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Lactation induces bone loss to provide sufficient calcium in the milk, a process that involves osteoclastic bone resorption but also osteocytes and perilacunar resorption. The exact mechanisms by which osteocytes contribute to bone loss remain elusive. Osteocytes express genes required in osteoclasts for bone resorption, including cathepsin K (*Ctsk*), and lactation elevates their expression. We show that *Ctsk* deletion in osteocytes prevented the increase in osteocyte lacunar area seen during lactation, as well as the effects of lactation to increase osteoclast numbers and decrease trabecular bone volume, cortical thickness, and mechanical properties. In addition, we show that *Ctsk* deletion in osteocytes increased bone parathyroid hormone–related peptide (PTHrP) and prevented the decrease in serum parathyroid hormone (PTH) induced by lactation, but amplified the increase in serum 1,25-dihydroxyvitamin D [1,25(OH)₂D]. The net result of these changes is to maintain serum and milk calcium levels in the normal range, ensuring normal offspring skeletal development. Our studies confirm the fundamental role of osteocytic perilacunar remodeling in physiological states of lactation and provide genetic evidence that osteocyte-derived Ctsk contributes not only to osteocyte perilacunar remodeling, but also to the regulation of PTH, PTHrP, 1,25(OH)₂D, osteoclastogenesis, and bone loss in response to the high calcium demand associated with lactation.

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Cathepsin K–deficient osteocytes prevent lactation-induced bone loss and parathyroid hormone suppression

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Lactation induces bone loss to provide sufficient calcium in the milk, a process that involves osteoclastic bone resorption but also osteocytes and perilacunar resorption. The exact mechanisms by which osteocytes contribute to bone loss remain elusive. Osteocytes express genes required in osteoclasts for bone resorption, including cathepsin K (Ctsk), and lactation elevates their expression. We show that Ctsk deletion in osteocytes prevented the increase in osteocyte lacunar area seen during lactation, as well as the effects of lactation to increase osteoclast numbers and decrease trabecular bone volume, cortical thickness, and mechanical properties. In addition, we show that Ctsk deletion in osteocytes increased bone parathyroid hormone–related peptide (PTHrP) and prevented the decrease in serum parathyroid hormone (PTH) induced by lactation, but amplified the increase in serum 1,25-dihydroxyvitamin D [1,25(OH)2D]. The net result of these changes is to maintain serum and milk calcium levels in the normal range, ensuring normal offspring skeletal development. Our studies confirm the fundamental role of osteocytic perilacunar remodeling in physiological states of lactation and provide genetic evidence that osteocyte-derived Ctsk contributes not only to osteocyte perilacunar remodeling, but also to the regulation of PTH, PTHrP, 1,25(OH)2D, osteoclastogenesis, and bone loss in response to the high calcium demand associated with lactation.

Introduction

Cathepsin K (Ctsk) is highly expressed in osteoclasts and is secreted in the bone-resorbing compartment, where it degrades the organic bone matrix after dissolution of the mineral phase at low pH during bone resorption (1, 2). Consequently, global deletion, osteoclast-targeted deletion, or pharmacological inhibition of Ctsk impairs bone resorption while increasing bone formation and results in a high bone mass (2–6). In all these situations, this is associated with high RANKL levels, increasing the number of otherwise functionally impaired osteoclasts (4–6) and mimicking the osteoclast-rich osteopetrotic phenotype observed in pycnodysostosis (7–9). Although osteocytes have been shown to resorb their perilacunar space (10–17), whether Ctsk is involved in this process is not known. Interestingly, during lactation in mice, a subpopulation of osteocytes expresses high mRNA and protein levels of Ctsk and other genes expressed in osteoclasts and linked to bone resorption such as tartrate-resistant acid phosphatase (Trap), carbonic anhydrase, or the proton pump V-ATPase (18, 19). This suggests that, at least under high systemic calcium demand, osteocytes are able to turn on a set of genes required for bone resorption to enlarge their lacunae. More recently, it was shown that these cells can also acidify their lacunar space (19), thus suggesting an osteoclast-like resorptive phenotype and supporting the potential relevance of osteocytes in calcium mobilization during lactation (15).

It is known that in order to provide adequate amounts of calcium to rapidly growing pups, lactation induces an increase in osteoclast numbers and bone resorption, resulting in a substantial mobilization of calcium into the milk at the expense of the skeleton (15, 20). Accordingly, lactating mice lose up to 30% of their bone mass over a 2-week period of breastfeeding (21–23). This rapid decrease in bone mass is, however, reversible, and bone mass is rapidly restored after weaning in many species (20, 23–26). Although this mobilization of calcium and the resulting bone loss have for the most part been attributed to low estrogen levels and an increase in parathyroid hormone–related peptide (PTHrP) production by the mammary glands, both resulting in an increase in osteoclast numbers (23, 24), the exact mechanisms underlying bone loss during lactation are incompletely understood. In particular, whether osteocyte perilacunar resorption plays a role in calcium mobilization and in the induction of osteoclastogenesis is not known. Thus, like osteoclastic bone resorption, Ctsk may be involved in bone matrix degradation during the process of osteo-

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cyte perilacunar resorption and, therefore, in the mobilization of calcium for the milk during lactation. In addition to its potential role in calcium mobilization, lactation-induced osteocytic perilacunar resorption negatively affects bone biomechanical properties by increasing the volume of the osteocytic network (27).

Although several studies have examined osteocyte responses to hormones, cytokines, mechanical loading, and lactation, as well as the influence of osteocytes on bone remodeling and the bone marrow (BM) microenvironment (18, 28–34), the feedback influence of perilacunar resorption on osteocytes themselves and the possibility that osteocyte-mediated changes in the periosteocytic matrix contribute to the way in which they regulate osteoclast differentiation and bone remodeling had not been investigated. Recently, a study by Dole et al. (17) reported that TGF-β receptor 2 (TGF-βR2) in osteocytes is required to maintain perilacunar remodeling, bone mass, and bone quality, but whether perilacunar remodeling affects osteocyte regulatory behavior was not explored.

Here, we hypothesized that while performing perilacunar resorption during lactation, osteocytes may be affected by the changes occurring in their lacunar microenvironment as a consequence of perilacunar resorption itself. This may in turn affect the way in which osteocytes regulate osteoclast differentiation and thereby calcium mobilization and bone loss during lactation. According to this hypothesis, deletion of Ctsk in osteocytes could not only prevent matrix degradation around osteocytes and perilacunar resorption but also alter the recruitment of osteoclasts, bone loss, and calcium metabolism responses during lactation.

