ. Osteoprotegerin ligand modulates murine osteoclast survival in vitro and in vivo. Am J Pathol. 2000; 157:435-448. [PubMed: 10934148]
10. Wu X, Pan G, McKenna MA, Zayzafoon M, Xiong WC, McDonald JM. RANKL regulates Fas expression and Fas-mediated apoptosis in osteoclasts. J Bone Miner Res. 2005; 20:107-116. [PubMed: 15619676]
11. Sobacchi C, Frattini A, Guerrini MM, Abinun M, Pangrazio A, Susani L, Bredius R, Mancini G, Cant A, Bishop N, Grabowski P, Del FA, Messina C, Errigo G, Coxon FP, Scott DI, Teti A, Rogers MJ, Vezzoni P, Villa A, Helfrich MH. Osteoclast-poor human osteopetrosis due to mutations in the gene encoding RANKL. Nat Genet. 2007; 39:960-962. [PubMed: 17632511]

12. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ, Simonet WS. osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes Dev.* 1998; 12:1260–1268. [PubMed: 9573043]
13. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R, Boyle WJ. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell.* 1997; 89:309–319. [see comments]. [PubMed: 9108485]
14. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. *Nat Med.* 2011; 17:1235–1241. [PubMed: 21909103]
15. Kim S, Yamazaki M, Zella LA, Meyer MB, Fretz JA, Shevde NK, Pike JW. Multiple enhancer regions located at significant distances upstream of the transcriptional start site mediate RANKL gene expression in response to 1,25-dihydroxyvitamin D3. *J Steroid Biochem Mol Biol.* 2007; 103:430–434. [PubMed: 17197168]
16. Kim S, Yamazaki M, Zella LA, Shevde NK, Pike JW. Activation of Receptor Activator of NF- κ B Ligand Gene Expression by 1,25-Dihydroxyvitamin D3 Is Mediated through Multiple Long-Range Enhancers. *Mol Cell Biol.* 2006; 26:6469–6486. [PubMed: 16914732]
17. Galli C, Zella LA, Fretz JA, Fu Q, Pike JW, Weinstein RS, Manolagas SC, O'Brien CA. Targeted deletion of a distant transcriptional enhancer of the receptor activator of nuclear factor- κ B ligand gene reduces bone remodeling and increases bone mass. *Endocrinology.* 2008; 149:146–153. [PubMed: 17932217]
18. Kondo T, Kitazawa R, Maeda S, Kitazawa S. 1 α ,25 dihydroxyvitamin D3 rapidly regulates the mouse osteoprotegerin gene through dual pathways. *J Bone Miner Res.* 2004; 19:1411–1419. [PubMed: 15312241]
19. Kartsogiannis V, Zhou H, Horwood NJ, Thomas RJ, Hards DK, Quinn JMW, Niforas P, Ng KW, Martin TJ, Gillespie MT. Localization of RANKL (Receptor activator of NF κ B ligand) mRNA and protein in skeletal and extraskelatal tissues. *Bone.* 1999; 25:525–534. [PubMed: 10574572]
20. Silvestrini G, Ballanti P, Patacchioli F, Leopizzi M, Gualtieri N, Monnazzi P, Tremante E, Sardella D, Bonucci E. Detection of osteoprotegerin (OPG) and its ligand (RANKL) mRNA and protein in femur and tibia of the rat. *J Mol Histol.* 2005; 36:59–67. [PubMed: 15704000]
21. Ikeda T, Utsuyama M, Hirokawa K. Expression profiles of receptor activator of nuclear factor κ B ligand, receptor activator of nuclear factor κ B, osteoprotegerin messenger RNA in aged and ovariectomized rat bones. *J Bone Miner Res.* 2001; 16:1416–1425. [PubMed: 11499864]
22. Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, Bonewald LF, Kodama T, Wutz A, Wagner EF, Penninger JM, Takayanagi H. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med.* 2011; 17:1231–1234. [PubMed: 21909105]
23. Keller H, Kneissel M. SOST is a target gene for PTH in bone. *Bone.* 2005; 37:148–158. [PubMed: 15946907]
24. Bellido T, Ali AA, Gubrij I, Plotkin LI, Fu Q, O'Brien CA, Manolagas SC, Jilka RL. Chronic elevation of parathyroid hormone in mice reduces expression of sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis. *Endocrinology.* 2005; 146:4577–4583. [PubMed: 16081646]
25. O'Brien CA, Plotkin LI, Galli C, Goellner JJ, Gortazar AR, Allen MR, Robling AG, Bouxsein M, Schipani E, Turner CH, Jilka RL, Weinstein RS, Manolagas SC, Bellido T. Control of bone mass and remodeling by PTH receptor signaling in osteocytes. *PLoS ONE.* 2008; 3:e2942. [PubMed: 18698360]
26. Saini V, Marengi DA, Barry KJ, Fulzele KS, Heiden E, Liu X, Dedic C, Maeda A, Lotinun S, Baron R, Pajevic PD. 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; 288:20122–20134. [PubMed: 23729679]
27. Powell WF Jr, Barry KJ, Tulum I, Kobayashi T, Harris SE, Bringhurst FR, Pajevic PD. Targeted ablation of the PTH/PTHrP receptor in osteocytes impairs bone structure and homeostatic calcemic responses. *J Endocrinol.* 2011; 209:21–32. [PubMed: 21220409]

