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MOLECULAR BIOLOGY OF SKELETAL DEVELOPMENT (T BELLIDO, SECTION EDITOR)

Bone Lining Cells: Normal Physiology and Role in Response to Anabolic Osteoporosis Treatments

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Abstract
Purpose of Review The goal of this chapter is to review the proposed roles for bone lining cells in bone homeostasis. We will focus on how these cells contribute to normal bone remodeling and how they might participate in bone anabolic responses to osteoporosis therapies.
Recent Findings Lineage tracing methodologies have recently demonstrated that quiescent bone lining cells can directly convert into active matrix-forming osteoblasts in the setting of treatment with parathyroid hormone and anti-sclerostin antibody.
Summary Bone lining cells are an abundant yet poorly studied cell type in bone. They most likely participate in normal bone remodeling and have important roles in responses to osteoanabolic osteoporosis treatments and in skeletal repair after injury. Novel models are needed to selectively ablate and interrogate the function of specific genes in bone lining cells.

Keywords Bone lining cells · Osteoblasts · Parathyroid hormone · Sclerostin · Osteoporosis · Lineage tracing

Active matrix-producing osteoblasts undergo one of at least three fates: they can die by apoptosis, become embedded within mineralized matrix as osteocytes, or remain quiescent on bone surfaces as bone lining cells [1, 2]. Although bone lining cells have been observed histologically for quite some time, their physiologic function in normal bone homeostasis has remained elusive. Histomorphometric analysis has demonstrated that the vast majority of cancellous and endocortical bone surfaces are covered by flat bone lining cells (BLCs) [3]. Many proposed functions of flat BLCs have been posited in the decades since their initial description. Here, we will review our current understanding of BLCs, focusing primarily on recent lineage tracing studies demonstrating that these cells serve as a pool of osteoblast precursors that may participate in bone formation stimulated by anabolic osteoporosis treatment strategies.

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Introduction: Bone Lining Cell Function During Normal Physiology

When bone surfaces are examined by electron microscopy, a flat layer of cells with a thin seam of non-mineralized matrix is apparent [4]. Based on possible physical homotypic connections between these thin BLCs and between osteocytes via gap functions, one proposed function of these cells is to form a functional "membrane" that separates bone and interstitial fluids [5, 6].

Beyond forming a functional barrier between bone and bone marrow, ample evidence exists to suggest that BLCs participate in skeletal remodeling during normal physiology. During bone remodeling, an initial step of matrix degradation is thought to precede osteoclastic recruitment to resorptive sites [7]. In addition, after vigorous resorption by osteoclasts occurs, it is thought that Howship's lacunae must be "cleaned" in a more precise manner [8]. Based on their widespread distribution throughout surfaces that may undergo active remodeling, BLCs are ideally poised to accomplish these catabolic functions. Recently, this hypothesis has been tested via

immunohistochemistry and laser capture microdissection. Immunostaining has revealed robust expression of matrix-degrading enzymes such as MMP13, MMP14, TIMP1, and TIMP2 in thin BLCs from human iliac crest bone biopsies [4]. Laser capture microdissection (LCM) from cryosections followed by RNA isolation confirmed expression of these genes in thin BLCs. Importantly, this LCM study confirmed expression of CBFA1/RUNX2 [9] in thin lining cells, an observation that supports the notion that these cells belong to the osteoblastic lineage [4].

Indeed, ultrastructural analysis of murine bones has demonstrated that BLCs wrap and destroy protruding collagen fibrils. Ex vivo experiments suggest an important role for matrix metalloproteases in this process. In addition to "cleaning up" Howship's lacunae after osteoclastic resorption, BLCs have been suggested to synthesize a thin layer of collagenous matrix, which may serve as a template for subsequent osteoblast activity. Importantly, in pycnodysostosis, a condition in which osteoclasts cannot resorb bone due to cathepsin K mutations [10], some matrix catabolism can be rescued by proteolytic activity from BLCs as well as other osteoclastic proteases other than cathepsin K [8].

