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Calcitonin Plays a Critical Role in Regulating Skeletal Mineral Metabolism during Lactation

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The maternal skeleton rapidly demineralizes during lactation to provide calcium to milk, responding to the stimuli of estrogen deficiency and mammary-secreted PTH-related protein. We used calcitonin/calcitonin gene-related peptide-α (*Ctcgrp*) null mice to determine whether calcitonin also modulates lactational mineral metabolism. During 21 d of lactation, spine bone mineral content dropped 53.6% in *Ctcgrp* nulls *vs.* 23.6% in wild-type (WT) siblings ($P < 0.0002$). After weaning, bone mineral content returned fully to baseline in 18.1 d in *Ctcgrp* null *vs.* 13.1 d in WT ($P < 0.01$) mice. Daily treatment with salmon calcitonin from the onset of lactation normalized the losses in *Ctcgrp* null mice, whereas calcitonin gene-related peptide-α or vehicle was without effect. Compared with WT, *Ctcgrp* null mice had increased circulating levels of PTH and up-regulation of mammary gland PTH-related protein mRNA. In addition, lactation caused the *Ctcgrp* null skeleton to undergo more trabecular thinning and increased trabecular separation compared with WT. Our studies confirm that an important physiological role of calcitonin is to protect the maternal skeleton against excessive resorption and attendant fragility during lactation and reveal that the postweaning skeleton has the remarkable ability to rapidly recover even from losses of over 50% of skeletal mineral content. (*Endocrinology* 147: 4010–4021, 2006)

THE SKELETON FULFILLS several obvious and important roles; it protects the vital organs, it is the scaffold from which organs and tissues are hung, it enables locomotion on land in a biped or quadruped posture, and it houses the bone marrow. Common clinical concerns about the skeleton include traumatic fractures, fragility fractures from osteoporosis, and a multitude of disorders that crowd the marrow space. Far less well appreciated is the function of the skeleton to serve as a storehouse of minerals and alkali, even to the point of compromising its integrity and strength. Among the times of great demand for calcium and other minerals are lactation, egg laying, and antler formation (1, 2). In each of these time periods, the skeleton undergoes a significant, rapid, and reversible demineralization to provide mineral, respectively, to the neonate, egg shell, and antlers.

Clinical studies have demonstrated (by dual x-ray absorptiometry) that a woman will lose an average of 5–10% of trabecular mineral content during 6 months of near-exclusive lactation and then fully regain it within several months of weaning (reviewed in Refs. 1, 3, and 4). Depending upon a woman's peak bone mass, these losses during lactation are sufficient enough to temporarily reduce her bone density into the osteopenic or osteoporotic range. In contrast, mice and rats will lose approximately 30% of skeletal mineral content (by dual x-ray absorptiometry or by ash weight) during 3 wk of lactation and then fully restore it within 2–3 wk after weaning (1). The rapid and complete recovery from the large losses of skeletal mineral induced by lactation is a clinically important finding, because the adult skeleton normally recovers slowly and incompletely from insults induced by glucocorticoids, prolonged bed rest, menopause, GnRH analog treatment, and weightlessness (1, 5–10).

The mechanism by which the skeleton demineralizes during lactation is not fully understood (1, 11–13). Mammary tissue dramatically up-regulates the expression and release of PTH-related protein (PTHrP), which enters the mother's bloodstream to stimulate bone resorption (12, 14, 15) and, in so doing, mimics the actions of sustained high levels of PTH. At the same time, hyperprolactinemia and other factors suppress the release of GnRH within the hypothalamus, thereby suppressing in sequence the pituitary gonadotropins, ovaries, ovulation, and the sex steroids (especially estradiol) (16). In addition, low estradiol levels of lactation permit enhanced osteoclast-mediated bone resorption and, thereby, potentiate the effect of PTHrP on the skeleton (13).

Calcitonin was originally thought to be an important regulator of calcium and bone metabolism in adults (17), but evidence gathered over the years has largely forced the conclusion that it is a vestigial hormone in mammals (18). The effects of calcitonin to reduce osteoclast-mediated bone resorption (to treat osteoporosis or Paget's disease) or to enhance urinary calcium excretion (to treat hypercalcemia of malignancy) require pharmacological doses of the hormone and not physiological levels (19, 20). However, several investigators had originally proposed that calcitonin might protect the skeleton against excessive resorption during pregnancy and lactation (21–24). The experimental approach

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Abbreviations: BMC, Bone mineral content; BMD, bone mineral density; CaT1, calcium transport protein subtype 1; CGRPα, calcitonin gene-related peptide-α; μCT, microcomputed tomography; WT, wild type.

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to test this hypothesis had been to remove the thyroid glands from sheep, goats, or rats, to replace thyroid hormone, and to determine the effect of a full cycle of pregnancy and lactation on the maternal bone mineral content (BMC) or ash weight of these thyroidectomized *vs.* sham-operated animals (21–24). Although some investigators determined that these presumably calcitonin-deficient animals had reduced BMC at the time of weaning, other investigators found no such effect (21–24). It wasn't appreciated at the time that thyroidectomized animals were not devoid of calcitonin because of extrathyroidal sources of calcitonin in mammary tissue (25, 26), placenta (27, 28), and pituitary (29). Thyroidectomized women experience increases in circulating calcitonin during lactation and express normal levels of calcitonin in milk (26).

We have retested the hypothesis that physiological levels of calcitonin are required to protect the maternal skeleton against excessive resorption during pregnancy and lactation. To do this, we used an established murine model in which exons 3–5 of the calcitonin/calcitonin gene-related peptide-α gene (*Ctcgrp*) have been ablated (30). Calcitonin gene-related peptide-α (CGRPα) is an alternative splice product of the calcitonin gene, whereas a second gene produces CGRPβ. Therefore, *Ctcgrp* null mice completely lack calcitonin in all tissues but still produce CGRP through the CGRPβ gene. Using this model, we determined that ablation of the gene doubled the losses of maternal mineral content during lactation and that the effect was specific to loss of calcitonin and not CGRPα.

Materials and Methods

Animal husbandry

Ctcgrp null mice were obtained by targeted ablation of exons 3–5 of the murine gene and genotyped by PCR as previously described (30). The original strain was back-crossed into Black Swiss (Taconic, Germantown, NY) for at least six generations, and the colony was maintained by breeding heterozygous-deleted mice together. Virgin first-degree relative pairs of wild-type (WT) and *Ctcgrp* null females were selected for study after 10 wk of age (attainment of peak bone mass). Mice were mated overnight, and the presence of a vaginal mucus plug on the morning after mating marked gestational d 0.5. Normal gestation in these mice is 19 d. We planned to cull litters within 48 h of delivery if needed to maintain similar numbers of pups (8 ± 1) per mouse, and weaning was forced at 21 d by removing the pups from the mother. All mice were given a standard chow (1% calcium, 0.75% phosphorus) diet and water *ad lib*. All studies were performed with the previous approval of the Institutional Animal Care Committee of Memorial University of Newfoundland.

Reproductive cycles and data collection time points

These studies were completed according to an approximate 70-d reproductive cycle. Baseline scans were collected for 5–10 d before mating and subsequent pregnancy (prepregnancy interval). Pregnancy lasted 19 d, lactation lasted 21 d, and postweaning recovery was 21 d. The four main data collection points included prepregnancy, end of pregnancy (day before expected delivery), end of lactation (forced weaning at d 21 of lactation), and end of postweaning recovery of the skeleton (d 21 after weaning). Additional time points included start of lactation (24–48 h), early lactation (5–7 d), and mid-lactation (10–14 d).

