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

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Article

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Ana O. Hoff,[1] Philip Catala-Lehnen,[2] Pamela M. Thomas,[1] Matthias Priemel,[2] Johannes M. Rueger,[2] Igor Nasonkin,[3] Allan Bradley,[3] Mark R. Hughes,[3] Nelson Ordonez,[4] Gilbert J. Cote,[1] Michael Amling,[2] and Robert F. Gagel[1]

[1]Department of Endocrine Neoplasia and Hormonal Disorders, University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA
[2]Department of Trauma Surgery, Hamburg University School of Medicine, Hamburg, Germany
[3]Department of Molecular Genetics, Baylor College of Medicine, Houston, Texas, USA
[4]Department of Pathology, University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA

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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

Address correspondence to: Robert F. Gagel, 1400 Holcombe Boulevard, Box 433, Houston, Texas 77030, USA. Phone: (713) 792-6517; Fax: (713) 794-1818; E-mail: rgagel@mdanderson.org.
Pamela M. Thomas' present address is: Pediatric Specialty Physicians, Lutheran Hospital of Indiana, Fort Wayne, Indiana, USA.
Igor Nasonkin's present address is: Whitehead Institute, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA.
Allan Bradley's present address is: Sanger Centre, Wellcome Trust Genome Campus, Cambridge, United Kingdom.
Mark R. Hughes' present address is: Molecular Medicine and Genetics, Wayne State University, Detroit, Michigan, USA.
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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).

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

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, 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′-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.
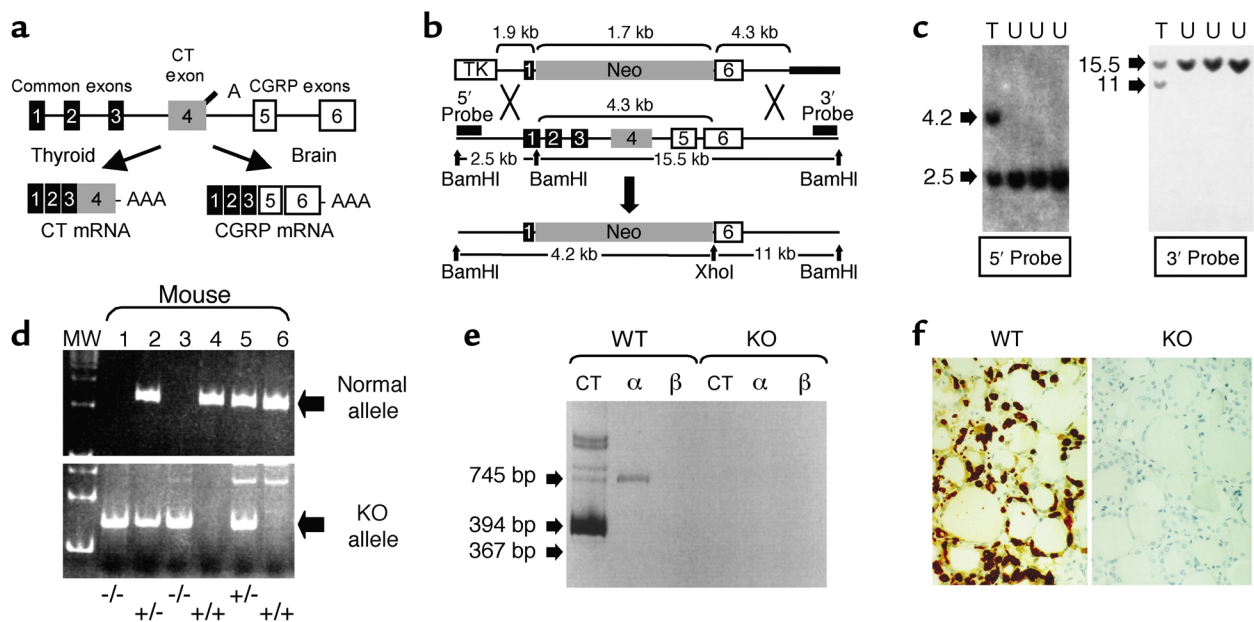
Response to PTH. Serum calcium and urine DPD was assessed in intact WT and KO animals by injection of vehicle (PBS with 0.1 mM HCl and 0.01% BSA) or human PTH (hPTH) (0.5 µg per gram body weight) into the abdominal peritoneal cavity (34). PTH powder (hPTH fragment 1-34; Sigma-Aldrich, St. Louis, Missouri, 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).