More recently, laser capture microdissection was performed to compare global gene expression patterns in osteoblasts, osteocytes, and BLCs in rats at baseline and in response to sclerostin antibody treatment [11]. Here, microdissection was facilitated by proximity of dissected cells to a fluorochrome injection just prior to sacrifice. Through this technique, it could be assured that osteoblasts were adjacent to active bone surfaces and BLCs were distant from active skeletal sites. A full discussion of the effects of sclerostin antibody treatment on BLC biology follows below. Prior to drug treatment, it is important to note that principal component analysis (PCA) [12] demonstrated clear global differences in gene expression profiles comparing osteoblasts, osteocytes, and BLCs. Therefore, while BLCs likely do represent cells within the osteoblastic lineage, their gene expression profile complements morphologic data, suggesting that these cells have an entirely distinct function than active, matrix-synthesizing osteoblasts [11]. Further bioinformatic analysis of these rich datasets will be necessary to better understand BLC-specific genes.

In summary, descriptive studies characterizing BLCs by histology, immunohistochemistry, and expression profiling indicate that these abundant osteoblast lineage cells may play an important role in matrix catabolism during normal bone remodeling cycles. Moving forward, novel tools will be necessary to dissect BLC function in vivo. For example, mice selectively lacking BLCs would be an invaluable reagent to better understand the function of these cells in bone development and remodeling. In addition, a BLC-specific Cre driver strain would greatly accelerate this field by providing investigators a way to manipulate gene function selectively in this cell type. Despite a potential role for BLCs in matrix catabolism, additional functions of these cells in bone anabolism likely do exist, as reviewed below.

Bone Lining Cells Participate in How Intermittent Parathyroid Hormone Stimulates New Osteoblastogenesis

The majority of existing osteoporosis medications work by slowing bone destruction by osteoclasts [13]. While effective, long-term suppression of bone resorption (with concomitant suppression of bone formation by osteoblasts) is associated with rare risks such as osteonecrosis of the jaw and atypical femoral fractures [14, 15]. Furthermore, anti-resorptive therapies reduce, but do not eliminate, fracture risk. Therefore, optimized anabolic treatment agents that stimulate new bone formation by osteoblasts are highly desired to treat this major public health problem in our aging population [14].

Currently, the only FDA-approved anabolic treatment for osteoporosis is teriparatide, a synthetic version of parathyroid hormone (PTH) amino acids 1–34. When given by once daily subcutaneous injection, teriparatide significantly boosts osteoblast numbers, bone formation, and bone density and reduces fracture risk [16].

While it has been long appreciated that both intermittent and continuous hyperparathyroidism increase osteoblast activity, the cellular mechanisms responsible have remained relatively obscure. Over the past two decades, several cellular mechanisms have been posited to explain how intermittent PTH treatment increases osteoblast numbers in vivo. On cancellous surfaces in vertebrae, intermittent PTH treatment significantly decreases osteoblast apoptosis [17], although it is unlikely that this effect fully accounts for the dramatic increase in osteoblast numbers seen following teriparatide administration. Direct effects of PTH on early cells in the osteoblast lineage have been proposed based on studies on cultured bone marrow-derived stromal cells (for example, [18]), but confirmation of these in vitro experiments in living animals has been limited due to inability to precisely label and track osteoblast precursors in vivo. Non-cell-autonomous effects of PTH on osteoblast activity may also occur. For example, PTH-induced osteoclastic bone resorption may liberate matrix growth factors that in turn recruit osteoblast progenitors to bone surfaces and stimulate their differentiation [19]. In addition, through effects on osteocytes, PTH reduces levels of the anti-osteoblastogenic WNT inhibitor sclerostin (see below for details) [20, 21], thus providing another paracrine mechanism through which PTH might stimulate osteoblast differentiation. In addition to possibly promoting osteoblastic differentiation, PTH may block adipogenic differentiation of early cells in the osteoblast lineage [22].

