Using geometric mean to compute robust mixture designs

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Title: Osteocyte remodeling of the lacunar-canalicular system: what's in a name?

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ABSTRACT

Purpose of Review

Osteocytes directly modify the bone surrounding the expansive lacunar-canalicular system (LCS) through both resorption and deposition. The existence of this phenomenon is now widely accepted, but is referred to as “osteocyte osteolysis,” “LCS remodeling,” and “perilacunar remodeling,” among other names. The uncertainty in naming this physiological process reflects the many persistent questions about why and how osteocytes interact with local bone matrix. The goal of this review is to examine the purpose and nature of LCS remodeling and its impacts on multiscale bone quality.

Recent Findings

While LCS remodeling is clearly important for systemic calcium mobilization, this process may have additional potential drivers and may impact the ability of bone to resist fracture. There is abundant evidence that the osteocyte can resorb and replace bone mineral and does so outside of extreme challenges to mineral homeostasis. The impacts of the osteocyte on organic matrix are less certain, especially regarding whether osteocytes produce osteoid. Though multiple lines of evidence point towards osteocyte production of organic matrix, definitive work is needed. Recent high-resolution imaging studies demonstrate that LCS remodeling influences local material properties. The role of LCS remodeling in the maintenance and deterioration of bone matrix quality in aging and disease are active areas of research.

Summary

In this review, we highlight current progress in understanding why and how the osteocyte removes and replaces bone tissue and the consequences of these activities to bone quality. We posit that answering these questions is essential for evaluating whether, how, when, and why LCS remodeling may be manipulated for therapeutic benefit in managing bone fragility.

1. Introduction

Osteocytes perform numerous functions, from coordinating osteoblast/osteoclast remodeling and mechanosensing (1–3), to regulating mineral homeostasis (4–6), communicating with organs far from bone (7–9) and directly remodeling the extracellular matrix of bone (10–12). Osteocytes are terminally differentiated osteoblasts that reside in lacunae, which are interconnected by small channels called canaliculi (**Figure 1A**)(1,13). Osteocytes are by far the most abundant cells in the skeleton (>90%) and can survive decades (1). Osteocytes coordinate bone resorption and bone formation by signaling that directs the recruitment, proliferation, and differentiation of both osteoclasts and osteoblasts (1–3). In addition, osteocyte apoptosis in response to fatigue microdamage triggers targeted remodeling to remove and replace the damaged matrix (14,15). Osteocytes also directly modify the bone surrounding their lacunar-canalicular system (LCS), though many questions persist about the reasons for this process and its impacts on bone tissue structure, function, health, and disease (1,4,11). Here, we provide a brief review of our current understanding of the purpose, the means, and the consequences of LCS remodeling by osteocytes.

Osteocyte osteolysis, the enlargement of osteocyte lacunae and their canaliculi, has been discussed in the literature for at least 60 years (16,17). The earliest reports were of perilacunar expansion in chicks fed low calcium diet or administered parathyroid hormone (PTH)(16,17). Osteocytic osteolysis was the topic of considerable research interest until Parfitt discounted this process and its study fell out of favor. Parfitt reasoned against osteocyte osteolysis because of technical limitations with available approaches and because the idea had been presented together with bone flow theory, which was refuted (18). While several papers over the next decades reported osteocytic bone resorption or deposition (19–25), more recent work convincingly demonstrated that osteocytes indeed remove and replace bone mineral in lactating mice (4,26). Since then, advances in imaging and study design reveal that osteocytes not only remove and replace bone mineral in the context of extreme challenges to mineral homeostasis, but also perform these activities under normal conditions (1,3,11,27). This direct matrix-altering osteocytic activity is currently referred to by many names, including osteocyte osteolysis, perilacunar remodeling, perilacunar-canalicular remodeling, lacunar-canalicular network remodeling, and lacunar-canalicular system (LCS) remodeling (11,27). We refer to the process as “LCS remodeling” in this review, though, as we discuss, the word ‘remodeling’ carries specific historical connotations in the field of bone and mineral metabolism and the naming of this physiological process will likely require re-visitation as our understanding of the purposes, means, and consequences of this process matures (28–30).