BMC and bone mineral density (BMD)

BMC and BMD were measured using the PIXImus 2 Bone Densitometer (GE Lunar, Madison, WI) and analyzed with PIXImus software version 2.1. A standard phantom (fat 11.9% and BMC 0.063 g) was used to calibrate the PIXImus daily. Anesthesia was induced with isoflurane (Baxter Corp., Toronto, Ontario, Canada) and maintained with a single ip injection of either thiopental sodium (Abbot Laboratories, Toronto, Ontario, Canada) or a 5:1 combination of ketamine hydrochloride (Wyeth Canada, Guelph, Ontario, Canada) and xylazine (Bayer Inc., Toronto, Ontario, Canada). The anesthetized mice were immobilized prone on a plastic tray with the spine straightened, and to maintain reproducibility with less than 1% precision error, the head was excluded in all scans. In additional quality control studies, mice on d 18.5 of pregnancy were scanned immediately before and after a cesarean section in which the pups were removed; this determined that the fetal skeletons contributed 1% or less to the apparent maternal BMC or BMD and were, therefore, negligible. Total body (minus head) and regional (spine and hind limb) BMC and BMD measurements were obtained for each mouse, and the absolute values were normalized to the nonpregnant baseline measurements. In the initial studies to determine the pattern of BMC and BMD change (comprising data in Fig. 1), each mouse was scanned every 2 d throughout the entire reproductive cycle. In later studies in which tissue would be harvested at specific time points, the mice were scanned at the main time points to ensure that the expected BMC and BMD changes were occurring. There were a few anesthetic-related deaths, but otherwise, the outcomes of the reproductive cycle (live pups and magnitude of BMC loss and recovery) were no different between dams that had been scanned every 2 d *vs.* those that had been scanned at the main time points. Pups that had received *in utero* exposure to x-rays were euthanized at weaning, whereas the dams were euthanized at the end of the postweaning recovery phase.

Chemical and hormone assays

Serial blood samples were taken from tail veins, whereas a cardiac puncture was done to obtain larger samples at the time that each mouse was euthanized. Urine was collected by allowing the mice to void into a clean, empty cage. Ionized calcium was measured on whole blood using a Chiron Diagnostics 634 Ca^{2+}/pH Analyzer (Chiron Diagnostics, East Walpole, MA). Urine calcium was measured using a colorimetric assay (Sigma-Aldrich, Oakville, Ontario, Canada). PTH was measured using a rat PTH 1–34 ELISA kit (Immutopics, San Clemente, CA). Osteocalcin was assessed using a two-site immunoradiometric assay (Immutopics). Prolactin was assessed using a RIA by Dr. A. F. Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA). Estradiol was measured with an enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI). Plasma PTHrP was measured using a sensitive RIA with an antibody directed to an amino-terminal epitope on samples that had been collected in a cocktail of aprotinin and EDTA (31). Deoxypyridinoline was measured using the METRA DPD enzyme immunoassay kit (Quidel Corp., San Diego, CA). Urinary measurements of deoxypyridinoline and calcium were expressed relative to creatinine to correct for variations in urine concentration. Creatinine was measured using an alkaline picrate colorimetric assay (Diagnostic Chemicals Ltd., Charlottetown, Prince Edward Island, Canada).

Milk collection and analysis

On d 7 of lactation, anesthetized mice were injected with 1.2 U oxytocin (Hospira Healthcare Co., Montreal, Quebec, Canada). After 1–2 min, milk was obtained using a self-designed pump consisting of a 1-ml pipette tip (Mandel Scientific Co. Inc., Guelph, Ontario, Canada) inserted into thick rubber tubing that in turn was connected to vacuum suction. The wide end of the pipette tip was placed over a nipple, and a gentle pumping action was used to obtain milk, which was then stored at −20 C until assayed. The milk was diluted 1:100 with distilled water and analyzed for calcium content using a Model 2380 Atomic Absorption Spectrophotometer (PerkinElmer, Norwalk, CT). Because much of the calcium content of milk is bound to proteins (32), and to control for differing milk concentrations, the protein content of milk was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL). Final results are expressed as micrograms calcium per microgram protein.

Duodenal calcium absorption

We adapted the method of Fleet and colleagues (33) to measure duodenal calcium absorption *in vivo* during mid-lactation and in non-

pregnant mice. Food was removed from the cages 3 h before the experiment to empty the duodenum and jejunum. Under anesthesia, an abdominal incision was made and a closed duodenal loop was created by tying two ligatures, one at the pyloric juncture and the second one about 1.5 cm distal to the first one. Forty microliters of ^{45}Ca buffer (150 nM NaCl, 2 mm $CaCl_2$, 30 mM Tris-HCl, and 16 μCi ^{45}Ca/ml at pH 7.4) were injected into the closed loop (^{45}Ca was obtained from Promega/Fisher Scientific Ltd., Burlington, Ontario, Canada). After 10 min, the loop was inspected to ensure that no areas of ischemia were evident. The loop was excised, solubilized in Scintigest (Promega/Fisher), and then counted on a liquid scintillation counter. To measure the total ^{45}Ca activity administered, and to control for the effect of biological tissue to quench the apparent level of radioactivity, additional scintillation vials were prepared in which the same amount of ^{45}Ca and an identical length of nonradioactive duodenum were added. Efficiency of ^{45}Ca absorption was expressed as (1 − ^{45}Ca remaining in each loop/amount of ^{45}Ca injected into the loop) × 100.

Biomechanical analysis

Left hind limbs were stored at −20 C after first removing the soft tissues and then thawed to room temperature in normal saline before analysis. Cortical bone strength was assayed using an Instron Series 3340 electromechanical test instrument (Instron, Norwood, MA). In brief, the femur was immobilized at each end in a fixture, and a load cell with maximum capacity of 10 N of force was positioned 2–3 cm above the mid-shaft of the tibia. The force required to break the femur (failure) was recorded, as was the load-displacement curve from which other biomechanical parameters could be derived.

Rescue experiments

Beginning on the day of delivery and for 21 d of lactation, selected WT and *Ctcgrp* null mice received once daily im injections of 0.9% saline (vehicle), salmon calcitonin (Rhone-Poulenc Rorer, Collegeville, PA), or rat CGRPα (American Peptide Co., Sunnyvale, CA). Mice were briefly anesthetized with isoflurane to receive the injection. Bone densitometry was used every second day throughout the experiment to precisely determine the excursion of BMC.

Microcomputed tomography (μCT) analysis

Right hind limbs and lumbar spines were harvested, adjacent flesh was removed, and then the samples were fixed in paraformaldehyde for 18 h. The bones were then rinsed and stored in 10× PBS at room temperature until analysis. The quantitative and qualitative μCT analysis for each femur and for the first and second lumbar vertebrae was completed at the Centre for Bone and Periodontal Research at McGill University using the SkyScan 1072 (SkyScan, Aartselaar, Belgium), as previously described (34).

Mammary tissue collection and storage

Mammary glands were harvested after a rapid cervical dislocation during late pregnancy, mid-lactation, and late lactation. Lymph nodes were removed and mammary tissue was washed with water containing 0.1% diethylpyrocarbonate. Tissues were either snap-frozen in liquid nitrogen and then placed at −70 C or placed immediately in 10% formalin [37% formaldehyde, 10× PBS (pH 7.3), and distilled water].

RNA extraction and cDNA synthesis

RNA was extracted from whole mammary glands using an RNeasy Midi Kit (QIAGEN, Mississauga, Ontario, Canada). Approximately 100 g of mouse mammary gland yielded 0.6–1.3 μg RNA/μl solution. cDNA synthesis was carried out with the SuperScript III First-Strand Synthesis System (Invitrogen Canada Inc., Burlington, Ontario, Canada).

