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The Osteocyte: An Endocrine Cell and More

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Few investigators think of bone as an endocrine gland, even after the discovery that osteocytes produce circulating fibroblast growth factor 23 (FGF23) that targets the kidney and potentially other organs. In fact, until the last few years, osteocytes were perceived by many as passive, metabolically inactive cells. However, exciting recent discoveries have shown that osteocytes encased within mineralized bone matrix are actually multifunctional cells, with many key regulatory roles in bone and mineral homeostasis. In addition to serving as endocrine cells and regulators of phosphate homeostasis, these cells control bone remodeling through regulation of both osteoclasts and osteoblasts, are mechanosensory cells that coordinate adaptive responses of the skeleton to mechanical loading, and also serve as a manager of the bone's reservoir of calcium. Osteocytes must survive for decades within the bone matrix, making them one of the longest lived cells in the body. Viability and survival are therefore extremely important to ensure optimal function of the osteocyte network. As we continue to search for new therapeutics, in addition to the osteoclast and the osteoblast, the osteocyte should be considered in new strategies to prevent and treat bone disease.

I. Introduction

OF THE MAJOR CELL types in bone, osteoblasts and osteoclasts have been defined by their respective functions of bone formation and bone resorption, but osteocytes were defined primarily by their morphology and location embedded within mineralized bone matrix rather than by their function. This is because until the past decade or so there has been a lack of clear understanding about the properties of these cells and their important functions in the skeleton. This perception persisted in spite of the fact that osteocytes make up over 95% of the bone cells in the adult skeleton, with this ratio increasing with age and with the size of the bone.

Osteocytes reside in lacunae within the mineralized bone matrix and send their dendritic processes (ranging from 40–100 per cell (1)) through tiny tunnels called canaliculi to form the osteocyte lacunocanalicular network (Figures 1 and 2), which connects to cells on the bone surface and to the vasculature. A fluid, termed canalicular or bone fluid, that is still not well characterized, travels through the lacunocanalicular space and bathes the osteocyte, thereby providing oxygen and nutrients to maintain the viability of the cell in this enclosed environment.

Early bone histologists hypothesized various functions for osteocytes, but lacked the tools to test their hypotheses. Today with the use of transgenic technologies combined with molecular and cell biology and state-of-the-art instrumentation, dramatic advances have been made in identifying and understanding the functions of these previously overlooked bone cells. It is now clear that, far from being a passive cell as it has been historically described in the literature, the osteocyte is a key cell that is indispensible for the normal function of the skeleton. Although osteocytes most likely do not play a major role in embryonic bone development and may only play a secondary role in growth and development postnatally, they appear to play a critical role in bone homeostasis in the adult skeleton. Thus, the osteocyte plays multifunctional roles in orchestrating bone remodeling by regulating both osteoblast and osteoclast function. Osteocytes also act as mechanosensors to control adaptive responses to mechanical loading of the skeleton and they may be a key target cell for the actions of Parathyroid Hormone (PTH) in bone. The osteocyte therefore appears to integrate hormonal and mechanical signals in the regulation of bone mass.

Abbreviations:

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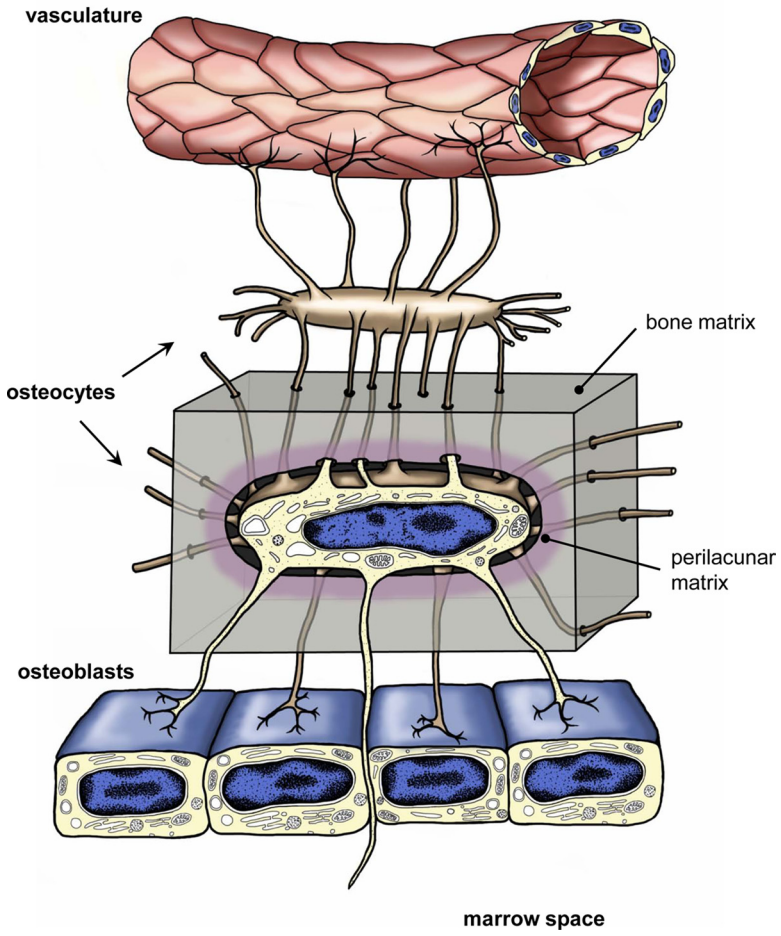
Figure 1.



The osteocyte

Schematic representation of an embedded osteocyte located within its lacuna, illustrating its dendritic processes passing through the bone matrix (gray shading) within narrow tunnels termed canaliculi. The osteocyte's dendritic processes interconnect with other osteocytes as well as surface osteoblasts. Note that some osteocyte processes may extend beyond the osteoblast layer to potentially interact with cells in the marrow and that osteocyte dendrites are also in intimate contact with the vasculature. The composition of the perilacunar matrix immediately adjacent to the osteocyte (mauve shading) is different from that of the rest of the bone matrix (gray shading), which may influence the magnitude of mechanical strains perceived by the osteocyte.

As osteocytes reside in an enclosed environment for extended periods of time, which can last up to decades, the viability of these cells becomes critical for their function. Not only does the viable osteocyte regulate bone homeostasis, but the dying or apoptotic osteocyte also may play key regulatory roles in sending signals to initiate bone remodeling, particularly when there is a need for bone repair. Autophagy (the process of controlled "self digestion" of the cell contents) also appears to be necessary to sustain the cell within the enclosed environment of the mineralized matrix.

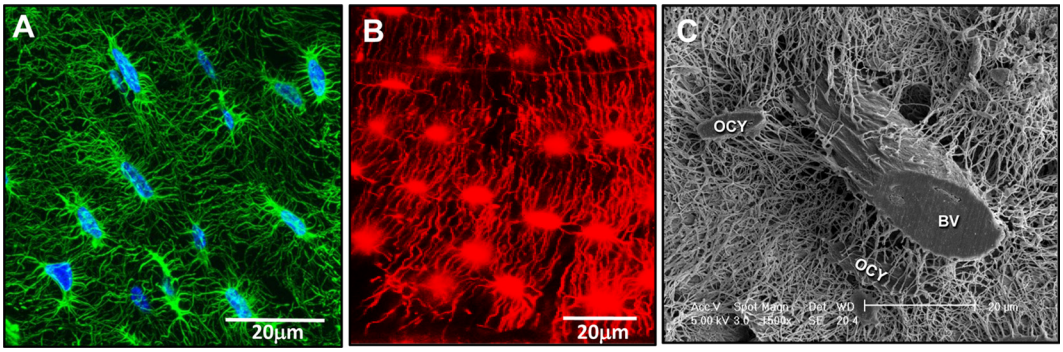
Another exciting and unexpected recent discovery is that osteocytes may function in an endocrine manner to regulate phosphate homeostasis through secretion into the circulation of fibroblast growth factor 23 (FGF23). When one thinks of an endocrine organ, tissues such as the pituitary or adrenal glands come to mind but one would not normally ascribe this function to bone. However, kidney, liver, and heart have all been shown to have endocrine functions and now it appears that bone (and specifically the osteocyte network) can be added to this category. Criteria for designating an organ as an endocrine gland are that it must form a system that directly secretes hormones into the bloodstream to affect distant target organs and cells. Endocrine organs must also be highly vascularized. Bone meets these criteria, since it is a highly vascular organ and the osteocyte lacunocanalicular network is in intimate connection with this vascular supply (see figures 1 and 2). The kidney produces factors such as erythropoietin and the liver produces insulin-like growth factor, both of which can be considered hormone-like. Similarly, the circulating factors produced by osteocytes, such as FGF23 and sclerostin, should also be considered in this category.

In summary, there has been an explosion in research on the properties and functions of osteocytes over the past few years, leading to many exciting discoveries and the assignment of new functions to this intriguing cell. This review aims to summarize the current state of knowledge of the osteocyte and to review its many functions, including its novel function as an endocrine cell. The review will also discuss the implications for treatment of bone diseases and future directions for research of this fascinating and unique cell.

Osteocyte Differentiation and Embedding

It is well accepted that osteocytes are derived from osteoblasts, which in turn are derived from osteogenic precursors residing in the bone marrow. The mature osteocyte represents a terminally differentiated stage of the

Figure 2.



Osteocyte morphology and interaction with the vasculature

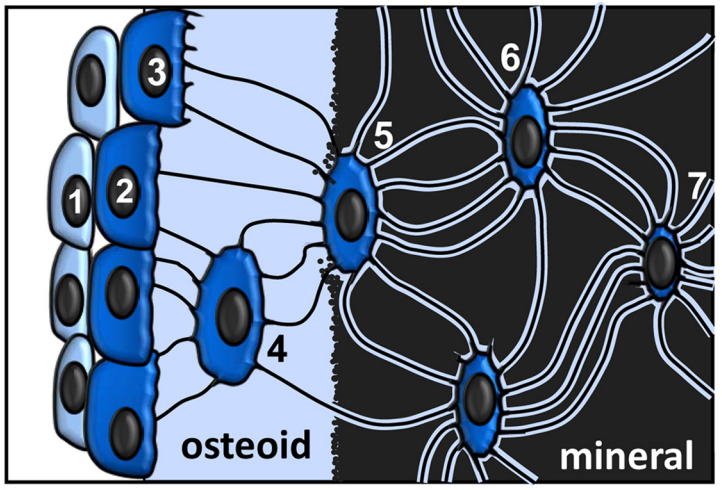
A) Confocal fluorescence image of osteocytes in the cortex of an adult mouse femur stained with Alexa Fluor 488 phalloidin, which stains the actin cytoskeleton of the cell. Note the highly dendritic morphology of the osteocyte with many actin-positive dendrites extending from the cell in all directions. Note also the extensive dendritic interconnections between adjacent osteocytes. **B)** Confocal fluorescence image of the osteocyte lacunocanalicular system in mouse cortical bone as revealed by the tracer dye, procion red. This dye is dispersed throughout the lacunocanalicular system within only 5 min of intravenous (IV) injection, demonstrating the intimate connection of the canalicular fluid with the circulation (as modified from 154). **C)** Scanning electron micrograph of an acid-etched resin casted mouse cortical bone specimen, revealing the 3D organization of the osteocyte lacunocanalicular system and its interaction with a blood vessel (BV). Note the two osteocyte lacunae (OCY) and extensive canaliculi, which are intimately connected to the surface of the blood vessel. Note also the extensive, interconnected canaliculi throughout the bone matrix.

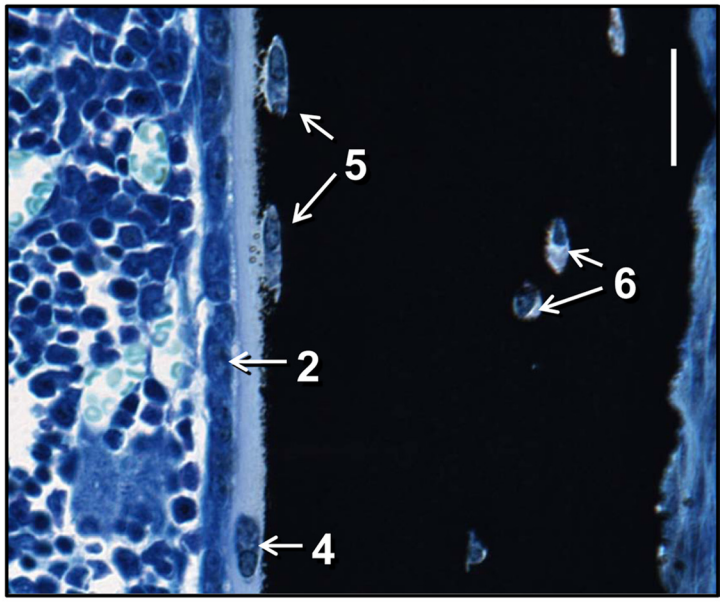
osteoblast lineage. However, the precise mechanisms by which an osteoblast becomes embedded in bone matrix to begin a new life as an osteocyte, and the genetic and molecular mechanisms that regulate the differentiation and maturation of the osteocyte are still not fully understood (reviewed in (2–4)). Once an osteoblast stops actively forming new bone matrix, it is thought to have one of three potential fates. It can become a quiescent cell on the bone surface, known as a lining cell (5), it can undergo programmed cell death via the process of apoptosis (5, 6), or it can become embedded in its own osteoid and differentiate into an osteocyte. Several different transitional stages between osteoblasts and osteocytes have been reported in the literature (7–10), based mainly on morphological observations (reviewed in (2)). Figure 3 shows a schematic representation of these transitional stages, compiled from these various articles.