The general purposes of LCS remodeling remain unresolved. Osteocytic osteolysis is clearly important for the systemic mobilization of calcium (4,6,31–35). LCS network geometries change with aging, disuse, osteoarthritis, and some therapies against metabolic bone disease, which implies that LCS remodeling is also dysregulated in these conditions (11,36–43). For these reasons, LCS remodeling is under consideration as a potential mechanism to modulate osteocyte mechanosensitivity or promote bone tissue fracture resistance. Whether these impacts are achieved and, if so, whether they are specifically directed by the osteocyte or secondary to calcium mobilization are unresolved. Here, we summarize the known and proposed purposes of LCS remodeling and their potential impacts on the skeleton.

Many fundamental questions exist about what components of the bone mineral-organic composite osteocytes can resorb and rebuild. The greatest certainty is that osteocytes can readily demineralize and later remineralize bone, although many questions remain about the location, spatial extent, timescales, and impacts of these processes. There are even more questions about whether and how the osteocyte removes and replaces extracellular matrix (ECM), including collagen and

noncollagenous proteins. These impacts to LCS bone mineral and matrix have the potential to influence bone quality from the scale of tissue toughening mechanisms to whole bone fracture resistance.

In this review, we highlight the current progress in understanding the purpose or purposes of LCS remodeling and its consequences to bone mineral, matrix, and multiscale bone quality. We also review key challenges and new approaches for surmounting long-standing questions about the osteocyte and its many possible impacts.

1. Purpose of osteocytic lacunar-canalicular system (LCS) remodeling

LCS remodeling is most prominently observed during systemic mobilization of calcium for reproductive purposes (4,5,10,35,44). Osteocytes actively resorb both perilacunar and pericanalicular matrix to meet the demands of milk production in mammals (4). Osteocytic bone resorption is a faster strategy for mobilizing calcium than recruiting and differentiating osteoclasts and occurs over a much larger surface area. To wit, the LCS surface is several orders of magnitude greater than the surface along Haversian systems available to osteoclasts and osteoblasts (26). Interestingly, canalicular width is also increased soon after fracture at non-fractured skeletal sites in mice, indicating a potential role of LCS remodeling in systemic calcium mobilization to assist the formation of a fracture callous (45). Following the removal of resorption pressure (e.g., cessation of either lactation of eggshell production), mineral is re-deposited, enabling peri-osteocytic matrix homeostasis (4,6,10,32). We will discuss existing data on the nature of the re-deposited matrix below, but whether matrix re-deposition is actively coupled or merely passive homeostasis secondary to lifting of the resorption pressure remains unknown.

PTH is a ubiquitous inducer of LCS remodeling (46). Both lactation and egg production elevate PTH and PTHrP in the maternal circulation to induce osteocyte osteolysis (47). In mice, mammary gland-specific deletion of PTHrP abrogates lactation-induced LCS remodeling (48). Exercise-induced LCS resorption is also mediated by PTH signaling (34). Continuous PTH signaling promotes osteocytic osteolysis by inducing expression of proton pumps, such as ATPase H+ Transporting V0 Subunit D2 (ATP6V0D2), which acidify and demineralize bone matrix and matrix-degrading enzymes including cathepsins and matrix metalloproteinases that degrade the organic matrix (4,35,49). PTH signaling is largely systemic, but LCS remodeling can also be regulated locally via osteocytic TGF-β signaling. Deletion of the TGF-β receptor from osteocytes impairs LCS remodeling, reducing osteocytic expression of both proton pumps and matrix degrading enzymes (50). We found that osteocyte-conditional deletion of the mechanosensitive transcriptional regulators, Yes-associated protein (YAP) and Transcriptional co-activator with PDZ-binding motif (TAZ) similarly disrupted LCS remodeling (51). Together, these observations suggest that despite diverse signals from both systemic and local factors, shared mechanisms mediate osteocytic signaling for both mechanotransduction and LCS remodeling.

