The Osteocyte: An Endocrine Cell . . . and More

Sarah L. Dallas, Matthew Prideaux, and Lynda F. Bonewald

Department of Oral and Craniofacial Sciences, School of Dentistry, University of Missouri-Kansas City, Kansas City, Missouri 64108

Few investigators think of bone as an endocrine gland, even after the discovery that osteocytes produce circulating fibroblast growth factor 23 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. (*Endocrine Reviews* 34: 658–690, 2013)

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 despite 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

Abbreviations: ASARM, acidic serine aspartate-rich MEPE-associated motif; DKK1, Dickkopf1-related protein 1; DMP1, Dentin matrix protein 1; ER-α, estrogen receptor-α; FAK, focal adhesion kinase; FGF, fibroblast growth factor; FGFR, FGF receptor; GFP, green fluorescent protein; kb, kilobase; LRP, low-density lipoprotein receptor-related protein; MEPE, matrix extracellular phosphoglycoprotein; NO, nitric oxide; NOS, NO synthase; 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; OPG, osteoprotegerin; PC1, polycystin 1; PGE$_2$, prostaglandin E$_2$; PHEX, phosphate-regulating gene with homologies to endopeptidases on the X chromosome; PKA, protein kinase A; PKD1, polycystic kidney disease 1; PTH1R, PTH receptor type 1; RANK, receptor activator of nuclear factor κ-B; RANKL, RANK ligand; SFRP1, secreted frizzled-related protein 1; TRAP, tartrate-resistant acid phosphatase.
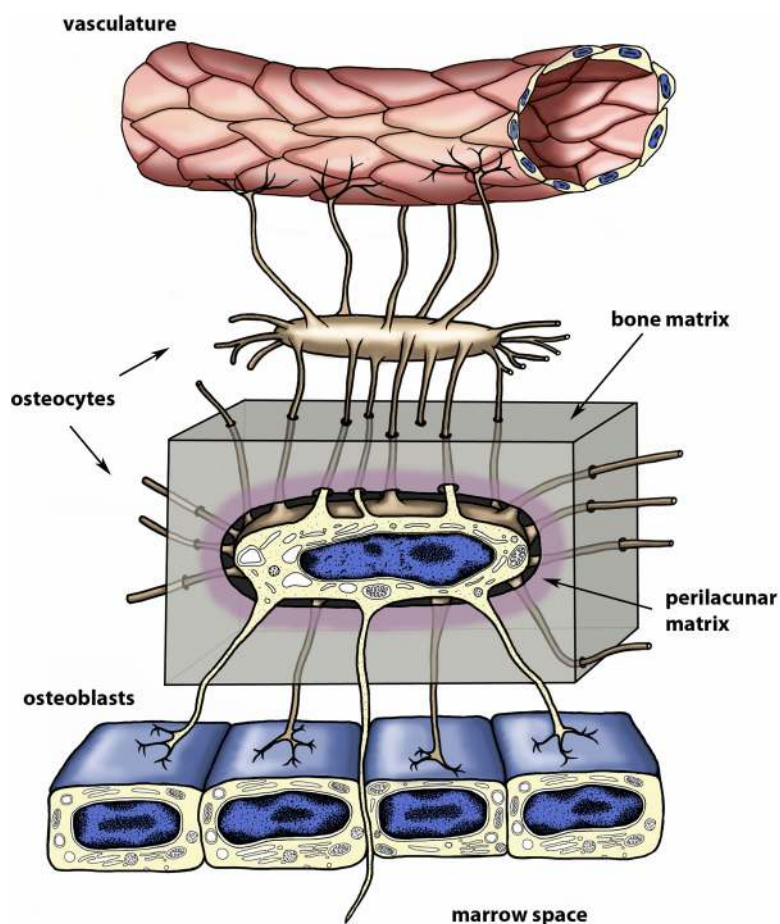
Figure 1.

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.

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 indispensable 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 os-

teoblast 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 PTH in bone. The osteocyte therefore appears to integrate hormonal and mechanical signals in the regulation of bone mass.

Because 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 because it is a highly vascular organ and the osteocyte lacunocanicular network is in intimate connection with this vascular supply (Figures 1 and 2). The kidney produces factors such as erythropoietin, and the liver produces IGF, 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, includ-
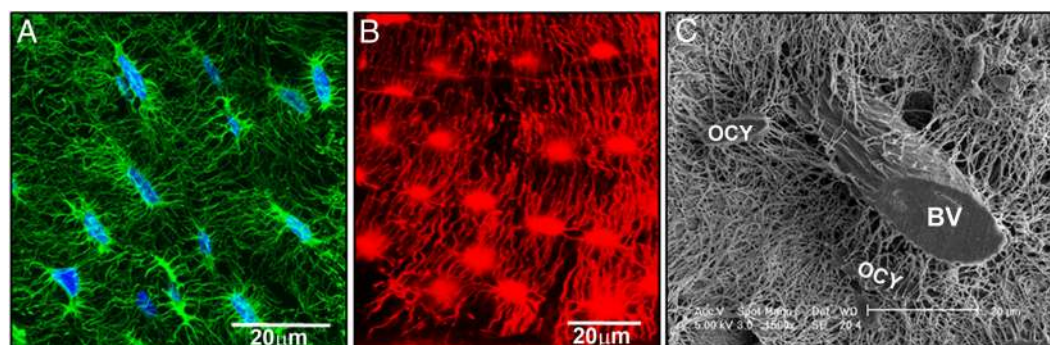
Figure 2.

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 minutes of iv injection, demonstrating the intimate connection of the canalicular fluid with the circulation (as modified from Ref. 231). C, Scanning electron micrograph of an acid-etched resin-casted mouse cortical bone specimen, revealing the 3-dimensional organization of the osteocyte lacunocanalicular system and its interaction with a blood vessel (BV). Note the 2 osteocyte lacunae (OCY) and extensive canaliculi, which are intimately connected to the surface of the blood vessel. Also note the extensive, interconnected canaliculi throughout the bone matrix.

ing 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.