The embedding of an osteoblast to become an osteocyte has been viewed as a passive process in which a subpopulation of osteoblasts on the bone surface slows down their matrix production relative to adjacent cells. These osteoblasts then become "buried alive" under the matrix produced by neighboring osteoblasts (2, 8, 11). However, research from two different groups suggests that the embedding mechanism may actually be an active and invasive process, in which matrix degradation is required for the formation of the osteocyte lacuna and canaliculi (12, 13).

During the transition process one would expect the differentiating osteocytes to share many markers with their osteoblast precursors, but also to express unique marker genes that play key regulatory roles in altering their morphology and function. Until recently, characterizing the molecular and genetic changes that occur during osteoblast to osteocyte differentiation was challenging due to the lack of appropriate osteocyte cell lines and difficulties in isolating sufficient numbers of osteocytes from tissue sources. However, within the past two decades, more and more markers of the osteocyte have been identified (reviewed in (3, 14, 15)). Furthermore, the generation of transgenic mouse lines in which GFP variants are selectively expressed in osteoblasts and osteocytes (16–18), and the use of proteomic and gene array technologies (19–21) has enabled comparisons of gene and protein expression profiles between osteoblasts and osteocytes, which has provided clues to help identify the key mediators of osteocyte differentiation and function. The changes in gene expression that represent a signature for transition towards an osteocytic phenotype include down regulation of type I collagen and alkaline phosphatase and upregulation of genes including Dentin matrix protein 1 (*DMP1*), E11/gp38/podoplanin, Fibroblast growth factor 23 (*FGF23*), Matrix extracellular phosphoglycoprotein (*MEPE*), and Phosphate-regulating gene with homologies to endopeptidases on the X chromosome (*PHEX*) (2, 22–25). Induction of *SOST*, which encodes the protein, sclerostin (26), indicates differentiation to a mature osteocyte phenotype. Compared to osteoblasts, osteocytes are also enriched in proteins associated with resistance to

Figure 3.





C

	1	2	3	4	5	6
RUNX2	+	+	-	-	-	-
OCN	-	+	+	+	+	-
E11	-	-	+	+	+	-
DMP1	-	-	+	+	+	+
PHEX	-	-	+	+	+	+
MEPE	-	-	-	+	+	+
Sclerostin	-	-	-	-	-	+
CapG	-	-	+	+	+	+
ORP150	-	-	-	-	-	+

Osteocyte Differentiation

hypoxia, such as ORP150 (20) and proteins important in cytoskeletal function, including destrin, CAPG, CDC42 and E11 (19–21, 27, 28). Osteocytes also show high expression of CD44 (29) and neuropeptide Y (30). This diverse set of genes has functions ranging from regulation of mineralization (DMP1, SOST), to phosphate homeostasis (PHEX, MEPE, FGF23) and cytoskeletal arrangement and/or dendrite formation (E11, CD44, CAPG, CDC42, destrin). The specific functions of these osteocyte marker proteins are discussed in more detail in this review in the section on osteocyte selective genes and their functions.

Although considerable progress has been made in generating a list of osteocyte marker genes, it remains unclear which of these are actually the key mediators that control osteocyte differentiation. Notable among them is the membrane bound protein, E11, which is expressed in early osteocytes (22, 31) and has been shown to play a role in the formation of dendrites in osteocytes (32), a critical step in their differentiation. Dmp1 may also play a role in osteocyte differentiation, as deletion of this gene in mice leads to a delay in osteocyte maturation (33). Sclerostin has also recently been implicated as a regulator of the differentiation from late osteoblast to preosteocyte through its regulation of Phex and Mepe (34). Interestingly, oxygen tension may also regulate the differentiation of osteoblasts into osteocytes (35), suggesting that some of the genes associated with resistance to hypoxia may play a role in osteocyte differentiation. In addition, mineralization of the extracellular matrix surrounding the osteocyte has also been shown to induce osteocyte maturation, both in vitro (36) and in vivo (37, 38).

The embedding of osteocytes does not seem to be a random process.

When viewed three dimensionally, osteocytes appear to be regularly spaced within the bone matrix with approximately equal distances between adjacent osteocytes. It remains unclear what dictates this highly ordered 3D organization of the osteocyte network. One interesting hypothesis comes from the work of Marotti (39), who has proposed that newly formed osteocytes secrete an osteoblast inhibitory signal once their dendrites have reached their maximal length. This inhibits osteoid production of the nearest, most intimately connected osteoblast, which then becomes buried in matrix produced by its neighbors. Marotti's theory is based entirely on morphological observations but nevertheless, it is an attractive hypothesis that potentially explains the ordered spacing of osteocytes in three dimensions. Our own studies using live cell imaging techniques have suggested that the osteocyte embedding process is highly dynamic and that the embedding cells repeatedly extend and retract their dendrites, making transient connections with already embedded osteocytes (4, 40, 41). This "sampling" of the local environment through the use of "exploratory dendrites" may allow the osteocyte to position itself with an appropriate spacing from other embedded osteocytes to maintain the ordered three dimensional spacing of the osteocyte network.

Although the molecular and genetic mechanisms that regulate osteocyte differentiation are slowly being unraveled, there remain many unanswered questions. These include the question of whether every osteoblast has an equal chance of becoming an osteocyte or whether there are specific subpopulations of osteoblasts with predefined fates. Aubin and colleagues have reported extensively on the heterogeneity of surface osteoblasts in terms of their gene and protein expression profiles (42, 43). It therefore seems plausible that there are specific subpopulations of osteoblasts on the bone surface that are already committed to becoming an osteocyte. Another unresolved issue is whether the decision for an osteoblast to differentiate into an osteocyte is a cell autonomous response or one that is controlled through signaling to surface osteoblasts from osteocytes that are already embedded. Imai and colleagues proposed that osteocytes can recruit osteoblasts and stimulate their differentiation by expressing osteoblast stimulating factor-1 (OSF-1, also known as heparin-binding growth associated molecule, HB-GAM) (44). They postulated that these osteoblasts then further differentiate into osteocytes, thereby providing a new source of OSF-1 for the next cycle of osteoblast recruitment.

The life span of the osteocyte is most likely determined by rates of bone turnover, the process by which osteoclasts resorb bone and osteoblasts replace the resorbed bone. Osteocytes may have a half-life of decades if they are located within a bone that has a slow turnover rate. An important question is whether the differentiation into an osteocyte is a "one way trip" – i.e., is it an irreversible process or do osteocytes have the ability to dedifferentiate back to an osteoblast or potentially give rise to other lineages, such as adipocytes and chondrocytes? The fate of osteocytes released by osteoclastic resorption is presently unknown. Some of the osteocytes that are only partially exposed by resorptive activity, may be re-embedded when new bone is formed after resorption (45). Resorption could also potentially liberate the osteocyte, but there is little clear evidence that osteocytes dedifferentiate back into the osteoblastic state (46). It seems most likely that most the osteocytes exposed during bone resorption either die by apoptosis and/or become phagocytosed by osteoclasts, as has been described in several reports (47–49). With the availability of transgenic mouse models in which lineage targeted GFP variants are expressed in osteoblasts and osteocytes (16–18), and the availability of transgenic tools for gene deletion and manipulation of gene expression in osteocytes (50, 51), it seems likely that the answers to some of these unresolved questions will soon be known and that we will gain deeper and deeper insight into the mechanisms controlling osteocyte differentiation.

Morphology of Osteocytes, Their Dendrites and Lacunocanalicular System

Osteocytes are one of an elite group of cells within the body, which also includes cementocytes and hypertrophic chondrocytes, that are completely encased within a mineralized extracellular matrix. This presents significant challenges with regards to communication with other cells and access to sufficient nutrients and oxygen. However, the osteocyte and its lacunocanalicular system are exquisitely adapted for this role and have developed a

FIGURE 3 Continued. . . A) Schematic diagram depicting the transitional stages that occur as osteoblasts differentiate into mature osteocytes. During this process, the volume of the cell body and the number of cell organelles decreases. 1= preosteoblast; 2= osteoblast; 3= embedding osteoblast; 4= osteoid osteocyte; 5= mineralizing osteocyte; 6, 7= mature osteocytes. B) Tetrachrome stained section of an adult mouse tibia illustrating several of the osteoblast-osteocyte transitional stages depicted schematically in (A) (bar = 25 μm). C) Table illustrating the relative temporal expression of various osteogenic markers during the transition from osteoblast to osteocyte as depicted in (A) and (B). RUNX2 directs early osteoblast differentiation and is expressed in both preosteoblasts and osteoblasts. OCN is expressed by mature osteoblasts and early osteocytes. E11 is the earliest osteocyte marker to be expressed during differentiation, but is not found in mature osteocytes in vivo. DMP1, CapG and MEPE expression is observed in mineralizing and mature osteocytes, whereas sclerostin expression is confined to mature osteocytes. ORP150 is also only found in mature osteocytes within the hypoxic environment of the mineralized bone matrix.

unique physiology to cope with the challenges of being "imprisoned" within a mineralized matrix.

Unlike the molecular regulatory mechanisms, the morphological changes that occur during the transition from an osteoblast to an osteocyte have been well characterized (2, 10, 11, 52, 53). During differentiation, osteoblast morphology changes from a polygonal to a more stellate shape. One of the first changes to take place in the embedding cell is the formation and elongation of numerous cellular projections, or dendritic processes, which are extended in a polarized manner towards the mineralizing front. This is followed by dendrites extending towards the vascular space or bone surface. These long, slender cytoplasmic processes radiate in all directions around the cell body of the osteocyte, with the highest density perpendicular to the bone surface (see figures 2A,B). Once embedded, the osteocyte maintains this polarity in the directionality of its dendrites and the directionality of mineralization such that mineral deposition occurs on one side of the embedding cell rather than equally all around it (see figure 3B). We have previously proposed that the osteoid osteocyte (i.e., the cell that is in the process of transitioning from osteoblast to osteocyte), is the cell that is primarily responsible for mineralization as opposed to osteoblasts on the bone surface (40, 54).

As the osteocyte embeds and further differentiates, there is a reduction in cell volume of up to 70% (10). Upon mineralization of the osteoid, there are also changes to osteocyte ultrastructure, such as a reduction in the ER and Golgi apparatus (55). In the past, this has been interpreted to imply reduced protein synthesis and metabolic activity of the osteocyte, leading to the misconception that osteocytes are passive, inactive cells and are of little importance during bone growth and development. However, it is now clear, as emphasized throughout this review, that osteocytes are highly active cells and play major roles in many key physiological processes both within and beyond the bone microenvironment.

The transition from a plump polygonal osteoblast to a stellate, highly dendritic osteocyte represents a profound change, which presumably requires extensive reorganization of cytoskeletal proteins. The characteristic polarization of the osteoblast is lost (56) and this is accompanied by striking changes in the expression and localization of actin-binding proteins, such as fimbrin, filamin and α-actinin, as shown by Kamioka and colleagues (57). These authors proposed that the organized expression of tubulin, vimentin and actin in the cell bodies and dendrites of osteocytes are important to establish and maintain their unique dendritic morphology (58).