28. Lu Y, Xie Y, Zhang S, Dusevich V, Bonewald LF, Feng JQ. DMP1-targeted Cre expression in odontoblasts and osteocytes. *J Dent Res*. 2007; 86:320–325. [PubMed: 17384025]
29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001; 25:402–408. [PubMed: 11846609]
30. Onal M, Piemontese M, Xiong J, Wang Y, Han L, Ye S, Komatsu M, Selig M, Weinstein RS, Zhao H, Jilka RL, Almeida M, Manolagas SC, O'Brien CA. Suppression of autophagy in osteocytes mimics skeletal aging. *J Biol Chem*. 2013; 288:17432–17440. [PubMed: 23645674]
31. O'Brien CA, Jia D, Plotkin LI, Bellido T, Powers CC, Stewart SA, Manolagas SC, Weinstein RS. Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. *Endocrinology*. 2004; 145:1835–1841. [PubMed: 14691012]
32. Onal M, Galli C, Fu Q, Xiong J, Weinstein RS, Manolagas SC, O'Brien CA. The RANKL distal control region is required for the increase in RANKL expression, but not the bone loss, associated with hyperparathyroidism or lactation in adult mice. *Mol Endocrinol*. 2012; 26:341–348. [PubMed: 22207718]
33. Grey A, Mitnick MA, Masiukiewicz U, Sun BH, Rudikoff S, Jilka RL, Manolagas SC, Insogna K. A role for interleukin-6 in parathyroid hormone-induced bone resorption in vivo. *Endocrinology*. 1999; 140:4683–4690. [PubMed: 10499526]
34. Hanada R, Hanada T, Sigl V, Schramek D, Penninger JM. RANKL/RANK beyond bones. *J Mol Med (Berl)*. 2011; 89:647–656. [PubMed: 21445556]
35. O'Brien CA, Gubrij I, Lin SC, Saylors RL, Manolagas SC. STAT3 activation in stromal osteoblastic cells is required for induction of the receptor activator of NF-kappa B ligand and stimulation of osteoclastogenesis by gp130-utilizing cytokines or interleukin-1 but not 1,25-dihydroxyvitamin D-3 or parathyroid hormone. *J Biol Chem*. 1999; 274:19301–19308. [PubMed: 10383440]
36. Choi Y, Woo KM, Ko SH, Lee YJ, Park SJ, Kim HM, Kwon BS. Osteoclastogenesis is enhanced by activated B cells but suppressed by activated CD8(+) T cells. *Eur J Immunol*. 2001; 31:2179–2188. [PubMed: 11449372]
37. Horwood NJ, Kartsogiannis V, Quinn JMW, Romas E, Martin TJ, Gillespie MT. Activated T lymphocytes support osteoclast formation in vitro. *Biochem Biophys Res Commun*. 1999; 265:144–150. [PubMed: 10548505]
38. Zhao S, Kato Y, Zhang Y, Harris S, Ahuja SS, Bonewald LF. MLO-Y4 osteocyte-like cells support osteoclast formation and activation. *J Bone Miner Res*. 2002; 17:2068–2079. [PubMed: 12412815]
39. Onal M, Xiong J, Chen X, Thostenson JD, Almeida M, Manolagas SC, O'Brien CA. Receptor Activator of Nuclear Factor kappaB Ligand (RANKL) Protein Expression by B Lymphocytes Contributes to Ovariectomy-induced Bone Loss. *J Biol Chem*. 2012; 287:29851–29860. [PubMed: 22782898]
40. Gao Y, Wu X, Terauchi M, Li JY, Grassi F, Galley S, Yang X, Weitzmann MN, Pacifici R. T cells potentiate PTH-induced cortical bone loss through CD40L signaling. *Cell Metab*. 2008; 8:132–145. [PubMed: 18680714]
41. Tawfeek H, Bedi B, Li JY, Adams J, Kobayashi T, Weitzmann MN, Kronenberg HM, Pacifici R. Disruption of PTH receptor 1 in T cells protects against PTH-induced bone loss. *PLoS ONE*. 2010; 5:e12290. [PubMed: 20808842]
42. Lieben L, Masuyama R, Torrekens S, Van LR, Schrooten J, Baatsen P, Lafage-Proust MH, Dresselaers T, Feng JQ, Bonewald LF, Meyer MB, Pike JW, Bouillon R, Carmeliet G. Normocalcemia is maintained in mice under conditions of calcium malabsorption by vitamin D-induced inhibition of bone mineralization. *J Clin Invest*. 2012; 122:1803–1815. [PubMed: 22523068]
43. Usui M, Xing L, Drissi H, Zuscik M, O'Keefe R, Chen D, Boyce BF. Murine and chicken chondrocytes regulate osteoclastogenesis by producing RANKL in response to BMP2. *J Bone Miner Res*. 2008; 23:314–325. [PubMed: 17967138]
44. Kalajzic I, Matthews BG, Torreggiani E, Harris MA, Divieti PP, Harris SE. In vitro and in vivo approaches to study osteocyte biology. *Bone*. 2013; 54:296–306. [PubMed: 23072918]