II. 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 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 Refs. 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 Ref. 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 subpop-

ulation 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 Refs. 3, 14, and 15). Furthermore, the generation of transgenic mouse lines in which green fluorescent protein (GFP) variants are selectively expressed in osteoblasts and osteocytes (16–18) and the use of proteomic and gene array technologies (19–21) have 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 toward an osteocytic phenotype include down-regulation of type I colla-
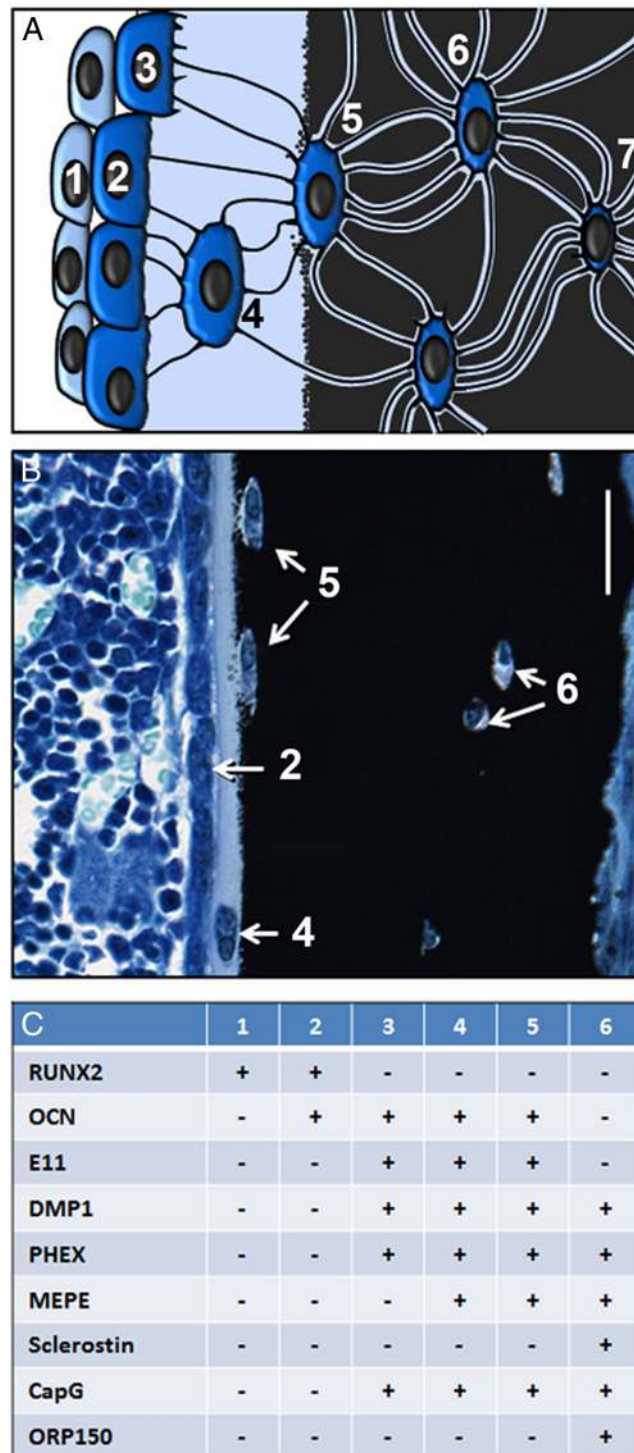
Figure 3.

Figure 3. Osteocyte differentiation. 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 and 7 = mature osteocytes. B, Tetrachrome-stained section of an adult mouse tibia illustrating several of the osteoblast-osteocyte transitional stages depicted schematically in panel 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 panels A and B. Runt-related transcription factor 2 (RUNX2) directs early osteoblast differentiation and is expressed in both preosteoblasts and osteoblasts. Osteocalcin (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.

gen and alkaline phosphatase and up-regulation 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 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, and destrin). The specific functions of these osteocyte marker proteins are discussed in more detail in *Section IV*.

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 because 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 3-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 3-dimensional 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 3 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 3-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 (42, 43) have reported extensively on the heterogeneity of surface osteoblasts in terms of their gene and protein expression profiles. 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 et al (44) 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). 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," ie, 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 the osteocytes exposed during bone resorption 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.

III. 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 regard 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 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 shape 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 toward the mineralizing front. This is followed by dendrites extending toward 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 (Figure 2, A and 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 1 side of the embedding cell rather than equally all around it (Figure 3B). We have previously proposed that the osteoid osteocyte (ie, 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 endoplasmic reticulum 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 that 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 et al (57). These authors proposed that the organized expression of tubulin, vimentin, and actin in the cell bodies and dendrites of osteocytes is 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 15–20 μm in diameter, and the cell processes pass through the bone matrix through narrow canals called canaliculi, approximately 250–300 nm in diameter (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 Refs. 15, 59, and 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 os-

teocyte 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 toward 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) (Figure 2, B 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 of less than 40 kDa can readily reach the lacunocanalicular system from the circulation but that molecules as large as 440 kDa cannot (67). The upper cutoff appears to be less than 70 kDa, and a filter of some sort appears to exist between the vascular system and the osteocyte lacunocanalicular system (68). As discussed in *Section VI*, 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 Refs. 14, 60, 68, and 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 Refs. 59 and 70). These may function as a molecular sieve that allows the rapid passage of smaller molecular mass molecules (eg, <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

osteocyte's 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.

IV. 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-moesin complexes; 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, small interfering RNA-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 ezrin-radixin-moesin 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 because 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 (23). 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 et al (33) showed no effect of overexpressing full-length DMP1 in bone under control of the 3.6-kilobase (kb) type I collagen promoter, although it did completely rescue the phenotype when overexpressed on a *Dmp1*-null background. In contrast, Bhatia et al (88) reported increased bone mineral density in transgenic mice that overexpressed DMP1 ubiquitously under control of the cytomegalovirus promoter. 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 the regulation of phosphate homeostasis. Interestingly, osteocytes within the osteomalacic 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 the regulation of mineralization and osteocyte maturation appear to be predominantly due to its role in the regulation of phosphate homeostasis because 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.