At first glance, the osteocyte may appear to be isolated from neighboring osteocytes in the mineralized matrix and from osteoblasts on the bone surface. However, these cells show a high degree of interconnectivity. The cell body is enclosed within a lacuna of 15–20um in diameter and the cell processes pass through the bone matrix through narrow canals called canaliculi, approximately 250–300nm in diameter, (see figures 1 and 2). Together, the osteocyte lacunae and canaliculi are referred to as the lacunocanalicular system. Numerous dendritic processes connect osteocytes to other osteocytes and allow osteocytes near the bone surface to be connected to osteoblasts and bone lining cells. This characteristic morphology allows for the passage of nutrients and biochemical signals from one osteocyte to another, and through these interconnections, the embedded osteocytes form a functional network (reviewed in (15, 59, 60). This interconnectivity of the osteocytes facilitates communication and helps maintain cell viability and is therefore essential for the normal functioning of the osteocyte. Disruption of the network can have negative consequences for bone health. To enable osteocytes to communicate with one another, gap junctions are present on the tips of the dendrites connecting them (61). These are transmembrane channels that connect the cytoplasm of adjacent cells and allow the cells to exchange molecules of less than 1 kDa (62).

Intriguingly, the work of Kamioka et al. (63) suggests that some osteocyte cell processes extend beyond the osteoblast layer, establishing direct contact between the osteocyte network and the marrow space. Through these dendrites there is the potential for direct signaling between osteocytes and cells in the bone marrow compartment. Our studies using live cell imaging approaches suggest that osteocytes, particularly those that have recently embedded, can extend and retract their dendritic processes, not only towards other osteocytes and osteoblasts, but also into marrow spaces (64). This suggests that osteocytes have the potential to make and break contact with other cell types.

The morphology of the lacunocanalicular system can be demonstrated using techniques such as scanning electron microscopy (EM) of plastic embedded, acid etched bone samples (65) or injection of mice with dyes such as the small molecular weight dye, procion red (65, 66) (see figures 2B and C). These techniques reveal the intimate connection of the osteocyte lacunocanalicular system with the vasculature and illustrate an important property of the lacunocanalicular system, namely that the canalicular space between the osteocyte dendrite and the canalicular wall is filled with circulating fluid, canalicular fluid, that carries solutes to and from the osteocyte via the circulation. Dye injection studies have shown permeation of the osteocyte canalicular space from the bloodstream within only a few minutes after injection (66). Tracer studies have

shown that molecules less than 40KDa can readily reach the lacunocanalicular system from the circulation but that molecules as large as 440KDa cannot (67). The upper cut-off appears to be less than 70KDa and a filter of some sort appears to exist between the vascular system and the osteocyte lacunocanalicular system (68). As discussed later in this review, mechanical loading of bone results in the flow of canalicular fluid around the osteocyte and its dendrites, which may provide a key stimulus for mechanotransduction (reviewed in (14, 60, 68, 69). It is thought that the lacunar and canalicular spaces are not completely open, but are filled with proteoglycans that constitute the glycocalyx of the cell (reviewed in (59, 70). These may function as a molecular sieve that allows the rapid passage of smaller molecular weight molecules (e.g., $<$70 kDa) but much slower passage of larger molecules (66).

Interestingly, the morphology of the lacunocanalicular system may not be permanent. Several studies suggest that osteocytes can modify their microenvironment by depositing and resorbing bone around their lacunae (71–75). Furthermore, there is evidence that the number of canaliculi increases with age (13, 76). This suggests either that the new bone formed in older animals produces osteocytes with more canaliculi or that existing osteocytes can generate new dendrites after they have embedded, presumably through a matrix degradative process (13). This adds an interesting changing dimension to considerations of the osteocytes' relationship with its lacunocanalicular system. Nevertheless, by developing such an intricate network of lacunae and canaliculi and by adopting its unique morphology with extensive dendritic connections, the osteocyte has been able to overcome the challenges of living in the hostile environment of the mineralized bone matrix.

Osteocyte Selective Genes/Proteins and their potential functions

Determining genetic markers for osteocytes has proven to be difficult due to the relatively low number of known osteocyte-specific genes. The recent characterization of proteins such as E11, sclerostin, DMP1 and MEPE as osteocyte markers has, however, enabled easier identification of osteocytes as well as providing insight into the functions of these cells.

The earliest marker known to be expressed by the differentiating osteocyte is E11 (22, 31). In the adult mouse, this marker is specifically expressed in osteocytes but not osteoblasts (32). Peak expression is observed in the young osteocytes, in comparison with the more mature cells, deeper in the mineralized bone matrix. Regulation of E11 is poorly understood, however, the transcription factors SP1/3 (77) and AP1 (78) are known to modulate expression and E11 is susceptible to post-translational degradation by the calpain family of proteases (79). E11 is known to bind to CD44 (80) and ezrin-radixin-moiesin complexes (ERMs), therefore these molecules may function together to regulate the formation of dendritic processes in osteocytes, a critical step in their differentiation. Overexpression of E11 in vitro has been shown to induce dendrite formation in an osteoblast-like cell line (81) and similar cytoskeletal rearrangements were observed in keratinocytes, with relocalization of ezrin to cell surface projections (82). Furthermore, siRNA-mediated knockdown of E11 prevented the fluid flow induced elongation of the cell processes of MLO-Y4 osteocyte-like cells (32). In addition to regulating dendrite elongation, binding of E11 to the ERM complexes activates the small GTPase RhoA and this was shown to result in increased motility of an epithelial cell line (83). In vivo, E11 expression was found to be increased in osteocytes after mechanical loading (32). Deletion of E11 in mice did not significantly affect embryonic bone development, however the effects of E11 on postnatal bone formation are unknown as these mice die soon after birth due to respiratory failure (84).

Several members of the SIBLING (Small, Integrin-Binding LIgand, N-linked Glycoprotein) family of proteins are also highly expressed by osteocytes and are known to have important functions within the bone environment. Dmp1 expression has been described in hypertrophic chondrocytes and osteoblasts during embryonic bone formation (85), however, its expression is more restricted to osteocytes during postnatal development (86). One of the main functions of Dmp1 is thought to be in the regulation of mineralization, consistent with its pattern of expression within the bone (87). Further evidence for this is provided by Dmp1-null mice, which show hypophosphatemia and highly elevated levels of Fgf23, leading to a severe impairment in mineralization and resulting in an osteomalacic phenotype (65). In contrast to loss of function, the gain of function effects of Dmp1 in vivo are less clear. Lu and colleagues showed no effect of overexpressing full length Dmp1 in bone under control of the 3.6Kb type I collagen promoter, although it did completely rescue the phenotype when overexpressed on a Dmp1-null background (33). In contrast, Bhatia and colleagues reported increased bone mineral density (BMD) in transgenic mice that overexpressed Dmp1 ubiquitously under control of the CMV promoter (88). However, in this model, one must take into account the fact that the Dmp1 is expressed ectopically in tissues in which it is not normally expressed. DMP1 loss of function mutations in humans have been associated with autosomal recessive forms of hypophosphatemic rickets (65). These findings provided the first evidence that osteocytes may play a major role in regulation of phosphate homeostasis. Interestingly, osteocytes within the osteoma-

lacic bone of Dmp1-null mice maintained their expression of alkaline phosphatase, type I collagen, osterix and E11 and failed to express mature osteocyte markers, such as sclerostin (65). These studies indicated that osteocyte differentiation was arrested at an early stage, therefore suggesting a key role for Dmp1 in osteocyte maturation. The effects of Dmp1 in regulation of mineralization and osteocyte maturation appear to be predominantly due to its role in regulation of phosphate homeostasis, since the mineralization defects and the impairment in osteocyte maturation can be rescued by restoration of normal phosphate homeostasis (38). These studies also highlight the importance of phosphate regulation in control of osteocyte differentiation.

Another protein that is highly expressed in osteocytes is PHEX, which was found to be the target of the osteocyte-specific antibody Mab OB7.3 used to isolate avian osteocytes (89). Mutation of PHEX results in X-linked hypophosphatemic rickets (90) and *Phex* is the mutated gene in the Hyp mouse, which is also widely used as a model of X-linked hypophosphatemic rickets (91). Although the exact function of PHEX remains to be determined, like DMP1, it is known to play a role in phosphate homeostasis and mineralization of the bone matrix. Loss of Phex expression, or mutations, such as in the Hyp mouse, lead to increased levels of Fgf23 through an unknown mechanism and this leads to subsequent hypophosphatemia (92). Interestingly, Fgf23 is not normally expressed at high levels in osteocytes in the healthy state but its expression in osteocytes is dramatically upregulated in both Dmp1 and Phex associated hypophosphatemic rickets (92). Moreover, osteocytes appear to be the main source of the elevated circulating levels of Fgf23 seen in these mouse models, giving support to the notion that they act as endocrine cells.

Fgf23 expression can also be regulated by another SIBLING protein, Mepe which is predominantly expressed by osteocytes (25). Mepe is not believed to act on Fgf23 directly, but increases Fgf23 levels via inhibition of Phex enzymatic activity (93). Mepe can be proteolytically cleaved by Cathepsin B or D to release the highly phosphorylated ASARM peptide fragment (94) which in addition to antagonizing Phex, can also inhibit matrix mineralization by binding directly to hydroxyapatite (95, 96). Evidence is growing that modulation of FGF23 expression by osteocyte proteins is of crucial importance in maintaining phosphate homeostasis and the high levels of expression of all these proteins in osteocytes further confirms that phosphate regulation is a vital function of these cells.

One of the most exciting osteocyte-specific proteins to be discovered is sclerostin, the protein product of the *SOST* gene (97, 98), which is expressed by mature osteocytes (26, 99). Sclerostin is a negative regulator of bone formation which antagonizes the Wnt/β-catenin signaling pathway by binding to the Wnt coreceptors, low density lipoprotein receptor related proteins 5 and 6 (Lrp5 and Lrp6) (100). When Wnt ligand binds to Lrp5/6 the receptor is phosphorylated, resulting in the activation of dishevelled, which in turn represses glycogen synthase kinase 3 (Gsk3). This results in the release of axin from its complex with β-catenin. The β-catenin accumulates and translocates to the nucleus, where it binds to transcription factors, leading to the activation of Wnt target gene expression (reviewed in (101)). However, in the presence of sclerostin, Wnt-receptor interaction is inhibited, β-catenin is phosphorylated by Gsk3 and targeted for ubiquitination and degradation via the proteosome pathway. Studies using loss of function and gain of function mouse models of *Sost* have demonstrated increased and decreased bone mass respectively (26, 102). Sclerostin expression is decreased by mechanical loading (103) and it was recently reported that the anabolic effects of loading are dependent on this decrease in expression (104). The profound gain of bone mass observed after the deletion of *Sost* has led to the development of monoclonal antibodies directed against sclerostin as a potential anabolic treatment for osteoporosis. Animal studies and early results from clinical trials look very promising and suggest that such antibodies may be an effective therapeutic for increasing bone mass (105–107).

Microarray comparisons of the gene expression profiles of ex vivo osteoblasts and osteocytes has been performed to identify other osteocyte-expressed genes which may play important roles in bone (21). Increased expression of genes associated with neurons was reported, such as neuropeptide Y. Further studies have shown that osteocyte-expressed neuropeptide Y can inhibit osteoblast activity and differentiation (30) and that neuropeptide Y expression can be modulated by mechanical loading. Proteomic studies have also been used to identify proteins which are highly expressed by osteocytes. The expression of ORP150, destrin and Macrophage-capping protein (CAPG) were found to be increased in MLO-Y4 cells compared to MC3T3 preosteoblasts and increased expression was confirmed in osteocytes relative to osteoblasts in vivo (20). Destrin and CAPG are believed to play a role in dendrite formation, whereas ORP150 is likely to protect the cells from the hypoxic conditions encountered within the mineralized bone matrix.

Figure 3C summarizes some of the main marker genes that are differentially expressed during the transition from an osteoblast to an early and late osteocyte and indicates their temporal expression patterns. The further identification of such genes is imperative to provide more genetic

markers for the osteocyte and to provide better tools for defining the steps in the osteocyte differentiation pathway. A greater understanding of the function of these genes is essential to fully elucidate the key roles that the osteocyte is playing, not only in the maintenance of bone mass, but also during endocrine signaling.

Tools for Studying Osteocytes

While osteocytes remain one of the more challenging cells to study due to their inaccessible location within the mineralized bone matrix, recent advances both in vivo and in vitro have facilitated investigation into their function. The development of the Dmp1-Cre mouse line, in which Cre recombinase expression is driven by the 10 kb Dmp1 promoter, has enabled researchers to delete genes specifically in Dmp1-expressing cells (50). The authors found that Cre recombinase activity was predominantly confined to osteocytes and odontoblasts with some activity in a subpopulation of late surface osteoblasts in these mice. Although only recently developed, these mice have already been successfully used to investigate the functions of several molecules in osteocytes, including β-catenin (108), parathyroid hormone receptor type 1 (Pth1r) (109), polycystic kidney disease 1 (Pkd1) (110), Mef2C (111) and Rankl (112, 113).