To test this hypothesis, we targeted the deletion of Ctsk in osteocytes by crossing 9.6-kb Dmp1Cre-transgenic mice with Ctsk<sup>fl/fl</sup> mice (Ctsk<sup>ocy</sup> mice). Deletion of Ctsk in osteocytes prevented the increase in the osteocyte lacunar area, osteoclast numbers, and bone resorption, preventing the bone loss seen in lactating control mice at both cortical and cancellous sites. In addition, we found that Ctsk deletion in osteocytes protected against the alteration of some biomechanical properties of the femur. Interestingly, higher levels of serum parathyroid hormone (PTH), bone PTHrP, and serum 1,25-dihydroxyvitamin D [1,25(OH)2D] in Ctsk<sup>ocy</sup> lactating mice compared with levels in control lactating mice allowed the appropriate mobilization of the calcium required for milk production and skeletal development in offspring.

**Results**

At steady state, Ctsk<sup>ocy</sup> mice exhibit normal skeletal homeostasis and remodeling. As previously reported (18), a number of osteocytes expressed Ctsk at steady state in control mice (Figure 1A). To determine whether Ctsk in osteocytes affects bone homeostasis at steady state, we crossed Ctsk<sup>fl/fl</sup> mice with transgenic mice expressing Cre recombinase under the control of 9.6-kb Dmp1 regulatory elements. Dmp1Cre Ctsk<sup>fl/fl</sup> (Ctsk<sup>ocy</sup>) mice were born at the expected Mendelian ratio and had no obvious skeletal phenotype at birth, as assessed by gross examination, compared with control littermates (Ctsk<sup>fl/fl</sup>) (data not shown). Ctsk<sup>ocy</sup> mice showed efficient Ctsk exon 5 genomic excision and a significant Ctsk deletion in long bones and muscle, but not in other tissues (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI22936DS1), as previously reported (35), and a significant reduction of Ctsk protein levels in osteocytes and Ctsk mRNA levels in marrow-depleted long bone midshafts (Figure 1, A and B). Immunohistochemistry and quantitative reverse transcription PCR (qRT-PCR) confirmed the specificity of Dmp1Cre to osteocytes, with osteoclast Ctsk remaining unaffected (Figure 1, C and D). Furthermore, in our hands, there was no Cre recombination (Supplemental Figure 1B) or Ctsk deletion in the mammary glands of Ctsk<sup>ocy</sup> mice, whether or not the mice were lactating (virgin Ctsk<sup>fl/fl</sup> = 1.0 ± 0.1, virgin Ctsk<sup>ocy</sup> = 0.72 ± 0.1, lactating Ctsk<sup>fl/fl</sup> = 0.6 ± 0.2, and lactating Ctsk<sup>ocy</sup> = 1.1 ± 0.4; n = 4–5, mean ± SEM). MicroCT (μCT) and histomorphometric analyses indicated that deletion of Ctsk in osteocytes did not alter cortical or cancellous bone in 12-week-old female mice under steady-state conditions (Figure 1E and Supplemental Tables 1 and 2). As seen in the female mice, except for an increase in trabecular thickness, we observed no changes in other parameters in the Ctsk<sup>ocy</sup> females compared with control littermates (Supplemental Table 3 and data not shown). Interestingly, however, and as detailed below, Ctsk deletion in osteocytes altered the level of expression of several genes relevant to bone homeostasis in osteocytes themselves.

**Osteocyte-specific deletion of Ctsk prevents lactation-induced osteocytic perilacunar resorption.** We then determined whether deletion of Ctsk in osteocytes would affect the skeletal response during lactation in female mice. Deletion of Ctsk in osteocytes had no effect on the femoral diaphysis osteocyte lacunar area in virgin mice as assessed by BSEM, nor on osteocyte numbers or density (Figure 2A and Supplemental Table 4). In contrast, and as previously shown (15, 18–20), osteocyte perilacunar resorption was increased during lactation in control mice, resulting in a significant increase in the osteocyte lacunar area and osteocyte lacunar perimeter (Figure 2B and Supplemental Table 4). Importantly, these changes were prevented by deletion of Ctsk in osteocytes (Figure 2B and Supplemental Table 4). In control mice, the number of osteocytes per unit bone volume (osteocyte density) was significantly increased (Supplemental Table 4) during the experimental period (which always covers 19 days of pregnancy and 12 days of lactation) (18, 19, 36), suggesting a decrease in the amount of matrix produced by each cell during the process of bone formation that led to embedding of the osteocytes. This increase in osteocyte density was partially prevented by Ctsk deletion (Supplemental Table 4). Histologically, no striking changes in the canalicular network were observed at steady state, during lactation, or between Ctsk<sup>ocy</sup> and Ctsk<sup>fl/fl</sup> females, as indicated by silver nitrate staining (Figure 2C). These findings therefore provide direct evidence that during lactation, a time of high calcium demand, Ctsk is required in osteocytes to enable the process of perilacunar resorption.

**Osteocyte-specific deletion of Ctsk prevents bone loss in lactating mice.** To examine whether Ctsk in osteocytes also contributes to bone loss during lactation, we measured bone turnover and homeostasis in virgin and lactating Ctsk<sup>ocy</sup> mice and control litter-
mice. As shown in Supplemental Table 1 and Figure 3, μCT analysis revealed that when Ctsk was present in osteocytes, a 12-day lactation period significantly decreased cortical bone volume and cortical thickness in control females. Cancellous bone volume was also significantly (Supplemental Table 1 and Figure 3) lower in the lactating control mice vs. −6.4% ± 5.5% in virgin mice (Supplemental Table 1 and Figure 3). These changes were not observed in the virgin mice (Supplemental Table 1 and Figure 3). CT analysis in osteocytes prevented the effects of lactation on the skeleton. We also measured vertebral bone mineral content by dual-energy x-ray absorptiometry (DEXA) and found a similar protection from bone loss in lactating control females, Ctskocy mice, indicating that the absence of Ctsk in osteocytes prevented the effects of lactation on the skeleton. We also measured trabecular bone mineral content by dual-energy x-ray absorptiometry. As expected, lactation significantly decreased trabecular bone surface remodeling, we performed dynamic histomorphometry. As expected, lactation significantly decreased trabecular bone volume and trabecular thickness, confirming the μCT results (Supplemental Table 2 and Figure 4). This loss of bone was due to a significant increase in osteoclast numbers, with no significant changes in the bone formation rate in control lactating mice (Supplemental Table 2 and Figure 4). These results demonstrated that Ctskocy mice are protected from lactation-induced increases in osteoclastogenesis. Although we observed no significant changes in procollagen type 1 N-terminal propeptide (P1NP) between genotypes during lactation, serum CTX1 levels were significantly increased in control lactating mice but remained at basal levels in Ctskocy lactating mice, consistent with the lower perilacunar resorption and osteoclast numbers observed in Ctskocy lactating mice (Figure 5 and Supplemental Table 5).