Mutations in *Phex*, such as in the Hyp mouse, or loss of PHEX expression leads to increased levels of FGF23 through an unknown mechanism and this leads to subsequent hypophosphatemia. Interestingly, FGF23 is not normally expressed at high levels in osteocytes in the healthy state, but its expression in osteocytes is dramatically up-regulated 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 that 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 disheveled, 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 Ref. 101). However, in the presence of sclerostin, Wnt-receptor interaction is inhibited, and β-catenin is phosphorylated by glycogen synthase kinase 3 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 ana-

bolic 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 have been performed to identify other osteocyte-expressed genes that may play important roles in bone (21). Increased expression of genes associated with neurons, such as neuropeptide Y, was reported. 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 that 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.

V. Tools for Studying Osteocytes

Although 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),

PTH receptor type 1 (PTH1R) (109), polycystin 1 (PC1) (110), Mef2C (111), and receptor activator of nuclear factor κ-B ligand (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 the usefulness of these techniques. 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 that 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

Table 1. 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; 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* (184, 212)
		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 (304)
		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 toward 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 8-kb-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 8-kb *Dmp1* promoter
		Temporally express early and late osteocyte markers such as E11, *Dmp1*, *Phex*, *Mepe*, *Sost*, and *Fgf23* (125)

Abbreviation: IFN-γ, interferon-γ.

cell lines have been described that differentiate toward 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.

VI. Osteocyte Mechanosensation and Transduction

Two of the earliest functions ascribed to osteocytes were 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 the distribution of osteocytes throughout the bone matrix and their complex interconnected network, early investigators hypothesized that they 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.

A. How osteocytes sense loading

When a bone is mechanically loaded, there are several possible stimuli that could be detected by the mechanosensory 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 are 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 3 types of stimuli because 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 physiological 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 5 Pa (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 relatively 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 cyclooxygenase 2 and the osteoprotegerin (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 (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 10-kb *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 $\alpha5\beta1$ 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 $\alpha5$ 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 2 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 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.

B. How loading affects osteocyte signaling

A true mechanotransducer must be able to convert the perceived mechanical strain signal into a biological output (ie, a cellular response). Although the precise biochemical pathways have yet to be fully unraveled, considerable progress has been made toward 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 protein kinase A (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, ie, 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 regard 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 milliseconds, 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 prostaglandin E_2 (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 P2X7 nucleotide receptor, an ATP-gated ion channel, may also play a role because 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 (155). Blockers of P2X7 receptors suppressed prostaglandin release, whereas agonists enhanced release in bone cells, suggesting that the P2X7 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 Ref. 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 hind limb unloading increases sclerostin expression (173). It has also been suggested that sclerostin must be down-regulated in order for the osteogenic response to anabolic loading to occur (104). Down-regulation of DKK1 and sclerostin 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 1 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 cross talk with the Wnt/β-catenin signaling pathway. Mice lacking the estrogen receptor-α (ER-α) isoform exhibit a defective response to loading (175). Lanyon and co-workers (176) suggest that the ER-α may play a role in shuttling β-catenin into the nucleus in response to mechanical strain in osteoblasts. 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 up-regulated in response to me-

chanical 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 hind limb 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 up-regulated 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 whereas 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 because 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.

VII. 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 that can integrate both hormonal and mechanical signals to regulate bone mass. This type of mechanism makes sense because 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 that 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 se-

cretion 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 NO synthase (NOS) is believed to be the main source of NO from osteocytes (183), although the inducible form of NOS has been detected in osteocytes after mechanical loading (153, 185). Neural NOS was also detected in human osteocytes (186). NO released by osteocytes in vivo has been shown to be important for bone formation during reloading (153), 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 Ref. 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 *Section VI*, 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 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 SFPR1 expression in vivo results in increased bone mass and mineral density and in vitro enhances osteoblast proliferation and differentiation into osteocytes (197). Peak SFRP1 expression is observed in the immature osteocyte and was decreased in the mature cell, indicating a role for SFRP1 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 (116), as few as 50 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 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, 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 (113) and Nakashima et al (112) 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) that 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-dimensional 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,
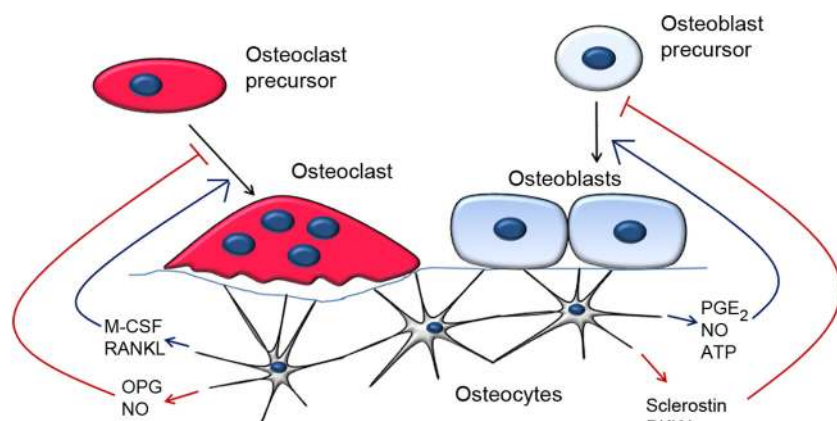
Figure 4.

Figure 4. Osteocyte regulation of bone remodeling. Osteocytes express RANKL and macrophage-colony stimulating factor (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. PGE$_2$, 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.

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.

