

The Osteocyte Network as a Source and Reservoir of Signaling Factors

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Within the past few years, information regarding osteocyte function has been emerging and expanding significantly. No longer is the osteocyte considered a passive cell acting simply as a 'placeholder' within mineralized bone. Osteocytes are derived from osteoblast progenitors and in the adult skeleton compose 90-95% of all bone cells. Therefore, the function of these cells in the adult and aging skeleton has become the focus of recent investigation. These cells are proving to be multifunctional, ranging from mechanotransduction, to regulation of mineral homeostasis, to control of bone remodeling. The osteocyte as a source and reservoir of signaling factors important in health and maintenance of the adult skeleton is addressed in this review.

OSTEOCYTES AS MULTIFUNCTIONAL CELLS

Osteocytes are thought to be derived from matrix producing osteoblasts. Osteoblasts are dynamic cells that move along the bone surface before localizing in a targeted, predetermined location before becoming embedded in the newly forming osteoid bone matrix. However, once embedded, these cells can continue to move; their dendritic processes can extend and retract and the cell body can undulate within their lacunae [8]. Once embedded and differentiated into dendritic cells, osteocytes are clearly extremely sensitive to mechanical stimulation in the form of bone fluid flow shear stress. These cells signal not only other osteocytes, but both osteoblasts and osteoclasts and their precursors on the bone surface (Fig. 1). Osteocytes are 'orchestrators' of bone remodeling sending signals of resorption and formation. The Wnt/ β -catenin signaling pathway, recognized as an important regulator of bone mass and bone cell function, is also important in osteocytes to transmit signals of mechanical loading to cells on the bone surface [16]. The Wnt/ β -catenin pathway in osteocytes is triggered by crosstalk with the prostaglandin pathway in response to loading leading to a decrease in expression of negative regulators of the pathway such as Sost/sclerostin and Dkk1 [6].

Osteocytes appear to regulate both calcium and phosphate metabolism. Osteocytes can regulate calcium availability. The osteo-

cyte can remove and replace their mineralized matrix under normal, healthy conditions such as lactation [21]. We propose that the term 'osteocytic remodeling' of their perilacunar and pericanalicular matrices be applied to pathological conditions such as hyperparathyroidism, whereas 'osteocytic remodeling' is the hallmark of a healthy osteocyte. The osteocyte appears to play a role in release of calcium from the bone matrix [2] especially in response to parathyroid hormone and parathyroid hormone related peptide. In addition to regulation of calcium, osteocytes appear to also regulate phosphate. Molecules such as phosphate regulating neutral endopeptidase on chromosome X (PheX), dentin matrix protein 1 (Dmp1), and fibroblast growth factor 23 (FGF23), are expressed by osteocytes [5]. Both Dmp1 and PheX appear to down regulate FGF23 expression which in turn allows reabsorption of phosphate by the kidney thereby maintaining sufficient circulating phosphate to maintain normal bone mineral content. In the absence of either Dmp1 or PheX, FGF23 is elevated in the osteocyte and systemically, leading to phosphate excretion by the kidney thereby lowering circulating phosphate resulting in osteomalacia and rickets. Based on these observations, we proposed that the osteocyte lacuna-canalicular network can function as an endocrine system, targeting distant organs such as kidney [9].

OSTEOCYTES ARE HORMONALLY RESPONSIVE CELLS TO PARATHYROID HORMONE (PTH)

Mice expressing a constitutively active PTH/PTHrP receptor targeted to osteocytes have a dramatic increase in bone cortical thick-

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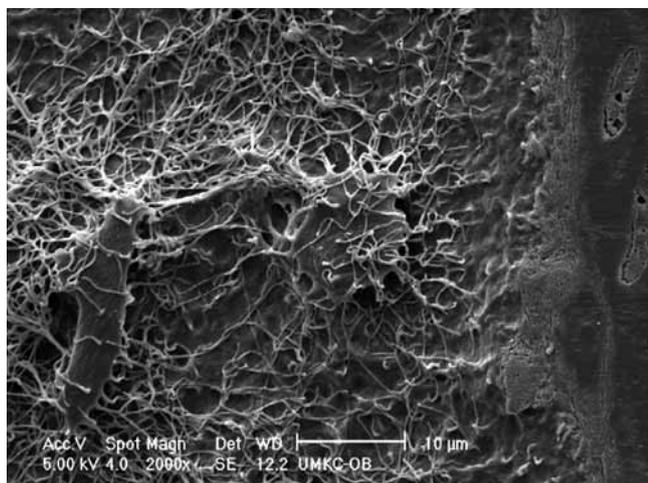