LCS remodeling may have developmental origins. In canalicular network development, osteocytes actively arrange collagen and excavate the matrix to form the porous LCS. There is compelling evidence that disruption of osteocyte LCS development can be rescued by postnatal activation of LCS remodeling (52). Wang and coauthors showed that osteocyte expression of Osterix (*Sp7*) is required for proper dendrite formation and canalicular network development (52). Remarkably, they found that both the dendrites and canalicular networks could be rescued within three weeks after an adenoviral gene-therapy to express the Osterix target gene, Osteocrin, injected at the time of weaning (52). These data demonstrate that postnatal activation of LCS remodeling can dramatically alter the LCS even in robustly-mineralized cortical bone. Recent transcriptomic data from adult mouse long bones further support this premise. The osteocyte transcriptome is enriched for genes implicated in mineral and matrix resorption (e.g., cathepsin K, tartrate resistant acid phosphatase, vacuolar ATPase

family), demonstrating that osteocytes retain the capacity to modify their surrounding canalicular architecture in a manner consistent with development (53).

An intriguing potential role of LCS remodeling, which would further integrate LCS remodeling and mechanoadaptation, is a mechanism to achieve strain amplification to engage remodeling (54–56). Digital image correlation studies show that strains are amplified near osteocytes, but the reasons for this result are not clear (57). In particular, it is plausible that osteocytes could engage LCS remodeling to amplify or dampen mechanical signals by altering the shape of lacunae and canaliculi or the compliance of the surrounding bone (56). However, whether the osteocyte can actively modulate its own mechanosensitivity through LCS remodeling is not determined.

Another question is whether LCS remodeling contributes to bone fracture resistance (13). Genetic mouse models that interfere with TGFβ or YAP/TAZ signaling decrease LCS remodeling and produce a phenotype similar to skeletal aging, including decreased fracture toughness (49,51,58). These results, together with truncated LCS geometry in aging (37,38,59), suggest that LCS remodeling may have a role in maintaining bone fracture resistance in youth and that this process is decreased in aging. However, whether LCS remodeling serves to toughen bone is not determined. The LCS surface area is enormous, with a surface area on part with a tennis court (215 m^2) and an end-to-end length of 175 km (13). LCS remodeling may thus result in the frequent turnover of an immense quantity of bone and decrease overall bone tissue maturity (i.e., increases the quantity of bone tissue with lower mineralization, less crosslinking, and less microdamage), which could serve to increase bone fracture resistance (11). Additionally, the loss of viable osteocytes and consequent micropetrosis could also deleteriously impact bone toughness (60–63). Establishing the significance of LCS remodeling to bone fracture resistance necessitates first determining the specific impacts of LCS remodeling on bone mineral, matrix, and multiscale bone quality.

While many open questions remain (**Box 1**), we are excited for the developments that coming years will bring toward resolving the purposes of LCS remodeling. We anticipate that ascertaining how, when, and why LCS remodeling may be controlled for therapeutic benefit could have a significant impact on our understanding and treatment of bone diseases.

2. What are the impacts of LCS remodeling on bone mineral?

Osteocytes very clearly can demineralize bone. Qiu and Bonewald's seminal work demonstrated that osteocytes expand their surrounding lacunae in response to lactation in C57Bl/6 mice fed low calcium diet and that weaning reverses the lacunar expansion (4). Demineralization also occurs around canaliculi (64–66), although the spatial extent to which acids and matrix degrading proteins produced by osteocytes can affect more distant locations along canaliculi is not known.