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 NOS 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 PTH 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 because these anabolic effects have been shown to be due, at least partly, to down-regulation 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 increases bone formation. This phenotype is dependent on increased Wnt signaling (214). Interestingly, increased osteoclast activity and bone turnover were also observed in

VIII. 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 os-

teocyte and its processes, thereby inducing the cell to send signals of osteoclast recruitment to initiate bone removal and repair. Microdamage and bone fatigue are associated with loss of osteocyte integrity (216). Verbogt et al (217) 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, BCL-2-associated X protein, 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. 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, TNF-α and 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 et al (208) showed that apoptotic bodies released by MLO-Y4 cells and primary osteocytes, but not osteoblasts, supported osteoclast formation. Other groups have shown that serum-starved MLO-Y4 cells secrete soluble RANKL (226) and that damaged MLO-Y4 cell networks embedded in 3-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 re-

ceptor modulators, bisphosphonates, calcitonin, CD40 ligand, and calbindin-D28k (for review, see Ref. 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, OPG also prevents glucocorticoid-induced apoptosis (236). Substrate stretching has also been shown to protect osteocytes from glucocorticoid-induced apoptosis, potentially through activation of FAK, ERK, and the extracellular matrix-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 because 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.

Although 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 TNF-α and that the same caspase pathways were not activated (244). 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 American Society for Bone and Mineral Research 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.

IX. 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 lacunocanicular system is estimated to be several orders of magnitude 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 canicular 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.

Although 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 et al (248) as "osteocytic osteolysis." This concept, although 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), uremic osteodystrophy (250, 251), and in response to 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 days (255), hibernating ground squirrels (256), snakes (257), and 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 (260) had described tartrate-resistant acid phosphatase (TRAP) activity in osteocytes in 1969, which was criticized as potentially being a diffusion artifact from osteoclasts. But this observation was later validated by Nakano et al (261) using in situ hybridization for *Trap* gene expression. 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, Zambonin-Zallone et al (263) also reported tetracycline labeling in osteocyte lacunae in egg-laying hens. Although there were numerous reports of osteocytic osteolysis, there had been few reports of osteocytes replacing their perilacunar matrix.

Using scanning electron microscopy, 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 PTH1R. 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 the osteocyte can not only remove its mineralized matrix but also replace this matrix. We propose that the term "osteocytic osteolysis" be re-
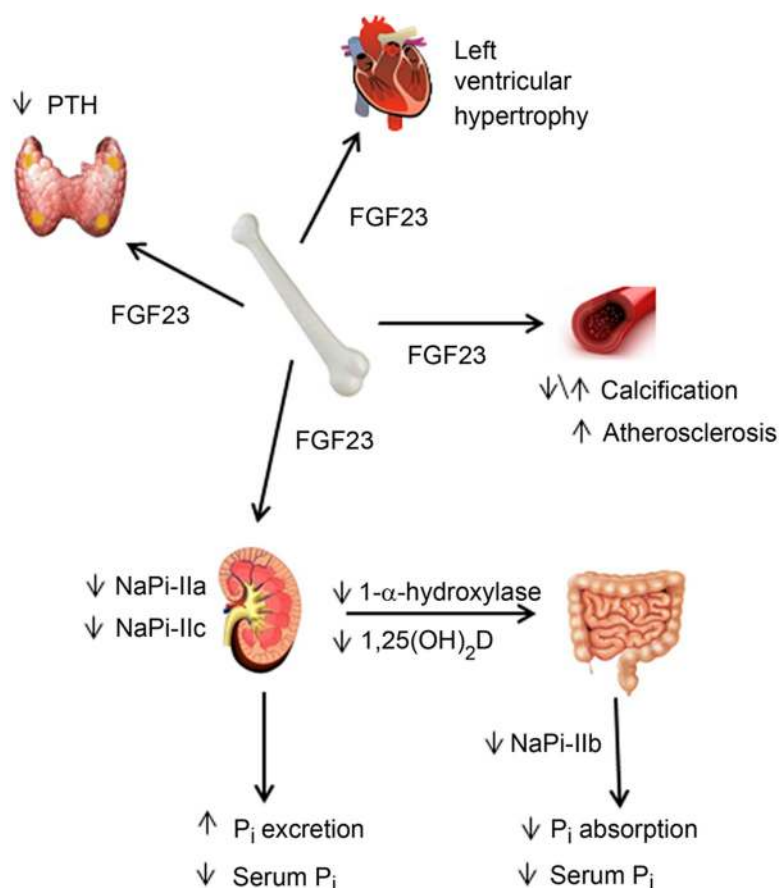
Figure 5.

Figure 5. Endocrine signaling by osteocytes. FGF23 secreted by the osteocyte regulates serum phosphate (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 left ventricular 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.

served 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.

X. 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 that permeates the bone matrix (Figure 2, B 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 *Section III*). Such connectivity between the canalicular fluid and vasculature allows osteocytes to be exposed to circulating hormones from distant tissues, but it 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 FGF family of proteins that binds to FGF receptors (FGFRs). This binding is dependent on the presence of Klotho. It has been shown that Klotho can convert the canonical FGFR, 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 that are of particular interest here.

One of the most important targets of FGF23 is the kidney (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-hydroxyvitamin D to the active vitamin D metabolite, 1,25-dihydroxyvitamin D [1,25(OH)$_2$D] (265). This reduction in 1,25(OH)$_2$D 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 because 1,25(OH)$_2$D 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)$_2$D, 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 days 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. In addition, 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 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 hypertrophy in human population studies (279, 280). FGF23 also induced hypertrophy in cultured mouse cardiomyocytes, and injection of FGF23 into wild-type mice resulted in left ventricular hypertrophy (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 in *Section XII*). 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 that 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 because 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 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.

XI. 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 left ventricular 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 because 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 IGF-I, IL-6, IL-8, IL-15, leukemia inhibitory factor, FGF21, and follistatin-like 1 (298). Pedersen (297) has proposed that skeletal muscle, the largest organ in the body, is an endocrine organ that exerts effects on other organs.

Previous studies have also demonstrated that skeletal muscle can secrete factors such as IGF-I, 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 (301) 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. Lara et al (302) 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). These findings suggest that this pathway plays a role not only in muscle development but also in muscle repair and myogenesis. Two factors produced by osteocytes, PGE_2 and Wnt3a, were found to enhance myogen-

esis. 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 they will require further in vivo validation.