Fig. 1. Osteocytes are connected to each other and to cells on the bone surface. Scanning electron micrograph of an acid etched resin embedded bone showing the extensive lacuno-canalicular network. Canaliculi connect to the bone surface and marrow space (right side of figure) (2,000 x-murine bone).

ness due to increased bone formation and intracortical remodeling [19]. Conversely, mice with an osteocyte-targeted deletion of the PTH/PTHrP receptor fail to show a bone anabolic response to intermittent PTH treatment as shown by Divieti-Pajevic and colleagues. PTH also cross talks with the Wnt/ β -catenin signaling pathway by altering expression of the SOST gene and its protein product, sclerostin, an inhibitor of Wnt signaling that binds to the Wnt co-receptors Lrp5 and Lrp6. PTH also appears to regulate FGF23 expression in osteocytes, demonstrating the interplay between these pathways in the control of phosphate homeostasis. Another example of this interplay is that circulating FGF23 was elevated in mice expressing a constitutively active PTH/PTHrP receptor in osteocytes. Treatment of wild-type animals with PTH or PTHrP also stimulated FGF23 expression in osteocytes, suggesting that inhibition of phosphate reabsorption by PTH is due to its actions not only on the kidney, but also on osteocytes to increase production of circulating FGF23 [7].

MECHANOTRANSDUCTION AND ACTIVATION OF SIGNALING PATHWAYS IN OSTEOCYTES

Clearly bone responds to both anabolic loading and to immobilization or unloading [14]. Several mechanisms have been proposed as to how osteocytes sense mechanical loading [4]. The osteocyte cell body, located within a lacunae, and its dendritic processes located within small tunnels called canaliculi are exposed to a 'bone fluid' that appears to shear stress the cell and its processes. The flow of this fluid appears to be driven by extravascular pressure as well

as applied cyclic mechanical loading. Integrins, though found on many cell types, appear to play a major role in osteocyte mechanosensation and appear to be perturbed by fluid flow shear stress. Integrins, heterodimers of α and β subunits, are receptors/transducers that connect the cytoskeleton to the extracellular matrix and appear to bridge osteocyte processes to their canalicular wall (You et al., 2004) but also appear to be responsible for the opening of connexin 43 hemichannels to release prostaglandin into the bone fluid surrounding the osteocyte [23]. Other early small signaling molecules in addition to prostaglandin include calcium, nitric oxide, and adenosine-5'-triphosphate (ATP). These small molecules act in concert to regulate signaling and gene expression in osteocytes [3].

The primary cilium plays a key role in mechanosensing in the kidney and may also be involved in mechanosensation in the osteocyte. Reducing the number of cilia in both MC3T3 and MLO-Y4 osteocyte cells reduces the induction of prostaglandin but not calcium flux in response to fluid flow shear stress [17]. In MLO-Y4 cells an increase of COX2 and the OPG/RANKL ratio in response to fluid flow shear stress occurred suggesting that the regulation of the OPG/RANKL ratio in osteocytes in response to mechanical loading may be a means by which bone formation and resorption can be maintained in equilibrium. Polycystin 1, encoded by the PKD1 gene, is a component of the mechanosensing complex in primary cilia. Conventional deletion of PKD1 results in mice with decreased bone mass [29], whereas osteocyte targeted deletion of PKD1 results in a dramatic decrease in bone formation in response to anabolic loading. To determine if PC1 plays a role in osteocyte mechanosensation, conditional deletion of PKD1 was performed using *Dmp1-Cre* mice. Only minor differences were observed in 16 week old *PKD1^{Dmp1-cKO}* mice by DEXA and μ CT analysis but a dramatic decrease in response to anabolic loading was observed showing that PC1 in osteocytes is essential for the bone anabolic response to load [28]. This was associated with reduced Wnt signaling. It is not clear if the cell body, cell processes and cilia work separately or in conjunction to sense and transmit mechanical stimuli.

