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Increased bone mass is an unexpected phenotype associated with deletion of the calcitonin gene

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Article

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Increased bone mass is an unexpected phenotype associated with deletion of the calcitonin gene

See the related Commentary beginning on page 1769.

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Introduction

In 1961, Harold Copp and colleagues identified a potent hypocalcemic substance that was released from the thyroid/parathyroid circulation in response to supraphysiologic calcium concentrations (1). This substance was named calcitonin (CT) because it was presumed to play a role in regulating calcium metabolism (2). Hirsch and coworkers (3) subsequently demonstrated that CT is produced by the thyroid gland. Later work confirmed its synthesis by the thyroidal C cell, its 32-amino-acid structure, and its interaction with specific receptors on the osteoclast to inhibit bone resorption (4–9).

Since the discovery of CT, multiple studies have confirmed its hypocalcemic effect, which is mediated by the interaction of CT with a specific receptor on the osteoclast (10, 11). This effect is sufficiently potent that salmon CT is widely used to treat conditions associated with increased osteoclastic activity, such as osteoporosis and Paget disease of bone (12–14). In almost all of the in vitro and in vivo studies conducted during the past 40 years, researchers concluded that CT is an inhibitor of bone resorption basally and in response to several activators of osteoclastic function including parathyroid hormone (PTH) (9, 15–19).

The primary RNA transcript of the *CT/CGRP* gene encodes two distinct peptides, CT and calcitonin gene–related peptide-α (CGRPα) (20–22). Cell-specific alternative RNA processing results in CT being produced almost exclusively by the thyroidal C cell and in

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Nonstandard abbreviations used: calcitonin (CT); human parathyroid hormone (hPTH); calcitonin gene–related peptide-α (CGRPα); wild-type (WT); knockout (KO); deoxypyridinoline (DPD); ovariectomized (OVX); 1,25-dihydroxy-vitamin D3 (1,25-D3).

CGRPα being produced throughout the central and peripheral nervous system (Figure 1a). The physiologic role of CGRPα is unclear, although it is postulated to regulate several processes in the central and peripheral nervous system and skin. Also, fragmentary evidence suggests that CGRP has effects on bone. Early studies focused on the inhibition of bone resorption by CGRPα, although effects were seen only at supraphysiologic concentrations (23–25). More recent studies have found that CGRPα stimulates bone formation, although the physiologic relevance of these findings is unclear (26–28).

One focus of our work has been to understand how *CT/CGRP* gene products affect bone metabolism. Many studies have documented that removing the thyroid gland (the main source of CT) has little or no effect on long-term calcium or bone metabolism (29–31). However, these experiments have been inconclusive because CT is also produced in other neuroendocrine cells (32). To better understand the physiologic and developmental role of endogenous CT and CGRPα, we deleted the mouse *CT/CGRP* gene by homologous recombination. We predicted either no effect of this deletion on bone mass or possibly an osteoporotic phenotype, since CT is known to inhibit osteoclast-induced bone resorption, and CGRPα is known to inhibit bone resorption (23) and stimulate bone formation (27). We were therefore surprised to observe a phenotype in which CT/CGRPα-deficient animals have a greater bone mass, increased bone formation, and maintain bone mass during estrogen deficiency by increasing bone formation.

Methods

Targeting strategy and confirmation of CT/CGRP-deficient status. The mouse calcitonin I (*CT/CGRP*) gene (Figure 1a) was cloned from a 129SvEv mouse genomic library (Stratagene, La Jolla, California, USA) and confirmed by DNA sequence analysis. We created a targeting vector in which PGKneoBPA replaced exons 2–5 of the *CT/CGRP* gene within the thymidine kinase plasmid MCITK-bpA (Figure 1b). The resulting vector has a 1.9-kb HindIII/BamHI fragment for the 5′ targeting arm and a 4.3-kb XbaI/HindIII fragment for the 3′ targeting arm. Neither targeting sequence contains coding sequences for the *CT/CGRP* gene (Figure 1b). This targeting construct was introduced into the embryonic stem cell line AB 2.1 using electroporation. Colonies were selected for resistance to both neomycin and ganciclovir. Targeted embryonic stem cell clones were identified by Southern blot hybridization (Figure 1c) and were injected into C57BL/6 blastocysts and transferred into pseudopregnant females, resulting in the generation of chimeras. We crossed overt chimeras with C57BL/6 mice to produce germline transmission of the targeted allele. Homozygous $CT/CGRP^{+/+}$ (wild-type; WT) and $CT/CGRP^{-/-}$ (knockout; KO) mice were generated by breeding animals heterozygous for the CT/CGRP deficiency. To expand the number of animals, F_2 or F_3 homozygous mice were mated to create WT or KO populations. WT and KO animals were identified by PCR analysis using a common forward primer (5′-CAGGATCAAGAGTCACCGCT-3′) that hybridized to *CT/CGRP* gene exon 1 and reverse primers that hybridized to either the *neomycin* gene coding region (5′-GGTGGATGTGGAATGTGTGC-3′) (KO) or CT/CGRP intron 1 (5′-GGAGCCTGCGCTCCAGCGAA-3′) (WT). We examined CT and CGRPα mRNA levels using an mRNA capture kit (Roche Molecular Systems, Branchburg, New Jersey, USA) followed by RT-PCR. Oligonucleotide primer pairs used for CT and CGRPα were 5′-CAGGATCAAGAGTCACCGCT-3′ (forward CT and CGRPα), 5′-GAGGTCTTGTGTGTACGTGC-3′ (exon 4 reverse), and 5′-ACGCCAGAACCAGCTGTCAT-3′ (exon 6 reverse). We detected CGRPβ mRNA using primers 5′-ACCCTCGGCGACCAGG-3′ (exon 3 forward) and 5′-TCCTTGAGGCCTTCACATCG-3′ (exon 6 reverse).

Mice. All animal experiments were approved by the Animal Care and Use Committee of The University of Texas M. D. Anderson Cancer Center. Mice were housed in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, with a standard light/dark cycle and fed a regular diet (LabDiet Rodent Diet; PMI Nutrition International, Richmond, Indiana, USA) containing 0.95% calcium without restrictions. Mice homozygous for the CT/CGRP deletion are born normally and weaned at the same age as WT mice, have body weights and physical features indistinguishable from WT mice, and are normally fertile.

Immunohistochemical analysis of the thyroid gland. The immunoperoxidase analysis was performed on formalin-fixed, paraffin-embedded tissues using the avidin-biotin-peroxidase complex method (33). Slides were incubated with a 1:50 dilution of the anti-CT antibody (Caltag Laboratories Inc., Burlingame, California, USA), and immunoperoxidase staining was performed using the LSAB2 peroxidase kit (DAKO Corp., Carpinteria, California, USA). The antigen-antibody reaction was visualized using 3-amino-9-ethylcarbazole as chromogen. Slides were counterstained with Mayer's hematoxylin.

