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

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Calcitonin (CT) is a known inhibitor of bone resorption. Calcitonin gene–related peptide-α (CGRPα), produced by alternative RNA processing of the CT/CGRP gene, has no clearly defined role in bone. To better understand the physiologic role of the CT/CGRP gene we created a mouse in which the coding sequences for both CT and CGRPα were deleted by homologous recombination. The CT/CGRP−/− knockout (KO) mice procreated normally, there were no identifiable developmental defects at birth, and they had normal baseline calcium-related chemistry values. However, KO animals were more responsive to exogenous human parathyroid hormone as evidenced by a greater increase of the serum calcium concentration and urine deoxypyridinoline crosslinks, an effect reversed by CT and mediated by a greater increase in bone resorption than in controls. Surprisingly, KO mice have significantly greater trabecular bone volume and a 1.5- to 2-fold increase in bone formation at 1 and 3 months of age. This effect appears to be mediated by increased bone formation. In addition, KO mice maintain bone mass following ovariectomy, whereas wild-type mice lose approximately one-third of their bone mass over 2 months. These findings argue for dual roles for CT/CGRP gene products: prevention of bone resorption in hypercalcemic states and a regulatory role in bone formation.


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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 1 (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 electroprojection. 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, F₂ or F₃ 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′-CAGGATCAAGTGCTACCAGCCT-3′) that hybridized to CT/CGRP gene exon 1 and reverse primers that hybridized to either the neomycin gene coding region (5′-GGTTGATGGAAGTGTGTGTCG-3′ (KO)) or CT/CGRP intron 1 (5′-GGACCTGACGCTTCAGCAGG-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′-CAGGATCAAGTGCTACCAGCCT-3′ (forward CT and CGRPα), 5′-GAGGTTTGGTTGTGACTGTGC-3′ (exon 4 reverse), and 5′-ACGCCAGAAACCAGCTGCTC-3′ (exon 6 reverse). We detected CGRPα mRNA using primers 5′-ACCCTGCGCAGACGACGG-3′ (exon 3 forward) and 5′-TCTCTGAGGACCTTCATCG-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., Carpenteria, 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 calcine (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.
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 fulcrum, 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

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<td>Baseline biochemical analysis of parameters of calcium metabolism in female mice</td>
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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.

**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.
both CT and CGRPα (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 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). 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).

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...
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/CGRP-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,
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 Cbfα1, 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 cGRPRα in the regulation of bone formation. It is known that cGRPRα-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 cGRPRα/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 cGRPRα precursor peptide has a biologic role. The primary transcript of the CT/cGRPα gene is processed to produce either proCT or proCGRPα. The mature CT or cGRPRα peptide is located centrally within the pro-peptide, and during cleavage, amino-terminal and carboxy-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/cGRPα 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/cGRPα gene product might prove useful in the prevention of bone loss associated with estrogen deficiency.

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