Real-time quantitative RT-PCR

cDNA synthesis was performed using the Superscript III First-Strand Synthesis System (Invitrogen) as previously described (35). Quantitative real-time RT-PCR data and analysis were obtained using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), also as previously described (35). For the calcitonin receptor, the calcium receptor, and the vitamin D receptor, TaqMan Gene Expression Assays were used that included predesigned primers and probes for optimal amplification (Applied Biosystems) along with TaqMan Universal PCR Master Mix (Applied Biosystems). All gene expression assays have a FAM reporter dye at the 5′ end of the TaqMan MGB probe and a nonfluorescent quencher at the 3′ end of the probe. Other gene-specific primers used for this study were as follows: for PTHrP, forward 5′-TCCACACAGCCGAAATCAGAGCTA-3′ and reverse 5′-TTCTCCTGTTCTCTGCGTTTCCCA-3′; for 1-α-hydroxylase, forward 5′-CCAGAGCGCTGTAGTTTCTCATCA-3′ and reverse 5′ATGAAGGTTTCTGTGTCAGGAGGG-3′; for calcium transport protein subtype 1 (CaT1), forward 5′-ATCGATGGCCCTGCGAACT-3′ and reverse 5′-CAGAGTAGAGGCCATCTTGTTGCTG-3′ (36); and for epithelial calcium channel (ECaC or CaT2), forward 5′-ATTGACGGACCTGCCAATTACAGAG-3′ and reverse 5′-GTGTTCAACCCGTAAGAACCAACGGTC-3′ (36). These primers were used along with SYBR Green PCR Master Mix (Applied Biosystems). The real-time PCR thermal cycler protocol included one cycle at 50 C for 2 min, one cycle at 95 C for 10 min, and 40 cycles of 95 C for 15 sec and 1 min at 60.0 C. For primers using SYBR Green PCR Master Mix, the real-time PCR program was the same but without the first cycle at 50 C for 2 min. All samples were analyzed in triplicate. Relative expression ratios were representative of the threshold cycle (the PCR cycle at which an increase in reporter fluorescence is above a baseline signal) normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and compared with the WT.

Statistical analysis

Data were analyzed using SYSTAT 5.2.1 for Macintosh (SYSTAT Inc., Evanston, IL). ANOVA was used for the initial analysis; a *post hoc* test (Tukey's) was used to determine which pairs of means differed significantly from each other. Real-time PCR results were analyzed by the $2^{-\Delta\Delta C}$T method where the target and reference are amplified in separate wells (37). Two-tailed probabilities are reported, and all data are presented as mean ± SE.

Results

BMC during pregnancy, lactation, and recovery

At the baseline age of 10–12 wk, and consistent with an earlier report (30), *Ctcgrp* null mice had a BMC of 0.397 ± 0.02 g *vs.* 0.383 ± 0.02 g in WT ($P = 0.61$). At the level of the whole body, WT and *Ctcgrp* null mice gained a similar degree of BMC during 18 d of pregnancy, but *Ctcgrp* null mice lost approximately twice the amount of BMC as WT during 21 d of lactation (Fig. 1A). The difference was present from early in lactation; WT mice lost 4.5 ± 3.6 and 15.7 ± 3.7% of baseline BMC by 7 and 14 d of lactation, respectively, whereas *Ctcgrp* null mice lost 10.1 ± 3.1 and 29.6 ± 3.1% of baseline BMC at the same time points. Both genotypes restored their BMC to their respective baseline values after weaning (Fig. 1A), although full recovery took several more days for *Ctcgrp* null mice (Fig. 1B). Closer examination of the BMC excursion indicated that the lactational losses were much more profound in the trabecular-rich vertebral bodies, such that *Ctcgrp* null mice lost more than half of their baseline BMC in the spine (Fig. 1C). At the hind limb, which contains relatively more cortical bone than the vertebral bodies, the losses were proportionately less, but *Ctcgrp* null mice still lost twice the WT value (Fig. 1D). The losses were not a result of any difference in litter sizes, which did not require any culling and were 8.9 ± 0.9 in WT and 7.6 ± 1.2 in *Ctcgrp* null (P = not significant).

The enhanced skeletal demineralization during lactation indicates increased bone resorption in the *Ctcgrp* null mice.

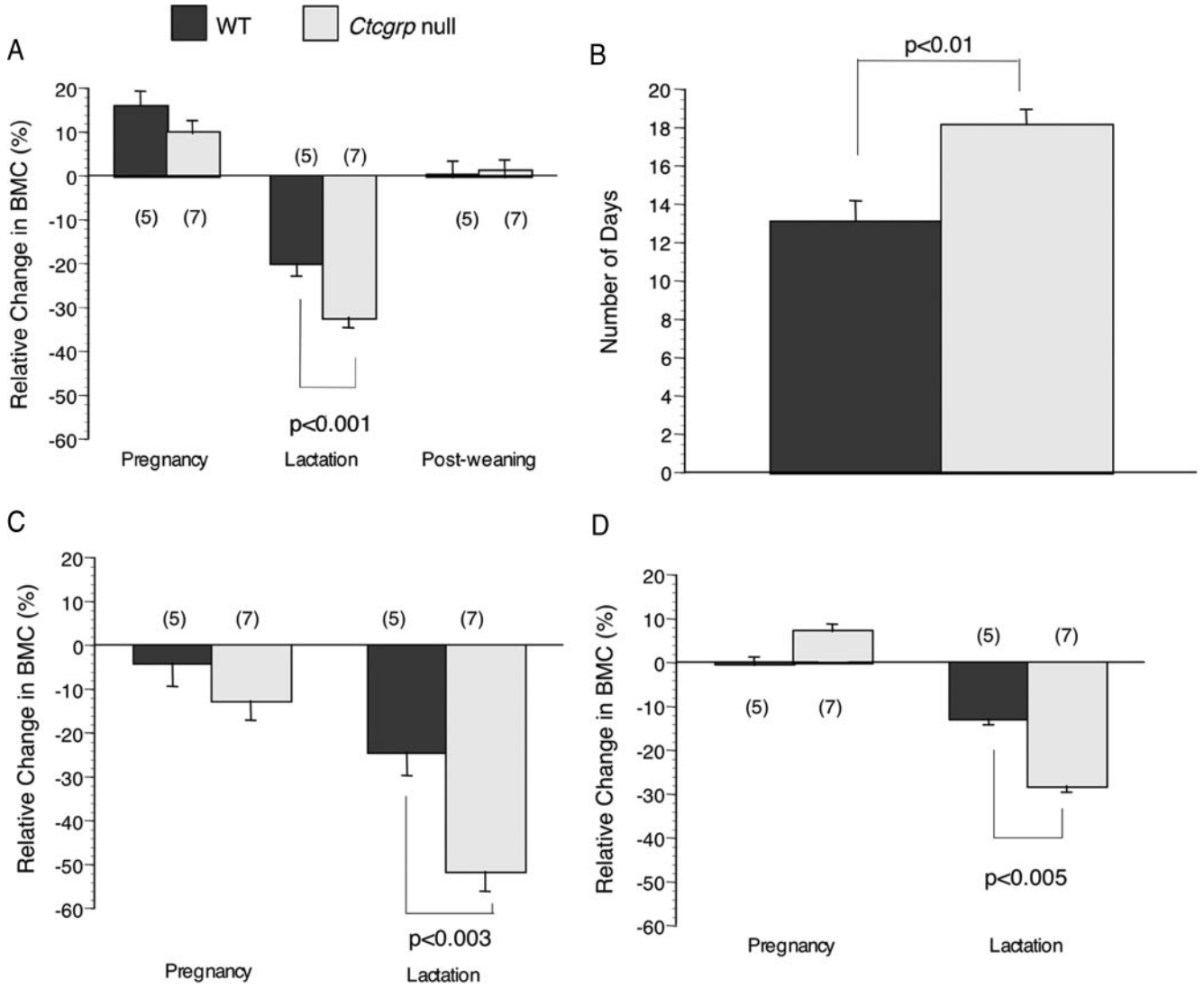

FIG. 1. *Ctcgrp* null mice lose more BMC during lactation but recover fully post weaning. A, Total-body BMC at end of pregnancy, lactation, and post weaning expressed as a percentage of prepregnancy baseline BMC; B, mean days taken to recover to baseline BMC post weaning; C, spine BMC; D, hind limb BMC. All values are mean ± SE. The *numbers in parentheses* indicate the numbers of mice studied.

Consistent with this, the urinary excretion of deoxypyridinoline/creatinine (a marker of bone resorption) was increased significantly in *Ctcgrp* null *vs.* WT mice during lactation (Fig. 2A). On the other hand, no difference was observed in the serum level of osteocalcin (a marker of bone formation) between *Ctcgrp* null and WT mice at any time point (Fig. 2B).