Osteocytes also deposit bone mineral. Recovery of LCS architecture after lifting resorption pressure (e.g., weaning) demonstrates that osteocytes do produce new bone (4,6,49). Perilacunar bone formation has been visualized in rodents and humans by the systemic injection of calcium-binding fluorochromes (4,12,40,49,51,67,68). While classically applied to quantify osteoblastic mineral deposition on bone surfaces, high resolution imaging reveals extensive fluorochrome labeling of both osteocyte lacunae and canaliculi (**Figure 1A**). Notably, the perilacunar fluorochrome signal is typically not visible at light intensity thresholds that are optimal for analysis of osteoblastic surface deposition. Perilacunar labeling is abundant, including in cases outside of extreme challenges to mineral homeostasis (12,49,51). In young adult C57Bl/6 female or male mice, the majority (60-80%) of cortical femur and tibia lacunae show a fluorochrome label when administered calcein or alizarin 2 days before euthanasia (12,51). Lacunae can also show sequential double labels (**Figure 1B,C**). Because fluorochrome

labels are present for newly formed bone or newly exposed bone surfaces (i.e., following resorption), interpreting double labels for osteocyte lacunae requires scrutiny. The presence of sequential double labels, such as after weaning in lactation studies, is strongly suggestive of perilacunar mineral deposition (4). Interestingly, osteocyte-specific deletion of the mechanotransducers, YAP and TAZ, or global MMP13 knockout, decrease the percentage of labeled lacunae (49,51). These data indicate that LCS mineralization is regulated by osteocyte signaling.

Osteocytes likely express mineralization promoters and inhibitors, but the details of their specific control over local mineralization are largely unknown. Most bone mass is spatially associated with regions of high canalicular density (66), which suggests osteocyte mineralization promotion. Osteocytes also participate in mineralizing osteoblast-produced osteoid (64) . Meanwhile, there is a 'halo' zone of lower mineralization immediately adjacent individual canaliculi that suggests local mineral inhibition (64). Additionally, lacunar infilling with mineral (i.e., micropetrosis) is a characteristic of aged human bone with fewer viable osteocytes that likely produce fewer mineral inhibitors (60–63). These data suggest that osteocyte-produced mineralization inhibitors and promotors influence the location and quantity of mineralization. The specific identities of mineralization inhibitors for early and mature osteocytes are not understood. Also uncertain is whether osteocyte-produced extracellular vesicles (EVs) participate in regulating local bone mineralization. EVs travel through the LCS and can affect structures as far afield as the brain (8,9,69). Matrix vesicles (MVs) secreted by osteoblasts are directly shown to promote mineral nucleation from within the MV and can also bind to collagen (70). Whether osteocytes secrete matrix vesicles to nucleate mineral along the LCS is undetermined.

The maturation dynamics of bone formed by the osteocyte are also uncertain. In osteoid formed by osteoblasts, primary mineralization takes 7-10 days and accounts for 70% of bone mineral (71,72). Osteoblast-formed bone also matures with regards to crystal perfection and carbonate substitution (73,74), but whether this is true of osteocyte-deposited bone has not been reported. Since bone mineralization and demineralization appear to be frequently activated by osteocytes, it is possible that matrix close to a healthy osteocyte infrequently achieves a highly mature state. Supporting this idea, synchrotron phase contrast studies from human and sheep bone show mass gradation around lacunae and canaliculi. The lowest mass (i.e., mineral) content is directly adjacent to the walls of lacunae and canaliculi and a peak is reached 200-400 nm away (66). Additional work demonstrates that mineral thickness is higher close to lacunar and canalicular walls within areas of dense osteocyte networks (75). These findings prompt comparisons with the 'lacunar brush border' of incompletely dense, needle-like minerals at the fringe of osteocyte lacunae witnessed nearly 50 years ago in electron micrographs by Bonucci and Gherardi (23). Together, these studies suggest that bone tissue maturation local to the LCS is likely, but the dynamics of this process and the connections to osteocyte resorption and deposition activity are much less certain.

While rough estimates of the relative amount of calcium mobilized by LCS remodeling relative to osteoclastic resorption exist, precise measurements are needed to understand the role of the osteocyte in participating in systemic mineral homeostasis in reproduction, health, aging, and disease. The most fundamental questions include how osteocytes regulate peri-LCS mineralization, where and when bone mineralization and demineralization occur, and over which spatial and timescales LCS bone matures (**Box 1**).