Osteocyte-expressed Ctsk regulates the expression of osteocytic genes involved in bone formation and resorption. The deletion of Ctsk was targeted to osteocytes, which are major regulators of bone remodeling (16, 37). Since we observed significant changes in remodeling activity, we determined whether deletion of Ctsk affects the expression of key regulatory genes in osteocytes. In agreement with previous observations in global or osteoclast-targeted Ctsk deletion (4–6), virgin Ctskocy mice had higher Rankl levels than did virgin Ctskfl/fl mice. However, Opg mRNA expression was also higher (2.6-fold) in virgin Ctskocy mice compared with expression in virgin Ctskfl/fl mice, explaining why the number of osteoclasts was not increased in Ctskocy mice. As expected, lacta-
Deletion of Ctsk in osteocytes decreases perilacunar remodeling during lactation. (A) Representative BSEM images (original magnification, ×500) of the femur diaphysis of virgin and lactating Ctsk\(^{fl/fl}\) and Ctsk\(^{cy}\) mice (n = 5–7 per group). (B) Measurements of the single osteocyte lacunar area and perimeter in virgin and lactating Ctsk\(^{fl/fl}\) (black dots and squares, respectively) and Ctsk\(^{cy}\) (red dots and squares, respectively) mice. Data are expressed as the mean ± SEM (n = 5–7 per group). IP < 0.05 versus virgin Ctsk\(^{fl/fl}\) mice; IP < 0.05 versus virgin Ctsk\(^{cy}\) mouse; and IP < 0.05 versus lactating Ctsk\(^{fl/fl}\) mice; 2-way ANOVA followed by Fisher’s PLSD. (C) Representative images of silver nitrate staining showing osteocyte canalicular networking in cortical bone from virgin and lactating Ctsk\(^{fl/fl}\) and Ctsk\(^{cy}\) mice (n = 3 per group). Scale bars: 20 μm.

Deletion of Ctsk in osteocytes partially protects lactating mothers against lactation-induced bone fragility. Finally, and because lactation has been shown to significantly increase bone fragility in mice (27), we sought to determine whether the deleterious effects of lactation on bone mechanical properties were prevented in Ctsk\(^{cy}\) lactating mice. For this purpose, we tested the mechanical prop-


tion led to a marked increase in Rankl mRNA expression in Ctsk\(^{fl}\)/\(^{\Delta}\) mice, whereas in Ctsk\(^{cy}\) mice its expression remained high but was significantly lower than in lactating control mice and at levels similar to those detected in virgin Ctsk\(^{cy}\) mice (Figure 6A and Supplemental Table 6). Furthermore, while Opg mRNA expression was not changed during lactation in Ctsk\(^{fl}\)/\(^{\Delta}\) mice, it decreased by approximately 2-fold in lactating Ctsk\(^{cy}\) mice (Figure 6A and Supplemental Table 6). Consequently, the Rankl/Opg ratio, which was not significantly increased in virgin Ctsk\(^{cy}\) mice, increased by 3- and 6-fold, respectively, during lactation. Histological evaluation of sclerostin confirmed the decrease seen in Sost mRNA levels in virgin Ctsk\(^{cy}\) and lactating Ctsk\(^{fl}\)/\(^{\Delta}\) and Ctsk\(^{cy}\) mice (Figure 6B). Interestingly, osteocyte Fg23 mRNA levels were significantly decreased by Ctsk deletion and by lactation in control mice, but we observed no difference in Ctsk\(^{cy}\) lactating mice (Figure 6A and Supplemental Table 6). Importantly, qRT-PCR evaluation of Ctsk mRNA and protein expression levels as well as TRAP activity confirmed a lactation-dependent increase in Ctsk levels and the percentage of TRAP osteocytes in lactating Ctsk\(^{fl}\)/\(^{\Delta}\) mice, whereas the number of TRAP osteocytes was high in Ctsk\(^{cy}\) mice, independent of lactation (Figure 6, A and C–E).

To determine which changes were cell autonomous, we next determined whether Ctsk regulates gene expression in osteocytes in vitro. For this purpose, and because Ctsk is involved in collagen degradation, we used the Ocy454 cell line cultured on collagen (39). Ocy454 cells express Ctsk, whose expression increases markedly during cell differentiation (Figure 7). Ctsk shRNA in Ocy454 cells significantly reduced the expression of Ctsk, as indicated by qRT-PCR, Western blotting, and immunohistochemical analyses (Figure 7, A–C). Similar findings were observed with 2 separate shRNAs targeting distinct regions of Ctsk (Supplemental Figure 2, A and B). The effect of Ctsk silencing on the expression of Rankl, osteoprotegerin (Opg), and Sost confirmed our in vivo cortical bone mRNA observations (Figure 7D), except after 14 days in culture, when Sost mRNA was unchanged by Ctsk knockdown. Together with the in vivo data, these results suggest that osteocyte-expressed Ctsk acts in a cell-autonomous autocrine manner to regulate osteocytic genes involved in the regulation of bone remodeling.