Mineral and hormones

We measured mineral and hormonal indices to examine possible mechanisms and consequences of the enhanced bone demineralization observed in *Ctcgrp* null mice. In our laboratory, WT mice in several inbred, outbred, and mixed strains maintain a normal ionized calcium during pregnancy and lactation and have suppressed levels of PTH during the same intervals compared with prepregnancy (not shown). Both WT and *Ctcgrp* null mice generally maintained a normal ionized calcium throughout pregnancy, lactation, and post weaning (Fig. 3A); however, *Ctcgrp* null mice were transiently hypercalcemic during the first 24–48 h postpartum at 1.40 ± 0.02 mmol/liter *vs.* 1.26 ± 0.02 mmol/liter in WT mice ($P < 0.001$). Urinary excretion of calcium normally decreases during lactation, likely because of the effect of PTHrP to enhance urinary calcium reabsorption. *Ctcgrp* null and WT mice both showed the same trend to decreased urinary calcium excretion during lactation but with no significant differences between the genotypes at any time point (Fig. 3B).

Serum PTH is normally suppressed to the lower end of the normal range during pregnancy and lactation, and this was observed in the WT mice (Fig. 3C). Although PTH was also reduced during pregnancy in *Ctcgrp* null mice, PTH became significantly elevated in *Ctcgrp* null mice during lactation and may have contributed to the enhanced demineralization observed in these mice (Fig. 3C). The elevation in PTH occurred during the interval of normocalcemia and not during the initial 24–48 h postpartum in which the ionized calcium was transiently elevated. In a separate analysis of serum

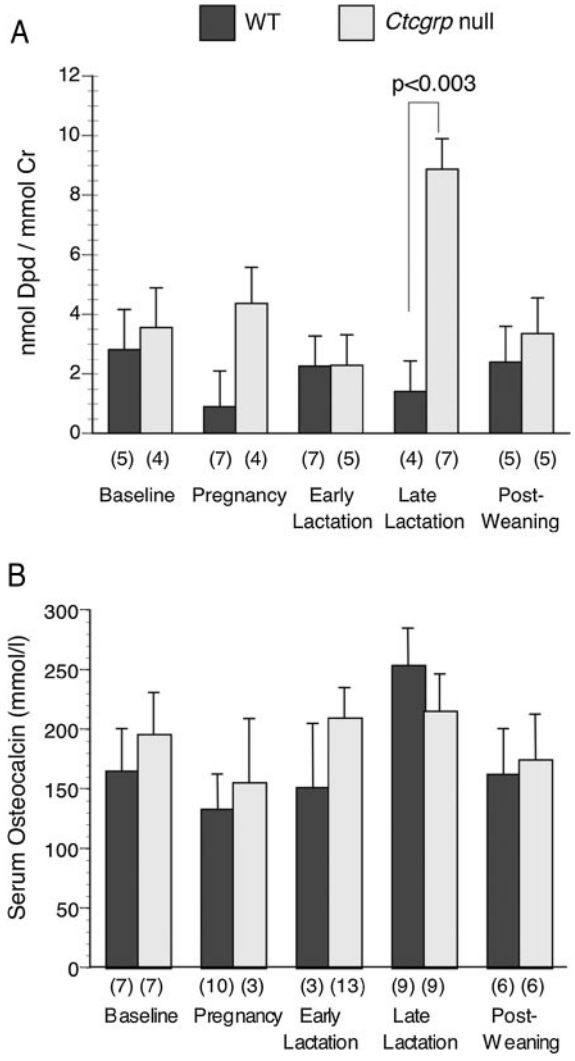

FIG. 2. Bone formation and resorption markers during the reproductive cycles. A, Urine deoxypyridinoline/creatinine (bone resorption marker) was elevated in *Ctcgrp* null mice *vs.* WT during lactation; B, serum osteocalcin (bone formation marker) showed no significant differences between genotypes at any time point. The *numbers in parentheses* indicate the numbers of mice studied.

obtained at 24–48 h (to correspond with the transient hypercalcemia), PTH levels were suppressed to the limit of detection (1.6 pg/ml) in both WT and *Ctcgrp* null mice (P = not significant). Estradiol is normally suppressed during lactation as a consequence of hyperprolactinemia, and low estradiol contributes to the skeletal resorption during this time period. We found that estradiol levels at mid-lactation were suppressed to the level of sensitivity of the estradiol assay (8 pg/ml) in all samples from both WT and *Ctcgrp* null mice (P = not significant). Prolactin levels (Fig. 3D) were also not significantly different between *Ctcgrp* null and WT mice at any time point.

We assayed duodenal ^{45}Ca absorption at mid-lactation in WT and *Ctcgrp* null mice to rule out impaired intestinal absorption as an indirect cause of the enhanced skeletal resorption in *Ctcgrp* null mice. Efficiency of absorption was greater than 80% at 10 min in both WT and *Ctcgrp* null mice and not significantly different from the nonpregnant WT value (Fig. 4A).

Given that the *Ctcgrp* null mice are resorbing more bone than WT but are not losing the excess calcium in urine and do not have impaired intestinal calcium absorption, we expected to find increased calcium content in milk. There are several challenges to measuring milk content in mice: milk content is highly variable within and between feeds (38, 39), much of the calcium is bound to proteins (32), and collecting milk is technically difficult. Despite these issues, we found an apparent (but not statistically significant) increase in milk calcium content corrected from the protein content (Fig. 4B).

PTHrP in lactating mammary tissue and circulation

Given the established importance of mammary-derived PTHrP to stimulate skeletal demineralization during lactation, we measured both mRNA and protein levels of this hormone. Within mammary tissue, PTHrP mRNA was significantly elevated as assessed by real-time quantitative RT-PCR (Fig. 5A), and the protein level was visibly increased by immunohistochemistry (Fig. 5B). Within the circulation, the plasma PTHrP level showed no significant difference during lactation compared with WT, with most values clustered near the assay detection limit of 2 pmol/liter (Fig. 5C).

Other calciotropic hormones and receptors within lactating mammary tissue

We also examined the expression of other calciotropic genes that are known to be expressed in mammary tissue, including the mRNAs for the calcium receptor, calcitonin receptor, 1-α-hydroxylase, and the vitamin D receptor. In addition, mRNAs for the epithelial calcium channels (CaT1 and CaT2) were also examined. There was no difference in the expression of any of these mRNAs between WT and *Ctcgrp* null mice, whereas CaT1 and CaT2 were not detected (data not shown).

Skeletal strength and microarchitecture

We also examined the consequences of enhanced demineralization during lactation on skeletal microarchitecture of the femur and lumbar vertebrae and on the strength of the tibia. μCT measurements demonstrated that *Ctcgrp* null mice had greater trabecular thickness and more closely spaced trabeculae at baseline compared with WT in both femora and lumbar vertebrae (Table 1). *Ctcgrp* null mice experienced more marked trabecular thinning and reached an equivalent level to WT at the end of lactation (Table 1). Trabecular spacing increased significantly in *Ctcgrp* null femora and approached the level of the WT at baseline, whereas trabecular spacing did not change significantly within vertebrae (Table 1). The μCT results also indicate a greater trabecular bone volume at baseline in *Ctcgrp* null mice *vs.* WT. Whereas WT trabecular bone volume increased significantly during lactation (a normal finding), the changes were blunted in *Ctcgrp* null bones at both the femora and vertebrae (Table 1).

