

Lactation Induces Site-Specific, Irreversible Osteocyte Lacunar Enlargement in Cortical Bone Formed After Pregnancy Onset

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INTRODUCTION: Pregnancy and lactation impose substantial demands on maternal calcium metabolism, leading to significant bone mineral loss and structural alterations during lactation, which are reversibly recovered after weaning [1,2]. Osteocytes (Ocys), the key regulators of bone mass, are thought to mediate these changes through perilacunar/canalicular remodeling (PLR), during which they resorb surrounding matrix and transiently enlarge their lacunae [3]. However, whether Ocys can reversibly remodel bone matrix remains poorly understood. Recent evidence suggests that lactation-induced lacunar enlargement may occur in a site-specific manner [4], raising questions about how Ocy PLR integrates with bone remodeling and mineral dynamics during lactation and post-weaning recovery. To address this, we performed *in vivo* imaging, multiple fluorochrome labeling, and site-specific analysis of Ocy lacunae using backscattered SEM (bSEM). We hypothesized that Ocy PLR during female reproduction is site-specific and correlates with spatially distinct bone remodeling patterns in cortical bone. This hypothesis was tested in both wild-type (WT) mice and Ocy-specific PTH/PTHrP receptor (PPR) knockout mice, in which lactation-induced Ocy PLR is abolished [3].

METHODS: All procedures were approved by the IACUC. Eight-week-old female C57BL6 mice with Ocy-specific PPR deletion (cKO: 14kb-Dmp1-Cre; PPR^{fl/fl}) and WT controls (WT: PPR^{fl/fl}) were assigned to (1) Virgin vs. Reproduction (Repro, n=3/group) for longitudinal analysis (timeline shown in Fig.1A), and (2) Virgin, Lactation, or Post-weaning groups (n=3-6/group, timeline shown in Fig.2A). ***In vivo* imaging:** DXA and μ CT (left femur midshaft-distal, 14- μ m voxel) were performed at pre-pregnancy, post-labor (Preg), end-lactation (Lac), and end-weaning (Wean, Fig.1A). Femoral aBMD and cortical thickness (Ct.Th; total, anterior, posterior) were quantified. ***Dynamic labeling and immunohistochemistry:*** To trace bone formation, calcein (green), alizarin (red), and tetracycline (yellow) were administered as indicated in Fig.1A. Longitudinal femoral cryosections (8 μ m; midshaft-to-distal, including anterior and posterior cortices) were scanned by Zeiss Axioscan (Fig.1F-I). Paraffin sections of tibial midshafts collected from the cross-sectional study (Fig.2A) were stained for MMP13, and % of MMP13-positive Ocys was quantified by ImageJ. ***bSEM:*** PMMA-embedded femoral midshafts were longitudinally sectioned and polished for bSEM imaging (FEI Quanta 250, 15kV, 9 mm WD). Ocy lacunar area was quantified in anterior and posterior cortices (140-160 lacunae/side/sample) and further stratified into five equal zones (Fig.3C,D; n=20-30 lacunae/zone/sample). ***Statistics:*** Differences relative to *Pre-preg* (Fig.1) and among groups (Fig.2) were tested by Student's *t*-test and one-way ANOVA with Bonferroni correction, respectively. Significance was indicated by asterisks (p<0.05).

RESULTS: Cortical bone remodeling and mineral dynamics: Virgin mice exhibited continuous skeletal accrual, evidenced by progressive increases in femoral aBMD and thickening of femoral midshaft Ct.Th (Fig.1B,C). In contrast, Repro mice displayed pronounced fluctuations (Fig.1B-E), with significant gains during pregnancy (aBMD +19%, Ct.Th +20%), sharp declines during lactation (aBMD -17%, Ct.Th -17% vs. *Preg*), and recovery post-weaning (Ct.Th +14% vs. *Lac*). Notably, lactation-induced cortical loss at femoral midshaft was largely confined to the posterior cortex (Ct.Th -25% vs. *Preg*, Fig.1E), with no significant change at the anterior cortex (Fig.1D). Mineral labeling confirmed the μ CT findings. At the anterior cortex, mineral deposition occurred primarily at the endosteal surface, with a slower rate in Repro mice compared to Virgins during pregnancy and lactation (Fig.1F,G). The anterior periosteum remained quiescent, showing no differences between Virgin and Repro mice (Fig.1F,G). In contrast, the posterior cortex exhibited active remodeling at both the endosteal and periosteal surfaces. At the posterior periosteum, Repro mice demonstrated continuous and greater mineral deposition vs. Virgins from pregnancy through post-weaning (Fig.1H,I). Bone resorption at the posterior endosteum was evident during lactation, indicated by the absence of green and red labels, followed by renewed bone formation post-weaning (yellow label, Fig.1I). The overall pattern of cortical bone changes and mineral dynamics in cKO mice did not differ significantly from WT mice (data not shown). ***Ocy PLR and site-specific lacunar changes:*** In WT mice, active PLR was indicated by a 44% increase in MMP13-positive Ocys during lactation, followed by a 32% decline after weaning (Fig.2B). This was accompanied by pronounced lacunar enlargement at the

anterior cortex during lactation, with no recovery in either anterior or posterior cortices post-weaning (Fig.2C,D). These changes were absent in cKO mice, confirming effective suppression of PLR through Ocy PPR deletion. Moreover, lactation-induced lacunar enlargement displayed site-specific patterns, being most prominent in the anterior central-to-endosteal zone and posterior central-to-periosteal zone (Fig.3C,G). These regions corresponded to newly formed bone after pregnancy onset. After weaning, lacunae remained enlarged in the anterior central cortex, whereas lacunar size decreased near the endosteal surfaces, likely due to newly embedded Ocys (Fig.3A-C). No changes in lacunar area were observed in the posterior central-periosteal zone (Fig.3E-G). These spatially distinct lacunar changes were absent in cKO mice (Fig.3D,H).