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Deletion of Ctsk in osteocytes prevents the lactation-induced decrease in serum PTH, maintaining high 1,25(OH)2D levels and ensuring normal serum calcium. Since bone remodeling and calcium metabolism are also affected by hormonal changes during lactation, we next explored the effects of deletion of Ctsk in osteocytes on hormonal regulation. During lactation, and in addition to the increase in PthrP mRNA expression in osteocytes that we observed (Figure 6A), the mammary gland produces PTHrP to mobilize calcium, in part from the skeleton, for milk production (20, 40). As a consequence of the circulating levels of PTHrP, PTH levels decrease, and, in mice, 1,25(OH)2D levels increase to maintain appropriate serum calcium levels via intestinal absorption in addition to calcium mobilized from bone (20, 41). To determine how osteocyte Ctsk affects these regulatory mechanisms, we measured serum calcium, PTH, and 1,25(OH)2D, and PTHrP plasma levels in lactating Ctskocy and control mice. As expected, serum calcium was maintained within the normal range in both lactating control mice and lactating Ctskocy mice (Supplemental Table 5). In contrast, serum PTH levels were decreased in lactating control mice (Figure 5 and Supplemental Table 5), but deletion of Ctsk in osteocytes prevented this decrease, maintaining PTH levels in Ctskocy lactating mice within the normal, nonlactating range (Figure 5 and Supplemental Table 5). We observed the expected increase in PTHrP mRNA expression in osteocytes that we observed (Figure 6A), the mammary gland produces PTHrP to mobilize calcium, in part from the skeleton, for milk production (20, 40). As a consequence of the lactation-induced decrease in serum PTH, maintaining high 1,25(OH)2D levels and ensuring normal serum calcium, when Ctsk is deleted in osteocytes in mice, PTH and 1,25(OH)2D levels increase to maintain appropriate serum calcium levels. This increase in 1,25(OH)2D levels is due to the increased production of PTHrP in lactating Ctskocy mice, which mobilizes calcium from the skeleton for milk production, and the increased intestinal absorption of calcium due to the increased levels of 1,25(OH)2D. As a result, serum calcium levels are maintained within the normal range in both lactating control mice and lactating Ctskocy mice (Supplemental Table 5). In contrast, serum PTH levels were decreased in lactating control mice (Figure 5 and Supplemental Table 5), but deletion of Ctsk in osteocytes prevented this decrease, maintaining PTH levels in Ctskocy lactating mice within the normal, nonlactating range (Figure 5 and Supplemental Table 5). We observed the expected increase in PTHrP mRNA expression in osteocytes that we observed (Figure 6A), the mammary gland produces PTHrP to mobilize calcium, in part from the skeleton, for milk production (20, 40). As a consequence of the lactation-induced decrease in serum PTH, maintaining high 1,25(OH)2D levels and ensuring normal serum calcium, when Ctsk is deleted in osteocytes in mice, PTH and 1,25(OH)2D levels increase to maintain appropriate serum calcium levels. This increase in 1,25(OH)2D levels is due to the increased production of PTHrP in lactating Ctskocy mice, which mobilizes calcium from the skeleton for milk production, and the increased intestinal absorption of calcium due to the increased levels of 1,25(OH)2D. As a result, serum calcium levels are maintained within the normal range in both lactating control mice and lactating Ctskocy mice (Supplemental Table 5). In contrast, serum PTH levels were decreased in lactating control mice (Figure 5 and Supplemental Table 5), but deletion of Ctsk in osteocytes prevented this decrease, maintaining PTH levels in Ctskocy lactating mice within the normal, nonlactating range (Figure 5 and Supplemental Table 5). We observed the expected increase in PTHrP mRNA expression in osteocytes that we observed (Figure 6A), the mammary gland produces PTHrP to mobilize calcium, in part from the skeleton, for milk production (20, 40). As a consequence of the lactation-induced decrease in serum PTH, maintaining high 1,25(OH)2D levels and ensuring normal serum calcium, when Ctsk is deleted in osteocytes in mice, PTH and 1,25(OH)2D levels increase to maintain appropriate serum calcium levels. This increase in 1,25(OH)2D levels is due to the increased production of PTHrP in lactating Ctskocy mice, which mobilizes calcium from the skeleton for milk production, and the increased intestinal absorption of calcium due to the increased levels of 1,25(OH)2D. As a result, serum calcium levels are maintained within the normal range in both lactating control mice and lactating Ctskocy mice (Supplemental Table 5).
Deletion of Ctsk in osteocytes does not affect milk calcium or skeletal development and density in the offspring of lactating Ctsk<sup>fl/fl</sup> mothers. Given that these hormonal changes ensured that serum calcium was maintained in the normal range, we then asked whether calcium concentration in the milk is also maintained and whether an appropriate supply of calcium from Ctsk<sup>fl/fl</sup> mothers to the offspring skeleton is ensured. For this purpose, we measured calcium content in milk and in the pup’s skeleton using μCT. We observed no changes in milk calcium content (2.19 ± 0.15 vs. 2.28 ± 0.32 mg/mg protein) and no differences in cancellous bone volume between the offspring of control and Ctsk<sup>fl/fl</sup> females (Figure 9 and Supplemental Table 5), indicating that, despite the decrease in osteocytic perilacunar resorption and the low osteoclast numbers and serum CTX1 levels, deletion of Ctsk in osteocytes had no effect on net calcium transfer via the milk to the pups and therefore on their bone development or homeostasis.