XII. Role of Osteocytes in Disease

The importance of osteocytes in maintaining bone health has been demonstrated by the diseases that 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 was due to a lower rate of osteocyte formation (305). A decrease in endothelial NOS and neural NOS 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 downregulation 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 bone mineral density 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 in *Section X*, the effects of osteocyte signaling are not merely confined to the bone microenvironment but can involve other tissues such as the kidney and the 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, 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, it was discovered recently 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 (86, 164). The importance of the osteocyte as a source of these factors in multiple myeloma, however, remains to be determined.

XIII. 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 only be "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 lacuno-canalicular 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.

Acknowledgments

References

1. **Beno T, Yoon YJ, Cowin SC, Fritton SP.** Estimation of bone permeability using accurate microstructural measurements. *J Biomech*. 2006;39(13):2378–2387.
2. **Franz-Odendaal TA, Hall BK, Witten PE.** Buried alive: how osteoblasts become osteocytes. *Dev Dyn*. 2006; 235(1):176–190.
3. **Bonewald LF.** The amazing osteocyte. *J Bone Miner Res*. 2011;26(2):229–238.
4. **Dallas SL, Bonewald LF.** Dynamics of the transition from osteoblast to osteocyte. *Ann NY Acad Sci*. 2010;1192(1): 437–443.
5. **Manolagas SC.** Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocr Rev*. 2000;21(2): 115–137.
6. **Jilka RL, Weinstein RS, Bellido T, Parfitt AM, Manolagas SC.** Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines. *J Bone Miner Res*. 1998;13(5):793–802.

7. **Holtrop ME.** Light and electron microscopical structure of bone forming cells. In: Hall BK, ed. *Bone. Vol. 1: The Osteoblast and Osteocyte.* Caldwell, NJ: The Telford Press; 1990:1–40.

8. **Nefussi JR, Sautier JM, Nicolas V, Forest N.** How osteoblasts become osteocytes: a decreasing matrix forming process. *J Biol Buccale.* 1991;19(1):75–82.

9. **Nijweide PJ, van der Plas A, Scherft JP.** Biochemical and histological studies on various bone cell preparations. *Calcif Tissue Int.* 1981;33(5):529–540.

10. **Palumbo C.** A three-dimensional ultrastructural study of osteoid-osteocytes in the tibia of chick embryos. *Cell Tissue Res.* 1986;246(1):125–131.

11. **Palumbo C, Palazzini S, Zaffe D, Marotti G.** Osteocyte differentiation in the tibia of newborn rabbit: an ultrastructural study of the formation of cytoplasmic processes. *Acta Anat (Basel).* 1990;137(4):350–358.

12. **Zhao W, Byrne MH, Wang Y, Krane SM.** Osteocyte and osteoblast apoptosis and excessive bone deposition accompany failure of collagenase cleavage of collagen. *J Clin Invest.* 2000;106(8):941–949.

13. **Holmbeck K, Bianco P, Pidoux I, et al.** The metalloproteinase MT1-MMP is required for normal development and maintenance of osteocyte processes in bone. *J Cell Sci.* 2005;118:147–156.

14. **Bonewald LF, Johnson ML.** Osteocytes, mechanosensing and Wnt signaling. *Bone.* 2008;42(4):606–615.

15. **Bonewald LF.** Osteocytes as dynamic multifunctional cells. *Ann NY Acad Sci.* 2007;1116:281–290.

16. **Kalajzic I, Braut A, Guo D, et al.** Dentin matrix protein 1 expression during osteoblastic differentiation, generation of an osteocyte GFP-transgene. *Bone.* 2004;35(1):74–82.

17. **Kalajzic I, Kalajzic Z, Kaliterna M, et al.** Use of type I collagen green fluorescent protein transgenes to identify subpopulations of cells at different stages of the osteoblast lineage. *J Bone Miner Res.* 2002;17(1):15–25.

18. **Kalajzic Z, Liu P, Kalajzic I, et al.** Directing the expression of a green fluorescent protein transgene in differentiated osteoblasts: comparison between rat type I collagen and rat osteocalcin promoters. *Bone.* 2002;31(6):654–660.

19. **Billiard J, Moran RA, Whitley MZ, et al.** Transcriptional profiling of human osteoblast differentiation. *J Cell Biochem.* 2003;89(2):389–400.

20. **Guo D, Keightley A, Guthrie J, Veno PA, Harris SE, Bonewald LF.** Identification of osteocyte-selective proteins. *Proteomics.* 2010;10(20):3688–3698.

21. **Paic F, Igwe JC, Nori R, et al.** Identification of differentially expressed genes between osteoblasts and osteocytes. *Bone.* 2009;45:682–692.

22. **Schulze E, Witt M, Kasper M, Lowik CW, Funk RH.** Immunohistochemical investigations on the differentiation marker protein E11 in rat calvaria, calvaria cell culture and the osteoblastic cell line ROS 17/2.8. *Histochem Cell Biol.* 1999;111(1):61–69.

23. **Toyosawa S, Shintani S, Fujiwara T, et al.** Dentin matrix protein 1 is predominantly expressed in chicken and rat osteocytes but not in osteoblasts. *J Bone Miner Res.* 2001; 16(11):2017–2026.

24. **Ubaidus S, Li M, Sultana S, et al.** FGF23 is mainly synthesized by osteocytes in the regularly distributed osteocytic lacunar canalicular system established after physiological bone remodeling. *J Electron Microsc (Tokyo).* 2009;58(6): 381–392.

25. **Igarashi M, Kamiya N, Ito K, Takagi M.** In situ localization and in vitro expression of osteoblast/osteocyte factor 45 mRNA during bone cell differentiation. *Histochem J.* 2002;34(5):255–263.

26. **Winkler DG, Sutherland MK, Geoghegan JC, et al.** Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *EMBO J.* 2003;22(23):6267–6276.

27. **Guo D, Keightley JA, Barragan L, Zhao J, Guthrie J, Bonewald LF.** Identification of proteins involved in cytoskeletal rearrangement, anti-hypoxia and membrane channels in osteocytes over osteoblasts. *J Bone Miner Res.* 2006; 21(suppl 1):S168.