Biochemical assays. We collected blood and urine samples from sex- and age-matched mice. Serum concentrations of total calcium and inorganic phosphorus were measured with an automated clinical chemistry analyzer in 2-month-old animals. Serum ionized calcium was measured with an ion-selective electrode in 18-month-old animals. Serum CT, thyroid CT, and intact PTH were analyzed by immunoradiometric assay (Immutopics Inc., San Clemente, California, USA).

The urinary excretion of deoxypyridinoline (DPD) crosslinks, a marker of bone resorption, was determined using the Pyrilinks-D ELISA (Metra Biosystems Inc., Mountain View, California, USA). Urinary creatinine concentration was determined using an alkaline picrate quantitative colorimetric assay, and DPD crosslinks were expressed relative to creatinine concentration as nM DPD per mM of urinary creatinine.

Response to PTH. Serum calcium and urine DPD was assessed in intact WT and KO animals by injection of vehicle (PBS with 0.1 mM HCl and 0.01% BSA) or human PTH (hPTH) (0.5 μg per gram body weight) into the abdominal peritoneal cavity (34). PTH powder (hPTH fragment 1-34; Sigma-Aldrich, St. Louis, Missouri, USA) was resuspended in 1 mM HCl and 0.1% BSA and the stock was diluted to its final concentration with PBS just prior to injection. Serum calcium and urine DPD were measured at baseline, 2 hours, and 4 hours. In rescue experiments, rat CT (Sigma-Aldrich), rat CGRPα (Sigma-Aldrich), or vehicle (PBS with 0.1 mM HCl and 0.01% BSA) was injected intramuscularly, followed 3 minutes later with an intraperitoneal injection of PTH (or vehicle, in the case of the control). Serum calcium was measured 1 hour after injection.

Ovariectomy. Ten KO and ten WT mice were randomized at age 3 months to receive a bilateral ovariectomy or a sham procedure in which the ovaries were exteriorized but not removed (five animals per group). Two months after the surgical procedure, the mice were injected with calcein (25 mg/kg) 10 days and 2 days before they were killed according to an established protocol (35).

Histomorphometry and radiographic analysis. Female and male mice killed at 1 month and 3 months of age were analyzed histomorphometrically and radiographically. After whole-animal contact radiography (Faxitron x-ray cabinet; Faxitron X-ray Corp., Wheeling, Illinois, USA) and autopsy, we dissected out the bones and fixed them in 3.7% PBS-buffered formaldehyde for 18 hours at 4°C. After dehydration, the undecalcified bone samples were embedded in methylmethacrylate, and 5-μm sections were cut in the sagittal plane on a Microtec rotation microtome (Techno-Med Biefeld, Munich, Germany) as previously described (35). Sections were stained with toluidine blue and modified von Kossa or Goldner trichrome stain and evaluated using a Zeiss microscope (Carl Zeiss Jena GmbH, Jena, Germany). To assess the dynamic histomorphometric parameters, we mounted 12-μm-thick, unstained sections in Fluoromount (Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA) to permit evaluation by fluorescence microscopy.

Quantitative histomorphometric analysis was performed in a blinded fashion on toluidine blue–stained, undecalcified proximal tibia and lumbar vertebra sections. For comparative histomorphometric analysis, samples from male and female WT and KO mice as well as ovariectomized (OVX) WT and KO female mice were used (five animals per group). Analysis of bone volume (percentage), trabecular thickness (μm), trabecular number (per mm), trabecular separation (μm), cortical thickness (μm), and number of osteoblasts and osteoclasts per mm of bone perimeter was carried out according to standardized protocols (36) using the OsteoMeasure

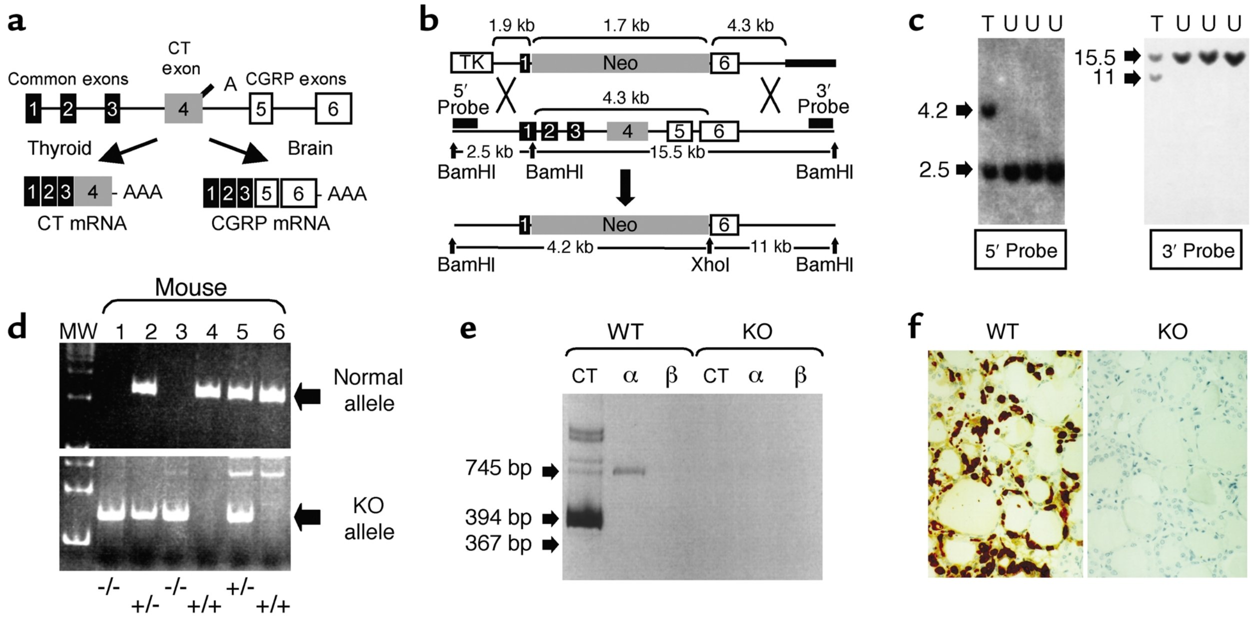

Figure 1

Creation of mice null for the *CT/CGRP* gene. (**a**) Schematic representation of *CT/CGRP* gene tissue-specific alternative RNA processing and precursor peptide processing. (**b**) Schematic of recombination of targeting vector with *CT/CGRP* gene. Black bars indicate general location of DNA probes used for Southern screening. (**c**) Representative Southern analysis of BamHI/XhoI-digested DNA isolated from doubly resistant embryonic stem cells showing targeted (T) and untargeted (U) clones. A restriction map outlining the origin of individual bands is provided in **b**. (**d**) Identification of mouse genotype by PCR analysis of tail-derived DNA (see Methods). PCR product in the upper gel shows the presence of normal WT allele (+/+); the lower gel shows rearranged KO allele (–/–). Both alleles are detected in heterozygous animals (+/–). MW, 100-bp ladder molecular weight marker. (**e**) RT-PCR analysis of mRNA isolated from the thyroid glands of WT or KO animals (see Methods). Primer pairs used detect mRNAs for CT, CGRPα (α) and CGRPβ (β), which have predicted band sizes of 394 bp, 745 bp, and 367 bp, respectively. (**f**) Calcitonin immunohistochemical staining of thyroid from WT and KO animals (see Methods).