The Dmp1 promoter has also been used to drive expression of the diphtheria toxin receptor in osteocytes in a murine transgenic model (51). This enabled the conditional ablation of up to 80% of the osteocytes upon injection of diphtheria toxin and has provided further insight into the importance of these cells in processes such as mechanotransduction and bone homeostasis, both of which were severely disrupted after osteocyte ablation. Transgenic mice in which GFP is under control of the 8 kb Dmp1 promoter have also been created and have provided an elegant and valuable new tool for osteocyte research (16). Calvarial cells from these mice were harvested and the GFP-positive osteocytes were isolated via fluorescence-activated cell sorting. The GFP positive cells were then used for gene expression analysis (21).

Before these sophisticated transgenic tools became available, more traditional methods were used for isolation of osteocytes and such methods still remain important research tools within the field. These methods include the isolation of osteocytes from avian bone by sequential EDTA and collagenase digestions and their purification using the monoclonal antibody OB7.3 (114). Similar digestion techniques have also been used on rat calvaria (115, 116) and long bones (56) to achieve osteocyte-enriched populations of cells. However, difficulties in obtaining substantial yields, in addition to maintaining the osteocyte phenotype of the ex vivo cells, have limited their usefulness. A recently described method has improved upon these techniques, enabling the isolation of greater numbers of osteocytes from mature or aged bone by grinding the bone fragments into a fine dust (117), thereby releasing osteocytes from the mineralized bone matrix.

Determination of osteocyte function in vitro has been greatly facilitated by the creation of immortalized osteocyte-like cell lines. Perhaps the best characterized of these cell lines are the MLO-Y4 cells, which are representative of an early osteocyte. These cells have been extensively studied (118–120) and their characteristics are outlined in Table 1. However, although MLO-Y4 cells have proved to be a very useful tool for studying osteocytes in vitro, they cannot provide information on the temporal changes in gene expression and morphology which occur as an osteoblast differentiates into an osteocyte. Primary calvarial osteoblasts have been widely used to study terminal osteoblast differentiation (121, 122), but the removal of these cells from the bone results in a highly heterogeneous cell population and may also result in contamination with fibroblasts and osteoclast precursors. Several osteoblastic cell lines have been described which differentiate towards an osteocyte-like phenotype in culture. These include HOB-01-C1 preosteocyte cells (123), MLO-A5 late osteoblast cells (124) and IDG-SW3 late osteoblast cells (125), which synthesize and mineralize a type-I collagen extracellular matrix and express known osteocyte markers. More information on these cell lines is provided in Table 1. These in vitro models form important tools which, alongside in vivo models, are essential for further investigation into osteocyte differentiation and function.

Osteocyte Mechanosensation and Transduction

One of the earliest functions ascribed to osteocytes was mechanosensation and mechanotransduction. It was first recognized by Julius Wolff that bone has the capacity to adapt to mechanical loading or lack of loading by adding or removing bone, thereby modifying bone mass (126). Because of their distribution throughout the bone matrix and their complex interconnected network, early investigators hypothesized that osteocytes are the cells responsible for sensing mechanical loading or lack of loading. By performing targeted deletion of osteocytes in mice expressing the diphtheria toxin receptor specifically in osteocytes, it was shown that these mice were resistant to unloading-induced bone loss (51). These findings further support the hypothesis that osteocytes are key mechanotransducers in bone.

(i) How Osteocytes Sense Loading

When a bone is mechanically loaded, there are several possible stimuli that could be detected by the mechano-

Table 1. The characteristics of the currently described osteocyte cell lines

Name	Origin	Characteristics
HOB-01-C1	Human cancellous bone	• Infected with temperature-sensitive large T antigen, cells proliferate at 34 C but do not divide at 40 C • Preosteocyte-like phenotype and express low levels of alkaline phosphatase and high levels of osteocalcin • Synthesize a mineralized matrix (123)
MLO-Y4	C57Bl/6 mouse long bone	• Express SV40 large T antigen driven by Osteocalcin-promoter • Immature osteocyte-like phenotype with dendritic morphology • Low alkaline phosphatase and high osteocalcin expression (120) • Express high levels of the early osteocyte marker E11 (32) but low levels of the mature osteocyte marker *Sost* (212,339) • Respond to fluid flow by activating Wnt/β-catenin signaling (131) and opening Cx43 hemichannels (163) • Support osteoclast formation and activation in co-culture systems (116) and form gap junctions with MC3T3-E1 cells (340) • Express primary cilia (137)
MLO-A5	C57Bl/6 mouse long bone	• Express SV40 large T antigen driven by the osteocalcin promoter • Late osteoblast phenotype and differentiate towards an osteocyte-like phenotype • Synthesize a mineralized matrix, which has a composition similar to normal bone (124) and is comprised of calcospherulites deposited along collagen fibrils (54) • Express *Sost* (212), *Dmp1*, *Phex* and *Cd44* (36)
IDG-SW3	Long bone cells derived from the Immortomouse crossed with the 8Kb-Dmp1-GFP mouse (16)	• IFN-γ and temperature-sensitive SV40 large T antigen, induces proliferation at 33 C with IFN-γ but not at 37 C • Synthesize a mineralized matrix • Express GFP under control of the 8Kb Dmp1 promoter • Temporally express early and late osteocyte markers such as E11, *Dmp1*, *Phex*, *Mepe*, *Sost* and *Fgf23* (125)

sensory osteocyte. These include the physical deformation of the bone matrix itself, the load-induced flow of canalicular fluid through the lacunocanalicular network, which results in fluid flow shear stress, or electrical streaming potentials that are generated from the flow of the canalicular fluid (which is an ionic solution) past the charged surfaces of the lacunocanalicular walls and/or cell membrane. The osteocyte and its dendritic processes are constantly exposed to canalicular fluid that flows through the lacunocanalicular system. It has been proposed that a baseline flow of canalicular fluid is driven by the extravascular pressure but that on top of this is superimposed rapid alterations in canalicular fluid flow that occur as a result of intermittent mechanical loading of the bone (127). This results in the cells being exposed to fluid flow shear stress. In vivo, it is difficult to separate out the three types of stimuli, as any mechanical loading input will result in the osteocyte being exposed to all three stimuli, including deformation of the bone matrix, fluid flow shear stress due to changes in canalicular fluid flow and associated streaming potentials.

Almost every cell responds to mechanical loading, however osteocytes appear to be one of the most sensitive cell types. Osteocytes are more sensitive than osteoblasts and fibroblasts to applied loading in the form of fluid flow shear stress and are more sensitive to fluid flow shear stress than to substrate stretching (128, 129). Theoretical models have been used to predict the fluid flow shear stresses exerted on osteocyte dendrites as a result of peak physiologic loads in vivo and have estimated these to be in the range of 8 to 30 dynes/cm^2 (127). Recently, real time measurement of load induced solute transport has been performed and these studies suggested a peak shear stress of 5Pa (approximately 30 dynes/cm^2) (130). MLO-Y4 osteocyte-like cells are several orders of magnitude more sensitive to fluid flow shear stress than 2T3 osteoblast-like cells (131) and MC3T3 osteoblast cells (132, 133).

There has been considerable debate within the field as to which part of the osteocyte is most important in sensing mechanical strain. Some investigators have proposed that the osteocyte only senses mechanical load through its dendritic processes and that the osteocyte cell body is rela-

tively insensitive to mechanical strain (134, 135). Others have proposed that osteocytes sense strain through both the cell body and dendritic processes (136), or that the primary cilium, a single hair-like projection found on every cell, is the primary strain sensing mechanism in the osteocyte (137, 138). There appears to be evidence for all three mechanisms and it remains unclear whether the cell body, cell processes and cilia work separately or in conjunction to sense and transmit mechanical stimuli. Recent studies using MLO-Y4 cells loaded by fluid flow shear stress on their dendrites or cell bodies suggest that the glycocalyx, present on the surface of dendritic processes, but not the cell body, plays an essential role in mechanotransduction by dendrites (139).

Removing the single primary cilium on either MC3T3 osteoblast or MLO-Y4 osteocyte cells by gene silencing approaches or using treatment with chloral hydrate reduced the amount of prostaglandin released by MLO-Y4 cells in response to fluid flow shear stress and also reduced increases in Cox2 and the Opg/Rankl ratio (138). The primary cilia in bone cells do not appear to mediate calcium flux in response to fluid flow, therefore the mechanism used by primary cilia in bone cells is distinct from that of kidney cells (138). Recently it has been shown that primary cilia signal through cAMP and adenyl cyclase 6 (140) and can mediate signaling between osteocytes and mesenchymal stem cells (MSCs) (141).

It is well known that polycystin 1 (PC1), encoded by the gene Pkd1, is part of a mechanosensing complex in renal cells that is associated with the primary cilium. Interestingly, mice with impaired PC1 function develop osteopenia (137) and deletion of Pkd1 in osteocytes using the 10kb Dmp1-Cre driver results in reduced bone mass in the young skeleton, which subsequently recovers in the adult skeleton. However, these adult mice are less responsive to anabolic load (110). The effects of deleting PC1 are reversed by deleting Kif3a, a transport protein known to play a role in the function of cilia (142).

Adhesion molecules are proposed to anchor the osteocyte cell body to the lacunar wall and the dendritic processes to the canalicular wall and to potentially interact with cytoskeletal proteins. These proteins therefore may play important roles in mechanotransduction. CD44, which is highly expressed on the osteocyte surface, was the first adhesion receptor to be described on osteocytes (29). The actin-bundling protein fimbrin is expressed at branches of dendritic processes (58). Both are known to connect to, and influence, the cell cytoskeleton. Integrins are well known to serve as major receptors/transducers connecting the cytoskeleton to the extracellular matrix in many cell types (143). It has been proposed that integrins bridge osteocyte processes to their canalicular wall and that they may play an important role in amplifying the strains perceived by osteocyte dendrites as a result of fluid flow (144). Recently it was found that Integrin $\alpha 5\beta 1$ interacts with Cx43 to mediate the opening of hemichannels in MLO-Y4 cells in response to mechanical stimulation. Surprisingly, this interaction appeared to be independent of the integrin association with fibronectin and its interaction with the extracellular matrix (145). This interaction between integrin $\alpha 5$ and Cx43 is important in the regulation and extracellular release of prostaglandin, an essential transducer of the effects of anabolic loading. Therefore, fluid flow shear stress may have two major effects on the osteocyte that are mediated through integrins; the first is the well-known effect of the integrin acting as a linker between the extracellular matrix and the intracellular cytoskeleton and the second, novel effect is through the opening of hemichannels that can release small molecules with autocrine and paracrine receptor mediated effects.

It has been shown that the osteocyte lacuna itself acts as a strain concentrator that amplifies the macroscopic strain applied over the whole bone. This strain amplification is strongly affected by the tissue material properties of the local perilacunar matrix immediately surrounding the osteocyte lacuna (136, 146, 147). Using a microstructural finite element analysis model, these investigators further showed that changes in the modulus of the osteocyte cell body and dendritic processes had little effect on the maximum strain transmitted to the osteocyte, the average strain in the cell processes, or on the maximum strain in the lacuna. In contrast, changing the material properties of the perilacunar matrix had the greatest impact on the strain transmitted to the osteocyte, with the maximum osteocyte strain relating inversely to the perilacunar tissue modulus. Therefore, any mechanism that changes the material properties of the perilacunar matrix, such as glucocorticoid induced hypomineralization (75), will have significant consequences to mechanosensation by osteocytes.

(ii) How Loading Affects Osteocyte Signaling

A true mechanotransducer must be able to convert the perceived mechanical strain signal into a biological output (i.e., a cellular response). Although the precise biochemical pathways have yet to be fully unraveled, considerable progress has been made towards delineating some of the response pathways in osteocytes that may mediate adaptive responses to mechanical loading and unloading in bone. In both primary osteocytes and MLO-Y4 osteocyte-like cells, fluid flow shear stress increases intracellular calcium, promotes the release of nitric oxide (NO), ATP and prostaglandins, induces opening of connexin 43 hemichannels and enhances gap junction functions. It has also

been shown to induce bending of cilia, and to initiate signaling pathways such as the Wnt/β-catenin and PKA pathways. Fluid flow shear stress also protects osteocytes against apoptosis, activates gene transcription and translation, and promotes dendrite elongation. It is still not clear what type of fluid flow shear stress occurs physiologically within the lacunocanalicular system, i.e., whether it is steady, pulsatile, oscillatory or a combination of one or more of these. The magnitude, frequency, and duration of the fluid flow stimulus in physiological conditions are also still unclear.