28. **Yang W, Harris MA, Heinrich JG, Guo D, Bonewald LF, Harris SE.** Gene expression signatures of a fibroblastoid preosteoblast and cuboidal osteoblast cell model compared to the MLO-Y4 osteocyte cell model. *Bone.* 2009;44(1): 32–45.

29. **Hughes DE, Salter DM, Simpson R.** CD44 expression in human bone: a novel marker of osteocytic differentiation. *J Bone Miner Res.* 1994;9(1):39–44.

30. **Igwe JC, Jiang X, Paic F, et al.** Neuropeptide Y is expressed by osteocytes and can inhibit osteoblastic activity. *J Cell Biochem.* 2009;108(3):621–630.

31. **Wetterwald A, Hoffstetter W, Cecchini MG, et al.** Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. *Bone.* 1996; 18(2):125–132.

32. **Zhang K, Barragan-Adjemian C, Ye L, et al.** E11/gp38 selective expression in osteocytes: regulation by mechanical strain and role in dendrite elongation. *Mol Cell Biol.* 2006;26(12):4539–4552.

33. **Lu Y, Yuan B, Qin C, et al.** The biological function of DMP-1 in osteocyte maturation is mediated by its 57-kDa C-terminal fragment. *J Bone Miner Res.* 2011;26(2):331–340.

34. **Atkins GJ, Rowe PS, Lim HP, et al.** Sclerostin is a locally acting regulator of late-osteoblast/preosteocyte differentiation and regulates mineralization through a MEPE-ASARM-dependent mechanism. *J Bone Miner Res.* 2011; 26(7):1425–1436.

35. **Hirao M, Hashimoto J, Yamasaki N, et al.** Oxygen tension is an important mediator of the transformation of osteoblasts to osteocytes. *J Bone Miner Metab.* 2007;25(5): 266–276.

36. **Prideaux M, Loveridge N, Pitsillides AA, Farquharson C.** Extracellular matrix mineralization promotes E11/gp38 glycoprotein expression and drives osteocytic differentiation. *PLoS One.* 2012;7(5):e36786.

37. **Irie K, Ejiri S, Sakakura Y, Shibui T, Yajima T.** Matrix mineralization as a trigger for osteocyte maturation. *J Histochem Cytochem.* 2008;56(6):561–567.

38. **Zhang R, Lu Y, Ye L, et al.** Unique roles of phosphorus in endochondral bone formation and osteocyte maturation. *J Bone Miner Res.* 2011;26(5):1047–1056.

39. **Marotti G.** The structure of bone tissues and the cellular control of their deposition. *Ital J Anat Embryol.* 1996; 101(4):25–79.

40. **Dallas SL, Veno PA, Bonewald LF, Rowe DW, Kalajzic I.** Dynamic imaging of fluorescently tagged osteoblast and

osteocyte populations integrates mineralization dynamics with osteoblast to osteocyte transition. *J Bone Miner Res*. 2007;22(suppl 1):S13.

41. **Veno PA, Lu Y, Kamel S, Feng J, Bonewald LF, Dallas SL.** A membrane targeted GFP selectively expressed in osteocytes reveals cell and membrane dynamics in living osteocytes. *J Bone Miner Res*. 2010;25(suppl 1):S56.

42. **Candeliere GA, Liu F, Aubin JE.** Individual osteoblasts in the developing calvaria express different gene repertoires. *Bone*. 2001;28(4):351–361.

43. **Liu F, Malaval L, Aubin JE.** The mature osteoblast phenotype is characterized by extensive plasticity. *Exp Cell Res*. 1997;232(1):97–105.

44. **Imai S, Heino TJ, Hienola A, et al.** Osteocyte-derived HB-GAM (pleiotrophin) is associated with bone formation and mechanical loading. *Bone*. 2009;44(5):785–794.

45. **Suzuki R, Domon T, Wakita M.** Some osteocytes released from their lacunae are embedded again in the bone and not engulfed by osteoclasts during bone remodeling. *Anat Embryol (Berl)*. 2000;202(2):119–128.

46. **van der Plas A, Aarden EM, Feijen JH, et al.** Characteristics and properties of osteocytes in culture. *J Bone Miner Res*. 1994;9(11):1697–1704.

47. **Suzuki R, Domon T, Wakita M, Akisaka T.** The reaction of osteoclasts when releasing osteocytes from osteocytic lacunae in the bone during bone modeling. *Tissue Cell*. 2003;35(3):189–197.

48. **Bronckers AL, Goei W, Luo G, et al.** DNA fragmentation during bone formation in neonatal rodents assessed by transferase-mediated end labeling. *J Bone Miner Res*. 1996;11(9):1281–1291.

49. **Elmardi AS, Katchburian MV, Katchburian E.** Electron microscopy of developing calvaria reveals images that suggest that osteoclasts engulf and destroy osteocytes during bone resorption. *Calcif Tissue Int*. 1990;46(4):239–245.

50. **Lu Y, Xie Y, Zhang S, Dusevich V, Bonewald LF, Feng JQ.** DMP1-targeted Cre expression in odontoblasts and osteocytes. *J Dent Res*. 2007;86(4):320–325.

51. **Tatsumi S, Ishii K, Amizuka N, et al.** Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab*. 2007;5(6):464–475.

52. **Palumbo C, Ferretti M, Marotti G.** Osteocyte dendrogenesis in static and dynamic bone formation: an ultrastructural study. *Anat Rec A Discov Mol Cell Evol Biol*. 2004; 278(1):474–480.

53. **Palumbo C, Palazzini S, Marotti G.** Morphological study of intercellular junctions during osteocyte differentiation. *Bone*. 1990;11(6):401–406.

54. **Barragan-Adjemian C, Nicolella D, Dusevich V, Dallas MR, Eick JD, Bonewald LF.** Mechanism by which MLO-A5 late osteoblasts/early osteocytes mineralize in culture: similarities with mineralization of lamellar bone. *Calcif Tissue Int*. 2006;79(5):340–353.