3. What are the impacts of LCS remodeling on organic bone matrix?

Osteocytes have the ability to degrade organic matrix, as indicated by the lack of lacunar expansion for lactating MMP13-null mice (49). Demineralized bone is found around lacunae, as can be observed from histological studies. In a comparison of Wistar rats treated with either 4 weeks of

subcutaneous PTH or vehicle, the vehicle rats show a thin band of matrix surrounding cortical tibia osteocyte lacunae positive for both hematoxylin and toluidine blue. These bands appeared as prominent perilacunar belts in PTH-treated rats, which also showed lacunar expansion (21). However, it is unclear whether this tissue was residual matrix after mineral resorption or instead new osteoid produced by osteocytes.

Whether osteocytes produce osteoid is currently debated. Several lines of evidence support that osteocytes likely have this ability. Multiple studies from mouse long bones show abundant transcripts for ECM production, including type 1 collagen (*Col1a1, Col1a2*), osteocalcin (*Bglap*), and osteonectin (*Sparc*) (53,76). In some cases, the expression of these ECM genes (e.g., *Col1a1, Bglap*) can be even higher for osteocytes than for osteoblasts, such as shown in a recent laser capture microscopy analysis of rat vertebrae (77). Additional evidence for the osteocyte production of osteoid comes from fluorochrome labeling and histological studies. Serial double labeling has been reported for osteocyte lacunae from post-lactation mice, which implies the formation of osteoid (**Figure 1 B-C**)(4). Serial osteopontin bands around lacunae were seen for Wistar rats, also suggesting serial matrix formation events (20). Radiolabeling studies showed [^{3}H] proline-labeled collagen around cortical lacunae from egg-laying hens during a period of calcium repletion (32,78,79). The irregular edges around these lacunae suggested, but did not confirm, prior bone removal. In humans, osteocyte-centered histological measurements were compared for Villanueva osteochrome-stained transiliac bone biopsies from hemodialysis (CKD) and osteoarthritic (OA) groups (40). Both CKD and OA groups showed osteoid-positive lacunae, but the number density of osteoid-positive lacunae was much greater for CKD patients with high PTH than either CKD-low PTH or OA. Dallas and colleagues recently developed a mouse model in which the topaz variant of green fluorescent protein (GFP*tpz*) was inserted into the mouse pro α2(I) collagen N-terminus with expression driven by the 3.6-kb type I collagen promoter (35,80). These mice exhibit bright bands of GFP signal around osteocytes (80). Lactating mice, fed a low-calcium diet to maximize skeletal calcium mobilization, had significantly reduced collagen-GFP signal (35). On recovery, serial bands of GFP-collagen appeared in the perilacunar matrix (S. Dallas, personal communication). While these several studies provide evidence for osteocyte ECM production, there is still considerable uncertainty about what osteocyte produces and when. Very few osteocyte investigations considered organic matrix, perhaps because this information is not readily available using methods commonly utilized to study LCS geometry (e.g., high resolution CT).

If osteocytes do produce osteoid, several questions become pertinent. There may be important compositional and functional differences between osteocyte- and osteoblast-produced osteoid. It would be valuable to discern which osteocyte-produced proteins are incorporated into mineralizing osteoid produced by osteoblasts versus into new matrix around osteocytes. The maturation of the collagen matrix is also of interest. Does LCS bone resorption and deposition decrease the maturity of the collagen matrix, and would insufficient turnover decrease the quality of this matrix? Whether osteocytes directly influence or regulate the post-translational modifications of proteins in bone extracellular matrix is unknown. It is possible, but not yet shown, that the osteocyte could participate in the regulation of enzymatic and nonenzymatic collagen crosslinking and therefore impact collagen maturity. Answering these questions will require focused investigations at measurement scales relevant to osteocyte bone resorption and reformation (i.e., hundreds of nanometers) (**Box 1**).