Table 1
Baseline biochemical analysis of parameters of calcium metabolism in female mice

Parameter	$CT/CGRP^{-/-}$	$CT/CGRP^{+/+}$
Calcium (mg/dl)	9.15 ± 0.99	8.98 ± 0.45
Ionized calcium (mmol/l)	0.99 ± 0.14	1.01 ± 0.13
Phosphorus (mg/dl)	10.1 ± 1.1	10.0 ± 1.0
Intact PTH (pg/ml)	25 ± 33	40 ± 48
Estradiol (pg/ml)	50.1 ± 6.2	54.0 ± 5.5
1,25-D3 (pg/ml)	34.5 ± 8.5	32.6 ± 10.1
Thyroxine (μg/dl)	3.6 ± 0.5	3.4 ± 0.7

All assays were measured using three or more animals. The levels represent mean ± SD. There was no statistical difference between groups in any of these parameters. See Methods for the assays used.

histomorphometry system (Osteometrics Inc., Atlanta, Georgia, USA). To assess dynamic histomorphometric indices, mice were given two injections of calcein 8 days apart and killed 2 days later according to a standard double-labeling protocol (35). Fluorochrome measurements were made on two nonconsecutive 12-μm-thick, unstained sections per animal. Statistical differences between groups (n = 5) were assessed by the Student t test. P values below 0.05 were accepted as significant, and error bars represent SD.

Biomechanical testing. Both femurs of mice that underwent ovariectomy or a sham procedure (five animals per group) were dissected free of soft tissue and stored in 50% ethanol-saline. The bones were transferred to isotonic saline and stored at 4°C for 12 hours before testing. A three-point bending test was performed as previously described (35), using a commercial high-precision instrument (Z2.5/TN 1S testing machine; Zwick GmH & Co., Ulm, Germany). In brief, the ends of the bone were supported on two fulcra located 5 mm apart. With the posterior aspect of the femur resting on the fulcra, a load was applied from above to the anterior midshaft midway between the two fulcra at a constant speed of 10 mm/min until bone failure. A chart recorder was used to generate a force-deformation curve. Experiments were performed in a blind fashion.

Results

Alternative RNA processing of the *CT/CGRP* gene makes selective disruption of either CT or CGRPα difficult (Figure 1a). Therefore, we opted to create a targeting vector that disrupts the entire coding region for

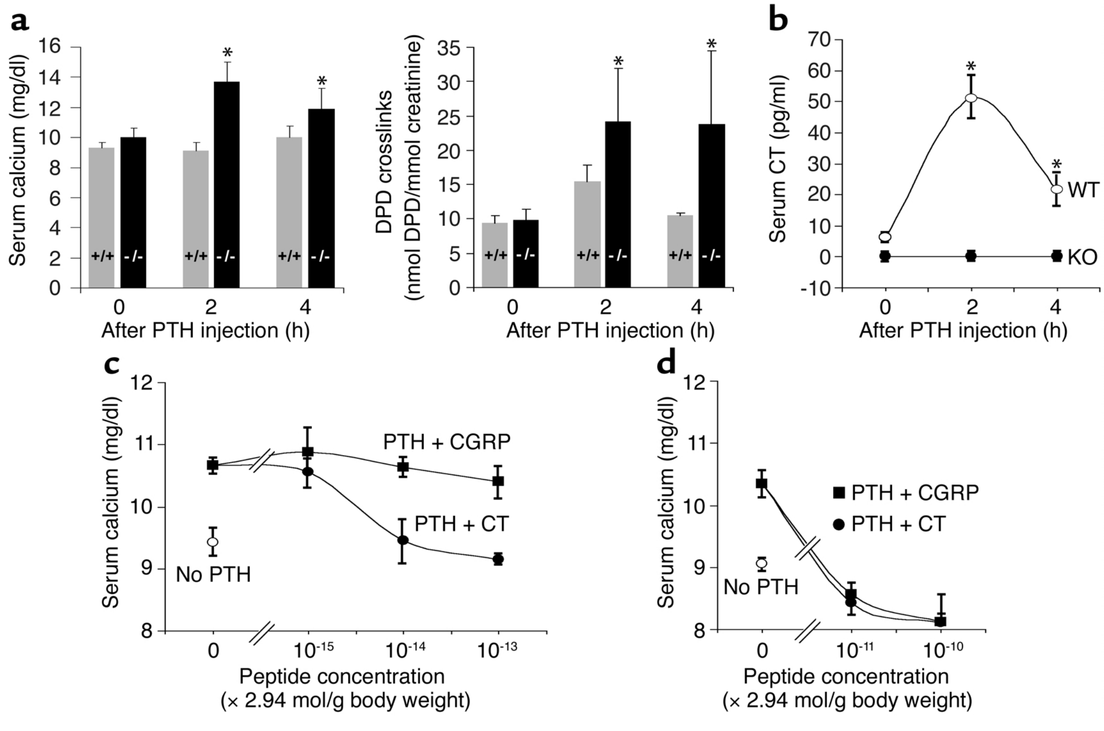

Figure 2
PTH-stimulated bone resorption in KO mice causes hypercalcemia and is blocked by CT administration. Groups of five WT (gray bars) or KO (black bars) male mice were injected intraperitoneally with human PTH or vehicle (PBS with 0.1 mM HCl and 0.01% BSA) and sacrificed at the indicated times with collection of serum and urine. (**a**) In KO animals, human PTH stimulates a 4 mg% rise in serum calcium concentration and a doubling of urine DPD during the first 2 hours of the experiment. No significant change of serum calcium or urine DPD was seen in WT animals treated with PTH or vehicle at any timepoint. (**b**) Measurement of serum CT demonstrated a significant increase in WT animals (open circles) and no detectable CT in KO animals (filled circles). (**c**) Groups of male mice were injected intramuscularly in the thigh with vehicle, rat CT (10, 100, or 1,000 pg/g mouse body weight) (filled circles), or rat CGRP (11.2, 112, or 1,120 pg/g mouse body weight) (filled squares), followed 3 minutes later by an intraperitoneal injection with human PTH (or vehicle, in the case of the control). Blood was collected 1 hour after the initial intramuscular injection. (**d**) Experiments were performed as in **c** but at higher dosage. All experiments included five animals per group with bars representing mean ± SD. *P < 0.05.