With regards to the temporal sequence of osteocyte responses to mechanical loading, one of the earliest events to occur in response to mechanical stimulation is the increase in intracellular calcium. Voltage-operated calcium channels that can be regulated by hormones, were shown to be expressed in osteoblasts and osteocytes (148, 149). Expression of voltage-gated potassium (K^+) channels during the differentiation from osteoblasts to osteocytes leads to different K^+ currents during osteoblast to osteocyte transition (150). Because of their contribution to maintenance of the cell membrane potential, and their fast response within 20 msec, K^+ channels and other ion channels may be involved in the earliest initiation of the mechanical response in osteocytes. The T-type voltage sensitive calcium channel is more highly expressed in osteocytes compared to the L-type channel, which is more highly expressed in osteoblasts (151). The T-type channel therefore may play a role in the unique kinetics of calcium signaling in osteocytes (133).

Soon after this rapid change in calcium signaling, within seconds to minutes, NO, ATP, and prostaglandin are released. Deleting or inhibiting any one of these three early small molecules will inhibit bone's anabolic response to loading (152–156). One of the earliest molecules released in response to shear stress is NO, which appears to correlate with PGE_2 release from osteocytes (128). In bone, NO inhibits resorption and promotes bone formation. Both osteoblasts and osteocytes release NO in response to mechanical strain or fluid flow shear stress (157). NO may also reduce osteocyte apoptosis (158, 159).

ATP is also rapidly released from osteocytes in response to extracellular calcium or mechanical stimulation (160, 161). There is evidence that this occurs through functional "hemichannels" that are formed by Cx43 on unopposed osteocyte cell membranes. The opening of these hemichannels by mechanical loading appears to provide a mechanism for ATP and NAD^+ release (162, 163). The P2 × 7 nucleotide receptor, an ATP-gated ion channel, may also play a role as its deletion in mice resulted in a 70% reduction in bone anabolic response to applied mechanical loading and fluid flow shear stress did not induce prostaglandin release in cells isolated from these mice (164). Blockers of P2 × 7 receptors suppressed prostaglandin release, whereas agonists enhanced release in bone cells, suggesting that the P2 × 7 receptor is necessary for release of prostaglandin in response to mechanical load.

Prostaglandins may be one of the key effectors of load related osteogenic responses. Treatment with prostaglandins in vivo enhances new bone formation in response to mechanical load (165) and inhibitors of prostaglandin production, such as indomethacin, block the effects of anabolic loading (152, 166). Fluid flow-induced shear stress stimulates the rapid release of PGE_2 by osteocytes (163). Interestingly, these authors have shown that PGE_2 is likely released through Cx43 hemichannels, which serve as a portal for the exit of intracellular PGE_2 in osteocytes stimulated with fluid flow shear stress. This released PGE_2 then signals in an autocrine/paracrine manner to the osteocytes to enhance gap junction function and increase Cx43 expression. It remains to be confirmed whether PGE_2 release via Cx43 hemichannels is important for in vivo load related responses. So far, in vivo loss of function studies suggest that the in vivo role of Cx43 is complex. Cx43 deficiency can either inhibit or enhance the bone anabolic response to loading (167, 168) and it has been suggested that the specific promoter used to delete Cx43 at different stages of the osteoblast lineage can affect not only the bone phenotype, but also the responses to loading (169).

As would be surmised, numerous signaling pathways are activated by mechanical loading of osteocytes. One of the most important appears to be the Wnt/β-catenin pathway, important for osteoblast differentiation, proliferation and matrix production. In osteocytes this pathway plays a role in transmitting signals of mechanical loading from osteocytes to cells on the bone surface (reviewed in (14)). Interestingly, it has been shown that PGE_2 released by mechanical loading acts through the β-catenin pathway to enhance Cx43 expression and gap junction function (170) and to protect osteocytes from glucocorticoid induced apoptosis (171).

Deletion of various components of the Wnt/β-catenin pathway has effects on bone responses to loading and unloading. In vivo, deletion of Lrp5, a major coreceptor for Wnt signaling, resulted in mice that showed impaired osteogenic responses to anabolic loading (172). Negative regulators of the Wnt/β-catenin pathway such as Dickkopf1-related protein 1 (Dkk1) and sclerostin are highly expressed in osteocytes (99). Sclerostin is expressed in the mature osteocytes and mechanical loading has been shown to reduce sclerostin levels in bone (103) whereas hindlimb unloading increases sclerostin expression (173).

It has also been suggested that Sost must be downregulated in order for the osteogenic response to anabolic loading to occur (104). Downregulation of Dkk1 and Sost may create a permissive environment in which Wnt proteins that are already present can activate the Wnt/β-catenin pathway. Whereas targeted deletion of β-catenin using the Dmp1-Cre driver results in dramatic bone loss (108), deletion of only one allele results in mice with a normal skeleton but a completely abrogated response to anabolic loading (174). Taking all these studies together, it appears that β-catenin plays an important role not only in osteocyte gap junction function and communication, osteocyte viability, and bone integrity, but also in bone responses to loading.

Other signaling pathways are activated in response to mechanical loading and may also crosstalk with the Wnt/β-catenin signaling pathway. Mice lacking the estrogen receptor alpha isoform (ER-α) exhibit a defective response to loading (175). Lanyon and coworkers suggest that the ER-α may play a role in shuttling β-catenin into the nucleus in response to mechanical strain in osteoblasts (176). This may in part explain how estrogen regulates bone mass via a functional intersection of the estrogen signaling pathway with Wnt/β–catenin signaling through ER-α.

Other genes known to be regulated by loading and by unloading include E11, a marker for the early osteocyte. This was shown to be upregulated in response to mechanical loading (32), as are regulators of mineralization and phosphate homeostasis such as Phex, Mepe, and Dmp1 (177–179). As mentioned previously, *Sost*/sclerostin, a marker for the late osteocyte and an inhibitor of osteoblast function, is down-regulated by anabolic mechanical loading and is increased in response to hindlimb unloading (103, 173). Recently, it has been shown that unloading increases Rankl expression in osteocytes (113), which may be responsible for the bone loss associated with unloading. It would follow that genes involved in bone formation would be upregulated in response to anabolic load and vice versa for unloading. Ideally, it would be important to identify genes that are rapidly activated in response to anabolic load to deduce the earliest pathways activated by mechanical loading.

Overall, there is considerable evidence that osteocytes play key roles in mechanosensation and mechanotransduction in bone and while some of the pathways and regulatory mechanisms are beginning to be elucidated there is still much that remains to be determined. A complete understanding of these pathways may lead to exciting opportunities for bone anabolic therapeutics, since a drug that could mimic the effects of mechanical loading or amplify bone anabolic responses to loading would be a valuable therapeutic for diseases such as osteoporosis.

Osteocytes as Orchestrators of Bone Formation and Resorption

A key feature of osteocytes is their ability to regulate the function of both osteoblasts and osteoclasts. It is therefore becoming clear that in contrast to the historical view of osteocytes as passive cells, they may actually function as central orchestrators of bone remodeling which can integrate both hormonal and mechanical signals to regulate bone mass. This type of mechanism makes sense, as through the osteocyte's centralized role in regulating responses to these stimuli, the skeleton would be able to meet not only the demands of its mechanical environment but also its other demands in relation to calcium and phosphate homeostasis.

The lacunocanalicular system is the ideal network for enabling the transmission of biochemical signals from deeply embedded osteocytes to osteoblasts at the bone surface and thereby allowing osteocytes to influence osteoblast activity. Indeed, in vitro studies have shown that avian osteocytes (180) and MLO-Y4 cells (181) release factors which enhance osteoblast differentiation and alkaline phosphatase activity. Osteocytes have also been shown to induce new bone formation at sites of fracture damage by recruiting mesenchymal stem cells via the secretion of Osteopontin (182). However, much is still to be learned about the mechanisms by which osteocytes signal to osteoblasts.

As discussed earlier, signaling molecules such as NO, PGE_2 and ATP are known to be rapidly released by osteocytes in response to external stimuli such as mechanical strain and many of these have direct effects on osteoblasts. NO generation by endothelial nitric oxide synthase (eNOS) is believed to be the main source of NO from osteocytes (183), although the inducible form of nitric oxide synthase (iNOS) has been detected in osteocytes after mechanical loading (184, 185). Neural NOS (nNOS) was also detected in human osteocytes (186). NO released by osteocytes in vivo has been shown to be important for bone formation during reloading (184) and in vitro, osteocyte-derived NO inhibits proliferation of early osteoblasts and promotes osteoblast differentiation (180). The effects of PGE_2 on osteoblasts and its anabolic effects on bone have also been extensively documented (see (187) for review). PGE_2 is known to induce bone formation (188) and osteoblastic differentiation of primary calvarial cell cultures in vitro (189) and to increase overall bone mass in vivo (190, 191). As discussed in the previous section, osteogenic responses to mechanical loading are impaired if production of prostaglandins is inhibited and are enhanced by treatment with PGE_2.

One of the most important signaling pathways used by osteocytes to regulate bone formation is the Wnt/β-

catenin pathway. Osteocytes express several modulators of Wnt signaling and therefore may regulate osteoblast activity via this mechanism. The activation of canonical Wnt signaling in early osteoblasts promotes osteoblast differentiation and bone formation (192) with opposing effects observed when Wnt signaling is disrupted (193). Several osteocyte-secreted Wnt antagonists have been described, with sclerostin in particular being the focus of many recent studies. In addition to sclerostin, osteocytes also express the Lrp5/6 inhibitor Dkk1, which inhibits osteoblast differentiation and bone formation (194–196). Similar to sclerostin, Dkk1 expression is down-regulated by mechanical loading in vivo (103), leading to enhanced bone formation. Another Wnt pathway inhibitor expressed by osteocytes is secreted frizzled-related protein 1 (sfrp1), which is a competitive antagonist of Wnt ligand binding. Loss of sfrp1 expression in vivo results in increased bone mass and mineral density and in vitro enhances osteoblast proliferation and differentiation into osteocytes (197). Peak sfrp-1 expression is observed in the immature osteocyte and was decreased in the mature cell, indicating a role for sfrp-1 in the negative regulation of cell survival during matrix embedding (198).

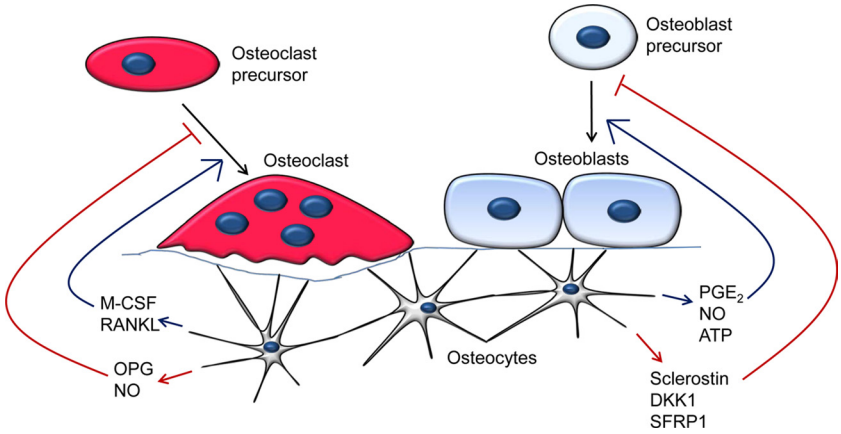
The control of bone remodeling requires regulation of not only osteoblast but also osteoclast activity. Coculture studies performed on both MLO-Y4 cells (116) and primary chick osteocytes (199) show that osteocytes are potent supporters of osteoclast formation and activation in vitro. In the study by Zhao et al. as few as fifty MLO-Y4 cells per well were required to promote robust osteoclast formation even in the absence of osteotropic factors. Osteoclast precursors express receptor activator of nuclear factor factor κ-B (Rank) on their cell surface. This receptor binds to its ligand (Rankl), which is expressed on the surface of cells of the osteoblast lineage, and it is this binding interaction between Rank and Rankl that promotes the differentiation of osteoclast precursors to form mature osteoclasts. Importantly, Rankl expression has been detected in osteocytes in vivo (200, 201) and MLO-Y4 cells in vitro (116, 202, 203), consistent with their ability to support osteoclast formation. Osteocytes and MLO-Y4 cells are also known to express another factor required for osteoclast activation, macrophage-colony stimulating factor (M-Csf) in addition to Opg, a decoy receptor for Rankl (116, 204). Deletion of β-catenin in osteocytes using Dmp1-Cre resulted in decreased expression of Opg and a subsequent increase in the Rankl/Opg ratio (108). These mice were characterized by severe osteoporosis, which was a consequence of enhanced osteoclast activity and excessive bone resorption. The importance of osteocytes in regulating osteoclastic activity was recently confirmed in elegant in vivo studies performed by Xiong et al. and Nakashima et al. in which Rankl was deleted specifically in osteocytes. These mouse models developed an osteopetrotic phenotype, which led the authors to conclude that osteocyte-derived Rankl is essential for normal bone remodeling in adult mice (112, 113). Therefore, there is a fairly strong body of evidence that Wnt signaling in osteocytes, via regulation of Rankl/Opg acts as a negative regulator of bone resorption.