Bone strength was assessed by the three-point bend test of the femur, a measurement that reflects cortical bone strength more than trabecular strength. Ultimate load required to

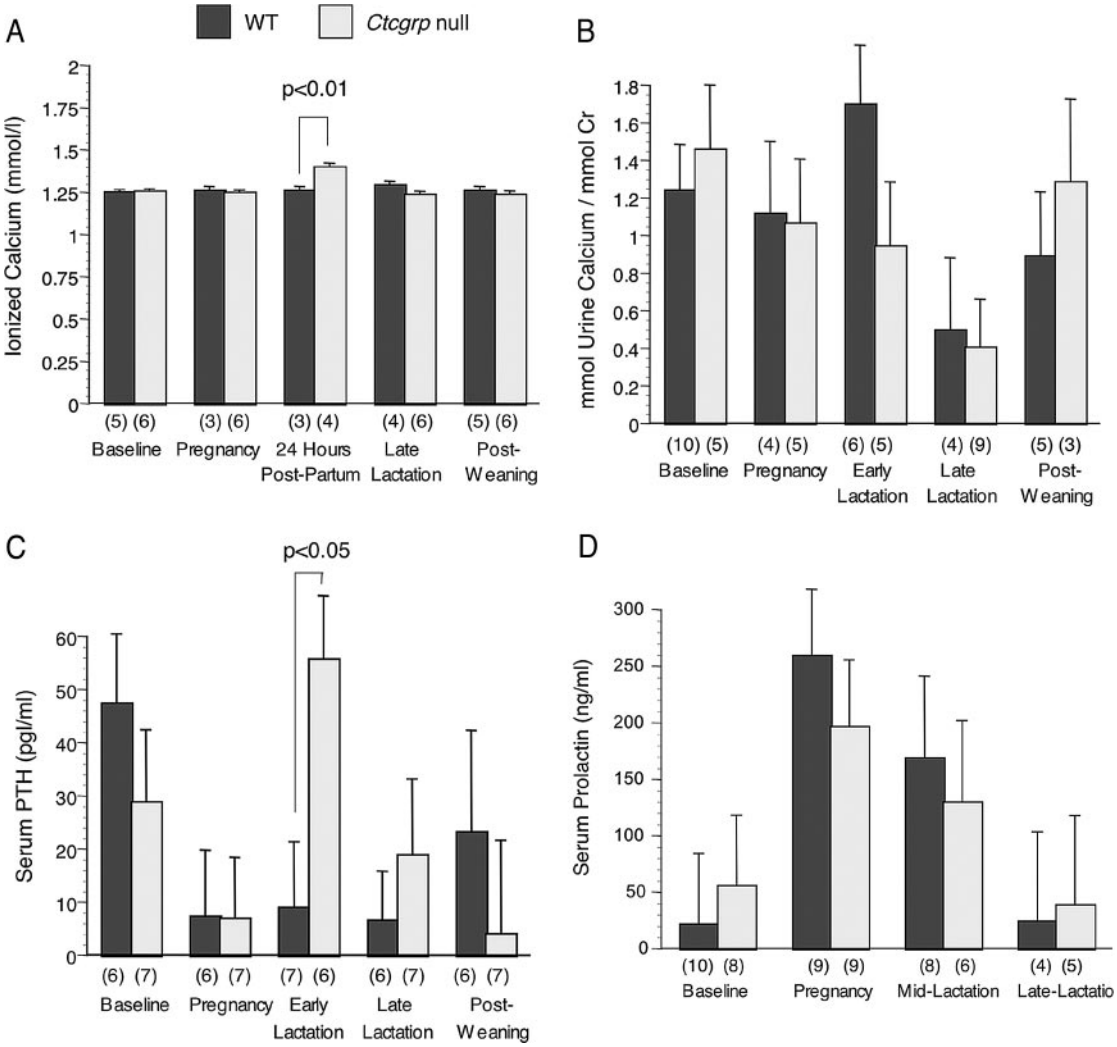

FIG. 3. Chemistry and hormone measurements. A, Ionized calcium remained constant throughout the reproductive cycle and showed no difference between *Ctcgrp* null and WT at the main time points apart from 24–48 h postpartum; B, urine calcium/creatinine showed a trend to decreased calcium excretion during lactation as expected, but no significant difference between genotypes; C, serum PTH was elevated during lactation in *Ctcgrp* null *vs.* WT but suppressed at 24–48 h postpartum (see text); D, serum prolactin was increased during pregnancy and early lactation but was no different between the genotypes. The *numbers in parentheses* indicate the numbers of mice studied.

cause breakage showed an apparent decrease from baseline to the end of lactation in both WT and *Ctcgrp* null mice but was not significantly different within or between groups (Table 2). Not included in the calculations were two *Ctcgrp* null femurs that were evidently fragile because they broke upon being placed into the device and thus could not be subjected to the three-point bend test. Additional intrinsic and extrinsic bone parameters are shown in Table 2 that suggest that cortical bone strength is not significantly different between WT and *Ctcgrp* null mice at any time point.

Rescue of skeletal phenotype during lactation

Lastly, we determined whether the enhanced skeletal losses during lactation were a consequence of absence of calcitonin, CGRPα, or both. Preliminary dose-finding experiments indicated that a mean dose of 10 IU salmon calcitonin daily would prevent the excessive losses of lactation in *Ctcgrp* null mice; larger doses reduced the normal losses during lactation, whereas 1 IU daily had minimal effect (not shown). Starting at the day of delivery and continuing for 21 d, WT and *Ctcgrp* null mice were treated with daily injections of calcitonin or vehicle, and several *Ctcgrp* null mice were treated with an equimolar dose (255 μg) of CGRPα. The mean losses of BMC observed in saline or CGRPα-treated mice were similar, showing enhanced losses approximately double that of WT (Fig. 6). In contrast, treatment with calcitonin resulted in lactational losses that were no different from those observed in saline-treated WT mice (Fig. 6). Calcitonin treatment was without visible effect in WT mice (Fig. 6).

Discussion

We have compared *Ctcgrp* null mice to their WT siblings to test the hypothesis that calcitonin protects the maternal skeleton from excessive resorption during pregnancy and lactation. We found that calcitonin was not required during pregnancy, which is an interval of net bone formation as

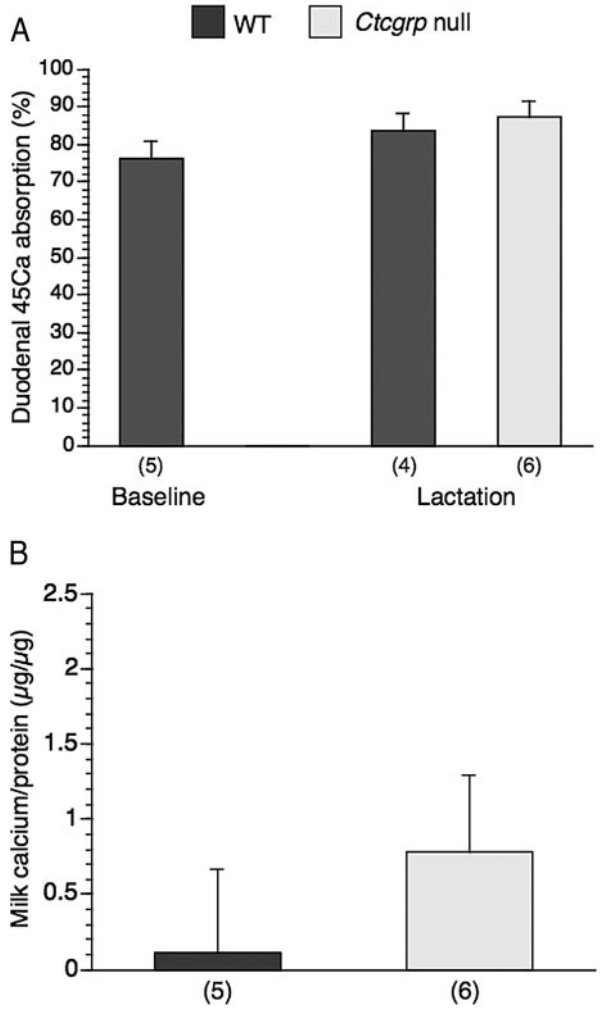

FIG. 4. Duodenal calcium absorption and milk calcium content. A, The efficiency of ^{45}Ca absorption at 10 min was no different between WT and *Ctcgrp* null during mid-lactation, and both showed a nonsignificant increase over the nonpregnant WT value; B, the calcium content of expressed milk, corrected for protein content, showed a higher mean value that was not statistically different from the WT value. *Numbers in parentheses* indicate the numbers of mice studied.

indicated by the rise in maternal BMC between mating and delivery. On the other hand, we found that calcitonin is required during lactation because *Ctcgrp* null mice lost about twice the amount of BMC during lactation as their WT relatives, with more profound losses at the trabecular-rich spine to the extent that over half of baseline BMC was lost. The enhanced losses were accompanied by increased circulating PTH and mammary expression of PTHrP, transient hypercalcemia in the first 24 h postpartum, increased bone resorption, normal intestinal calcium absorption, and apparently increased milk calcium content. Despite the dramatic losses in BMC, cortical bone strength was not significantly different between *Ctcgrp* null and WT mice at the end of lactation. The excessive resorption in *Ctcgrp* null mice was prevented by treatment with daily injections of calcitonin but not by treatment with CGRPα, confirming that the skeletal phenotype is specific to loss of calcitonin and not to loss of CGRPα. Finally, *Ctcgrp* null mice fully regained to their baseline BMC after weaning, demonstrating the skeleton's tremendous ability to repair itself from a greater than 50% deficit.