45. Murayama A, Takeyama K, Kitanaka S, Kadera Y, Kawaguchi Y, Hosoya T, Kato S. Positive and negative regulations of the renal 25-hydroxyvitamin D3 1 α hydroxylase gene by parathyroid hormone, calcitonin, and 1 α ,25(OH) $_2$ D $_3$ in intact animals. *Endocrinology*. 1999; 140:2224–2231. [PubMed: 10218975]
46. O'Brien CA. Control of RANKL gene expression. *Bone*. 2010; 46:911–919. [PubMed: 19716455]
47. Bonewald LF. The amazing osteocyte. *J Bone Miner Res*. 2011; 26:229–238. [PubMed: 21254230]
48. Manolagas SC, O'Brien CA, Almeida M. The role of estrogen and androgen receptors in bone health and disease. *Nature Reviews Endocrinology*. 2013; 9:699–712.
49. Emerton KB, Hu B, Woo AA, Sinofsky A, Hernandez C, Majeska RJ, Jepsen KJ, Schaffler MB. Osteocyte apoptosis and control of bone resorption following ovariectomy in mice. *Bone*. 2010; 46:577–583. [PubMed: 19925896]

Highlights

- Secondary hyperparathyroidism induces RANKL production by osteocytes
- Osteoclastogenesis and bone loss induced by secondary hyperparathyroidism were blunted in mice lacking RANKL in osteocytes
- Secondary hyperparathyroidism controls bone resorption, in part, by acting on osteocytes

Figure 1.

