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## Recent Progress in Osteocyte Research

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The last decade has seen an exponential increase in our understanding of osteocytes function and biology. These cells, once considered inert by-standers trapped into the mineralized bone, has now risen to be key regulators of skeletal metabolism, mineral homeostasis, and hematopoiesis. As tools and techniques to study osteocytes improved and expanded, it has become evident that there is more to these cells than initially thought. Osteocytes are now recognized not only as the key responders to mechanical forces but also as orchestrators of bone remodeling and mineral homeostasis. These cells are the primary source of several important proteins, such as sclerostin and fibroblast growth factor 23, that are currently target as novel therapies for bone loss (as the case for antisclerostin antibodies) or phosphate disorders. Better understanding of the intricate cellular and molecular mechanisms that govern osteocyte biology will open new avenue of research and ultimately identify novel therapeutics to treat bone and mineral disorders. This review summarizes novel findings and discusses future avenues of research.

**Keywords:** Osteocytes; Sclerostin; Mineral homeostasis; Bone homeostasis

### INTRODUCTION

Our understanding of the function of osteocytes has expanded dramatically over the last decade primarily due to the identification of osteocytes specific markers, such as dentin matrix protein 1 (DMP1) and SOST/sclerostin, that has allowed, for the first time a closer look at the biology of these cells. Osteocytes, the cells deeply entrapped into the mineralized matrix, have emerged as key regulators not only of skeletal and mineral homeostasis, but also hematopoiesis. This review will summarize novel findings in osteocyte biology and future avenues of research.

### THE OSTEOCYTES

Osteocytes are postmitotic, terminally differentiated osteo-

blasts that during the process of matrix mineralization remain entrapped in the mineralizing matrix that they are actively synthesizing. They reside both in the mineralized matrix and in the newly formed osteoid. Earlier work of Gaillard et al. [1] and Rutishauser and Majno [2], described two stages into the life of an osteocyte; an early stage (young osteocyte) in which the cell is smaller in size, reside into the osteoid and do not express alkaline phosphatase and a late stage (mature osteocyte) in which the larger cell re-express alkaline phosphatase and is deeply embedded in the mineralized bone. The larger cell will then degenerate and leave an empty lacuna [1]. This classification has been recently revised to include an additional stage of differentiation: according to their spatial localization and gene expression, these cells are now divided into osteoid, mineralizing, and the mature osteocytes [3]. The osteoid, or nascent osteocyte is characterized by a relative proxim-

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ity to the endosteal (and possibly periosteal) surface and the expression of transcripts such as E11/gp38/podoplanin [4], matrix extracellular phosphoglycoprotein (MEPE) and phosphate-regulating gene with homologies to endopeptidases on the X chromosome (Phex). This cell, opposite to the more mature osteocyte, is negative for sclerostin and fibroblast growth factor (FGF)-23 expression but does express DMP1, as elegantly demonstrated by Kalajzic et al. [5] in a recent review. It is not clear, however, if osteoid, mineralizing and mature osteocytes exert different biological functions or if they all work as mechanosensor and skeletal regulators. Indeed recent studies suggest that cortical and trabecular osteocytes might have distinct roles with the former being the sensor of load while the latter being the controller of bone metabolism. Windahl et al. [6], for example, reported that estrogen receptor- $\alpha$  ablation in osteocytes differentially affects trabecular and cortical bone compartments in males mice.

Marotti et al. [7] and Palumbo et al. [8,9] have extensively described the morphological changes that accompany the transformation from a motile osteoblast into an entombed osteocyte. They demonstrate, by histological analysis of newborn rabbit bones, that the formation of osteocyte cytoplasmic processes is asynchronous and asymmetrical and precede the mineralization of the organic matrix. Moreover, osteocytes are evolutionary highly conserved and the organized structure of these cells within a mineralized matrix is present in bone specimens from *Tyrannosaurus rex*, dating back more than 80 million of years ago, clearly indicating an important role for these cells in skeletal metabolism [10].