Discussion
The skeleton is a storehouse for minerals, including calcium, which is mobilized in situations of high calcium demand such as lactation (20, 40). Lactation induces a large transfer of bone calcium to the circulation of lactating mothers to allow for the proper amount of calcium in milk, a physiological process that induces significant bone loss (40). A well-defined mechanism by which bone loss occurs during lactation involves the upregulation of osteoclastogenesis and osteoclast-dependent bone resorption (42). However, recent studies have confirmed that osteocytes can remodel their perilacunar space and resorb the perilacunar matrix, enlarging their lacunae during lactation (15, 17, 18, 27, 36) and potentially contributing to calcium mobilization from the skeleton. Indeed, the total osteocyte lacunar surface area has been estimated to be at least 10 times that of the trabecular bone surfaces upon which osteoclasts can act (11, 12, 43). Hence, small changes in osteocyte lacunae can mean large changes in mineral content at the level of the whole animal or human. On the one hand, the importance of perilacunar resorption (osteocytic osteolysis) in ensuring calcium mobilization from the skeleton, especially at times of high calcium demand such as during lactation, is still controversial. On the other hand, the influence of the matrix degradation that occurs during perilacunar resorption on the regulation of osteocyte behavior, bone remodeling and skeletal homeostasis is only beginning to be explored (17, 27, 36). In this study, we show that osteocyte-derived Ctsk plays a key role in the mechanism by which osteocytes regulate not only the size...
of their lacunae but also their own gene expression profile, affecting the regulation of skeletal homeostasis and calcium metabolism in response to lactation (Figure 10). Indeed, our results show that Ctsk-dependent degradation of its surrounding matrix by the osteocyte is required not only for perilacunar resorption and enlargement of osteocyte lacunae, but also for the induction of osteoclast differentiation, which ultimately lead to bone loss and fragility. Consequently, preventing matrix degradation around osteocytes by the targeted deletion of \( Ctsk \) decreases not only perilacunar resorption, but also prevents the increase in osteoclast numbers and the mild decrease in bone formation, bone loss and, in part, the increase in bone fragility that normally occur during lactation in mice. This was due in part to a lower \( \text{Rankl}/\text{Opg} \) ratio in osteocytes in the absence of \( Ctsk \), in the presence of low levels of \( Sost \). The inability of \( Ctsk \)-deleted osteocytes to efficiently enlarge their lacunae and generate the appropriate signals to increase osteoclast numbers would, if not compensated by other mechanisms, be expected to at least partially impair calcium mobilization from bone. In lactating women, suppression of serum PTH is usually observed (42) as a consequence of the high calcium levels induced by mammary gland–derived PTHrP (40). We show here that the same regulatory phenomenon occurs in control lactating mice when they are not simultaneously subjected to a low-calcium diet, as done in many previously published reports (19, 36). Here, however, PTH levels failed to decrease in \( Ctsk^{\text{floxed}} \) lactating mice, in the presence of low levels of \( \text{Sost} \). The inability of \( Ctsk \)-deleted osteocytes to efficiently enlarge their lacunae and generate the appropriate signals to increase osteoclast numbers would, if not compensated by other mechanisms, be expected to at least partially impair calcium mobilization from bone. In lactating women, suppression of serum PTH is usually observed (42) as a consequence of the high calcium levels induced by mammary gland–derived PTHrP (40). We show here that the same regulatory phenomenon occurs in control lactating mice when they are not simultaneously subjected to a low-calcium diet, as done in many previously published reports (19, 36). Here, however, PTH levels failed to decrease in \( Ctsk^{\text{floxed}} \) lactating mice, indicating that the negative feedback loop that normally downregulates PTH during lactation is impaired in the absence of Ctsk in osteocytes, and this occurs despite the high mRNA levels of \( \text{Pthrp} \) in the bones of \( Ctsk^{\text{floxed}} \) lactating mice. We interpret these data as showing that calcium mobilization from bone is indeed impaired in the absence of Ctsk in osteocytes but that this impairment is compensated by significant hormonal changes. Instead of being partially suppressed in control lactating mice, we found that the parathyroid gland continued to secrete PTH during lactation in \( Ctsk^{\text{floxed}} \) mice in order to maintain serum calcium levels. However, in the absence of Ctsk, osteocytic osteolysis and osteoclastogenesis failed to respond appropriately to the demand for calcium. Consequently, this process required not only higher PTH levels but also higher \( 1,25(\text{OH})_2\text{D} \) as well as higher local PTHrP levels to maintain serum and milk calcium levels in the normal range (Figure 10). Together, PTH and PTHrP, and possibly the low levels of bone \( \text{Fgf}_23 \), induced by high \( 1,25(\text{OH})_2\text{D} \) (44), allowed sufficiently effective intestinal calcium absorption in these mice that were not deprived of dietary calcium. As shown by others, \( 1,25(\text{OH})_2\text{D} \) protects mice from bone loss during lactation (45). The higher levels of circulating PTH and \( 1,25(\text{OH})_2\text{D} \), in \( Ctsk^{\text{floxed}} \) versus control lactating mice, together with the high levels of \( \text{Pthrp} \) in bone itself, may also explain the limited increase in circulating PTHrP levels we detected in the lactating \( Ctsk^{\text{floxed}} \) mice. Altogether, this mechanism ensured the maintenance of sufficient calcium levels in the maternal serum and in milk, allowing normal skeletal development of the offspring of \( Ctsk^{\text{floxed}} \) mothers, despite the prevention of perilacunar resorption and osteoclast recruitment. Most important, this led to the prevention of bone loss in \( Ctsk^{\text{floxed}} \) mothers, while sufficient calcium was provided to the offspring. In contrast, as shown by Ardeshirpour et al. (46), when the mother is deprived of dietary calcium in addition to a blockade of osteoclastogenesis, maternal calcium, PTH, and pup growth are affected. Together, these findings further establish that in mice, in which the demand for calcium is high, bone resorption, whether by osteocytes or osteoclasts, and dietary calcium absorption back each other up to ensure proper levels of calcium in the milk (42). Although our study clearly establishes these links in lactating mice, it should be noted that in humans,

![Graph](https://example.com/graph.png)

**Figure 5. Deletion of Ctsk in osteocytes affects serum hormone and CTX1 levels.** P1NP, CTX1 PTH, PTHrP, and 1,25(OH)\_2 vitamin D\_3 levels in serum or plasma from virgin and lactating Ctsk\textsuperscript{floxed} (black dots and squares, respectively) and Ctsk\textsuperscript{cyo} (red dots and squares, respectively) mice. For PTHrP, the dotted line represents the limit of detection of the assay. Data are expressed as the mean ± SEM (n = 5–9 per group). \( \text{†} P < 0.05 \) compared with virgin Ctsk\textsuperscript{floxed} mice; \( \text{‡} P < 0.05 \) versus virgin Ctsk\textsuperscript{cyo} mice; \( \text{#} P < 0.05 \) versus lactating Ctsk\textsuperscript{floxed} mice; 2-way ANOVA followed by Fisher’s PLSD test.
The increase in PTHrP (18) and 1,25(OH)2D (48) that occurs during lactation. However, perilacunar resorption and matrix degradation appear to be required for more than just lacunar enlargement, and our results suggest that these are required steps for PTH and PTHrP to induce an increase in osteoclast numbers (Figure 10). Indeed, as observed previously with global or osteoclast-targeted deletion of Ctsk (4–6), Rankl expression and the Rankl/Opg ratio were increased in osteocytes of Ctskocy mice at steady state, independent of lactation. During lactation, Rankl and the Rankl/Opg ratio were, 1,25(OH)2D levels and calcium absorption have been reported to be unaltered in lactating mothers (42, 47). Indeed, randomized interventional trials and observational studies have shown that women generally maintain the same degree of skeletal resorption during lactation, regardless of low or high calcium intake, suggesting that dietary calcium absorption is not as crucial in lactating women as it is in mice (20, 42).