Osteocyte death via apoptosis is also thought to result in local bone remodeling via the recruitment of osteoclasts to sites of bone damage. Viable osteocytes have been proposed to secrete as yet unknown factor(s) which inhibit osteoclast activity and a loss of production of these factors due to osteocyte death, may release the osteoclasts from inhibition (205). Consistent with this hypothesis, the targeted in vivo ablation of osteocytes using the osteocyte-specific Diphtheria toxin receptor mouse model was shown to induce osteoclast formation and activation, leading to bone loss (51). Additionally, targeted damage of MLO-Y4 cells cultured in a 3-D collagen gel induced osteoclast formation specifically at sites of injury (206) and apoptotic MLO-Y4 cells release Hmgb1 (High mobility group box 1), which is chemotactic to osteoclasts (207). Osteocyte-derived apoptotic bodies have also been shown to induce osteoclast formation in vitro and in vivo, whereas osteoblast-derived apoptotic bodies had no effect (208). This effect was not mediated by Rankl but was shown to be TNF-α-dependent.

As mentioned above, NO signaling can influence bone mass by increasing bone formation however, it is also known to inhibit osteoclasts. Conditioned media from mechanically-stimulated chick osteocytes inhibited osteoclast formation and bone resorption in vitro, whereas this effect was blocked upon the addition of an NO synthase inhibitor (209). Osteocyte production of NO therefore appears to promote bone formation while at the same time inhibiting bone resorption.

In addition to their role in mediating adaptive responses to mechanical loading, osteocytes can also respond to hormonal signals to affect bone formation and resorption. The presence of the parathyroid hormone receptor (Pth1r) has been detected on the surface of osteocytes as well as osteoblasts (210, 211). Signaling by PTH and PTHrP is known to have either anabolic or catabolic effects on the skeleton, depending on whether the administration is intermittent or continuous. It now appears that osteocytes may be a key target cell for the anabolic actions of PTH in the skeleton, since these anabolic effects have been shown to be due, at least partly, to downregulation of osteocyte *Sost* expression (212, 213). Constitutive activation of the Pth1r in osteocytes in vivo, under control of the Dmp1 promoter, also inhibits *Sost* expression and dramatically

Figure 4.



Osteocyte regulation of bone remodeling

Osteocytes express RANKL and M-CSF to promote, and OPG and NO to inhibit, osteoclast formation and activity. Osteocytes also regulate bone formation via the secretion of modulators of the Wnt signaling pathway. PGE2, NO and ATP act to activate Wnt signaling, whereas sclerostin, DKK1 and sFRP1 all inhibit Wnt signaling and subsequent osteoblast activity. Maintenance of this balance between resorption and formation by the osteocyte is essential for bone homeostasis.

increases bone formation. This phenotype is dependent on increased Wnt signaling (214). Interestingly, increased osteoclast activity and bone turnover were also observed in this mouse model but the net result was an increase in bone mass. Conversely, in a lactating mouse model in which there is a chronic elevation in PTHrP, Rankl was shown to be elevated in osteocytes (215) and most likely to be responsible for increased osteoclast activity.

Overall, it is becoming clear that it is not just the osteoblasts and osteoclasts that are the critical players in bone remodeling, but that the osteocyte also plays a key role in regulating the activity of these two cell types, as summarized in figure 4. Moreover, the osteocyte appears to be playing a centralized role in integrating mechanical loading and hormonal signals to regulate bone mass in the skeleton.

Osteocyte Life, Death, and in Between

Not only the healthy, viable osteocyte, but also the dying or dead osteocyte can have important regulatory effects on other cell types in bone. Whereas some osteocytes appear to sacrifice themselves by apoptosis to initiate bone remodeling or bone resorption, others remain viable in the bone matrix until the death of the organism. Unlike osteoclasts that only live for days and osteoblasts that live for weeks, osteocytes are an extremely long lived cell, surviving for up to decades in bone matrix in adults. The life span of the osteocyte is most likely determined by rates of bone turnover, the process by which osteoclasts resorb bone and osteoblasts replace the resorbed bone. Osteocytes may have a half-life of decades if they are located within a bone that has a slow turnover rate. The cell is nonmitotic while it is encased in bone and appears to have developed protective mechanisms to ensure its survival, especially under stress conditions such as immobilization, hypoxia, and certain disease conditions. The osteocyte can undergo necrosis, apoptosis, and autophagy, each of which may have specific regulatory effects on other cells in bone. With age, the dying osteocyte appears to undergo a process called micropetrosis, in which the lacuna fills with mineral. Each of these states most likely has a significant impact on the canalicular fluid circulation within the lacunocanalicular network and on osteocyte cell functions such as mechanosensation, signaling, and release of soluble mediators.

Damaged bone requires replacement. It was proposed that microcracks that occur in bone can damage the osteocyte and its processes, thereby inducing the cell to send signals of osteoclast recruitment to initiate bone removal and repair. Microdamage and bone fatigue is associated with loss of osteocyte integrity (216). These authors have also shown that the antiapoptotic factor, B cell lymphoma 2 (Bcl-2), is found in osteocytes around the cutting cone, whereas the proapoptotic factor, Bcl2-associated X protein (Bax) is found in osteocytes in the path of the osteoclast within the cutting cone, suggesting that these osteocytes are undergoing programmed cell death. They therefore proposed that the dying (apoptotic) osteocytes were sending factors to recruit and direct osteoclasts to remove the damaged bone (217). More recently, however, it has been shown that the highest expression of osteoclast supportive signals is found in a subpopulation of osteocytes surrounding areas of microdamage. These osteocytes have a higher Rankl/Opg ratio than that observed in apoptotic osteocytes within the areas of microdamage, suggesting that these viable cells are the ones responsible for inducing osteoclastogenesis (218).

It has been shown that osteocytes express markers of apoptosis in response to withdrawal of estrogen (219), to oxygen deprivation as occurs during immobilization (220), and in response to glucocorticoid treatment, (221, 222). Increased osteocyte apoptosis was shown to play an

important role in the decreased bone strength observed with glucocorticoid treatment (223). Further studies have indicated that dexamethasone treatment of MLO-Y4 osteocytes in vitro causes detachment of the cells from the substrate by interfering with focal adhesion kinase (FAK) signaling, resulting in cell death (224). Interestingly, TNFalpha and Interleukin-1 (IL-1) have both been reported to increase with estrogen deficiency and also are known inducers of osteocyte apoptosis (225). A number of in vitro studies also support the concept that the apoptotic osteocyte can support osteoclast formation. Kogianni and colleagues showed that apoptotic bodies released by MLO-Y4 cells and primary osteocytes, but not osteoblasts supported osteoclast formation (208). Other groups have shown that serum starved MLO-Y4 cells secrete soluble Rankl (226) and that damaged MLO-Y4 cell networks embedded in three dimensional gels have increased Rankl and decreased Opg production (227). Recently, thiazolidinediones have been shown to induce osteocyte apoptosis (228), as does high dose alcohol (229), and methotrexate used for cancer treatment (230). Therefore, a major research focus has been on osteocyte viability and approaches to prevent osteocyte cell death.

Searches for inhibitors that would protect osteocytes from apoptosis induced by estrogen deprivation or glucocorticoid treatment have identified several candidates. These include PTH, estrogen and selective estrogen receptor modulators, bisphosphonates, calcitonin, CD40 Ligand, and Calbindin-D28k (For review see (231)). Intermittent PTH treatment has been shown to increase osteocyte density in mice and block osteocyte apoptosis induced by glucocorticoid treatment (232). Increased osteocyte death is also observed after parathyroidectomy in patients with secondary hyperparathyroidism (233), suggesting a protective role for PTH on osteocytes in vivo. Estrogen inhibits etoposide induced apoptosis (234) and the estrogen receptor is required for the antiapoptotic effect of mechanical stimulation (235). Mechanical loading in the form of fluid flow shear stress was also shown to protect against glucocorticoid induced apoptosis through the release of prostaglandin and subsequent activation of the Wnt/β-catenin pathway (171). A target of the Wnt/β-catenin pathway, osteoprotegerin, also prevents glucocorticoid-induced apoptosis (236). Substrate stretching has also been shown to protect osteocytes from glucocorticoid induced apoptosis, potentially through activation of focal adhesion kinase, ERK, and the ECM-integrin/FAK signaling that is linked with the Wnt/β-catenin pathway (237). Cx43, the major component of gap junctions and hemichannels in osteocytes is also important for osteocyte survival, as Cx43 is required for the antiapoptotic effects of bisphosphonates (238) and deletion of Cx43 in osteoblasts results in animals with increased osteocyte apoptosis (239). Bisphosphonate treatment was shown to reduce osteocyte apoptosis in response to fatigue loading (240) and to protect against glucocorticoid induced apoptosis by transiently increasing ERK phosphorylation (241). A similar effect was also observed with calcitonin and mechanical stimulation (242). Treatment of ovariectomized mice with a pan-caspase inhibitor, QVDOPh, was shown to inhibit ovariectomy-induced osteocyte apoptosis and reduce osteoclastic bone resorption (243). Inhibitors of osteocyte apoptosis may therefore prove to be useful therapeutics for the treatment of diseases of bone loss.

While much attention was being focused on osteocyte apoptosis, one group of investigators noted that the magnitude of glucocorticoid-induced apoptosis in MLO-Y4 cells was much less than that induced by cytokines such as TNFalpha and that the same caspase pathways were not activated. They decided to investigate whether glucocorticoid treatment could be inducing autophagy in these cells (244) and showed that glucocorticoid was associated with induction of autophagy related markers in osteocytes in vitro and in vivo. Autophagy is a mechanism in which the cell selectively degrades some of its own contents in an attempt to maintain viability under conditions of stress. It is a tightly regulated process of lysosomal "self-degradation" in which the cell degrades and recycles nonessential cellular products such as parts of the cytoplasm and intracellular organelles to reallocate limited cell resources to processes that are critical for survival. Autophagy can therefore protect cells from apoptosis and eventually cell death, thereby preserving viability until the stress can be relieved. However, should the stress continue or worsen, the outcome can be cell death. Other investigators have also shown glucocorticoid induced osteocyte autophagy in vivo, (245) where it was shown that low dose glucocorticoid induced autophagy and high dose glucocorticoid induced apoptosis. At the most recent ASBMR meeting it was shown that targeted deletion of the autophagy gene, ATG7, using the Dmp1-Cre driver, which reduced autophagy in osteocytes, resulted in mice with low bone mass (246). These data suggest that the long-lived osteocyte requires numerous protective mechanisms, one of which is the capacity to enter the state of autophagy, at least periodically, to enable it to survive for decades within its enclosed environment.

Osteocytic Perilacunar Remodeling: An Old Concept Rediscovered

Bone is a porous tissue. Whereas blood vessels and haversian canals are obvious porosities, the osteocyte lacunae are smaller and more numerous, and the canaliculi contribute to bone porosity to an even greater extent. The

surface area of the osteocyte lacunocanalicular system is estimated to be several orders of magnitudes greater than the bone surface area (247). Osteocytes and their dendritic processes therefore have access to an extremely large surface area of bone mineral and the removal of only a few angstroms of mineral from their lacunar and canalicular walls would be expected have significant effects on circulating, systemic ion levels. Just as osteoclasts and osteoblasts remodel the bone surface, osteocytes may have the capacity to remodel the surfaces to which they are constantly exposed.

While exciting new functions of osteocytes have been discovered within recent years, old functions previously proposed for osteocytes have also been rediscovered. One of these 'old functions' is that of osteocytes being able to remove their perilacunar matrix, a process that was previously referred to by Belanger as 'osteocytic osteolysis' (248). This concept, though proposed in the 1960s, soon disappeared from the literature, most likely because investigators lacked the tools and technology to validate the original descriptive observations. 'Osteocytic osteolysis' was previously viewed as being a feature of pathological conditions, especially due to high or continuous PTH. Enlarged lacunae have long been described in patients with rickets or osteomalacia (249), uraemic osteodystrophy (250, 251) and in response to parathyroid hormone (PTH) (252, 253). An increase in the number of lysosomal vesicles was documented in response to PTH (71, 248, 254). Enlarged lacunae have also been described in rats sent into space for 22 d (255), hibernating ground squirrels (256) and snakes (257) and in glucocorticoid-treated mice (75). Glycoproteins have been detected around the osteocytes of rats treated with parathyroid extract (258) and in lactating rats fed with a calcium-free diet (259) and the interpretation of these data was that the glycoproteins were exposed with the removal of mineral.