DISCUSSION: Our study revealed site-specific patterns of cortical bone loss at the femoral midshaft during lactation, driven primarily by enhanced resorption at the posterior endosteum. Continuous bone formation occurred mainly in the anterior central-endosteal and the posterior central-periosteal regions from pregnancy through post-weaning, coinciding with sites of active Ocy PLR and lacunar enlargement during lactation. This site-specific lacunar enlargement observed at both anterior and posterior cortices appeared irreversible, suggesting the limited restorative capacity of pre-existing osteocytes. The apparent post-weaning "recovery" likely results from the incorporation of newly embedded osteocytes rather than remodeling of existing ones. Consistent with previous reports [3], deletion of Ocy PPR effectively inhibited Ocy PLR and abolished these spatially distinct lacunar changes, likely through blockade of PTHrP signaling in Ocys during lactation.

SIGNIFICANCE: This is the first study to longitudinally track maternal bone dynamics in mice during reproduction using *in vivo* imaging combined with mineral labeling. We demonstrate that lactation-induced lacunar enlargement is highly spatial-specific and may be irreversible. These findings redefine osteocyte plasticity during reproductive cycles, revealing functional heterogeneity by cortical location and highlighting the lasting skeletal impact of lactation.

REFERENCES: [1] Kovacs, *Physiol Rev*, 2015; [2] Liu X *et al.*, *Biomech*, 2019; [3] Qing H *et al.*, *JBMR*, 2012; [4] Sieverts M *et al.*, *Sci Rep*, 2024

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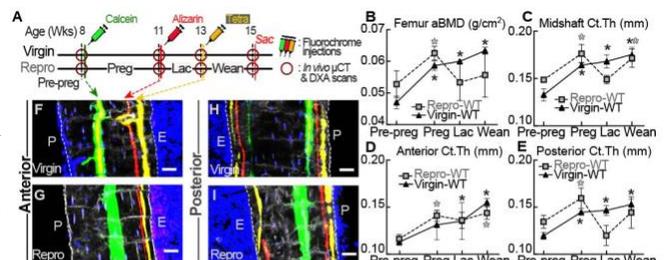


Figure 1. (A) Experimental timeline. Longitudinal tracking of Femur (B) aBMD and midshaft Ct.Th of the (C) total, (D) anterior, and (E) posterior femur. Mineral labeling at (F, G) anterior and (H, I) posterior cortices (P:periosteal, E:endosteal, dashed lines; blue:nucleus; bar: 30 μ m). Asterisks: significant differences vs. Pre-preg (solid; WT, void; Repro; p<0.05).

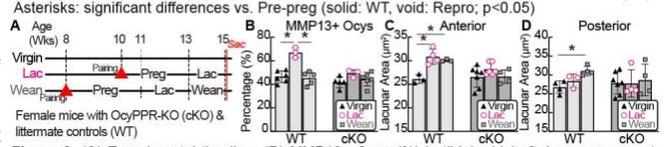


Figure 2. (A) Experimental timeline. (B) MMP13+ Ocys (%) in tibial midshaft. Lacunar area at anterior (C) and posterior (D) cortices.

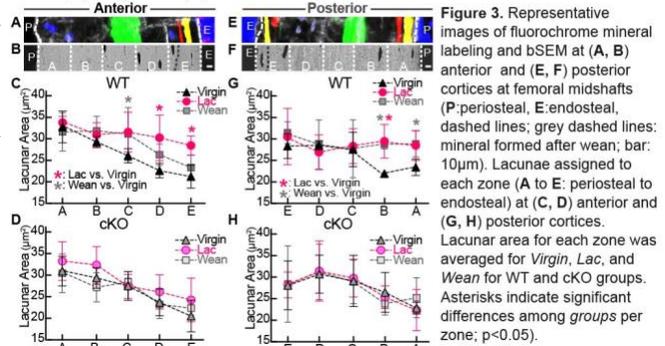


Figure 3. Representative images of fluorochrome mineral labeling and bSEM at (A, B) anterior and (E, F) posterior cortices at femoral midshafts (P:periosteal, E:endosteal, dashed lines; grey dashed lines: mineral formed after wean; bar: 10 μ m). Lacunae assigned to each zone (A to E: periosteal to endosteal) at (C, D) anterior and (G, H) posterior cortices. Lacunar area for each zone was averaged for *Virgin*, *Lac*, and *Wean* for WT and cKO groups. Asterisks indicate significant differences among groups per zone; p<0.05).