Thus, perilacunar resorption and matrix degradation around the osteocyte are an integral part of the response of osteocytes to the increase in PTHrP (18) and 1,25(OH)2D (48) that occurs during lactation. However, perilacunar resorption and matrix degradation appear to be required for more than just lacunar enlargement, and our results suggest that these are required steps for PTH and PTHrP to induce an increase in osteoclast numbers (Figure 10). Indeed, as observed previously with global or osteoclast-targeted deletion of Ctsk (4–6), Rankl expression and the Rankl/Opg ratio were increased in osteocytes of Ctskocy mice at steady state, independent of lactation. During lactation, Rankl and the Rankl/Opg ratio were, 1,25(OH)2D levels and calcium absorption have been reported to be unaltered in lactating mothers (42, 47). Indeed, randomized interventional trials and observational studies have shown that women generally maintain the same degree of skeletal resorption during lactation, regardless of low or high calcium intake, suggesting that dietary calcium absorption is not as crucial in lactating women as it is in mice (20, 42).

Thus, perilacunar resorption and matrix degradation around the osteocyte are an integral part of the response of osteocytes to
components, such as collagen. In fact, these molecules have already been implicated in the regulation of osteocytes in vitro and, more recently for MMP13 and TGF-β signaling, in vivo (13, 17, 49). Since it has been shown that Ctsk can fully degrade TGF-β (50) as well as IGF1 (51), Ctsk deletion in osteocytes could simply prevent the complete degradation of these growth factors once perilacunar resorption is initiated, allowing activation of signaling from the osteocyte membrane. Alternatively, similar to some other proteolytic enzymes (52–54), Ctsk could bind to cell-surface receptors such as LDL receptor–related protein 1 (LRP1) (52–54) once secreted into the perilacunar space and act as an autocrine ligand. Further work will be required to test these hypotheses and identify whether matrix components and/or Ctsk itself acting as a ligand are critical for allowing osteocytes to generate and transmit the signals required for recruitment of osteoclast precursors and regulation of bone remodeling.

Methods

Blinding. To avoid bias, data from all studies were collected in a blind fashion, in which the observer was unaware of the experimental group. Key studies were performed by more than 1 individual to determine whether there was consistency in the observations.

Mice. Generation of the Ctskfl/fl mice was previously described (4, 6), and the 9.6KbDmp1-Cre mice were provided by J.Q. Feng (Department of Biomedical Sciences, Texas A&M University Baylor College of Dentistry, Dallas, Texas, USA) (55). Both mouse models were on a C57BL/6J background. Mice hemizygous for Dmp1-Cre were crossed with Ctskfl/fl mice to generate Dmp1-Cre Ctskfl/fl however, markedly increased in control mice but remained at the same, albeit high, levels in the absence of Ctsk. This suggests that the inability of osteocytes to efficiently induce osteoclastogenesis when depleted of Ctsk cannot be simply attributed to a lack of Rankl or a low Rankl/Opg ratio. Instead, these findings suggest that matrix degradation by osteocytes is dependent on Ctsk in osteocytes and that this process initiates a feedback-signaling loop that regulates osteoclastogenesis.

How can Ctsk-dependent matrix degradation by osteocytes contribute to the regulation of osteocytic genes that in turn regulate bone remodeling? Although the present study does not provide a definitive answer to this question, it clearly demonstrates that gene expression is altered in osteocytes after targeted Ctsk deletion. Our study showed that Rankl and Opg mRNA levels, as well as TRAP activity, were increased in nonlactating Ctsk−/− osteocytes relative to control mice, whereas Sost and Fgf23 mRNA levels were decreased, consistent with the increase in PthrP expression by osteocytes in Ctsk-deleted osteocytes (38), independent of lactation. Mechanistically, we hypothesize that the absence of Ctsk could prevent the degradation of products from the matrix, allowing these products to be present in the osteocyte lacunar microenvironment at high enough concentrations to signal back to receptors on the osteocyte membrane. Alternatively, lack of Ctsk may prevent the activation of factors such as TGF-β. According to this hypothesis, the osteocyte would induce matrix degradation-dependent signaling cascades via mobilized and/or activated matrix-derived growth factors, such as TGF-β or insulin growth factor 1 (IGF1), and/or proteolytically cleaved fragments of matrix

Figure 7. Ctsk silencing in Ocy454 cells affects the expression of genes involved in bone remodeling. (A) Efficiency of Ctsk deletion in Ocy454 cells. Control shRNA (black dots) and Ctsk shRNA (red dots) (catalog TRCN0000054624). Data shown represent the mean of triplicate experiments ± SEM and were normalized to the expression of 18S mRNA (n = 3). *P < 0.05 by Student’s 2-tailed t test. (B) Densitometric analysis of Western blotting showing the efficiency of Ctsk silencing. β-Tubulin was used as a loading control (n = 3). (C) Ctsk immunostaining in control shRNA and Ctsk shRNA Ocy454 cells showing Ctsk (red), actin (green) and nucleus (blue) (n = 3). Scale bars: 20 μm. (D) qRT-PCR analysis of mRNA expression in control shRNA (black dots) and Ctsk shRNA (red dots) Ocy454 cells. Data shown are the mean of triplicate experiments ± SEM and were normalized to the expression of 18S mRNA (n = 3). *P < 0.05, **P < 0.01, and ***P < 0.001, by Student’s t test for comparisons of control shRNA with Ctsk shRNA at each time point.
femurs. Freshly dissected femurs were embedded in methyl methacrylate and sectioned and the blocks sequentially polished with sandpaper and carbon coated. Images of osteocyte lacunae in cortical bone at the diaphysis were captured at ×500 magnification using Backscatter scanning electron microscopy (BSEM) imaging technique was used to quantify osteocyte lacunae cortical bone at the mid-diaphysis of femurs. Freshly dissected femurs were embedded in methyl methacrylate and sectioned and the blocks sequentially polished with sandpaper and carbon coated. Images of osteocyte lacunae in cortical bone at the diaphysis were captured at ×500 magnification using BSEM (Zeiss Supra 55) in a standard 0.1-mm² cortical area 5-6 mm from the proximal growth plate. The “single osteocyte lacunar area” represents the mean of all (>100) individual osteocyte lacunae measurements within the predefined area. The osteocyte lacunar area, osteocyte perimeter, and osteocyte numbers were quantified using Fiji software in a blinded fashion. Analyses of bones from 5 to 7 mice per group were performed.