Osteocytes communicate with each other and with cells at the endosteal and periosteal surface through an extensive and intricate system of canaliculi. These cells are also in close proximity of capillary and vessels, raising the hypothesis that osteocytes might function as an endocrine organ and directly secrete proteins, such as FGF-23, Phex, or sclerostin into the circulation [11]. Moreover, the osteocytic network, with its extensive system, is an ideal structure to sense mechanical loading and control mineral homeostasis. It has been postulated that osteocytes can send signals for both bone resorption and formation and thus orchestrate a proper cycle of remodeling, as discussed in details below.

## OSTEOCYTE FUNCTIONS: MECHANOTRANSDUCTION

What are the functions of an osteocyte? It has been recognized

for over 30 years that osteocytes are mechanosensor of bone. They are indeed the cells capable of sensing mechanical forces applied to the skeleton and transform these forces into biological stimuli. It is now widely accepted that the cell perceives forces in the form of shear stress created by the flow of fluid inside the lacuna-canalicular network. It has been proposed that the flow derives from both the compression induced by loading and the extravascular pressure. Efforts to physically measure and quantify this flow are currently ongoing and recent reports suggest that osteocytes are subjected to forces in the order of 5 Pa [12]. Although the theory that osteocytes are the mechanosensor of bone has been around for decades, the definite demonstration of this theory comes from work of Tsumi et al. [13]. Using a mouse model of targeted osteocytes ablation, they demonstrated resistance to disuse induces bone loss highlighting the central role that osteocytes play in mechanosensation. How an osteocyte can sense the fluid flow in the lacuno-canalicular network is still not completely understood. Primary cilia have been described as important mechanosensor and mice lacking Kif3a or ITF88 (polaris), both key components of the cilia, in bone cells, display skeletal abnormalities. Targeted ablation of Kif3a from osteoblasts, and consequently from osteocytes, using the Col1 $\alpha$  2.3 promoter, affects skeletal anabolic responses to cyclic axial compression, suggesting that primary cilia are needed for proper skeletal mechanosensory [14,15]. Similarly, mice in which PDK1 is ablated in osteocytes, using the 10kb-DMP1 promoter have impaired anabolic response to mechanical loading, further supporting the importance of cilia in skeletal mechanosensation [16,17].

Osteocytes responses to load, both *in vivo* and *in vitro*, include secretion of prostaglandin E<sub>2</sub> and nitric oxide and the expression of several mechanosensitive transcripts such as DMP1, MEPE, and Phex [18,19]. Another osteocytic gene highly regulated by mechanical forces is SOST. SOST/sclerostin is suppressed during load whereas it is increased during hindlimb unloading [20]. Moreover mice lacking SOST are resistant to disuse induced bone loss and antisclerostin antibodies can prevent bone loss associated with hindlimb unloading [21]. This demonstrates that osteocytes are key orchestrators not only of skeletal responses to loading but also to unloading, as occurs during paralysis, prolonged bed rest or space flight. Frost [22] postulated that osteocytes are the mechanostat of bone, ascribing to these cells the role of driving skeletal adaptation to mechanical forces. Bone adapts its form to mechanical demands through a mechanisms known as bone modeling. During modeling, bone resorption,

driven by osteoclasts, and bone formation, osteoblasts-mediated, occurs on different surfaces of the skeleton such as the bone undergoes reshaping to adapt to different loading conditions. Bone remodeling, on the other hand, is the continuous and spatially coupled, resorption and formation of the skeleton to preserve functional integrity. According to Frost [22], the mechanostat distinguishes between bone modeling (shape change) and remodeling (replacement only). Examples of bone adaptation to loading are evident in professional athletes, where the high load applied to the skeleton results in stronger and bigger bones, whereas in astronauts or in paralyzed patients there is severe bone loss due to unloading. As described above, compelling evidences ascribe to sclerostin an important role in this disuse induced bone loss.