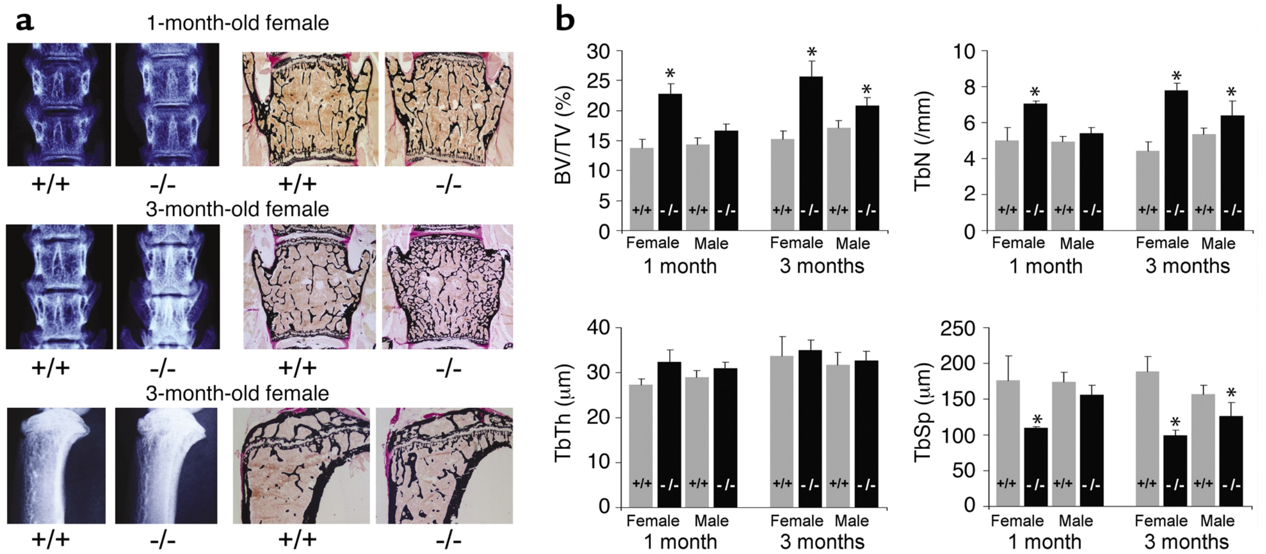

Figure 3
KO of the *CT/CGRP* gene results in increased bone in mice. (**a**) Radiographic and histologic analysis of vertebrae from 1-month-old and 3-month-old WT and KO mice, and of the proximal tibiae from 3-month-old WT and KO mice. (**b**) Histomorphometric analysis of trabecular bone volume in vertebrae of WT (gray bars) and KO (black bars) mice. Graphs provide data for trabecular bone volume as a ratio of total bone volume (BV/TV), trabecular number (TbN), trabecular thickness (TbTh), and trabecular spacing (TbSp). Bars represent mean ± SD (n = 5). *Statistically significant difference between WT and KO animals ($P < 0.05$) as determined by Student t test.

both CT and CGRPα (Figure 1b) (Figure 1b). Targeted embryonic stem cells identified by Southern analysis (Figure 1c) were used to generate chimeric founder animals that were bred with C57BL/6 mice. Animals with a homozygous KO of the *CT/CGRP* locus were born and developed normally. Male and female KO animals were fertile when mated with heterozygous or homozygous CT/CGRP KO animals. There was no difference in the size, weight, or general appearance of WT and KO animals. PCR analysis using a common *CT/CGRP* gene exon 1 primer and primers specific for *CT/CGRP* gene intron 1 or the *neomycin* gene demonstrated the absence of the *CT/CGRP* gene in homozygous animals (Figure 1d). The CT/CGRP-deficient state was confirmed by the absence of CT and CGRPα in RT-PCR analysis of the thyroid (Figure 1e), immunohistochemical analysis for CT (Figure 1f) or CGRP (37), and serum and thyroid immunoradiometric assays (Figure 2b and data not shown). In addition, no CT or CGRPα was detected in other tissues (brain, adrenal glands, and liver) (data not shown).

Baseline parameters of calcium metabolism. Serum calcium, ionized calcium, phosphorus, intact PTH, thyroxine, and 1,25-dihydroxy-vitamin D3 (1,25-D3) did not differ between KO and WT animals (Table 1).

Exogenous PTH stimulates bone resorption in KO mice. To determine whether CT/CGRP deficiency causes abnormalities in mineral metabolism that were not detected under basal conditions, we treated KO and WT animals with hPTH (0.5 μg/g body weight) (34). hPTH stimulated a significant increase in the serum calcium and urine DPD concentrations 2 hours and 4 hours following injection in KO but not WT animals (Figure 2a). Parallel measurements of serum CT during this experiment show a significant increase in serum CT in WT but not KO animals (Figure 2b). One plausible explanation for this, which we tested experimentally, is that the rise in serum CT in the WT animals treated with PTH prevented osteoclast-mediated bone resorption and the subsequent rise in serum calcium. This led us to examine whether pretreatment with CT immediately prior to the PTH injection would prevent the hypercalcemic response. Rat CT but not rat CGRP, at doses between 10 and 100 pg/g (2.94×10^{-15} to 2.94×10^{-14} mol/g) body weight, inhibited the PTH-mediated rise in serum calcium concentration (Figure 2c). The half-maximal effect of approximately 30 pg/g body weight is nearly identical to that described for the effect of CT to lower the serum calcium concentration under basal conditions in two prior reports (38, 39). Effects of CGRP were observed only at higher concentrations, similar to previously reported results (Figure 2d) (38, 39).