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
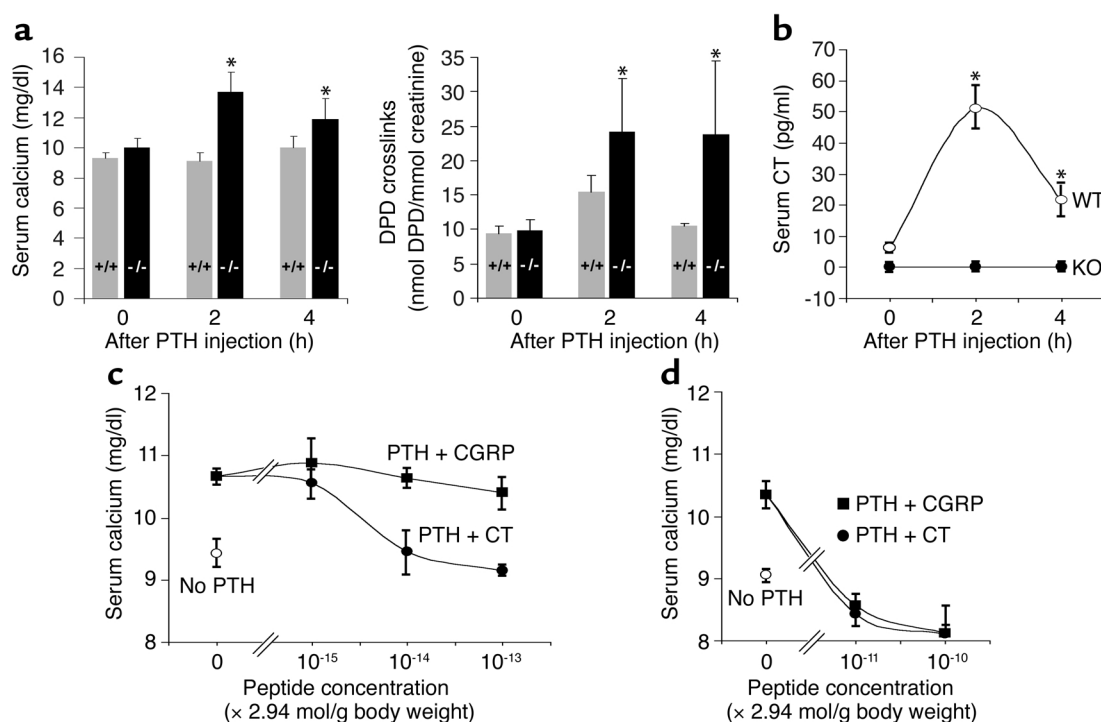parameters. See Methods for the assays used.

histomorphometry system (Osteometrics Inc.,
Atlanta, Georgia, USA). To assess dynamic histomor-
phometric indices, mice were given two injections of
calcein 8 days apart and killed 2 days later according
to a standard double-labeling protocol (35). Fluo-
rochrome measurements were made on two noncon-
secutive 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
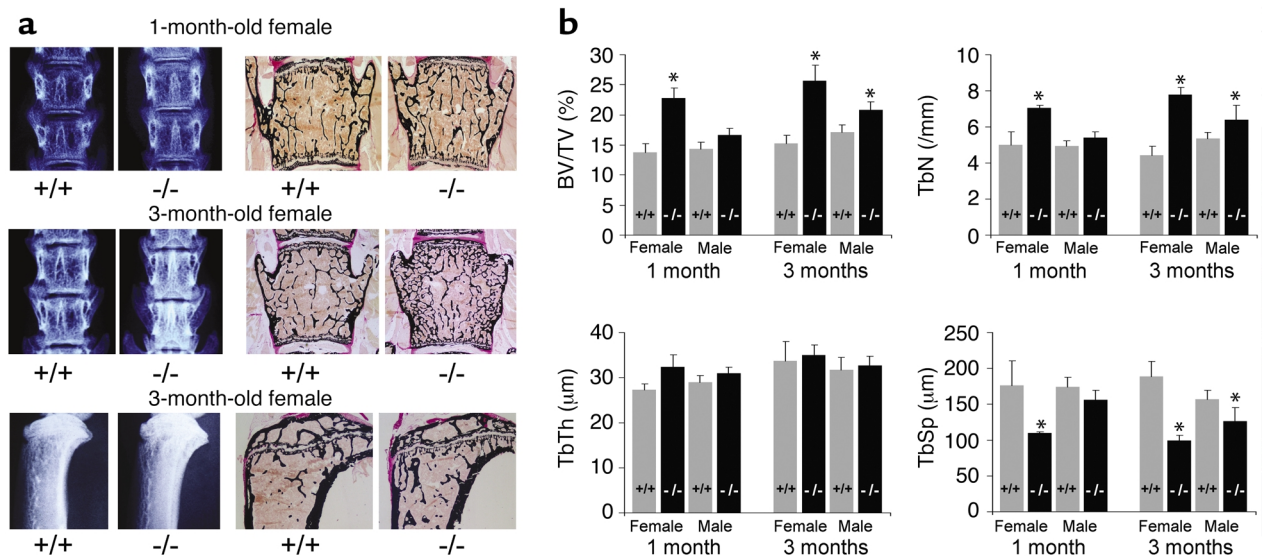accepted as significant, and error bars represent SD.