Deletion of RANKL in Dmp1-Cre expressing cells blunts the bone loss associated with dietary calcium deficiency. A, Five-month-old male RANKL^{Ot} mice and their RANKL^{f/f} littermates were fed control diet (open bars) or calcium-deficient diet (filled bars) for 30 days. Percent change of BMD in the spine, femur, and total body were determined by comparison of values at the start and end of 30 days of experimental diet. Values represent the mean of 6–7 animals per group. B, Seven-month-old female RANKL^{Ot} mice and their RANKL^{f/f} littermates were treated and analyzed as in A. Values represent the mean of 10–13 animals per group. *, $P < 0.05$.

Figure 2.

Deletion of RANKL in Dmp1-Cre expressing cells inhibits cortical bone loss. A–B, Cortical thickness and cortical porosity measured in femurs from 5-month-old male RANKL^{f/f} and RANKL^{Δot} mice fed control diet (open bars) or calcium-deficient diet (filled bars) for 30 days. C, μ CT images of femoral cortical bone (arrow indicates position of pores). D–F, Cortical thickness, cortical porosity, and bone strength measured in femurs of 7-month-old female RANKL^{f/f} and RANKL^{Δot} mice fed control diet (open bars) or calcium-deficient diet (filled bars) for 30 days. Values represent the mean of 6–13 animals per group. *, $P < 0.05$.

Figure 3.

Deletion of RANKL from Dmp1-Cre expressing cells blunts the increase of RANKL induced by dietary calcium deficiency. A, Quantitative RT-PCR of RANKL mRNA in the indicated tissues of 5-month-old male RANKL^{Δot} and RANKL^{fl/f} littermates fed control diet (open bars) or calcium-deficient diet (filled bars) for 30 days. All mRNA levels were normalized to ribosomal protein S2 mRNA levels. Values represent the mean of 6–7 animals per group. B, Immunoblot of RANKL in protein extracted from tibial shafts of 7-month-old female RANKL^{Δot} mice and their RANKL^{fl/f} littermates fed control diet (open bars) or calcium-deficient diet (filled bars) for 30 days. Each lane contains protein from two animals

of the same group. The intensity of the RANKL band normalized to actin is shown on the right. Values represent the mean of 5–6 samples per group. * $P < 0.05$.

Figure 4.

Deletion of RANKL from Dmp1-Cre expressing cells blunts the increase in osteoclast number caused by dietary calcium deficiency. A–B, Osteoclast number per bone perimeter (Oc.N/B.Pm) and osteoclast perimeter per bone perimeter (Oc.Pm/B.Pm) in cancellous bone (A) and at the endocortical surface (B) of decalcified femurs from 5-month-old male mice (n = 6–7 animals per group). C–D, Histological sections of femurs stained for TRAP activity (osteoclasts are stained red) and counterstained with methyl green (scale bar = 20μm) in cancellous bone (C) and endocortical bone (D). *, P < 0.05.

Figure 5.

Deletion of RANKL from Dmp1-Cre expressing cells blunts the increase in bone resorption caused by dietary calcium deficiency. A, Circulating CTX levels in the blood plasma of 5-month-old male RANKL^{f/f} and RANKL^{Δot} mice fed control diet (open bars) or calcium-deficient diet (filled bars) for 30 days. B–D, Calcium, phosphorous, and PTH concentrations in the blood plasma of the same groups shown in A. E, Quantitative RT-PCR of Cyp27b1 mRNA in kidney and Sost mRNA in tibia shafts from the same groups as in A. Values represent the mean of 6–7 animals per group. *, P < 0.05.