4. What are the effects of LCS remodeling on bone quality?

LCS remodeling alters perilacunar bone quality and impacts tissue-level material behavior (11). Most investigations of LCS remodeling on bone quality have employed microscale tools (e.g., Raman spectroscopy, backscattered SEM, nanoindentation) to assess the gradation of bone stiffness or

composition with distance from lacunae. In young adult mice, bone properties are usually similar between bone 1-5 micrometers from lacunae and farther away (e.g., 7-15 μm)(10,34,81). In circumstances that challenge mineral homeostasis, such as lactation, kidney disease, glucocorticoid treatment, or PTH administration or endogenous expression from exercise, bone close to osteocyte lacunae is less mineralized or less stiff than at farther distances (4,34,81,82). However, outside of these contexts, resolving the impact of LCS remodeling on the surrounding tissue requires zooming in by at least an order of magnitude (11). Synchrotron phase contrast and transmission electron microscopy studies show that bone composition near the LCS is graded at the scale of hundreds of nanometers (66). In these studies, the lowest mineralization was seen immediately adjacent to lacunar and canalicular walls, which increased to a peak value 200-400 nm away. These gradations were reduced for lacunae from necrotic, glucocorticoid-treated bone compared with healthy mandibles, suggesting that osteocyte health may influence LCS bone material properties (66).

We recently found a more direct connection between osteocyte LCS activity and local bone quality (12). We mapped bone modulus at nanometer-scale resolution using atomic force microscopy (AFM) in defined regions around fluorochrome labeled and nonlabelled lacunae in the cortical femur for 5 mo and 22 mo C57Bl/6 female mice. A similar profile of modulus versus distance from LCS walls was found from these AFM maps as for mineral gradation seen in prior synchrotron studies (12,66). Modulus initially increased from a minimum value along the lacunar wall to a maximum value 200-400 nm away. Labeled and non-labelled lacunae had a similar shape of gradation (i.e., similar initial rise and also location of peak value), but for labeled lacunae at both ages the gradation was shifted downwards towards lower moduli. Of note, perilacunar modulus gradation depends on hydration. For dehydrated bone, perilacunar modulus gradation closely corresponds to those seen in synchrotron microscopy and likely indicates gradation in mineralization. For hydrated bone, there is also a sharp modulus increase over the first ~400 nm from the lacunar wall, followed by a gradual increase to a peak modulus at approximately 1 micrometer away. Notably, this characteristic length corresponds with the distance from which 60% of bone mineral is located from the nearest lacunar or canalicular surface (75). An interesting question is if mineral and modulus gradations local to osteocytes influence strain experienced by the osteocyte. The more compliant tissue close to the LCS would be expected to amplify strain (56). Control over material gradation near the LCS may constitute a mechanism by which osteocytes regulate strain amplification. If this mechanism exists, and if it is impaired with decreased osteocyte viability in aging, are unresolved questions.

Several persistent questions need to be resolved about the impacts of LCS remodeling on bone quality. It is important to identify which tissue-scale toughening mechanisms, if any, are specifically impacted by LCS remodeling. Measurement of how much mineral and matrix are each resorbed and replaced by the osteocyte, and how these activities vary with skeletal location, sex, and across the lifespan, are needed to assess whether LCS remodeling 'adds up' as a mechanism by which the osteocyte can promote bone fracture resistance (**Box 1**).

6. Discussion

Fundamental questions about the role and impacts of LCS remodeling on bone currently limit our ability to interact with this system for therapeutic benefit. These questions are also implicit in the lack of consensus about what to call this physiological process. Since Belanger coined the term 'osteocytic osteolysis', the names used to describe this phenomenon have evolved and now include perilacunar remodeling, perilacunar-canalicular remodeling, lacunar-canalicular network remodeling, and lacunar-canalicular system remodeling. Notably, whether ‘remodeling’ is an appropriate description of the bone resorption and deposition activities of the osteocyte is unresolved. Remodeling, as

understood from the perspective of conventional bone histomorphometry, implies the coupled removal and replacement of bone tissue by the basic multicellular unit (29,30,83). While we now agree that the osteocyte performs bone resorption and replacement, whether these activities are coordinated has not been demonstrated and the nature of the re-deposited matrix is under debate. 'Modeling' may be more appropriate, which refers to the uncoordinated removal or deposition of bone. This process serves to adapt and optimize the shape of bone in response to changing loading demands (or disuse) (30). It could be that LCS remodeling should be thought of as serial modeling activities. Another candidate is 'turnover', indicating that bone is removed and replaced, but without the coordination of remodeling nor the specific functionality of modeling. This wording is conventionally used in the context of mineral exchange (30). At the moment, 'LCS turnover' is probably the most conservative description for these osteocyte bone resorption and formation process, but the name can and should evolve with our understanding of why, when, and how the osteocyte interacts with its surrounding mineral and matrix.