Physiological role for calcitonin during lactation

Our confirmation of a physiological role for calcitonin during lactation brings closure to a hypothesis that had been raised more than 30 yr ago but left unresolved (21–24). The inconsistent results among these early investigations likely reflected the confounding effects of thyroid ablation and thyroid hormone replacement, but especially the previously unrecognized fact that extrathyroidal sources of calcitonin existed (25, 27, 29). Only through the use of a gene ablation model has it become possible to examine the effect of complete absence of calcitonin on skeletal metabolism during pregnancy and lactation.

Ctcgrp null mice have enhanced skeletal resorption as demonstrated by the large drop in BMC from either prepregnancy or peak of pregnancy to end of lactation, the trabecular thinning and increased trabecular separation on μCT, and the elevated excretion of urinary deoxypyridinoline/creatinine. The transient hypercalcemia in the first 24 h postpartum may be a consequence of enhanced bone resorption flooding the maternal circulation with calcium at a time when milk production is not fully established; the ionized calcium was normal throughout the rest of lactation.

Calcitonin evidently plays a central role in modulating skeletal responsiveness during lactation, a role that may not be limited to its direct actions on osteoclasts. The presence of calcitonin and its receptor in mammary tissue (25) and pituitary (29, 40) indicates that there are several possible routes through which absence of calcitonin may lead to enhanced bone resorption in *Ctcgrp* null mice. We will consider the osteoclast, pituitary, and mammary tissue in turn.

Calcitonin, osteoclasts, and increased trabecular bone resorption

During lactation, enhanced osteoclast-mediated bone resorption is driven by a hormonal milieu in which circulating PTH is low, PTHrP is elevated, estradiol is low, and calcitonin is elevated (1, 3, 4). In *Ctcgrp* null mice, circulating PTH was elevated, mammary gland expression of PTHrP was up-regulated above normal, estradiol was low, and calcitonin was absent. The circulating level of PTHrP was not significantly increased in *Ctcgrp* null mice, but the assay sensitivity (2 pmol/liter) was likely insufficient to detect a true difference given that most values were clustered near the detection limit. PTH and PTHrP are equivalent in their ability to activate the PTH/PTHrP receptor on osteoblasts and communicate through the RANK-RANKL-OPG pathway to stimulate osteoclast-mediated bone resorption (41, 42); in contrast, calcitonin acts directly on osteoclasts to inhibit bone resorption (18). Thus, the mechanism for enhanced bone resorption in the *Ctcgrp* null mice may be simply a result of exaggerated stimulation of PTH/PTHrP receptor-mediated bone resorption by both PTH and PTHrP, coupled with absence of the effect of calcitonin to directly inhibit the activity of osteoclasts. In turn, pituitary and mammary tissue may

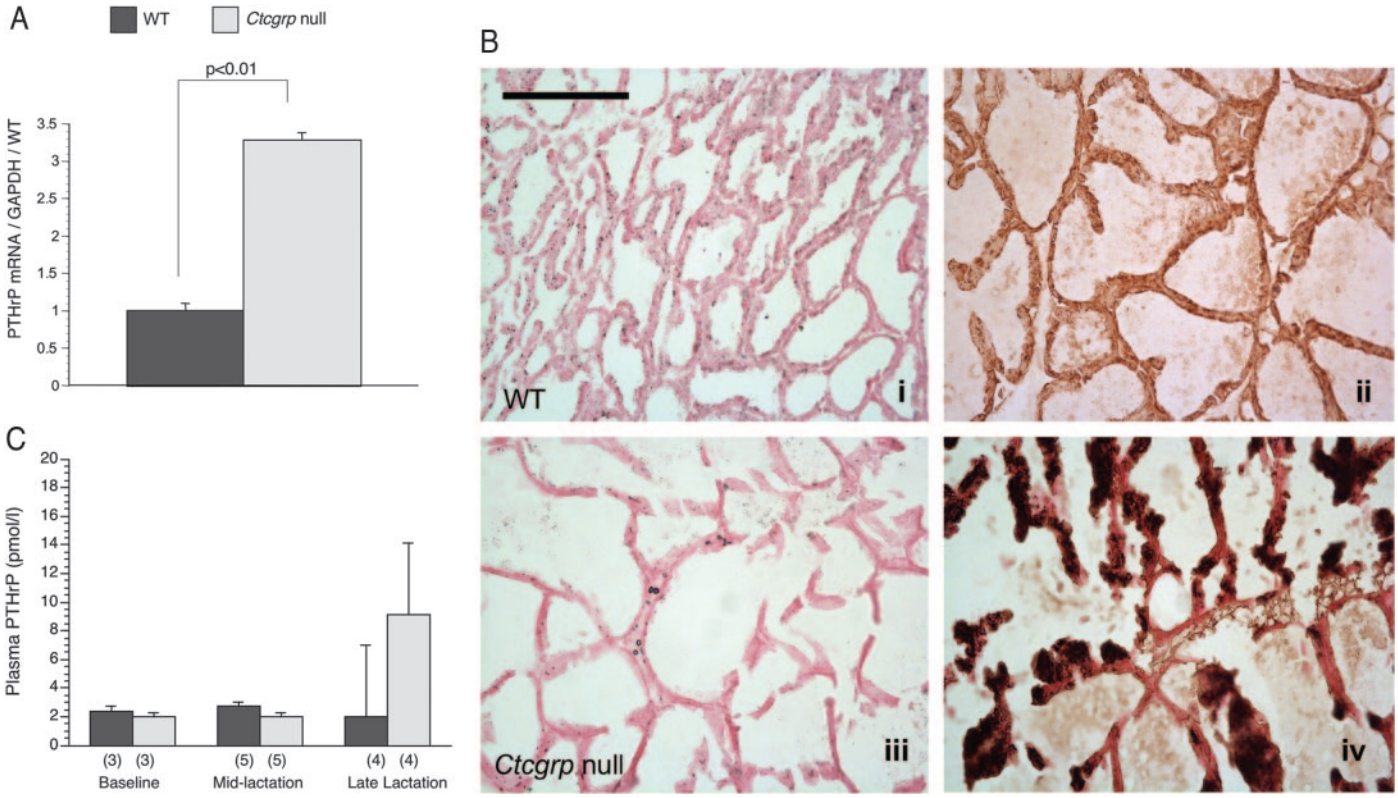

FIG. 5. Mammary gland production of PTHrP. A, Real-time quantitative RT-PCR demonstrated a significant increase in PTHrP mRNA of *Ctcgrp* null mammary tissue *vs.* WT; B, immunohistochemistry of mammary tissue using a rabbit anti-PTHrP antibody. PTHrP was significantly increased in *Ctcgrp* null mammary tissue (*scale bar*, 100 μm); C, plasma PTHrP showed no significant difference between WT and *Ctcgrp* null mice at any time point studied. The *numbers in parentheses* indicate the numbers of mice studied. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

both contribute to the up-regulation in PTH and PTHrP as described below.