Interestingly, Wergedal and Baylink had described Tartrate Resistent Acid Phosphatase, TRAP, activity in osteocytes in 1969 (260), which was criticized as potentially being a diffusion artifact from osteoclasts. But this observation was later validated by Nakano and coworkers using in situ hybridization for *Trap* gene expression (261). After describing TRAP activity in osteocytes, Baylink went on to show tetracycline binding to the perilacunar matrix, which led him to suggest that osteocytes have the ability not only to remove bone from their perilacunar matrix, but also to add it back (262). In 1983, Zallone and colleagues also reported tetracycline labeling in osteocyte lacunae in egg-laying hens (263). Whereas there were numerous reports of 'osteocytic osteolysis', there had been few reports of osteocytes replacing their perilacunar matrix.

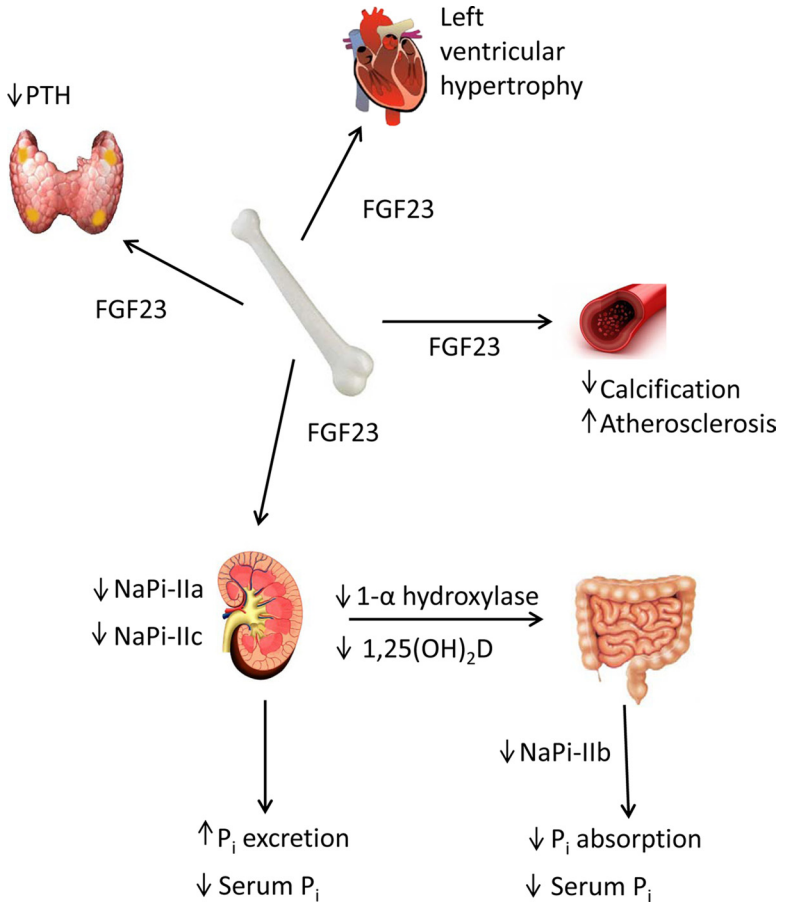
Using scanning EM, gene arrays, and transgenic technology to examine osteocytes in virgin, lactating, and postlactation mice, it was recently found that osteocyte lacunar area increases with lactation and returns to normal with forced weaning (215). Genes thought to be osteoclast specific, such as *Trap* and Cathepsin K, were found to be elevated in osteocytes during lactation and returned to normal with weaning. PTHrP, which is known to be elevated in the circulation during lactation, reproduced these effects on lacunar enlargement and this effect of PTHrP was confirmed to be mediated through the PTH type 1 receptor. This study shows that *healthy* osteocytes can both remove and replace their perilacunar matrix during processes related to normal reproductive function, suggesting that osteocytes can potentially play a role in mineral homeostasis during conditions of high calcium demand, such as lactation. Interestingly, in this study, no effect on lacunar size was observed in the hind limbs of tail suspended mice, suggesting that removal of osteocyte perilacunar matrix is not initiated in response to unloading, but is targeted by specific signals, such as hormonal regulation.

Our work suggests that not only can the osteocyte remove its mineralized matrix, but it can also replace this matrix. We propose that the term "osteocytic osteolysis" be reserved for pathological conditions, whereas the term "perilacunar remodeling" be used for the function of the healthy osteocyte such as its function in the healthy lactating animal. It is interesting to see that an old and apparently forgotten function for osteocytes has now been resurrected and that the observations of early osteocyte research pioneers have been validated with modern research approaches.

The Osteocyte as an Endocrine Cell

It may initially seem counterintuitive to describe the osteocyte as an endocrine cell considering its location deep within the bone matrix and its appearance as an isolated cell, cut off from other cells and tissues. However, the lacunocanalicular system allows for the flow of canalicular fluid that reflects the composition of the circulation and exposes the osteocyte to the hormones and factors that circulate within the blood. A variety of imaging techniques have demonstrated the connectivity of osteocyte processes, not only with other bone cells but also with the vasculature which permeates the bone matrix (see figure 2B and C). Injection of dyes such as procion red into the tail vein of a mouse results in the dye being distributed via the circulation to the osteocyte lacunocanalicular system within a matter of minutes and studies have shown that molecules up to 70 kDa in size can readily reach the lacunocanalicular space from the bloodstream (see earlier

Figure 5.



Endocrine signaling by osteocytes

FGF23 secreted by the osteocyte regulates serum P_i by down-regulating the expression of sodium/phosphate cotransporters in the kidney. There is also a decrease in 1-α hydroxylase production by the kidney, resulting in decreased NaPi-IIb in the intestine and reduced P_i uptake. High serum FGF23 has also been linked with an increased risk of LV hypertrophy in the heart, atherosclerosis and vascular calcification. However, vascular and soft tissue calcification is increased in Fgf23-null mouse models, suggesting that FGF23 can promote and inhibit vascular calcification. FGF23 is also known to negatively regulate the secretion of PTH via the parathyroid.

section on osteocyte morphology and lacunocanalicular system). Such connectivity between the canalicular fluid and vasculature allows osteocytes to be exposed to circulating hormones from distant tissues, but would also provide a conduit for the passage of hormones and other molecular mediators secreted by osteocytes into the circulation to exert effects on distant target organs. Exciting recent research has discovered the presence of such osteocyte-produced endocrine factors, with the result that the osteocytic network should now be viewed as an endocrine tissue that plays a vital role in the regulation of phosphate homeostasis.

Since its identification in 2000, FGF23 has emerged as one of the most important osteocyte-secreted endocrine factors. Initially identified in the ventrolateral thalamic nucleus of the brain (264), Fgf23 was found to be most highly expressed in bone, predominantly by the osteocyte (65, 265–267) and highly elevated in conditions of hypophosphatemia. FGF23 is a 32-kDa member of the fibroblast growth factor family of proteins that binds to FGF receptors. This binding is dependent on the presence of Klotho. It has been shown that Klotho can convert the canonical FGF receptor, FGFR1 (IIIc), into a receptor that is specific for Fgf23 (268) and that a monoclonal antibody targeted against Klotho can prevent activation of Fgf23 receptor-mediated signaling. FGF23 is known to have effects on the musculoskeletal system and increased levels of circulating FGF23 result in hypophosphatemic disorders however, it is the effects of osteocyte-secreted FGF23 beyond the bone which are of particular interest here.

One of the most important targets of FGF23 is the kidney (see Figure 5). Signaling between bone and kidney plays a vital role in the maintenance of serum phosphate levels, which is dependent on the circulating levels of FGF23. FGF23 has been shown to decrease expression of the sodium/phosphate cotransporters NaPi-IIa and NaPi-IIc in the kidney, which are required for renal phosphate reabsorption (269, 270). This leads to increased urinary excretion of phosphate. Additionally, excess FGF23 levels are known to down-regulate the expression of 1-α hydroxylase, which is required for the conversion of 25(OH)D to the active vitamin D metabolite, $1,25(OH)_2D$ (265). This reduction in $1,25(OH)_2D$ levels results in decreased expression of NaPi-IIb in the intestine, therefore reducing phosphate absorption and leading to hypophosphatemia (271). This signaling between the osteocyte and the kidney is bidirectional, as $1,25(OH)_2D$ induces expression of Fgf23 by the osteocyte in murine (272) and cell culture models (125, 272), suggesting a negative feedback system.

In addition to $1,25(OH)_2D$, several other factors have been shown to modulate FGF23 expression and activity.

For example, intake of dietary phosphorous was shown to regulate serum Fgf23 levels in adult mice, with increased phosphate supplementation correlating with increases in serum phosphate and Fgf23 levels (273). Likewise, an increase or decrease in dietary phosphate was shown to result in increased or decreased levels of circulating intact FGF23 respectively, in healthy human subjects (274). Recent data have also suggested that PTH may directly regulate FGF23 levels. Infusion of PTH for 3 d in adult mice resulted in increased *Fgf23* mRNA expression in the calvaria and increased serum Fgf23 (275). PTH was also shown to up-regulate expression of *Fgf23* mRNA in UMR106 cells, although this effect was abolished if sclerostin was added to the cultures. Additionally, the PKA activator Forskolin up-regulated expression of Fgf23 in these cells, indicating that both the Wnt and PKA pathway may modulate the effects of PTH on Fgf23. Similar results were observed in a recent study by Rhee et al. (276), in which they generated transgenic mice with constitutive activation of the PTH receptor (Pth1r) in osteocytes under control of the Dmp1 promoter. The authors observed increased Fgf23 expression specifically in osteocytes and this effect was abolished by crossing the mice with mice overexpressing *Sost*. In vitro studies showed that treatment with PTH, PTHrP and cAMP increased *Fgf23* mRNA in osteocyte-enriched cell cultures, again suggesting the importance of PKA and Wnt signaling in regulating FGF23.

As mentioned above, PTH secreted by the parathyroid gland increases FGF23 expression in vivo. Interestingly, FGF23 is also able to act reciprocally on the parathyroid gland to decrease PTH secretion, identifying the parathyroid gland as another endocrine target of osteocyte signaling. Addition of recombinant FGF23 to cultured bovine parathyroid cells resulted in decreased PTH mRNA expression and reduced the secretion of PTH into the culture media (277). Similar effects were observed in ex vivo rat parathyroid glands, where recombinant FGF23 decreased PTH mRNA expression and secretion of PTH into the media and in vivo, where FGF23 decreased serum PTH levels and PTH mRNA expression in the parathyroid (278). Increased expression of the FGF23 coreceptor klotho was also observed in the parathyroid in response to FGF23 administration (278).

Recent studies have linked raised levels of circulating FGF23 to an increased risk of heart disease. Elevated levels of FGF23 were found to be independently associated with left ventricular (LV) hypertrophy (LHV) in human population studies (279, 280). FGF23 also induced hypertrophy in cultured mouse cardiomyocytes and injection of FGF23 into wild-type mice resulted in LVH (280). Interestingly, these effects were found to be klotho-independent. Increased serum FGF23 has also been linked with impaired vascular function (279), vascular calcification (281) and increased fat mass (282), indicating the importance of osteocyte-regulated proteins in health and disease (as discussed later in this review). These findings of altered cardiac and vascular function also identify these tissues as target organs of osteocyte-secreted FGF23.

As discussed previously, the osteocyte is known to express several other factors which play a role in the regulation of phosphate homeostasis. Dmp1, Phex and Mepe have well characterized effects on the skeletal system, as demonstrated by loss of function mutations and knockout mouse models (65, 91, 283, 284). These secreted proteins coordinately regulate Fgf23 expression and activity and, by doing so, influence tissues outside of the bone environment. Dmp1 is a negative regulator of Fgf23 expression, as osteocytes in the Dmp1-null mouse express high amounts of Fgf23 mRNA and protein, compared to wild-type mice, resulting in elevated serum Fgf23 levels (65, 266).

In addition to DMP1, PHEX is also known to inhibit FGF23. Given that the PHEX protein is an enzyme with endopeptidase activity it was suspected that FGF23 could be proteolytically cleaved by PHEX. However, evidence has so far suggested that FGF23 is not a direct substrate for PHEX but PHEX instead regulates Fgf23 at the transcriptional level (284). The exact mechanism(s) by which DMP1 and PHEX regulate FGF23 are yet to be determined, however, recent research has suggested that their inhibitory effects are mediated by FGF receptor (FGFR) signaling (285). Dmp1-null and Hyp mouse models show evidence of enhanced FGFR signaling compared to wild-type control mice and the elevated levels of Fgf23 present in the bone marrow stromal cells from both mouse models were abolished by inhibition of FGFR signaling.