Most of the persistent knowledge gaps about the osteocyte can be classified in two categories with unique technical challenges. The first category is centered on how the osteocyte affects its local environment over space and time. While monitoring the size and shape of lacunae and canaliculi is useful for revealing large phenotypes (i.e., LCS expansion in lactation or truncation in aging) and is tractable via many common tools, this approach does not answer questions about how and when bone is resorbed or formed by osteocytes. A smaller lacuna could result from bone formation or, alternatively, but the cessation of resorption. Bone histomorphometry techniques are in many cases well-suited to improve our understanding about the temporal and spatial dynamics of LCS bone resorption and deposition. Also unresolved is how LCS remodeling impacts surrounding bone maturity and material properties. We now understand the need to 'zoom in' to witness the impacts of the osteocyte on its surrounding bone. Highly resolved tools, such as AFM, are useful for making progress in this space. We note that AFM on bone is technically challenging, requiring very smooth surfaces and stiff cantilevers (11,12). Performing AFM on hydrated bone introduces additional testing considerations (12).

A second category of questions is about the connection between osteocyte health and behavior on LCS remodeling. Because decalcification is necessary to assess osteocyte viability and protein production and is usually incompatible with studying the material properties of bone tissue, there are many longstanding questions about how osteocyte health and behavior influence LCS remodeling and, as a consequence, tissue properties. Our best tools are currently focused on affecting ensembles of osteocytes through genetic or pharmacological interventions and then assessing many of these cells. In complex processes such as aging, where osteocytes may be healthy, apoptotic, or senescent, the specific states of health of individual cells may differently affect LCS remodeling. Surmounting this major technical challenge is needed to link structure and function and accelerate our understanding of why and how osteocytes modify, or fail to modify, their surroundings.

While the prominence of studying LCS remodeling as a scientific pursuit has waxed and waned over the decades, now is a particularly exciting time to be studying this phenomenon. Many open questions regarding the purpose, the nature, the dynamics, and the impacts of LCS remodeling on bone tissue remain. We posit that answering these questions will be essential to ascertain whether, how, when, and why intervention in LCS remodeling may be exploited for therapeutic benefit. While our understanding to date of this scientifically fascinating and physiologically important phenomenon remains nascent, with the critical mass of researchers interested in this topic, new tools and models available, including cross-species comparative biology, we are enthusiastic about the insights the coming years will bring and their future impact on skeletal medicine.

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ETHICS DECLARATIONS

Conflicts of Interest

The authors declare no conflicts of interest.