Calcitonin, pituitary, and prolactin

With respect to the pituitary, both pharmacological administration of calcitonin (43) and targeted overexpression of calcitonin within pituitary lactotrophs have been found to inhibit production of prolactin (44). Therefore, loss of calcitonin might be expected to lead to enhanced synthesis and release of prolactin, which in turn would cause more profound ovarian suppression, lower estradiol, and enhanced bone resorption. Low estradiol (45) and high prolactin (15, 46–49) are factors that stimulate PTHrP production by mammary tissue *in vivo*. However, we found no significant difference in mean prolactin levels at any time point in *Ctcgrp* null *vs.* WT mice, and estradiol levels were undetectable in both genotypes during mid-lactation. Prolactin is released in pulsatile fashion during lactation (16), but it was not possible

TABLE 1. μCT analysis of trabecular bone parameters within femora and lumbar vertebrae

	BV (mm^3)	TV (mm^3)	BV/TV (%)	BS (mm^2)	SMI	Tb.Th. (μm)	Tb.Sp. (μm)
Femora							
Ctcgrp null baseline (n = 3)	0.43 ± 0.07^{a}	3.34 ± 0.22	12.8 ± 1.8^{a}	28.5 ± 4.3	1.83 ± 0.09^{a}	$72.7 \pm 5.3^{a,b,c}$	$277 \pm 26^{a,b}$
Ctcgrp null trough (n = 6)	0.42 ± 0.05	3.70 ± 0.16	11.6 ± 1.3^{b}	29.7 ± 3.0^{a}	1.79 ± 0.06^{b}	47.7 ± 3.6^{a}	$363 \pm 26^{a,c}$
WT baseline (n = 3)	0.19 ± 0.07^{a}	3.11 ± 0.22^{a}	$6.2 \pm 1.8^{a,b}$	15.0 ± 4.3^{a}	$2.21 \pm 0.08^{a,b,c}$	53.3 ± 5.1^{b}	413 ± 26^{b}
WT trough (n = 5)	0.34 ± 0.06	3.90 ± 0.17^{a}	8.8 ± 1.4	24.5 ± 3.3	1.92 ± 0.07^{c}	52.0 ± 3.9^{c}	495 ± 32^{c}
Vertebrae							
Ctcgrp null baseline (n = 3)	0.65 ± 0.05	2.71 ± 0.15^{a}	23.7 ± 1.1^{a}	$31.4 \pm 2.1^{a,b}$	1.11 ± 0.06	$54.5 \pm 2.0^{a,b}$	213 ± 12
Ctcgrp null trough (n = 6)	0.77 ± 0.04^{a}	2.93 ± 0.12^{b}	25.2 ± 0.9^{b}	$38.2 \pm 1.7^{b,c}$	1.10 ± 0.05^{a}	46.2 ± 1.6^{a}	201 ± 9
WT baseline (n = 3)	$0.56 \pm 0.05^{a,b}$	2.92 ± 0.13^{c}	$19.0 \pm 1.1^{a,b,c}$	$29.5 \pm 2.1^{c,d}$	1.31 ± 0.06^{a}	49.0 ± 2.0	225 ± 11
WT trough (n = 5)	0.82 ± 0.05^{b}	$3.54 \pm 0.12^{a,b,c}$	23.3 ± 1.1^{c}	$42.1 \pm 1.9^{a,d}$	1.15 ± 0.06	44.6 ± 1.8^{b}	217 ± 11

μCT analysis of femora and lumbar vertebrae was performed at baseline (nonpregnant) and at the end of lactation (trough). Within femora, *Ctcgrp* null mice showed higher trabecular bone volumes, greater trabecular thickness, and more densely packed trabeculae at baseline. *Ctcgrp* null mice underwent more pronounced thinning of trabeculae than WT by the end of lactation but did not increase trabecular bone volumes. Lumbar spine showed a similar pattern of changes. Lumbar spine values include results from both the first and second lumbar vertebrae. BS, Trabecular bone surface; BV, trabecular bone volume; BV/TV, trabecular bone volume relative to total volume; SMI, structure-model index; Tb.Sp., trabecular spacing; Tb.Th., trabecular thickness; TV, total volume including trabecular bone (but excluding cortical bone).

[a–d] *Shared letters within columns* indicate statistical significance of $P < 0.05$ (or lower) by Tukey analysis.

TABLE 2. Biomechanical parameters derived from the three-point bend test of femurs

	Ultimate load (g)	Stiffness (N/mm)	Toughness (J/mm^3)	Ultimate stress (mPA)	Work to failure (N × mm)	Moment of inertia (mm^4)
Ctcgrp null baseline (n = 3)	1678 ± 265	91 ± 14	3.56 ± 0.65	189 ± 27	2.26 ± 0.39	0.086 ± 0.001
Ctcgrp null trough (n = 6)	1430 ± 229	102 ± 14	3.46 ± 0.65	179 ± 27	2.17 ± 0.39	0.100 ± 0.001
WT baseline (n = 3)	1914 ± 266	132 ± 14	4.00 ± 0.65	208 ± 27	2.64 ± 0.39	0.088 ± 0.001
WT trough (n = 5)	1737 ± 226	96 ± 18	3.78 ± 0.80	143 ± 34	2.78 ± 0.48	0.120 ± 0.001

This test of cortical strength showed a nonsignificant trend for decreased bone strength between baseline (nonpregnant) and end of lactation (trough), with no significant difference between groups at any time point. Two *Ctcgrp* null femurs were clearly fragile because they broke upon being placed into the device but could not be included in the data analysis. *Numbers in parentheses in the first column* indicate the numbers of mice studied.

to collect multiple serum samples from each mouse to assess peak levels of this hormone. Our results indicate that the proposed role of calcitonin to regulate pituitary lactotrophs and systemic prolactin levels may not be a physiologically important pathway.

Calcitonin in mammary tissue

The presence of calcitonin and its receptor within mammary tissue (25) may indicate a local regulatory role for calcitonin in which the loss of it leads to enhanced PTHrP production. The calcium receptor has been shown to locally regulate the production of PTHrP within mammary tissue as well as the calcium and fluid content of milk (50). We did not observe a change in calcium receptor mRNA expression by real-time quantitative RT-PCR, and thus the pathway by which absence of calcitonin leads to stimulation of PTHrP may be independent of the calcium receptor.

The factors that control milk production are not well understood, although it is known that the calcium receptor, PTHrP, prolactin, and suckling all contribute to the regulation of the calcium and fluid content of milk (12, 50, 51). The numerous calcium regulatory genes that are expressed by lactating mammary tissue include the calcium receptor, calcitonin receptor, 1-α-hydroxylase, and vitamin D receptor

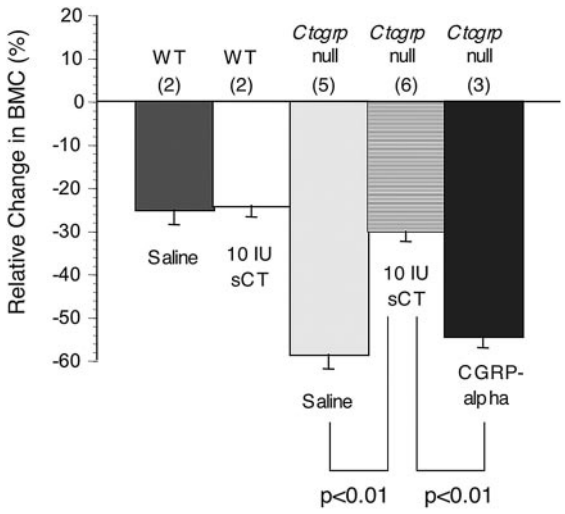

FIG. 6. Calcitonin but not CGRP rescues the *Ctcgrp* null phenotype. Salmon calcitonin administered im once daily throughout 21 d of lactation resulted in a loss of BMC in the *Ctcgrp* null mice that was similar to WT. *Ctcgrp* null mice that received saline (controls), as well as *Ctcgrp* null mice that received an equimolar dose of CGRPα, lost significantly more BMC than the *Ctcgrp* null mice receiving calcitonin treatment. The *numbers in parentheses* indicate the number of mice studied.

(25, 50, 52). We did not observe any alteration in the expression of these genes within mammary tissue; the only consistent finding was an elevation in PTHrP in mammary tissue that lacks calcitonin. No previous publications have examined CaT1 or CaT2 mRNA expression in lactating mammary tissue, although CaT1 protein had previously been detected by immunohistochemistry in nonlactating human breast tissue (53). We did not detect CaT1 or CaT2 mRNA in lactating mammary tissue of WT or *Ctcgrp* null lactating mice, which suggests that the epithelial calcium channels are not involved in milk production.