OSTEOCYTE CELL DEATH

Osteocyte cell death, either as necrosis or apoptosis leads to activation of osteoclastic bone resorption. Death signaling pathways are activated in osteocytes during necrosis [25] or apoptosis [15] that induce signals of bone resorption. Recently we have shown that autophagy ('auto' self; 'phagy', eat; i.e. 'eating self') is a major means

by which osteocytes respond to glucocorticoid stress to maintain their viability [27]. Autophagy is a lysosomal degradation pathway essential for survival, differentiation, development, and homeostasis for certain cell types. During autophagy, parts of the cytoplasm and intracellular organelles are sequestered within the autophagic vacuoles for delivery to lysosomes for degradation. Autophagosomes and expression of LC3II was shown in both MLO-Y4 cells and primary osteocytes in response to glucocorticoid treatment and the mTOR signaling pathway appeared to be responsible. We propose that programmed osteocyte cell death or apoptosis may be the predominant mechanism used by osteocytes to signal bone remodeling through the activation of osteoclasts such as in response to micro-damage. In contrast, autophagy may be used by osteocytes under conditions of stress such as glucocorticoid treatment, in an effort to simply maintain the survival of osteocytes within the bone matrix. However, there is a limited period of time or extent of autophagy that the osteocyte can sustain or endure before it undergoes apoptotic cell death. Autophagy can also lead to cell death. Different signaling pathways and the production of unique factors most likely accompanies the necrosis, apoptosis, or autophagy of osteocytes.

ACCESSIBILITY OF OSTEOCYTES

Because mature osteocytes are surrounded by a mineralized matrix they are difficult to isolate in significant numbers and purity. Osteocytes from immature bone such as chick and 1-2 week old mice can be isolated in reasonable numbers for experimentation [3,14]. However, the older the animal, the more difficult this procedure becomes. To begin to tackle this problem, which is most likely the reason why osteocytes were ignored for years, cell lines were generated. Mikuni-Takagaki et al. [18] described primary murine osteocytes as dendritic cells being low in alkaline phosphatase, high in osteocalcin and high in casein kinase 2. The MLO-Y4 osteocyte-like cell line [13] possess these characteristics in addition to high E11/gp38, a marker for early osteocytes, RANKL, OPG, TRAP, MEPE, PHEX, and FGF23, but low amounts of Dmp1 and Sost/sclerostin. We propose that this cell line represents an osteocyte in which Dmp1 has been down-regulated. Consequently, this cell line does not form a matrix, nor mineralize in culture. It has proved useful for the study of osteocyte apoptosis, osteocyte autophagy, osteocyte communication, osteocyte signaling to osteoclasts, osteoblasts, mesenchymal stem cells, osteocyte response to fluid flow shear stress, ATP, nitric oxide, prostaglandin production and signaling, β -catenin

signaling, PKD1 and cilia -- the list is extensive (search 'MLO-Y4' in PubMed). However, this cell line does not make bone matrix and is not surrounded by a matrix-so it does not completely replicate the mature osteocyte within a mineralized matrix.

The MLO-A5 cell line overcomes many of the limitations of the MLO-Y4 cell line. This cell line is referred to as a late osteoblast, early osteoid-osteocyte cell line that produces very large amounts of alkaline phosphatase, osteocalcin, collagen, bone sialoprotein and mineralizes in the absence of an external source of phosphate with 7-9 days of culture [12]. The mineralization process of MLO-A5s has been characterized using a variety of high resolution microscopy techniques, and energy dispersive spectrometry and shown to be more bone-like than that of other osteoblast cell lines [1]. This cell line has proved useful to study the mineralization process, specifically the formation of nanospherulites, the response of cells in a 3 dimensional matrix to loading [24], and recently, collagen and fibronectin assembly in the bone matrix [11].

Recently, a third cell line, IDG-SW3, has been generated by crossing the Immortomouse with the Dmp1-GFP mouse [26]. The cells are maintained in a non-differentiated state at 33 degrees and addition of gamma interferon, but upon incubation at 38 degrees in the absence of gamma interferon, the cells rapidly express GFP and differentiate into mature osteocytes. One major advantage of the SW3 over the MLO-A5 is that osteocyte differentiation can be tracked or monitored using the GFP signal. Also with differentiation this cell line will make higher amounts of matrix, nanospherulites, Sost/sclerostin and FGF23 than the MLO-A5 cells. This cell line should prove useful for numerous studies of osteocyte biology and function such as the hormonal and mechanical regulation of factors such as Sost and FGF23.