Roentgenographic and histomorphometric evaluation of bone. Contact x-rays of vertebral bodies and tibias (Figure 3a) demonstrated higher bone density in 1- and 3-month-old female KO animals than in female WT mice of the same age. Similar observations were made in 3-month-old male animals (data not shown). Histologic analysis showed a markedly higher trabecular bone volume in vertebral bodies at 1 and 3 months and in the proximal tibia at 3 months in female KO mice (Figure 3a). These findings were confirmed by histomorphometric analysis, demonstrating significantly higher bone volume and trabecular number in KO female animals (Figure 3b). Trabecular thickness

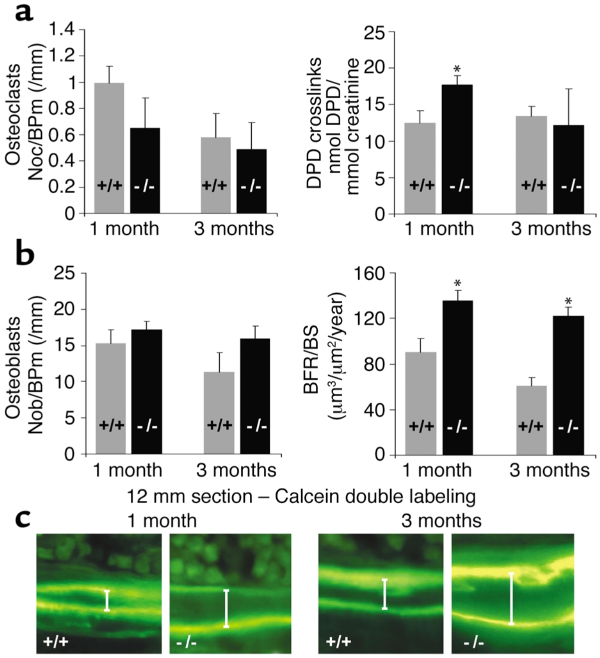

Figure 4

Comparison of bone resorption and formation in female WT and KO animals. (**a**) Parameters of bone resorption. Histomorphometric analysis of osteoclast number per mm bone perimeter (Noc/BPm) and urinary DPD crosslinks (a marker of bone resorption) was performed in 1- and 3-month-old WT (gray bars) and KO (black bars) mice. (**b**) Parameters of bone formation. Histomorphometric analysis of osteoblast number per mm bone perimeter (Nob/BPm) (left graph) and in vivo analysis of bone formation (right graph) in 1- and 3-month-old WT and KO mice. The rate of bone formation was determined by in vivo dual calcein labeling; the graphs provide quantification of the bone formation rate per bone surface (BFR/BS). (**c**) Representative fluorescent micrographs demonstrate the two labeled mineralization fronts, with the arrows indicating growth between labelings. Note the greater distance between the two labeled areas in the KO mice than in the WT animals. All experiments included five animals per group; bars represent mean ± SD. *Statistically significant difference between WT and KO mice ($P < 0.05$).

was the same in WT and KO animals, whereas trabecular spacing was significantly decreased in KO animals (Figure 3b). In males, the increase in trabecular bone volume and trabecular number and the decrease in trabecular spacing were identified only in 3-month-old animals (Figure 3b).

Dynamic histomorphometry and assessment of osteoclast function. The finding of increased bone volume and trabecular number in static histomorphometric measurements of KO mice was surprising to us, particularly in view of the greater sensitivity to exogenous PTH. To determine whether the effect was related to a decrease of osteoclast or increase of osteoblast function, we performed several analyses. First, there was no significant evidence for diminished osteoclast function. The number of osteoclasts was similar in female KO and WT animals at 1 and 3 months (Figure 4a). The urine DPD values at 1 month in KO animals were slightly greater than in WT, but the values did not differ at 3 months or at baseline as shown in Figure 2a. Second, there is clear evidence for increased osteoblast function, despite the fact that there is no significant increase in osteoblast number (Figure 4b). Double calcein labeling studies document a 1.5- and 2-fold increase in bone formation rates at 1 and 3 months, respectively (Figure 4, b and c; data not shown for males). These results argue persuasively that the observed increase of bone mass in the KO mice results from an increase of bone formation rather than a decrease in osteoclast resorption.

Effects of estrogen deficiency in KO or WT mice. Estrogen deficiency, like exposure to exogenous PTH, produces a perturbation of bone remodeling characterized by increased osteoclastic bone resorption and long-term bone loss. These findings led us to ask whether CT/CGRP-deficient mice would be more sensitive to the effects of estrogen deficiency. In the experiments conducted (see Methods), WT OVX animals had a 36% reduction in trabecular bone volume 2 months after ovariectomy (Figure 5, a and b). In contrast, KO animals maintained their trabecular bone volume following ovariectomy (Figure 5, a and b). There was no significant difference in sham or OVX osteoclast number in either WT or KO mice (Figure 5c). Osteoblast numbers were increased in both OVX WT and KO animals compared with sham WT or KO animals (Figure 5d). Bone formation rate, measured by double calcein labeling, was again higher in KO than WT sham-operated mice. It rose 1.6-fold in KO animals following ovariectomy compared with an increase of 1.3-fold in WT mice, providing the likely explanation for maintenance of bone mass in the OVX KO mice.

The compensatory increase in bone formation following estrogen deficiency results not only in maintenance of bone mass but also in preservation of bone quality. Biomechanical analysis of the femurs of KO and WT animals after ovariectomy was performed using a three-point bending test (35). There was a 35% reduction ($P < 0.05$) in the force needed to break the femurs of WT OVX animals, whereas in KO animals the reduction was 10% (not significant) (Figure 5e).

Discussion

During the 40 years since the discovery of CT there has been much speculation about its role in regulatory biology. Our studies show clearly that the *CT/CGRP* gene is not necessary for procreation, its absence is not associated with any profound developmental defect, and it does not appear to affect basal calcium or other mineral homeostasis. However, if one looks more carefully, there are indications of a role for CT in normal bone and mineral homeostasis.

The first is an enhanced responsiveness of CT/CGRPα-deficient mice to exogenous PTH administration. The KO animals have a greater calcemic response to exogenous PTH than WT animals, an effect that is caused by greater bone resorption in KO than WT animals. The greater calcemic response in KO mice extends to treatment with 1,25-D3; after 5 days of treatment,