The potential mechanisms for the PTH-induced osteoblastogenesis listed above are unlikely to account for the ability of intermittent PTH to increase osteoblast numbers of periosteal surfaces. An additional source of new osteoblasts may be BLCs, which, as discussed above, are present on the majority of quiescent periosteal, endosteal, and cancellous bone surfaces. Histomorphometric analyses revealed that PTH rapidly increases fluorochrome-labeled bone surfaces and incorporation of radiolabeled amino acids into bone surfaces. This is accompanied by increased osteoblast numbers without increased osteoblast proliferation [23]. EM studies further demonstrated that PTH treatment has no overall change in cell number on bone surfaces, since increased osteoblast numbers are accompanied by reduced numbers of quiescent BLCs [24]. Taken together, these observations support the hypothesis that PTH might directly convert quiescent BLCs into active osteoblasts.

More recently, lineage tracing technology has allowed investigators to label precisely defined cell populations in vivo and then to track their differentiation over time. In this approach, a tamoxifen-dependent Cre transgene is expressed using a cell type-specific promoter. Mice are bred to a Cre-dependent reporter, such that after tamoxifen administration, labeled cells can be easily identified [25]. Lineage tracing has been applied to study endochondral bone development, elegantly demonstrating that osteoblast precursors in the perichondrium invade the bone collar to give rise to trabecular osteoblasts, osteocytes, and stromal cells within developing bones [26]. More recently, Kim et al. used an analogous lineage tracing strategy to label mature osteoblasts and osteocytes in postnatal mice [27••]. The fate of osteoblasts on periosteal bone surfaces was then monitored over a subsequent chase period. As expected, the majority of initially labeled osteoblasts disappeared, likely due to death by apoptosis. However, some labeled cells remained on bone surfaces. The remaining labeled cells on bone surfaces were not actively synthesizing type I collagen and displayed ultrastructural features consistent with quiescent BLCs. Therefore, lineage tracing demonstrated that some BLCs derive from mature osteoblasts.

Then, Kim et al. treated mice with intermittent PTH for 3 days after a prolonged chase period. Compared to vehicle-treated mice, labeled cells on bone surfaces now assumed a plump, osteoblastic morphology and actively synthesized type I collagen [27••]. Importantly, labeled osteoblasts were not actively proliferating, as assessed by BrdU incorporation. Therefore, this approach demonstrated that intermittent PTH treatment converts BLCs into mature osteoblasts on periosteal surfaces. Since this method only examines the morphology of a rare population of labeled cells, it is impossible to determine the relative contribution of lining cell reactivation to the overall pool of PTH-stimulated osteoblastogenesis. Furthermore, the molecular mechanisms underlying PTH-induced lining cell reactivation remain to be determined. For example, it is unknown whether BLCs express PTH receptors or whether PTH-induced BLC activation occurs through direct effects of PTH on BLCs or involves paracrine mechanisms. Finally, whether continuous hyperparathyroidism also affects BLCs in a similar manner remains to be determined using a lineage tracing approach.

Sclerostin Antibody Increases Osteoblast Numbers on Quiescent Bone Surfaces and Reactivates Bone Lining Cells

Although teriparatide is currently the only approved bone anabolic osteoporosis treatment agent, several additional anabolic therapies are currently in development [28–30]. Sclerostin (encoded by the gene SOST) is a protein secreted by osteocytes that negatively regulates bone formation. Humans with mutations in or near SOST have high bone mass and resistance to fractures [31, 32], and common variation in this locus predicts bone density and fracture risk [33, 34]. By binding to the WNT co-receptor LRP5 and blocking its ability to bind ligands, sclerostin is a potent inhibitor of WNT signaling in osteoblasts [35]. Monoclonal anti-sclerostin antibodies potently boost osteoblast numbers, bone formation, and bone mass in rodents and humans [36]. Interestingly, in addition to stimulating new bone formation, sclerostin antibody treatment also reduces osteoclast activity [30]. Like PTH, the precise cellular mechanisms through which sclerostin controls osteoblast activity remain unclear. Since sclerostin antibody rapidly increases osteoblast numbers on previously quiescent bone surfaces, the possibility that lining cell reactivation might contribute is quite appealing.