THE OSTEOCYTE CAN TARGET AND CAN BE TARGETED BY BONE CELLS AND OTHER TISSUES

Can systemic factors reach the osteocyte? The osteocyte lacuno-canalicular system is connected to the bone surface (Fig. 1) and this system is maintained with bone remodeling (Fig. 2). Clearly if one injects procian red into the tail vein of a mouse, within minutes the osteocyte lacuno-canalicular system is filled with the dye. It has been shown that molecules less the 70 kDa, about the size of BSA, can travel through this network [10]. It has also been shown that bisphosphonates can target the osteocyte, as a fluorescent bisphosphonate has been shown to accumulate in osteocyte lacunae [22].

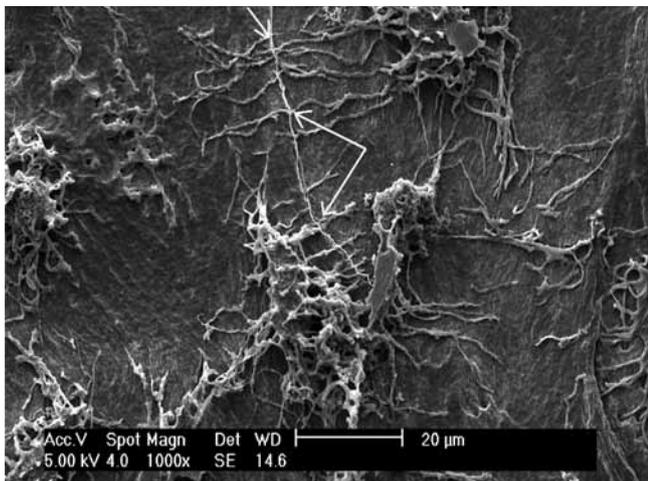


Fig. 2. Canaliculi bridge the reversal front. This suggests that during the remodeling process, osteocytes can connect their existing dendrites/canaliculi with newly formed dendrites/canaliculi of newly embedded osteocytes. In this manner, the connectivity of the osteocyte lacuno-canalicular network is maintained (1,000 x-human bone).

Therefore, one must assume that circulating factors, molecules, and drugs less than this particular size cutoff can reach the osteocyte.

One factor that appears to be produced by osteocytes is Sost/sclerostin, an inhibitor of the Wnt/ β -catenin pathway that functions by binding to Lrp 4, 5, and 6 [20]. Although produced in other cell types in the embryonic and developing skeleton, mainly mature osteocyte in the mature skeleton appear to produce this factor. Discovering that the increased bone mass condition, sclerosteosis, is caused by inactivating mutations of the Sost gene has led to novel therapeutics focused on neutralizing the protein product, sclerostin. Neutralizing antibodies to sclerostin has bone anabolic effects and appears useful in not only treating bone loss such as postmenopausal bone loss and immune disease, but also in accelerating fracture repair and orthopedic conditions. This is an exciting new area of investigation.

CONCLUSIONS

In summary, far from being the quiescent cell as it is often referred to in textbooks, the osteocyte is a highly active cell type that regulates functions as diverse as mechanosensation, mineral homeostasis, and skeletal responses to hormonal signals. The molecular signaling pathways that regulate these diverse functions of the osteocytes are being identified and characterized (Table. 1). These hold promise for the development of novel therapeutics for the treatment of bone disease. Can factors secreted by the osteocyte reach

Table 1. Osteocyte signaling factors

Factor	Target	Function
PGE2, ATP, NO	Other osteocytes, osteoblasts	Anabolic
Sclerostin, Dkk1	Osteoblasts	Inhibit bone formation
FGF23	Kidney	Phosphate regulation
RANKL, M-CSf	Osteoclasts	Activates osteoclasts
OPG	Osteoclasts	Inhibits osteoclast activation

PGE2, prostaglandin E2; ATP, adenosine-5'-triphosphate; NO, Nitric Oxide; FGF23, fibroblast growth factor 23; RANKL, receptor activator of NF- κ B ligand; M-CSf, monocyte chemotactic and stimulating factor; OPG, osteoprotegerin.

cells on the bone surface and cells distant from bone? Their close relationship and contact with the vascular system support this hypothesis. Therefore, osteocytes can be viewed as a source and potential reservoir of factors in bone.

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