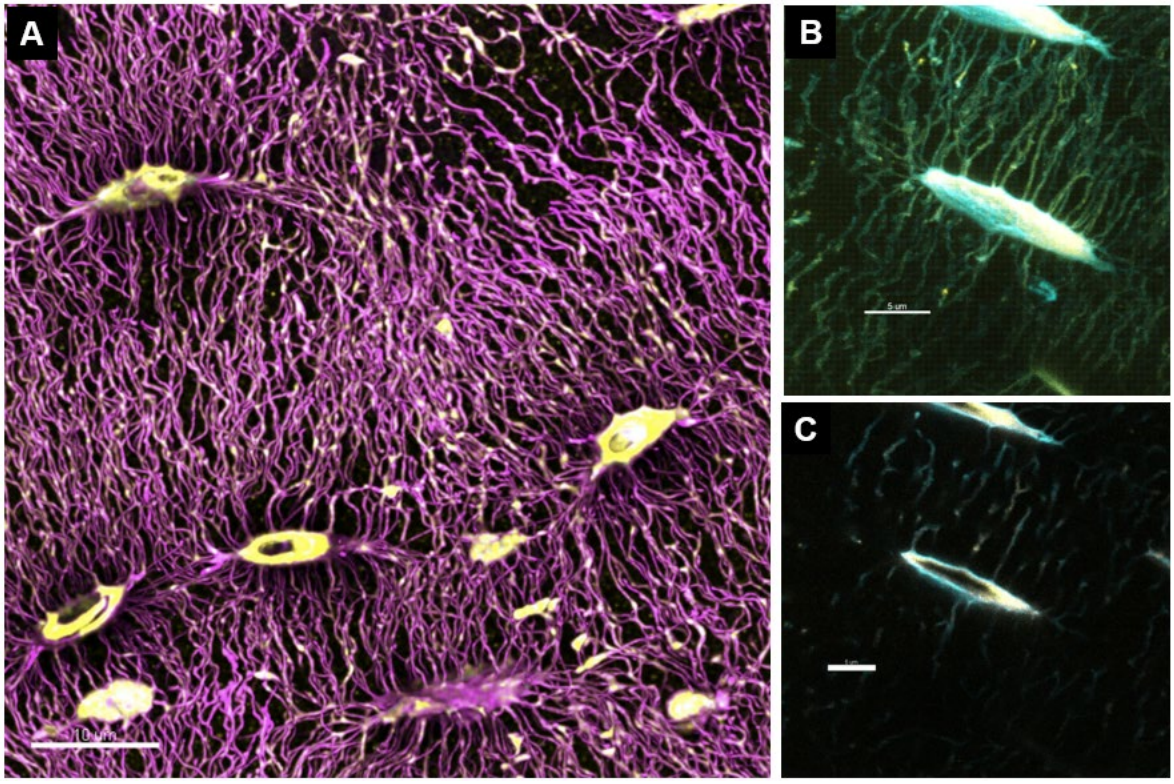

Figure 1. **Osteocytes reside in the highly-connected lacunar-canalicular system (LCS) and can exhibit progressive mineral deposition.** A) Osteocyte-resident lacunae and canaliculi, visualized by basic fuchsin staining (magenta). Calcein labels (yellow) indicate exposed perilacunar and canalicular mineral, either from mineral resorption or new deposition. B) Mice administered with sequential fluorochome labels - calcein (yellow) and alizarin (cyan) - identify double-labeled lacunae and labeled canaliculi (scale bar 10 µm). A calcein label was administered 2d before euthanasia. Three dimensional reconstructions, shown here in maximum-intensity projection, demonstrate serial 'shells' of new bone. C) A single confocal slice demonstrates separately-labeled rings of sequentially-deposited mineral (B-C scale bar 5 µm). Alizarin and calcein labels administered 8d and 2d before euthanasia, respectively. All images from 5-month female C57Bl6/J mice. Image credit Ghazal Vahidi, Montana State University.

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Osteocyte lacunar canalicular remodeling: persistent questions

What is the purpose?	What are the impacts on bone mineral?	What are the impacts on bone matrix?	What are the impacts on bone quality?
▪ Are LCS bone resorption and replacement processes coupled? ▪ Does LCS remodeling improve osteocyte mechanosensitivity? ▪ Does LCS remodeling improve bone fracture toughness?	▪ How do osteocytes promote and inhibit LCS bone mineralization? ▪ When and where do mineralization and demineralization occur? ▪ Over which spatial and timescales does LCS bone mature?	▪ Do osteocytes produce osteoid? ▪ How are osteocyte-produced factors incorporated into mineralizing versus mature bone matrix? ▪ How does peri-LCS matrix mature and to what extent?	▪ How much mineral and matrix are removed and replaced by the osteocyte? ▪ What tissue-scale toughening mechanisms are impacted by LCS remodeling? ▪ How does aging influence LCS remodeling activities?

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394 **Box 1**: Persistent questions about osteocyte lacunar-canalicular remodeling and the impacts of this
395 physiological process on bone tissue.

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