## BONE AND MINERAL HOMEOSTASIS

Osteocytes are not only the sensor of mechanical forces, they are also master controller of bone and mineral homeostasis. These cells are, postnatally, the main source sclerostin and receptor activation of nuclear factor- $\kappa$ B ligand (RANKL). Sclerostin, the product of the gene *SOST*, has emerged as a powerful inhibitor of bone formation. The protein binds to LRP5 and the related LRP6 and four receptors and block the canonical Wnt- $\beta$ catenin signaling pathway. Lack (or mutation) of sclerostin induces sclerosteosis whereas its over expression causes severe osteopenia (for review on Wnts) [23]. Osteocytes are also the main source of FGF-23, a key regulator, together with parathyroid hormone (PTH) of phosphate homeostasis [24,25]. Thus, it is not surprising that genetic manipulation of osteocytes induced both skeletal and mineral defects. Mice lacking RANKL in osteocytes have increased bone mineral density and reduced bone remodeling [26,27], as do mice lacking the receptor of PTH (PTH1R). Mice lacking *Atg7*, an autophagy gene, from osteocytes, display similar skeletal phenotype as aging mice, indicating that these cells are key regulator of skeletal metabolism [28]. Both DMP1-null mice and individuals with inactivating mutation of this gene have rickets and osteomalacia and are hypophosphatemic as a consequence of high levels of FGF-23 [29]. On the contrary, mice lacking FGF-23 are hyperphosphatemic, osteopenic, and die prematurely. It has been reported that osteocytes are also the major source of insulin-like growth factor (IGF)-1 and IGF-1 produced by these cells is required for proper anabolic responses to mechanical loading. Deletion of IGF-1 in osteocytes, (using the DMP1-Cre promoter) impairs skeletal growth, have re-

duced periosteal circumference and have a blunted response to mechanical forces. When IGF-1 conditional KO mice were subjected to mechanical loading, there was a significant reduction in osteoblastic bone formation response, indicating that IGF-1 is an important determinant of both bone size and bone strength [30].

Pioneering studies on osteocytes involving histological analysis of bone specimens raised the possibility that osteocytes could reabsorb bone and directly contribute to mineralion homeostasis. The finding that in various diseases (renal osteodystrophy, hyperparathyroidism, and immobilization) the size of the osteocytic lacunae was increased led to the hypothesis that these cells were indeed capable of modifying the perilacunar mineralized matrix. This theory was quickly abandoned when isolated osteocytes failed to reabsorb bone in a classical osteoclast pit-forming assay and few decades later, the concept that osteocytes can indeed reabsorb their perilacunar matrix has been revisited. The current osteocytic osteolysis concept, as proposed by Cullinane [31], suggests that osteocytes do not remove substantial amount of bone (as osteoclast), but rather modify the matrix minerals in the perilacunar areas. This perilacunar remodeling is evident during continuous infusion with PTH [32], treatment with prednisone [33] or during lactation [34]. As described above, osteocytes express receptor for PTH, a known regulator of calcium homeostasis, and they are the ideal candidates for systemic homeostasis regulation. Mice lacking the PTH1R specifically in osteocytes have indeed an impaired calcium homeostasis when subjected to a low calcium diet, indicating these cells also control calcium homeostasis [35]. Lastly, when the vitamin D receptor is ablated from osteocytes, the animals are unable to mobilize calcium from bone under a vitamin D stimulus, indicating that vitamin D signaling in osteocytes is critical for moving calcium from skeletal stores to the blood [36]. In light to recent report indicating that osteocytes are the major source of RANKL and therefore bone remodeling [26,27], we can speculate that the calcemic effect of vitamin D on bone is via osteocyte-derived RANKL although evidences for this pathway are still missing.

## HEMATOPOIESIS

Hematopoiesis, the process that continuously gives rise blood cells, is tightly regulated not only by intrinsic factors but also by extrinsic clues derived from various cells within the bone marrow microenvironment. The bone marrow is indeed the pri-