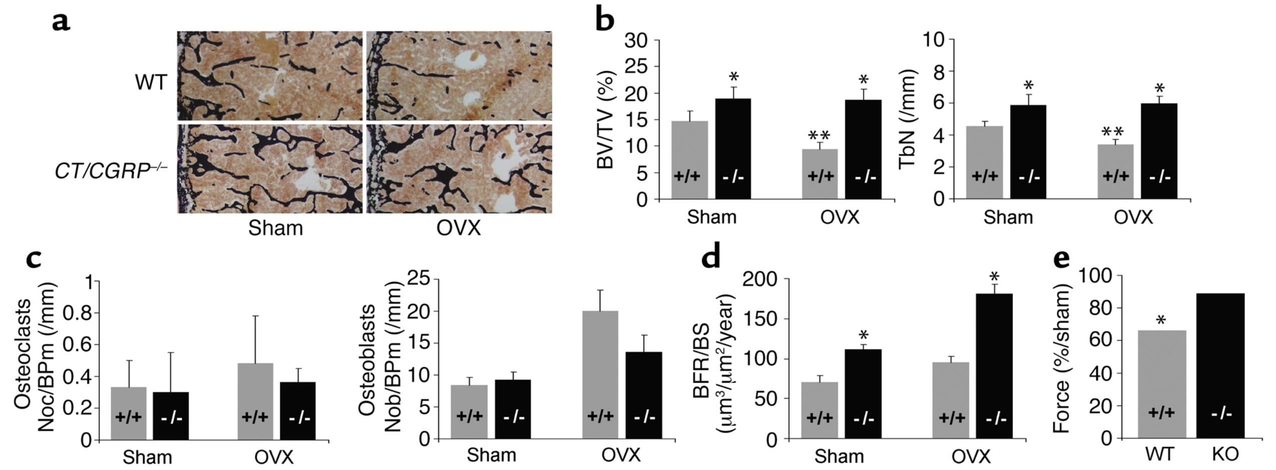

Figure 5
The absence of the *CT/CGRP* gene is protective against estrogen deficiency–mediated bone loss. At 3 months, WT and KO female mice received ovariectomy or a sham procedure. Animals were sacrificed at age 5 months following dual calcein labeling. (**a**) Histologic analysis of the vertebrae of WT and KO animals after ovariectomy or a sham procedure (see Methods). (**b**) Static histomorphometric analysis of vertebral bodies from WT (gray bars) and KO (black bars) mice. Graphs provide data for trabecular bone volume as a ratio of total bone volume (BV/TV) and trabecular number (TbN). Significant differences were observed between WT sham and WT OVX animals (**$P < 0.01$), and between WT and KO animals in both groups (*$P < 0.05$). (**c**) Histomorphometric analysis of osteoclast number and osteoblast number per mm bone perimeter. (**d**) Dynamic histomorphometric analysis of bone formation rate per bone surface in WT and KO mice after ovariectomy or a sham procedure. (**e**) Biomechanical properties of femora after ovariectomy or a sham procedure. Ovariectomy resulted in decreased force needed to break bone as determined by a three-point bending test (*$P < 0.05$). All experiments included five animals per group; bars represent mean ± SD.

KO animals had a serum calcium concentration that was 4 mg% higher than that in WT animals (data not shown). One likely explanation for these results is the absence of a countervailing inhibitory effect of CT in KO animals, a point buttressed by the finding that CT but not CGRP prevents the rise of serum calcium caused by PTH in KO mice. These findings confirm the long-held belief that CT inhibits bone resorption in the face of an acute hypercalcemic challenge (40).

The second effect of the *CT/CGRP* gene, not previously suspected, is to regulate bone formation. At the outset of this investigation, we hypothesized that CT/CGRP deficiency would either have no effect on bone mass or would cause a bone loss phenotype characterized by increased osteoclastic resorption. However, we found exactly the opposite. Further analysis demonstrated that there was no difference in osteoclast number or bone resorption markers (DPD), whereas bone formation rates were increased significantly. Although the data in Figure 4a show a trend toward a lower osteoclast number in KO animals, providing a potential explanation for the higher bone mass, the higher levels of urine DPD in these 1-month-old animals exclude this possibility. Basal parameters of calcium metabolism including serum ionized or total calcium, phosphorus, intact PTH, and 1,25-D3 levels did not differ between WT and KO mice, excluding other obvious potential causes. None of the experimental approaches we used in these studies could exclude subtle changes in osteoclast function that could, over time, provide an explanation for the observed differences in bone mass.

Dual tetracycline labeling studies demonstrate a greater bone formation rate in KO animals than WT controls. This finding suggests an important and novel function for a *CT/CGRP* gene product (or products) to regulate bone formation that was heretofore unrecognized. These observations are analogous in some respects to the rethinking of PTH action in recent years. Although there were strong hints of an effect by PTH of stimulating bone formation, these observations were largely dismissed because of the predominant belief that the major effect of PTH was to stimulate bone resorption. It was only when the anabolic effects of PTH in humans were demonstrated conclusively that the effects of PTH on osteoblast function were reexamined.

Current information indicates that CT binds to a specific receptor on the osteoclast, activating at least two different signaling pathways (41–43). This activation leads to retraction of the osteoclast from the bone surface and reduced production of acid and other proteolytic enzymes. Our results confirm the importance of this peptide in osteoclastic bone resorption; however, the finding of increased bone formation suggests one of several additional possible effects. One is the possibility that activation of the CT receptor results in the production of an osteoclast signal that is responsible, in a physiologic sense, for inhibition of osteoblast function. Such a mechanism would make some sense. Conditions associated with increased bone resorption commonly have associated increases in bone formation. CT may not only inhibit bone resorption, but might also activate a signaling pathway that in parallel inhibits bone formation. This could be the reverse of the RANKL/RANK receptor system that leads to increased osteoclast resorption when activated by interaction of PTH with the osteoblasts. There is some evidence in the literature to support this possibility, although these reports use pharmacologic doses of CT (25, 44–47). A second possibility (one we think less likely) is that CT interacts either directly with the osteoblast or with another cell type that communicates with the osteoblast. There is little evidence for the presence of a CT receptor on osteoblasts (48, 49). The only known marrow cell with a CT receptor, other than the osteoclast, is the lymphocyte (50). Lymphocytes are known to produce the transcription factor Cbfa1, and the RANKL decoy receptor OPG, two factors involved in bone remodeling (51, 52). No direct link between CT treatment and expression of either of these factors is known. Finally, it is possible that there are subtle effects of CT deficiency on osteoclast number or function over the life of the animal that have altered the balance between resorption and formation. We think this explanation is unlikely because the double calcein labeling studies were performed over a short period (8 days) and the measurements were made at sites where there were no identifiable osteoclasts.

Just as the expected phenotype in the KO animals was one associated with loss of bone, we expected similarly to find bone loss in ovariectomized KO mice. Ovariectomy and subsequent estrogen deficiency normally leads to cytokine-mediated osteoclast resorption and bone loss. This was the result observed in WT ovariectomized mice. In contrast, there was no decrease in bone volume in KO animals, and analogous to the findings in the basal state in KO animals, bone formation was substantially increased. These findings demonstrate that the absence of the *CT/CGRP* gene is associated with increased bone formation not only in the basal state, but also in a condition characterized by increased osteoclastic resorption. Again our findings do not conclusively exclude the possibility that a subtle change in osteoclast function might contribute over the 2-month period to the observed phenotype. Additional studies will be required to definitively exclude this possibility.

An alternative mechanism for the observed effects on bone formation is a role for CGRPα in the regulation of bone formation. It is known that CGRPα-containing neurons terminate in bone, providing a potential delivery mechanism. There are two known effects of CGRP in bone. Reports from the period following its discovery in the early 1980s showed inhibitory effects on bone resorption, albeit at higher molar concentrations than are required for the same effect by CT, findings we have confirmed (Figure 2d) (23, 25). A reinterpretation of these data based on the elucidation of the CGRPα/RAMP receptor system (53, 54) suggests that CGRP at high concentrations cross-reacts with the CT receptor. CGRP has also been shown to stimulate osteoblastic bone formation (26, 27, 55). In vitro studies show the presence of CGRP receptors on osteoblasts and a stimulatory effect on bone formation (26, 55, 56). In other whole-animal studies, expression of CGRP specifically in osteoblasts (under the regulation of an osteocalcin promoter) resulted in increased bone formation (27). There have been no reports of an inhibitory effect of CGRP on bone formation, making it an unlikely candidate for the effects we observed.