Secondary hyperparathyroidism in Ctcgrp null mice

Normally PTH is suppressed during lactation in humans and rodents (1), but we observed a significant elevation in PTH in *Ctcgrp* null mice during mid-lactation and to a lesser degree during late lactation. The cause of the elevated PTH may be an indirect consequence of loss of calcitonin. It has previously been demonstrated that larger litter sizes or a restricted calcium intake will induce maternal hypocalcemia and secondary hyperparathyroidism during lactation (54–56). In such cases, loss of calcium into milk may overwhelm the mother's ability to maintain her own blood calcium, resulting in hypocalcemia that in turn acts through the calcium receptor to induce PTH release. *Ctcgrp* null mice have low urinary calcium excretion, normal duodenal calcium absorption, and enhanced skeletal resorption; these factors should obviously lead to increased availability of calcium for milk production. The fact that PTH is elevated in *Ctcgrp* null mice may indicate that the loss of calcium into milk exceeds the requirement to maintain a normal blood calcium in the mother. Milk production by mammary tissue is controlled by both local and systemic factors (16, 57) and can induce hypocalcemia and tetany (milk fever) in lactating animals (58, 59); therefore, it is conceivable that loss of calcitonin within mammary tissue leads to an up-regulation in milk production that, in turn, challenges the ability of the mother to maintain her own serum calcium.

Confounding influences on bone strength

Ctcgrp null mice lost twice as much mineral content as WT siblings, and yet skeletal strength (as assessed by the three-point bend test) was not significantly different from WT at the end of lactation. This likely reflects several factors. First, the three-point bend test examines the strength of cortical bone, which undergoes very modest resorption during lactation (1, 60). Second, it is trabecular bone that is rapidly

mobilized during lactation because of its large surface area and vascularity, factors that enable it to be resorbed by osteoclasts on multiple surfaces (60, 61). Third, absence of calcitonin from conception leads to increased trabecular bone mass, as indicated in the original report (30) and in our study in which the *Ctcgrp* null skeleton had thicker and more densely packed trabeculae compared with WT. Thus, even a 50% drop in skeletal mass was well tolerated by the *Ctcgrp* null mice because the large losses resulted in a trabecular thickness that was equivalent to the level of WT mice at the end of lactation. Finally, pregnancy and lactation are normally associated with other changes in skeletal architecture (increased bone volume and expansion of periosteal surfaces while endosteal bone is being lost) that will offset the weakening effect that skeletal demineralization during lactation would be expected to induce (62). *Ctcgrp* null mice have greater trabecular and total bone volumes than WT at baseline; although these volumes increased substantially in WT mice, the changes were comparably modest in *Ctcgrp* null mice. The larger bone volumes of the *Ctcgrp* null mice likely compensated for the loss of trabecular bone during lactation.

Relevance to lactation in humans

A few case series examined male and female thyroidectomized individuals who received thyroid hormone replacement and reported conflicting results as to whether such patients developed a long-term deficit in bone mass or an increase in fracture risk relative to matched individuals with intact thyroids (63–67). No study has examined the bone mass of thyroidectomized women after a cycle of pregnancy and lactation, and of course such women would retain the ability to make calcitonin in pituitary and mammary tissue and would not be fully calcitonin deficient (26). Vertebral compression fractures occasionally occur during lactation in otherwise healthy young women, a condition termed osteoporosis of lactation (68). In such cases, excess bone resorption during lactation is presumed to have led to increased fragility that resolves after weaning. Our results raise the possibility that at least some of these cases might represent the human equivalent of absence of calcitonin or its receptor.

Skeletal recovery after weaning

In contrast to the demineralization that occurs during lactation, the factors that are required to stimulate recovery of the skeleton after weaning remain unknown. Our results indicate that calcitonin is not required for skeletal recovery to be achieved in full, although its absence may contribute to a delay in recovery. Calcitonin is required in the short term during lactation to prevent excessive resorption of the skeleton and likely attendant fragility, but it is not required in the long term because the skeleton recovers fully despite its absence and despite the increased mineral deficit induced by lactation. Resolving the mechanism by which the skeleton recovers remains a clinically important and relevant task because the skeleton is able to recover so completely and rapidly from the large losses of skeletal mineral content that are induced by lactation. *Ctcgrp* null mice may be an ideal model to examine skeletal recovery because the factors stimulating recovery are likely to be up-regulated more in these mice compared with normal.

Breast, brain, and bone circuit

In summary, evidence from recent studies has pointed to the central role of mammary tissue to stimulate skeletal resorption during lactation by producing PTHrP under the control of the calcium receptor and also to the central role of the pituitary to secrete prolactin and, thereby, suppress estradiol and stimulate PTHrP (the breast, brain, and bone circuit, Fig. 7). The data in our manuscript indicate that calcitonin plays a key role by modulating the extent of skeletal resorption during lactation. Absence of calcitonin in the systemic circulation will lead to increased osteoclast activity through loss of its direct effects via the calcitonin receptor to inhibit osteoclast number and function. However, because the calcitonin receptor is also expressed in mammary tissue and pituitary (two tissues that also produce calcitonin), it is possible that calcitonin acts at all three sites to modulate lactation: directly to inhibit osteoclasts, within pituitary to

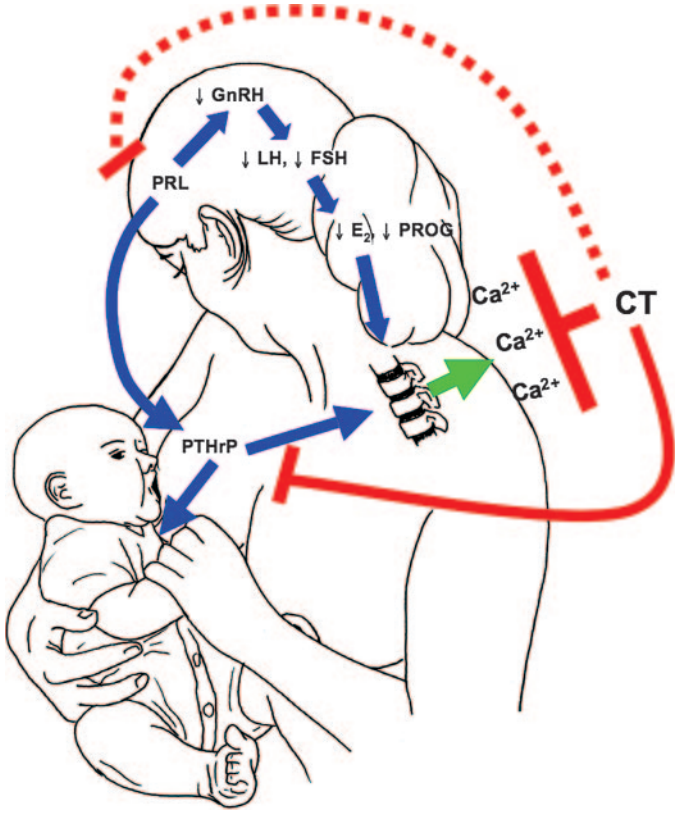

FIG. 7. Breast, brain, and bone circuit. High prolactin (PRL) levels during lactation stimulate PTHrP production by mammary tissue, and suppress the GnRH pulse center in the hypothalamus, in turn suppressing the pituitary gonadotropins (LH and FSH) and then the ovaries [ovulation, estradiol (E_2), and progesterone (PROG) production]. Low estradiol and mammary-derived PTHrP act synergistically to enhance osteoclast-mediated bone resorption. The presence of calcitonin (CT) and its receptor in mammary tissue, pituitary, and bone (*red lines*) allows for at least three pathways by which calcitonin could modulate BMC losses during lactation. Our observation that serum prolactin levels were no different between *Ctcgrp* null and WT indicates that the postulated role of calcitonin to inhibit pituitary lactotrophs (*dashed red line*) may not be physiologically important.

inhibit prolactin release, and within mammary tissue to modulate PTHrP or milk production. Additional work is needed to determine the relative importance of these three physiological loops.

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