mary site for hematopoiesis that is highly orchestrated by interactions between the hematopoietic stem cells (HSCs) and their niches on the endosteal bone surface. The HSC niche consists of cells from the osteolineage, sinusoidal endothelial cells, mesenchymal stromal and stem cells, sympathetic neurons, and the extracellular matrix [37,38]. In these niches, the HSC undergo self-renewal and/or differentiate into hematopoietic lineage with cues from the niche-supporting cells, which provide structural as well as signaling support for the hematopoietic microenvironment. Osteoblasts have been shown as critical regulators of HSC maintenance, proliferation, and maturation [39,40] via various cell-surface proteins such as Notch1 ligand Jagged1 and via secreted cytokines such as CXCL12. Osteoblasts also provide a niche for B-lymphopoiesis in part via interleukin-7 expression through Gs $\alpha$ -signaling [41]. Hypoxic osteoblasts are also a major producer of erythropoietin through which they regulate erythropoiesis [42]. Moreover, osteoclasts has been shown to be potential HSC regulators through degradation of endosteal components and promotion of mobilization of HSCs [43]. In contrast, the role of osteocytes in HSC and progenitor regulation, and hence hematopoiesis, has remained completely unexplored despite the fact that these cells comprise 90% to 95% of all bone cells in the adult skeleton and they produce an extensive canalicular network that reaches the endosteal bone surface, a preferred site for the HSC niche. Osteoblasts and osteocytes express several G-protein coupled receptor (GPCR) and signaling through these receptors has been shown to control the niche. Interestingly constitutive activation of the PTH/PTHrP receptor in osteoblasts increased the numbers of HSCs [39] whereas its expression in osteocytes does not [44], suggesting that cells early in the osteoblast lineage are critical for maintaining an intact HSC niche. To examine the role of osteocytes and GPCR signaling in regulation of hematopoiesis we recently generated mice lacking Gs $\alpha$  in osteocytes (Ocy-Gs $\alpha$ <sup>KO</sup>). Surprisingly, ablation of Gs $\alpha$  from these cells induces a marked neutrophilia, thrombocytopenia and splenomegaly. Ocy-Gs $\alpha$ <sup>KO</sup> mice displayed a profound myeloproliferative phenotype characterized by a dramatic increase in myeloid cells in bone marrow, spleen, and peripheral blood and marked [45]. Using a novel *ex vivo* coculture system using osteocyte-enriched bone explants we identified granulocyte colony-stimulating factor as the principal cytokine regulating granulopoiesis in these mice. Interestingly, PTH1R signaling in osteocytes is not responsible for the myeloproliferative phenotype since mice lacking receptor expression in osteocytes (Ocy-PPR<sup>KO</sup>) have normal hematopoiesis [45]. Moreover, the expression of

SOST/sclerostin, a Wnt inhibitor and suppressor of osteoblast proliferation and functions, was increased significantly in these mice. Treatment with antisclerostin antibody partially restored the number of osteoblasts in Ocy-Gs $\alpha$ <sup>KO</sup> mice without rescuing the bone marrow abnormalities demonstrating that the hematopoietic abnormalities present in these mice are independent on osteoblasts or increased SOST/sclerostin expression [45]. On the other hand, SOST/sclerostin can directly control the fate of B-cells, as recently reported [46]. Cain et al. [46] showed that SOST<sup>-/-</sup> mice, despite the high bone mass and increased number of osteoblasts, have no differences in the frequency or absolute number of HSCs, common lymphoid progenitors, common myeloid/megakaryocyte erythroid progenitors, or granulocyte/monocyte progenitors, confirming the findings in mice overexpressing the constitutive PTH/PTHrP receptor in osteocytes [44]. Interestingly, in these mice, B cells are significantly reduced in both their frequency and cell number in the bone marrow and the reduction was a consequence of increased apoptosis due to a reduction in Cxcl12 expression in the stromal cells.

## FUTURE DIRECTIONS

It is evident that there is a need for further investigations aimed at better understand osteocytes function. Several questions remain unanswered and areas in need of better understanding are numerous. For example, which are the molecular cues that signal an osteoblast to become an osteocyte? How can osteocytes sense mechanical forces applied to the skeleton and what is the role of primary cilia? What control the increased in SOST/sclerostin induced by unloading? How can an osteocyte sense phosphate? What is the role of osteocytes in hematopoiesis? These are many more other unanswered questions will require better tools and *in vitro* models to study and analyze these cells and advance our understanding of their complex functions even further.

## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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