μCT. μCT analysis was performed using a desktop μCT35 (Scanco Medical) according to recommended guidelines (56). Briefly, cortical bone parameters at the femoral midshaft and cancellous bone microarchitecture in the distal femoral metaphysis were determined using a 7-μm isotropic voxel size. Eighty-six transverse μCT slices of cortical bone were evaluated, and total cross-sectional volume (millimeter cubed), cortical volume (millimeter cubed), marrow volume (millimeter cubed), and cortical thickness (millimeter) were assessed. For cancellous bone, bone volume fraction (bone volume per tissue...
sue volume (BV/TV) (percentage), trabecular number (Tb.N) (per millimeter), trabecular thickness (Tb.Th) (millimeter), trabecular separation (Tb.Sp) (millimeter), connective tissue density (ConnD) (per millimeter cubed), and structural model index (SMI) were analyzed. Analyses of bones from 7 to 9 mice per group were performed. For the analysis of offspring bones, 8 female Ctsk\textsuperscript{fl/fl} pups were subjected to μCT analysis. Intracortical bone volume fraction was measured in a 600-μm-long region (100 transverse slices) beginning at the top of the proximal tibia metaphysis and extending distally. A threshold of 200 μCT analysis. Intracortical bone volume fraction was measured in a 600-μm-long region (100 transverse slices) beginning at the top of the proximal tibia metaphysis and extending distally. A threshold of 200

Histomorphometry. Twelve-week-old Ctsk\textsuperscript{fl/fl} and Ctsk\textsuperscript{ocy} mice were subcutaneously injected with 20 mg/kg calcine (MilliporeSigma) and demeclocycline (MilliporeSigma) on days 7 and 2 before necropsy, respectively. For lactation studies, virgin and lactating Ctsk\textsuperscript{fl/fl} and Ctsk\textsuperscript{ocy} mice were injected with fluorochrome on days 7 and 2 before necropsy. For phenotype analyses, femurs were fixed in 70% alcohol and embedded in methylmethacrylate. Toluidine blue and von Kossa staining was performed using 5-μm longitudinal sections. Histomorphometric measurements were carried out semiautomatically with the OsteoMeasur e image analyzer (OsteoMetric). A standard sampling site with an area of 2.5 mm\textsuperscript{2} was established in the secondary spongiosa of the distal metaphysis. All parameters were expressed according to standardized nomenclature (57, 58). Dynamic parameters including the mineralizing surface per bone surface (MS/BV) (percentage), the mineral apposition rate (MAR) (micrometer per day), and the bone formation rate (BFR/BV) (percentage per year) were quantified. Static parameters including BV/TV (percentage), Tb.Th (micrometer), Tb.Sp (micrometer), and Tb.N (per millimeter), osteoblast surface per bone surface (Ob.S/BS) (percentage), osteoblast number per bone perimeter (N.Ob/B.Pm) (per millimeter), osteoclast surface per bone surface (Oc.S/BS) (percentage), and osteoclast number per bone perimeter (N.Oc/B.Pm) (per millimeter) were measured. Analyses of bones from 6 to 9 mice per group were performed.

Cell culture. Ocy454 osteocyte cells were plated at 1 × 10\textsuperscript{4} cells/ml and allowed to reach confluence at 33°C in α-MEM supplemented with 10% FBS and 1% Anti-Anti (Life Technologies, Thermo Fisher Scientific) to induce proliferation as previously described (39). Subsequently, cells were transferred to 37°C and differentiated for the indicated durations. Ocy454 cells were infected with 2 distinct lentivirus particles expressing shRNAs targeting Ctsk (TRCN0000054624 and TRCN0000030591, Mission TRC, MilliporeSigma) for 24 hours, followed by puromycin (10 μg/ml) selection at 33°C. A nontargeted shRNA (Non-target/Control-SHC002,

Figure 9. Deletion of Ctsk in osteocytes does not affect offspring skeletal homeostasis. (A) Representative μCT images of the proximal tibia from 12-day-old Ctsk\textsuperscript{fl/fl} female offspring of lactating Ctsk\textsuperscript{fl/fl} and Ctsk\textsuperscript{ocy} mice. Scale bar: 1 mm. (B) μCT analysis of BV/TV and bone mineral density (BMD) bones from offspring of lactating Ctsk\textsuperscript{fl/fl} (black squares) and Ctsk\textsuperscript{ocy} (red squares) mice (n = 8 per group).

Figure 10. Schematic representation of the relationship between osteocytic osteolysis, bone remodeling, and calcium metabolism during lactation in mice in the presence or absence of Ctsk in osteocytes. (A) In the presence of Ctsk in osteocytes (control lactating mice), the changes induced by the lactating mammary gland, including increased secretion of PTHrP, switch the osteocytes to the resorbing mode, increasing TRAP activity and Ctsk expression and leading to increased perilacunar resorption (PLR) and lacunar enlargement. These changes in the osteocyte microenvironment lead to an increase in PTHrP and the Rankl/Opg ratio, thereby increasing osteoclastogenesis, bone resorption, and serum calcium. This increase in serum calcium is also secondary to higher PTHrP and 1,25(OH)\textsubscript{2}D acting on the kidney and the gut, resulting in both calcium supply to the milk and repression of endogenous PTH secretion. (B) In the absence of Ctsk in osteocytes (Ctsk\textsuperscript{ocy} lactating mice), although the changes induced in the mammary gland by lactation increase TRAP activity and Pthrp in osteocytes, deletion of Ctsk prevents PLR, enlargement of the lacuna, and an increase in the Rankl/Opg ratio and osteoclastogenesis. The lack of osteocytic osteolysis and osteoclastogenesis limits the supply of calcium to serum; this is sensed by the parathyroid gland, which continues to secrete PTH at prelactation levels and, together with PTHrP, enhances 1,25(OH)\textsubscript{2}D levels and thus calcium absorption from the gut, compensating for the limited supply of calcium coming from bone and ensuring appropriate calcium levels in the blood and milk.
MilliporeSigma) was used to generate control cells. Analyses were performed at least 3 times in duplicate.