Biomechanical testing. Both femurs of mice that under-
went 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-pre-
cision 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 dif-
ficult (Figure 1a). Therefore, we opted to create a tar-
geting 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 con-
centration 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 exper-
iments 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.

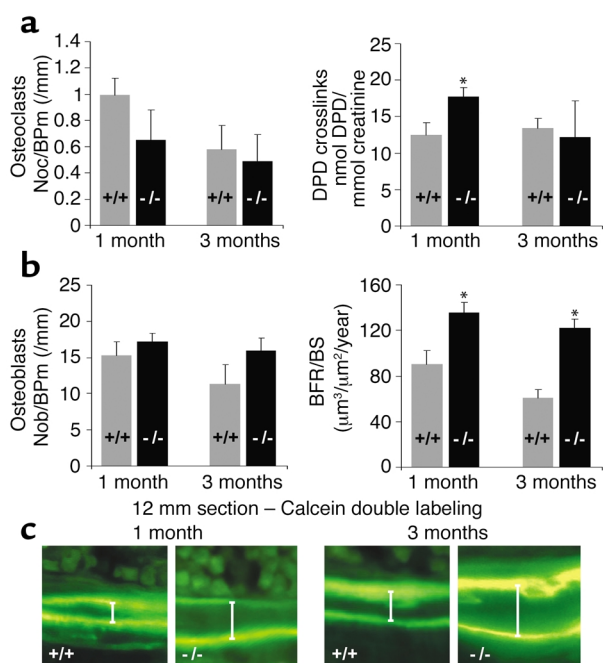both CT and CGRPα (Figure 1b) (Figure 1b). Targeted embryonic stem cells identified by Southern analysis (Figure 1c) were used to generate chimeric founder animals that were bred with C57BL/6 mice. Animals with a homozygous KO of the *CT/CGRP* locus were born and developed normally. Male and female KO 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

a

b

12 mm section – Calcein double labeling

c

1 month   3 months

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 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.
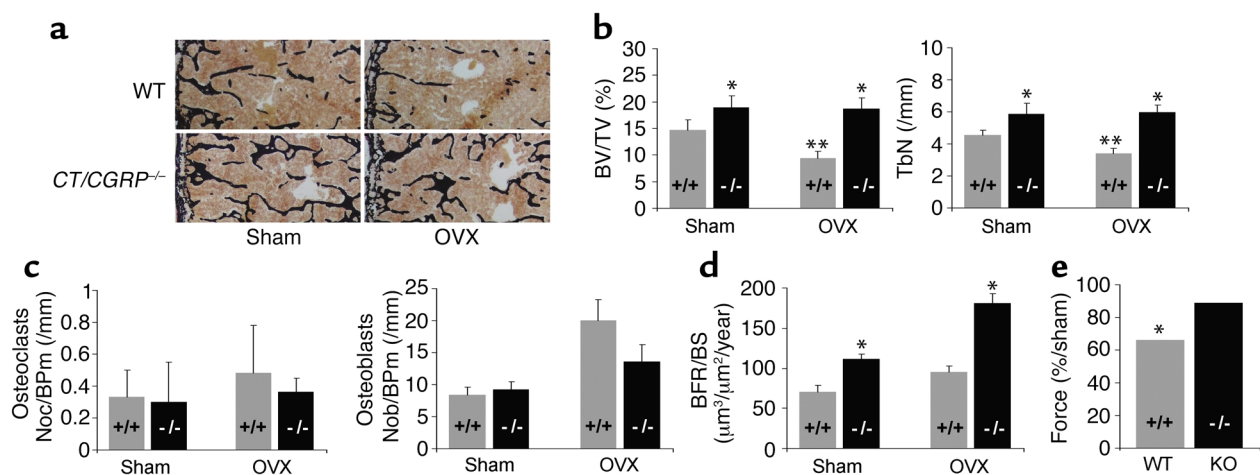
Effects of estrogen deficiency in KO or WT mice. Estrogen deficiency, like exposure to exogenous PTH, produces a perturbation of bone remodeling characterized by increased osteoclastic bone resorption and long-term bone loss. These findings led us to ask whether CT/CGRP-deficient mice would be more sensitive 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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