55. **Dudley HR, Spiro D.** The fine structure of bone cells. *J Biophys Biochem Cytol*. 1961;11:627–649.

56. **Gu G, Nars M, Hentunen TA, Metsikko K, Vaananen HK.** Isolated primary osteocytes express functional gap junctions in vitro. *Cell Tissue Res*. 2006;323(2):263–271.

57. **Kamioka H, Sugawara Y, Honjo T, Yamashiro T, Takano-Yamamoto T.** Terminal differentiation of osteoblasts to osteocytes is accompanied by dramatic changes in the dis-

tribution of actin-binding proteins. *J Bone Miner Res*. 2004;19(3):471–478.

58. **Tanaka-Kamioka K, Kamioka H, Ris H, Lim SS.** Osteocyte shape is dependent on actin filaments and osteocyte processes are unique actin-rich projections. *J Bone Miner Res*. 1998;13(10):1555–1568.

59. **Knothe Tate ML, Adamson JR, Tami AE, Bauer TW.** The osteocyte. *Int J Biochem Cell Biol*. 2004;36(1):1–8.

60. **Bonewald LF.** Mechanosensation and transduction in osteocytes. *Bonekey Osteovision*. 2006;3(10):7–15.

61. **Doty SB.** Morphological evidence of gap junctions between bone cells. *Calcif Tissue Int*. 1981;33(5):509–512.

62. **Bennett MV, Goodenough DA.** Gap junctions, electrotonic coupling, and intercellular communication. *Neurosci Res Program Bull*. 1978;16(3):1–486.

63. **Kamioka H, Honjo T, Takano-Yamamoto T.** A three-dimensional distribution of osteocyte processes revealed by the combination of confocal laser scanning microscopy and differential interference contrast microscopy. *Bone*. 2001;28(2):145–149.

64. **Veno PA, Nicolella DP, Kalajzic I, Rowe DW, Bonewald LF, Dallas SL.** Dynamic imaging in living calvaria reveals the motile properties of osteoblasts and osteocytes and suggests heterogeneity of osteoblasts in bone. *J Bone Miner Res*. 2007;22(suppl 1):S13.

65. **Feng JQ, Ward LM, Liu S, et al.** Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat Genet*. 2006;38(11):1310–1315.

66. **Knothe Tate ML, Niederer P, Knothe U.** In vivo tracer transport through the lacunocanalicular system of rat bone in an environment devoid of mechanical loading. *Bone*. 1998;22(2):107–117.

67. **Wang L, Ciani C, Doty SB, Fritton SP.** Delineating bone's interstitial fluid pathway in vivo. *Bone*. 2004;34(3):499–509.

68. **Fritton SP, Weinbaum S.** Fluid and solute transport in bone: flow-induced mechanotransduction. *Annu Rev Fluid Mech*. 2009;41:347–374.

69. **Weinbaum S, Duan Y, Thi MM, You L.** An integrative review of mechanotransduction in endothelial, epithelial (renal) and dendritic cells (osteocytes). *Cell Mol Bioeng*. 2011;4(4):510–537.

70. **Knothe Tate ML.** "Whither flows the fluid in bone?" An osteocyte's perspective. *J Biomech*. 2003;36(10):1409–1424.

71. **Belanger LF.** Osteocytic osteolysis. *Calcif Tissue Res*. 1969;4(1):1–12.

72. **Belanger LF.** Osteocytic osteolysis in a cretaceous reptile. *Rev Can Biol*. 1977;36(1):71–73.

73. **Doty SB, Nunez EA.** Activation of osteoclasts and the repopulation of bone surfaces following hibernation in the bat, *Myotis lucifugus*. *Anat Rec*. 1985;213(4):481–495.

74. **Steinberg B, Singh IJ, Mitchell OG.** The effects of cold-stress. Hibernation, and prolonged inactivity on bone dynamics in the golden hamster, *Mesocricetus auratus*. *J Morphol*. 1981;167(1):43–51.

75. **Lane NE, Yao W, Balooch M, et al.** Glucocorticoid-treated mice have localized changes in trabecular bone material properties and osteocyte lacunar size that are not observed

in placebo-treated or estrogen-deficient mice. *J Bone Miner Res*. 2006;21(3):466–476.

76. **Okada S, Yoshida S, Ashrafi SH, Schraufnagel DE.** The canalicular structure of compact bone in the rat at different ages. *Microsc Microanal*. 2002;8(2):104–115.

77. **Hantusch B, Kalt R, Krieger S, Puri C, Kerjaschki D.** Sp1/Sp3 and DNA-methylation contribute to basal transcriptional activation of human podoplanin in MG63 versus Saos-2 osteoblastic cells. *BMC Mol Biol*. 2007;8:20.

78. **Kunita A, Kashima TG, Ohazama A, Grigoriadis AE, Fukayama M.** Podoplanin is regulated by AP-1 and promotes platelet aggregation and cell migration in osteosarcoma. *Am J Pathol*. 2011;179(2):1041–1049.

79. **Martin-Villar E, Yurrita MM, Fernandez-Munoz B, Quintanilla M, Renart J.** Regulation of podoplanin/PA2.26 antigen expression in tumour cells. Involvement of calpain-mediated proteolysis. *Int J Biochem Cell Biol*. 2009;41(6):1421–1429.

80. **Ohizumi I, Harada N, Taniguchi K, et al.** Association of CD44 with OTS-8 in tumor vascular endothelial cells. *Biochim Biophys Acta*. 2000;1497(2):197–203.

81. **Sprague LW, Heinzman U, Atkinson MJ.** Phenotypic changes following over-expression of sense or antisense E11 cDNA in ROS 17/2.8 cells. *J Bone Miner Res*. 1996;11(suppl 1):S132.

82. **Scholl FG, Gamallo C, Vilaró S, Quintanilla M.** Identification of PA2.26 antigen as a novel cell-surface mucin-type glycoprotein that induces plasma membrane extensions and increased motility in keratinocytes. *J Cell Sci*. 1999;112:4601–4613.