Finally, there is the possibility that one of the other peptides derived from cleavage of the CT or CGRPα precursor peptide has a biologic role. The primary transcript of the *CT/CGRP* gene is processed to produce either proCT or proCGRPα. The mature CT or CGRPα peptide is located centrally within the propeptide, and during cleavage, amino-terminal and carboxyl-terminal fragments are produced. Available evidence suggests that neither the carboxyl terminal nor the amino-flanking peptides of proCT have a significant effect on bone metabolism (39, 55, 57), although this possibility has not been tested rigorously. There is little information available on the effect of N-proCGRPα on bone biology (55).

The unexpected finding of increased bone formation in KO mice suggests an unrecognized effect of a *CT/CGRP* gene product on regulation of bone formation. The mechanism by which this effect occurs remains unclear, but this animal model and others currently available (58) will be useful in examining the several possibilities. These findings also suggest that development of an antagonist to the causative *CT/CGRP* gene product might prove useful in the prevention of bone loss associated with estrogen deficiency.

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1. Copp, D.H., Davidson, A.G.F., and Cheney, B.A. 1961. Evidence for a new parathyroid hormone which lowers blood calcium. *Proc. Canad. Fed. Biol. Soc.* **4**:17.
2. Copp, D.H., Cameron, E.C., Cheney, B.A., Davidson, A.G.F., and Henze, K.G. 1962. Evidence for calcitonin—a new hormone from the parathyroid that lowers blood calcium. *Endocrinology.* **70**:638–649.
3. Hirsch, P.F., Gauthier, G.F., and Munson, P.L. 1963. Thyroid hypocalcemic principle and recurrent laryngeal nerve injury as factors affecting response to parathyroidectomy in rats. *Endocrinology.* **73**:244–251.
4. Foster, G.V., MacIntyre, I., and Pearse, A.G.E. 1965. Calcitonin production and the mitochondrion-rich cells of the dog thyroid. *Nature.* **203**:1029–1031.
5. Foster, G.V., et al. 1964. Thyroid origin of calcitonin. *Nature.* **202**:1303–1305.
6. Pearse, A.G.E. 1966. 5-Hydroxytryptophan uptake by dog thyroid "C" cells, and its possible significance in polypeptide hormone production. *Nature.* **211**:598–600.
7. Potts, J.T.J., Niall, H.D., Keutmann, H.T., Brewer, H.B., and Deftos, L.J. 1968. The amino acid sequence of porcine thyrocalcitonin. *Proc. Natl. Acad. Sci. USA.* **59**:1321–1328.
8. Neher, R., et al. 1968. Human calcitonin. *Nature.* **220**:984–986.
9. Friedman, J., and Raisz, L.G. 1965. Thyrocalcitonin: inhibitor of bone resorption in tissue culture. *Science.* **150**:1465–1467.