Osteoclasts were generated on coverslips. BM cells isolated from Ctsk<sup>fl/fl</sup> and Ctsk<sup>ov</sup> mice were cultured in α-MEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin for 24 hours to generate BM macrophages (BMMs). BMMs were cultured on coverslips in α-MEM for 2 days with macrophage-CSF (M-CSF) (20 ng/ml), and for an additional 6 days in the same medium with M-CSF (20 ng/ml) and RANKL (3.3 ng/ml) (R&D Systems). Likewise, for RNA isolation, BMMs were isolated from BM-flushedibia and femurs of 6- to 8-week-old Ctsk<sup>fl/fl</sup> and Ctsk<sup>ov</sup> mice. For osteoclast generation, cells were cultured with M-CSF and RANKL for 4 days.

**Immunohistochemistry.** Paraffin-embedded, decalcifiedibia were fixed in 10% neutral buffered formalin (NBF). Longitudinal sections (5-μm-thick) were incubated with antibodies specific to Ctsk (ab19027, Abcam) and sclerostin (AF1589, R&D Systems) at 4°C overnight and then treated with tyramide signal amplification-biotin (TSA biotin) (PerkinElmer) and streptavidin-HRP according to the manufacturer’s instructions. Analyses of bones from 3 to 4 mice were performed. Osteoclasts were fixed with 3.7% formaldehyde in PBS and permeabilized in 0.05% saponin followed by acetone. Cells were incubated in anti-Ctsk antibody (ab19027, Abcam) and incubated 24 hours later with fluorescent secondary antibody (Alexa Fluor 488, Green, A-11034, Thermo Fisher Scientific) and mounted in FluorSave (Calbiochem, MilliporeSigma). Nuclei were labeled with TO-PRO-3 (T3605, Thermo Fisher Scientific) in the secondary antibody solution. Ctsk-expressing osteoclasts were visualized using a 510 Meta laser scanning confocal microscope (Carl Zeiss).

**RNA extraction, cDNA synthesis, and qRT-PCR.** Total RNA was isolated fromibia and femurs flushed to remove BM and from mammary glands using a monophasic solution of guanidine isothiocyanate and phenol according to the manufacturer’s instructions (TRIzol Reagent, Invitrogen, Thermo Fisher Scientific), followed by RNA clean-up using an RNeasy Plus Mini Kit (Qiagen). RNA from Ocy454 cells and BMM-derived osteoclasts were isolated using the RNeasy Plus Kit (Qiagen). Total RNA (1 μg) was used to synthesize first-strand cDNA with SuperScript II (Invitrogen, Thermo Fisher Scientific) for reverse transcription PCR. mRNA levels encoding each gene of interest were normalized to actin mRNA in our laboratory. The 2<sup>-ΔΔCt</sup> method was used when calculating the estimated modulus of elasticity and bending strength. Polar moment of inertia and post-yield deformation analyses of bones from 6 to 7 mice per group were performed.

**Serum and plasma analysis.** For biochemical measurements, blood from virgin and lactating Ctsk<sup>fl/fl</sup> and Ctsk<sup>ov</sup> mice was obtained by cheek pouch puncture after a 3-hour fast, and serum was obtained by centrifugation using serum separator tubes. Biochemical measurements were done using commercially available ELISA kits for PTH (Immutopics), and 1,25 (OH) vitamin D<sub>3</sub> (Immunodiagnostic Systems). Serum PINP and CTX were measured using commercial kits from Immunodiagnostic Systems, according to the manufacturer’s instructions. PTHrP plasma levels were measured as previously reported (61). Briefly, blood was collected into commercially available anticoagulant-treated tubes (Thermo Fisher Scientific) containing a protease inhibitor cocktail of aprotinin, leupeptin, and pepstatin. The 2-site human PTHrP immunoradiometric sandwich assay (DSL 8100, Beckman Coulter) was modified by replacing the DSL 8100 antibody–coated tubes with a 96-well plate coated with anti-human PTHrP(1-36), which was affinity-purified from rabbit anti-PTHrP antibody. Standard curves were constructed using the kit standards diluted in mouse plasma that had been incubated overnight at room temperature to degrade endogenous PTHrP. Samples and standards were incubated overnight in the antibody-coated wells at 4°C, washed, and incubated again with the kit’s iodinated mid-region human PTHrP antibody for 20 hours. Wells were washed and counted in a Packard Cobra II gamma counter. Ample values were extrapolated from the standard curve using GraphPad Prism software and a nonlinear regression curve fit to a sigmoidal dose response.
response with a variable slope. Any given sample was defined as being above the limit of detection if it was both at least 2 SDs above the mean of 4 zero standards and if it fell within the linear portion of the standard curve. The analyses involved 5 to 9 mice per group.

**Milk calcium concentration.** Serum milk calcium concentration was determined using the Quantichrom Calcium Assay Kit (DICA-500, BioAssay Systems) according to the manufacturer’s protocol. In brief, milk was diluted 1:100 in distilled water before measuring calcium concentration, and the level of milk calcium was normalized to the protein content, which was measured using the Bradford protein determination assay (Bio-Rad Laboratories). The analyses involved 4–6 lactating Ctskfl/fl and Ctsk–/– mice.

**Statistics.** Multiple comparisons were analyzed using a 2-way ANOVA followed by Fisher’s protected least significant difference (PLSD) post hoc test comparing all groups. A 2-way ANOVA was used to determine an interaction between Ctsk and lactation. An unpaired, 2-tailed Student t test was used for comparisons of 2 groups. GraphPad Prism 7 software was used for statistical analyses. A P value of less than 0.05 was considered statistically significant.

**Study approval.** All animal procedures were performed in accordance with a protocol approved by the IACUC of Harvard Medical School and followed the US Public Health Service Policy on Humane Care and Use of Laboratory Animals.

**Author contributions**

SL, RB, and FG designed the study. SL, YI, and FG performed experiments. RK generated the floxed mice. SL, KN, VTC, LN, NI, DB, DH, and PD performed measurements. VP maintained the mice colonies and collected samples. SL, FG, and RB wrote the manuscript. SL, RB, FG, JW, and MLB edited the manuscript.

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Ejiri S, Ozawa H. Osteocytic osteolysis observed in rats to which parathyroid hormone was continuously administered. J Bone Miner Metab. 2004;22(6):524–529.