The effects of MEPE on FGF23 and matrix mineralization are dependent on its cleavage to release an acidic serine aspartate-rich MEPE-associated motif (ASARM) from its C-terminus. This 19 amino acid ASARM peptide is known to be a potent inhibitor of mineralization in vivo (94, 286) and Mepe-null mice are characterized by progressive increases in trabecular bone mass and mineral apposition rate with aging (287). Again, the exact mechanisms by which MEPE and the MEPE-ASARM peptide regulate phosphate homeostasis are still to be elucidated, however the ASARM peptide is known to bind specifically to Phex in vitro (288) and inhibit Phex enzymatic activity (93), which results in up-regulation of Fgf23 expression. Phex can also regulate Fgf23 by binding to Mepe and preventing its proteolytic degradation and the release of the ASARM peptide (288, 289). In addition, the phosphorylated MEPE-ASARM peptide itself is a substrate for

Phex, with cleavage of ASARM by Phex neutralizing its activity and restoring mineralization (95).

The effects of MEPE can also extend beyond the bone and may operate independently of FGF23. MEPE is present in the serum of adult humans, suggesting an endocrine function for MEPE and the levels of MEPE correlate with serum phosphate levels (290). Administration of full-length MEPE into a rat model was shown to directly decrease uptake of phosphate in the proximal tubule and lead to renal phosphate wasting (291). Likewise, administration of recombinant MEPE to renal cell cultures inhibited phosphate uptake (291) and short-term infusion of MEPE in rats inhibited renal and intestinal phosphate absorption independently of serum FGF23, PTH or 1,25(OH)2D (292). In addition to the full-length protein, the MEPE-ASARM peptide has also been shown to inhibit phosphate uptake in vivo (293). MEPE-ASARM is elevated in the serum of Hyp mice and was found to accumulate in the proximal tubules in the Hyp mouse kidney (294). Overexpression of Mepe in a transgenic mouse model was also shown to decrease renal calcification and increase blood vessel formation in the kidney (295), although Mepe expression in this model was also increased in the proximal tubules so the effects cannot be definitively attributed to osteocyte-derived Mepe. It is clear that interactions between FGF23, PHEX, MEPE, DMP1 and other SIBLING family members are essential for regulating phosphate levels, however further studies are required to fully elucidate how the interplay between these proteins occurs at the genetic and molecular level.

Crosstalk between Osteocytes and Muscle cells

As discussed above, osteocytes can signal to tissues other than bone via secretion of FGF23. One of these targets of pathological FGF23 signaling is cardiac muscle, with increases in FGF23 resulting in LV hypertrophy. This therefore raises an interesting possibility; can osteocytes directly influence muscle mass by secreting muscle regulatory factors? Such interplay between bone and muscle seems highly plausible, especially as muscle and bone mass are tightly correlated throughout life. During organogenesis, muscle and bone develop in close association from common mesodermal precursors. During exercise and disuse, changes in muscle and bone mass are also closely coupled and with aging there is a concomitant loss of both bone and muscle mass. The dogma has been that the major effect of muscle on bone is mediated through mechanical loading of bone via muscle contraction during exercise and locomotion. However, it has recently been proposed that muscle, in addition to the skeleton, can act as an endocrine tissue to secrete factors into the circulation that target other tissues. Like osteocytes, muscle is also a source of circulating signaling factors, termed 'myokines' (296, 297). These myokines include factors such as insulin-like growth factor 1, Interleukins 6, 8, 15, Leukemia Inhibitory Factor, FGF21 and Follistatin-like 1 (298). Pedersen has proposed that skeletal muscle, the largest organ in the body, is an endocrine organ that exerts effects on other organs (297).

Previous studies have also demonstrated that skeletal muscle can secrete factors such as IGF-1, FGF2 and myostatin, which can influence osteoblast activity and differentiation (299, 300). However, whether direct signaling occurs between muscle and osteocytes was unknown. Recently, several studies have suggested that such signaling may indeed play an important physiological role in osteocytes in processes such as regulation of cell viability and response to mechanical stimuli. Jähn et al. found that differentiated C2C12 myotubes and primary muscle secrete factors that protect osteocytes against apoptosis due to glucocorticoids and that muscle contraction enhanced factor production. The muscle secreted factor inhibited glucocorticoid induced apoptosis through activation of the Wnt/β-catenin pathway (301). Lara-Castillo and coworkers found that differentiated myotubes and primary muscle produce a factor that synergizes with fluid flow shear stress to increase prostaglandin production by osteocytes. Again, this was likely mediated through synergistic activation of the Wnt/β-catenin pathway (302). Conversely, MLO-Y4 osteocyte-like cells and primary osteocytes secrete factors that induce muscle myogenesis in C2C12 pluripotent cells and these factors activate the Wnt/β-catenin pathway (303) (304). These findings suggest that this pathway not only plays a role in muscle development but also in muscle repair and myogenesis. Two factors produced by osteocytes, PGE_2 and Wnt3a were found to enhance myogenesis. These early studies provide supporting data that cross-talk occurs between muscle and bone and suggest other potential endocrine and/or paracrine functions for osteocytes but will require further in vivo validation.

Role of Osteocytes in Disease

The importance of osteocytes in maintaining bone health has been demonstrated by the diseases which occur in their absence. A 34% reduction in osteocyte density was observed in cancellous bone of the iliac crest in patients who presented with fracture when compared to healthy controls, suggesting that osteocytes are important in preserving the mechanical properties of bone. These patients did not show an increase in the number of empty lacunae however, suggesting that the decrease in osteocyte number was not due to osteocyte apoptosis but due to a lower rate of osteocyte formation (305). A decrease in eNOS and

nNOS expression was also observed in the femoral neck of hip fracture patients, in comparison to nonfracture controls (306, 307). This is likely to result in a reduction of NO, a powerful anabolic stimulator (as previously discussed), potentially leading to bone fragility.

Apoptosis of osteocytes is commonly observed in patients with steroid and alcohol-induced osteonecrosis of the femoral neck (221, 308). Osteocyte death is also observed after renal transplant, possibly due to the administration of glucocorticoids and other immunosuppressive therapeutics (309). There has recently been concern over the possibility that therapeutic use of bisphosphonates may result in osteonecrosis of the jaw. This has been linked to an increase in osteocyte apoptosis in humans (310). In support of this, in a rat experimental model, the administration of zoledronic acid resulted in osteocyte cell death (311). However, opposing results were observed in mice, with zoledronic acid having no effect on osteocyte viability in the mandible and femur (312).

Abnormalities in osteocyte morphology have been noted in many musculoskeletal diseases. In osteoporotic bone, disruption of the orientation of the lacunocanalicular system is observed, with decreased connectivity between neighboring osteocytes. Osteoarthritic bone is characterized by a decrease in osteocyte connectivity; however the orientation of the processes is unchanged. In contrast, osteocytes in osteomalacic bone display high connectivity but with disorientation of the dendrites (59). Such disruption of the lacunocanalicular system will affect osteocyte signaling, likely leading to alterations in the bone mechanical properties.

Hyperparathyroidism has a profound effect on the skeletal system. Excessive PTH secretion results in a loss of bone mass due to increased osteoclast activity (313). Rankl has been shown to be an important mediator of this increase in osteoclast activity, which could be attenuated in a mouse model of secondary hyperparathyroidism by treatment with the endogenous Rankl inhibitor Opg (314). With recent studies suggesting that osteocytes are a major source of Rankl and Opg in vivo (112, 113), it seems likely that the detrimental effects on bone health observed in hyperparathyroidism are mediated, at least partly, by the osteocyte. In contrast to the effects of continuous PTH, intermittent PTH treatment is known to be anabolic and may be partially mediated through down-regulation of osteocyte-expressed sclerostin (315–317). In humans it has been reported that patients administered PTH have reduced sclerostin levels (317) and serum sclerostin levels in postmenopausal women negatively correlate with PTH (318). Overexpression of *Sost* in a mouse model attenuated the anabolic effects of intermittent PTH administration (316). However, these animals still showed a response indicating that the anabolic effect cannot be fully attributed to sclerostin regulation. Sclerostin itself has become a very attractive target for therapeutics to increase bone mass. Monoclonal antibodies have been created to target sclerostin and their efficacy has been tested in both animal and clinical trials. Sclerostin antibody was shown to protect against bone loss due to hind-limb immobilization in a rat model (107) and it also increased bone formation and mineral density in cynomolgus monkey (319) and rat models (320, 321). In a recently published human trial, a single treatment with sclerostin antibody increased the expression of bone formation markers such as Alkaline phosphatase, decreased markers of bone resorption and led to gains of up to 5.3% and 2.8% in BMD in the vertebrae and hip respectively (106). Sclerostin antibody has also shown therapeutic promise in the treatment of fractures (105) and has alleviated the loss of bone observed after dexamethasone treatment of young mice (322). Off target effects appear to be minimal, with the only reported issue being a decrease in platelet count and increased megakaryocytes in sclerostin antibody-treated rats. These effects were species-specific, with no reactivity between the antibodies and human, or monkey, platelets or megakaryocytes (323).

As discussed previously, the effects of osteocyte signaling are not merely confined to the bone microenvironment but can involve other tissues such as the kidney and cardiovascular system. FGF23 expression is known to be regulated by DMP1, PHEX and MEPE, which are all predominantly expressed by osteocytes. Elevated levels of circulating FGF23 lead to increased renal phosphate excretion and subsequent hypophosphatemia and osteomalacia (270, 324). In chronic kidney disease (CKD) serum levels of FGF23 are increased, particularly in the later stages of the disease (325, 326). Treatment with FGF23 antibody restored serum phosphate levels in the Hyp mouse model and corrected the osteomalacic phenotype (327). Likewise, treatment of Dmp1-null mice with FGF23 antibody ameliorated the bone defects observed in these mice by increasing levels of circulating phosphate (38). In addition to effects on the musculoskeletal system, high serum FGF23 levels have been linked with atherosclerosis (328), ventricular hypertrophy (329, 330), increased risk of cardiovascular disease (331) and vascular calcification (281). Treatments targeting FGF23 would therefore appear to offer significant therapeutic potential and not merely for bone homeostasis.

Decreased bone mass is a common feature in inflammatory bowel disorders, particularly in children (332, 333) and it was recently reported that the bone loss observed in Crohn's disease was associated with increased osteocyte death and decreased bone remodeling (334).

The role of osteocytes in cancer-induced bone disease has, so far, been relatively unexplored. However, recently it was discovered that there is an increase in osteocyte apoptosis, and subsequently a decrease in viable osteocytes, in the bones of patients suffering from multiple myeloma (335). This resulted in an increase in osteoclast formation and osteolytic lesions. A recent study has also suggested that circulating levels of sclerostin are elevated in patients suffering from multiple myeloma and correlate with decreased bone mass (336). Other Wnt signaling inhibitors secreted by osteocytes, such as DKK1 and sFRP1, are also known to be involved in myeloma-induced bone destruction (337, 338). The importance of the osteocyte as a source of these factors in multiple myeloma, however, remains to be determined.

Summary and Perspective

It is becoming clear that the osteocyte is a far more active and functional cell than dogma has previously suggested. Recent research has further proved many of the suspected functions of osteocytes, such as mechanosensation and regulation of bone remodeling, in addition to suggesting novel functions, such as the ability to act as an endocrine organ. The potential of the osteocyte to influence cells and tissues far beyond the bone microenvironment, suggests that we may be only 'scratching the surface' of what the osteocyte is capable of. Signaling between osteocytes and the parathyroid, kidney, cardiac and skeletal muscle indicates the importance of the endocrine function of osteocytes and the impact that maintaining osteocyte viability may have on other tissues. It also raises the question of which other cells and tissues could be communicating with the osteocyte? The extensive connectivity of the lacunocanalicular system with the vasculature permits the transmission of signals to and from osteocytes, from tissues throughout the body and it is highly likely that other targets of osteocyte signaling will be discovered in the near future.

The osteocyte is gaining increasing interest as a target of therapeutics to increase bone mass. Clinical and animal trials have shown that using monoclonal antibodies directed against sclerostin has a potent bone anabolic effect. Future research may yield additional osteocyte secreted factors, which may have therapeutic potential in disorders such as sarcopenia and chronic kidney failure, given the cross-talk between these organs. Clearly, the osteocyte must now be considered not only in the context of its immediate microenvironment but also in its far-reaching endocrine effects beyond the bone. Therefore, adding to a growing list of functions of these fascinating, dynamic cells.

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