10. Nicholson, G.C., Moseley, J.M., Sexton, P.M., Mendelsohn, F.A.O., and Martin, T.J. 1986. Abundant calcitonin receptors in isolated rat osteoclasts: biochemical and autoradiographic characterization. *J. Clin. Invest.* **78**:355–361.
11. Lin, H.Y., et al. 1991. Expression cloning of an adenylate cyclase-coupled calcitonin receptor. *Science.* **254**:1022–1024.
12. Singer, F.R. 1977. Human calcitonin treatment of Paget's disease of bone. *Clin. Orthop.* **127**:86–93.
13. Civitelli, R., et al. 1988. Bone turnover in postmenopausal osteoporosis: effect of calcitonin treatment. *J. Clin. Invest.* **82**:1268–1274.
14. Azria, M., Copp, D.H., and Zanelli, J.M. 1995. 25 years of salmon calcitonin: from synthesis to therapeutic use. *Calcif. Tissue Int.* **57**:1–4.
15. Holtrop, M.E., Raisz, L.G., and Simmons, H.A. 1974. The effects of parathyroid hormone, colchicine, and calcitonin on the ultrastructure and the activity of osteoclasts in organ culture. *J. Cell Biol.* **60**:346–355.
16. Baylink, D., Morey, E., and Rich, C. 1969. Effect of calcitonin on the rates of bone formation and resorption in the rat. *Endocrinology.* **84**:261–269.
17. Singer, F.R., Melvin, K.E., and Mills, B.G. 1976. Acute effects of calcitonin on osteoclasts in man. *Clin. Endocrinol.* **5**:333S–340S.
18. Cooper, C.W., Hirsch, P.F., and Munson, P.L. 1970. Importance of endogenous thyrocalcitonin for protection against hypercalcemia in the rat. *Endocrinology.* **86**:406–415.
19. Mittleman, R., Chausmer, A., Bellavia, J., and Wallach, S. 1967. Thyrocalcitonin activity in hypercalcemia produced by calcium salts, parathyroid hormone and vitamin D. *Endocrinology.* **81**:599–604.
20. Jacobs, J.W., et al. 1981. Calcitonin messenger RNA encodes multiple polypeptides in a single precursor. *Science.* **213**:457–459.
21. Amara, S.G., Jonas, V., Rosenfeld, M.G., Ong, E.S., and Evans, R.M. 1982. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature.* **298**:240–244.
22. Rosenfeld, M.G., et al. 1983. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature.* **304**:129–135.
23. Zaidi, M., et al. 1987. Calcitonin gene related peptide inhibits osteoclastic bone resorption: a comparative study. *Calcif. Tissue Int.* **40**:149–154.
24. Zaidi, M., Chambers, T.J., Gaines Das, R.E., Morris, R.E., and MacIntyre, I. 1987. A direct action of calcitonin gene-related peptide on isolated osteoclasts. *J. Endocrinol.* **115**:505–510.
25. Valentijn, K., et al. 1997. Effects of calcitonin gene-related peptide on bone turnover in ovariectomized rats. *Bone.* **21**:269–274.
26. Vignery, A., and McCarthy, T.L. 1996. The neuropeptide calcitonin gene-related peptide stimulates insulin-like growth factor I production by primary fetal rat osteoblasts. *Bone.* **18**:331–335.
27. Ballica, R., et al. 1999. Targeted expression of calcitonin gene-related peptide to osteoblasts increases bone density in mice. *J. Bone Miner. Res.* **14**:1067–1074.
28. Burns, D.M., Forstrom, J.M., Friday, K.E., Howard, G.A., and Roos, B.A. 1989. Procalcitonin's amino-terminal cleavage peptide is a bone-cell mitogen. *Proc. Natl. Acad. Sci. USA.* **86**:9519–9523.
29. McDermott, M.T., Kidd, G.S., Blue, P., Ghaed, V., and Hofeldt, F.D. 1983. Reduced bone mineral content in totally thyroidectomized patients: possible effect of calcitonin deficiency. *J. Clin. Endocrinol. Metab.* **56**:936–939.
30. Demeester-Mirkine, N., Bergmann, P., Body, J.-J., and Corvilain, J. 1990. Calcitonin and bone mineral status in congenital hypothyroidism. *Calcif. Tissue Int.* **46**:222–226.
31. Hurley, D.L., Tiegs, R.D., Wahner, H.W., and Heath, H. 1987. Axial and appendicular bone mineral density in patients with long-term deficiency or excess of calcitonin. *N. Engl. J. Med.* **317**:537–541.
32. Becker, K.L., Monaghan, K.G., and Silva, O.L. 1980. Immunocytochemical localization of calcitonin in Kulchitsky cells of human lung. *Arch. Pathol. Lab. Med.* **104**:196–198.
33. Hsu, S.M., Raine, L., and Fanger, H. 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* **29**:577–580.
34. Zhao, W., Byrne, M.H., Boyce, B.F., and Krane, S.M. 1999. Bone resorption induced by parathyroid hormone is strikingly diminished in collagenase-resistant mutant mice. *J. Clin. Invest.* **103**:517–524.
35. Amling, M., et al. 1999. Rescue of the skeletal phenotype of vitamin D receptor-ablated mice in the setting of normal mineral ion homeostasis: formal histomorphometric and biomechanical analyses. *Endocrinology.* **140**:4982–4987.
36. Parfitt, A.M., et al. 1987. Bone histomorphometry: standardization of nomenclature, symbols and units. *J. Bone Miner. Res.* **2**:595–610.
37. Gangula, P.R., et al. 2000. Increased blood pressure in alpha-calcitonin gene-related peptide/calcitonin gene knockout mice. *Hypertension.* **35**:470–475.
38. Zaidi, M., et al. 1988. Effects of peptides from the calcitonin genes on bone and bone cells. *Q. J. Exp. Physiol.* **734**:471–485.
39. Roos, B.A., Fischer, J.A., Pignat, W., Alander, C.B., and Raisz, L.G. 1986. Evaluation of the in vivo and in vitro calcium-regulating actions of noncalcitonin peptides produced via calcitonin gene expression. *Endocrinology.* **118**:46–51.
40. Roos, B.A., Yoon, M., Cutshaw, S.V., and Kalu, D.N. 1980. Calcium regulatory action of endogenous rat calcitonin demonstrated by passive immunization with calcitonin antibodies. *Endocrinology.* **107**:1320–1326.
41. Selander, K.S., et al. 1996. Calcitonin promotes osteoclast survival in vitro. *Mol. Cell. Endocrinol.* **122**:119–129.
42. Kanaoka, K., et al. 2000. A common downstream signaling activity of osteoclast survival factors that prevent nitric oxide-promoted osteoclast apoptosis. *Endocrinology.* **141**:2995–3005.
43. Sakai, H., Kobayashi, Y., Sakai, E., Shibata, M., and Kato, Y. 2000. Cell adhesion is a prerequisite for osteoclast survival. *Biochem. Biophys. Res. Commun.* **270**:550–556.
44. Monier-Faugere, M.C., Geng, Z., Qi, Q., Arnala, I., and Malluche, H.H. 1996. Calcitonin prevents bone loss but decreases osteoblastic activity in ovariohysterectomized beagle dogs. *J. Bone Miner. Res.* **11**:446–455.
45. Pienkowski, D., et al. 1997. Calcitonin alters bone quality in beagle dogs. *J. Bone Miner. Res.* **12**:1936–1943.
46. Okawa, A., Goto, S., and Moriya, H. 1999. Calcitonin simultaneously regulates both periosteal hyperostosis and trabecular osteopenia in the spinal hyperostotic mouse (twy/twy) in vivo. *Calcif. Tissue Int.* **64**:239–247.
47. Krane, S.M., Harris, E.D., Singer, F.R., and Potts, J.T. 1973. Acute effects of calcitonin on bone formation in man. *Metabolism.* **22**:51–58.
48. Kobayashi, T., Sugimoto, T., Saijoh, K., Fukase, M., and Chihara, K. 1994. Calcitonin directly acts on mouse osteoblastic MC3T3-E1 cells to stimulate mRNA expression of c-fos, insulin-like growth factor-1 and osteoblastic phenotypes (type 1 collagen and osteocalcin). *Biochem. Biophys. Res. Commun.* **199**:876–880.
49. Forrest, S.M., et al. 1985. Characterization of an osteoblast-like clonal cell line which responds to both parathyroid hormone and calcitonin. *Calcif. Tissue Int.* **37**:51–56.
50. Marx, S.J., Aurbach, G.D., Gavin, J.R., and Buell, D.W. 1974. Calcitonin receptors on cultured human lymphocytes. *J. Biol. Chem.* **249**:6812–6816.
51. Marie, P., Debiais, F., Cohen-Solal, M., and de Vernejoul, M.C. 2000. New factors controlling bone remodeling. *Joint Bone Spine*. **67**:150–156.
52. Horowitz, M.C., Xi, Y., Wilson, K., and Kacena, M.A. 2001. Control of osteoclastogenesis and bone resorption by members of the TNF family of receptors and ligands. *Cytokine Growth Factor Rev.* **12**:9–18.
53. Tilakaratne, N., Christopoulos, G., Zumpe, E.T., Foord, S.M., and Sexton, P.M. 2000. Amylin receptor phenotypes derived from human calcitonin receptor/RAMP coexpression exhibit pharmacological differences dependent on receptor isoform and host cell environment. *J. Pharmacol. Exp. Ther.* **294**:61–72.
54. McLatchie, L.M., et al. 1998. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature.* **393**:333–339.
55. Burns, D.M., Howard, G.A., and Roos, B.A. 1992. An assessment of the anabolic skeletal actions of the common-region peptides derived from the CGRP and calcitonin prohormones. *Ann. N.Y. Acad. Sci.* **657**:50–62.
56. Cornish, J., et al. 1999. Comparison of the effects of calcitonin gene-related peptide and amylin on osteoblasts. *J. Bone Miner. Res.* **14**:1302–1309.
57. Hillyard, C.J., et al. 1983. Katacalcin: a new plasma calcium-lowering hormone. *Lancet.* **1**:846–848.
58. Lu, J.T., et al. 1999. Mice lacking alpha-calcitonin gene-related peptide exhibit normal cardiovascular regulation and neuromuscular development. *Mol. Cell. Neurosci.* **14**:99–120.