Histomorphometry studies have strongly supported the notion that sclerostin antibody (Scl-Ab) treatment might convert lining cells into osteoblasts. In ovariectomized rats and cynomolgus monkeys, Scl-Ab treatment causes a dramatic reduction in quiescent bone surfaces and a concomitant increase in bone surfaces actively engaged in mineralization [37•]. Based on these data, Ominsky et al. have posited that the predominant mechanism through which Scl-Ab increases bone formation is by stimulated modeling-based formation at previously quiescent surfaces. At the cellular level, the same group used stereologic histomorphometry to demonstrate concomitant decreases in lining cells and increases in osteoblasts after Scl-Ab treatment in rat trabecular bone [38]. However, this methodology does not prove direct conversion of lining cells into active osteoblasts.

Recently, lineage tracing was used to assess the effects of Scl-Ab on BLCs [39••]. In these studies, two separate tamoxifen-dependent transgenes were used: osteocalcin-CreER and DMP1-CreER. This strategy allowed for visualization of osteoblasts on endocortical and periosteal surfaces and observation of their

subsequent differentiation into BLCs. After a prolonged chase period, mice were treated with Scl-Ab, and the morphology and characteristics of remaining cells on bone surfaces were examined. Like PTH treatment, Scl-Ab administration led to increases in the size and expression of the osteoblast marker osteocalcin in previously labeled cells. Again, labeled osteoblasts were not proliferating. While bone synthetic capacity was not directly assessed in this study, work by Kalajzic's group has demonstrated that increased cell thickness corresponds to increased matrix production as measured by calcein labeling [40•]. Taken together, these results support a model in which direct reactivation of quiescent BLCs contributes to the rapid increase in osteoblasts following Scl-Ab treatment [39••].

As more studies are performed using these lineage tracing strategies, it has become evident that several limitations exist. First, since the behavior of only those labeled cells is examined, it is impossible to use this approach to determine the relative contribution of BLC to Scl-Ab-stimulated osteoblastogenesis. Second, using sensitive fluorescent reporters, it is likely that CreER expression may be more promiscuous than initially assumed. For example, after tamoxifen administration, osteocalcin-CreER labels a subpopulation of cells in the bone marrow with a unique reticular morphology [39••]. Other investigators have independently made the same observation using constitutive osteocalcin-Cre and DMP1-Cre lines [41••]. When a heterogeneous population of cells is initially labeled in a lineage tracing experiment, it can be difficult to make strong conclusions about the initial origin of cells subsequently studied.

Although the mechanism through which Scl-Ab induces lining cell reactivation remains incompletely understood, laser capture microdissection studies have investigated changes in global gene expression in these cells in response to this anabolic treatment [11, 42]. Shortly after Scl-Ab treatment, a WNT-dependent pattern of gene expression is newly observed in BLCs, indicating that their activity may be kept in check by tonic inhibition from sclerostin. Future studies in which WNT signaling components are selectively deleted from BLCs will be necessary to determine if WNT signaling is necessary for Scl-Ab-induced BLC activation. Finally, lining cell reactivation may help to explain the fact that the anabolic efficacy of Scl-Ab rapidly wanes over time [30] if the available pool of lining cells is rapidly depleted without repletion.

A Role for Bone Lining Cells in Bone Regeneration

Multiple bone resident stem cell types have been proposed to participate in bone remodeling during normal physiology and skeletal regeneration after injury [43]. Recently, Matic et al. [40•] employed a lineage tracing to mark BLCs by treating DMP1-CreER mice with a Cre-dependent fluorescent reporter with tamoxifen followed by a prolonged chase period. These experimental animals also possessed a third transgene in which thymidine kinase is driven by a type 1 collagen promoter element active in osteoblasts [44]. Therefore, osteoblast ablation in these tri-transgenic mice could be achieved by treatment with ganciclovir. Following osteoblast ablation, a clear contribution of genetically labeled BLCs to osteoblast recovery was noted, particularly on cancellous bone surfaces. BLC contribution to osteoblast recovery was blunted by prednisolone treatment, suggesting that glucocorticoids may impair skeletal homeostasis in part by blocking BLC to osteoblast conversion.

At the mechanistic level, Matic et al. were able to isolate labeled BLCs by flow cytometry and assess gene and cell surface marker expression. Interestingly, the stem cell marker Sca-1 was expressed in very low levels in osteoblasts, but was present on ~40% of BLCs. Additional skeletal stem cell markers (CD51, CD44, and LepR) were also enriched in BLCs when compared to osteoblasts. Therefore, these authors suggest that BLCs represent a pool of committed progenitors that can give rise to osteoblasts under certain conditions. Despite the artificial nature of the injury repair model used, these provocative data further support a model from the aforementioned pharmacologic studies in which BLCs can serve as a reservoir for new osteoblast differentiation.

Additional Skeletal Stresses in Which Bone Lining Cells May Participate

Above, we have reviewed how BLCs might contribute to basal bone remodeling and to stimulated osteoblastogenesis in response to parathyroid hormone, sclerostin antibody, and osteoblast ablation. While lineage tracing represents the current "gold standard" to assess BLC/osteoblast conversion, several interesting histomorphometric studies are worthy of mention here.

First, mechanical loading is another important physiologic condition in which new osteoblastogenesis is evoked, primarily on the periosteal bone surfaces [45]. An important mechanism through which loading stimulates new osteoblast activation is via stimulating osteocytes to reduce sclerostin levels [46, 47]. Therefore, the strong possibility exists that loading-induced SOST downregulation stimulates new periosteal osteoblasts by converting existing periosteal BLCs into active osteoblasts. To date, lineage tracing to address this possibility has not been performed. However, when tail vertebrae of adult rats are loaded, a response similar to BLC activation is noted on cancellous surfaces [48]. In this model, cells with ultrastructural osteoblastic morphology appeared on previously quiescent surfaces without active proliferation.

In this study, the authors noted that the kinetics of loading-induced lining cell activation closely mirrored that of loading-induced matrix production.

Exposure to ionizing radiation leads to significant skeletal damage [49]. While ionizing radiation ultimately causes bone loss, immediately after treatment, there is a transient and exuberant increase in osteoblast activity [50]. Turner et al. carefully examined cancellous bone surfaces immediately after ionizing radiation exposure and observed concomitant increases in surfaces covered by osteoblasts and decreases in BLCs, consistent with transient activation of previously quiescent BLCs [51]. Interestingly, acute radiation exposure also dramatically increases resorption due to increased osteoclastic surfaces. Therefore, the nature of the stimulus through which radiation activates quiescent BLCs is likely to be different than how other stimuli that do not increase bone resorption (like Scl-Ab) activate BLCs.

A final pharmacologic agent which shows a potent anabolic effect is basic fibroblast growth factor (bFGF). Similar to the studies outlined above, histomorphometry analysis revealed that bFGF induces a rapid anabolic effect not associated with osteoblast proliferation or upregulation of growth factors that might be trophic for osteoblast progenitors such as TGF-beta or IGF-I [52]. Therefore, the authors proposed that rapid bFGF-induced gains in osteoblast may also be through direct conversion of BLCs to active osteoblasts.

Summary and Future Perspectives

Taken together, the studies summarized here support an important role for quiescent BLCs in bone homeostasis. Although lineage tracing remains the gold standard to assess BLC to osteoblast conversion, limitations to this technique do exist. Open areas for future investigation include gene profiling experiments to identify additional BLC-specific genes. A better understanding of the transcriptome of BLCs (versus osteoblasts and osteocytes) may offer new insights into the function of this cell type and allow for the development of new transgenic models to selectively ablate and manipulate genes in BLCs. Lineage tracing has clearly demonstrated that teriparatide and Scl-Ab stimulate BLC to osteoblast conversion. However, the relative contribution of this phenomenon to the overall pool of osteoblastogenesis stimulated by these agents remains unclear. In addition, the molecular steps required for these agents to activate BLCs are totally unknown. Finally, the ultimate fate of osteoblasts deriving from activated BLCs (versus other osteoblasts differentiating from other progenitor sources) is completely unknown. Future research into these fascinating problems will illuminate novel pathways controlling osteoblast homeostasis and identify novel treatment targets for osteoporosis.

Compliance with Ethical Standards

Conflict of Interest Marc N. Wein declares no potential conflict of interest.

Human and Animal Rights and Informed Consent This article contains no studies with human or animal subjects performed by any of the authors.

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