83. **Martin-Villar E, Megias D, Castel S, Yurrita MM, Vilaro S, Quintanilla M.** Podoplanin binds ERM proteins to activate RhoA and promote epithelial-mesenchymal transition. *J Cell Sci*. 2006;119:4541–4553.

84. **Schacht V, Ramirez MI, Hong YK, et al.** T1α/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *EMBO J*. 2003;22(14):3546–3556.

85. **Feng JQ, Zhang J, Dallas SL, et al.** Dentin matrix protein 1, a target molecule for Cbfa1 in bone, is a unique bone marker gene. *J Bone Miner Res*. 2002;17(10):1822–1831.

86. **Heath DJ, Chantry AD, Buckle CH, et al.** Inhibiting Dickkopf-1 (Dkk1) removes suppression of bone formation and prevents the development of osteolytic bone disease in multiple myeloma. *J Bone Miner Res*. 2009;24(3):425–436.

87. **Feng JQ, Huang H, Lu Y, et al.** The Dentin matrix protein 1 (Dmp1) is specifically expressed in mineralized, but not soft, tissues during development. *J Dent Res*. 2003;82(10):776–780.

88. **Bhatia A, Albazzaz M, Espinoza Orias AA, et al.** Overexpression of DMP1 accelerates mineralization and alters cortical bone biomechanical properties in vivo. *J Mech Behav Biomed Mater*. 2012;5(1):1–8.

89. **Westbroek I, De Rooij KE, Nijweide PJ.** Osteocyte-specific monoclonal antibody MAb OB7.3 is directed against Phex protein. *J Bone Miner Res*. 2002;17(5):845–853.

90. A gene (PEX) with homologies to endopeptidases is mutated in patients with X-linked hypophosphatemic rickets. The HYP Consortium. *Nat Genet*. 1995;11(2):130–136.

91. **Strom TM, Francis F, Lorenz B, et al.** Pex gene deletions in Gy and Hyp mice provide mouse models for X-linked hypophosphatemia. *Hum Mol Genet*. 1997;6(2):165–171.

92. **Liu S, Tang W, Zhou J, Vierthaler L, Quarles LD.** Distinct roles for intrinsic osteocyte abnormalities and systemic factors in regulation of FGF23 and bone mineralization in Hyp mice. *Am J Physiol Endocrinol Metab*. 2007;293(6):E1636–E1644.

93. **Liu S, Rowe PS, Vierthaler L, Zhou J, Quarles LD.** Phosphorylated acidic serine-aspartate-rich MEPE-associated motif peptide from matrix extracellular phosphoglycoprotein inhibits phosphate regulating gene with homologies to endopeptidases on the X-chromosome enzyme activity. *J Endocrinol*. 2007;192(1):261–267.

94. **Rowe PS, Kumagai Y, Gutierrez G, et al.** MEPE has the properties of an osteoblastic phosphatonin and minhibin. *Bone*. 2004;34(2):303–319.

95. **Addison WN, Nakano Y, Loisel T, Crine P, McKee MD.** MEPE-ASARM peptides control extracellular matrix mineralization by binding to hydroxyapatite: an inhibition regulated by PHEX cleavage of ASARM. *J Bone Miner Res*. 2008;23(10):1638–1649.

96. **Boskey AL, Chiang P, Fermanis A, et al.** MEPE's diverse effects on mineralization. *Calcif Tissue Int*. 2010;86(1):42–46.

97. **Balemans W, Ebeling M, Patel N, et al.** Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). *Hum Mol Genet*. 2001;10(5):537–543.

98. **Brunkow ME, Gardner JC, Van Ness J, et al.** Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. *Am J Hum Genet*. 2001;68(3):577–589.

99. **Poole KE, van Bezooijen RL, Loveridge N, et al.** Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. *FASEB J*. 2005;19(13):1842–1844.

100. **Li X, Zhang Y, Kang H, et al.** Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *J Biol Chem*. 2005;280(20):19883–19887.

101. **Ott SM.** Sclerostin and Wnt signaling—the pathway to bone strength. *J Clin Endocrinol Metab*. 2005;90(12):6741–6743.

102. **Li X, Ominsky MS, Niu QT, et al.** Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. *J Bone Miner Res*. 2008;23(6):860–869.

103. **Robling AG, Niziolek PJ, Baldridge LA, et al.** Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. *J Biol Chem*. 2008;283(9):5866–5875.

104. **Tu X, Rhee Y, Condon KW, et al.** Sost downregulation and local Wnt signaling are required for the osteogenic response to mechanical loading. *Bone*. 2012;50(1):209–217.

105. **Ominsky MS, Li C, Li X, et al.** Inhibition of sclerostin by monoclonal antibody enhances bone healing and improves bone density and strength of nonfractured bones. *J Bone Miner Res*. 2011;26(5):1012–1021.

106. **Padhi D, Jang G, Stouch B, Fang L, Posvar E.** Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody. *J Bone Miner Res*. 2011;26(1):19–26.

107. **Tian X, Jee WS, Li X, Paszty C, Ke HZ.** Sclerostin antibody

increases bone mass by stimulating bone formation and inhibiting bone resorption in a hindlimb-immobilization rat model. *Bone*. 2011;48(2):197–201.

108. Kramer I, Halleux C, Keller H, et al. Osteocyte Wnt/β-catenin signaling is required for normal bone homeostasis. *Mol Cell Biol*. 2010;30(12):3071–3085.

109. Powell WF Jr, Barry KJ, Tulum I, et al. Targeted ablation of the PTH/PTHrP receptor in osteocytes impairs bone structure and homeostatic calcemic responses. *J Endocrinol*. 2011;209(1):21–32.

110. Xiao Z, Dallas M, Qiu N, et al. Conditional deletion of Pkd1 in osteocytes disrupts skeletal mechanosensing in mice. *FASEB J*. 2011;25(7):2418–2432.

111. Kramer I, Baertschi S, Halleux C, Keller H, Kneissel M. Mef2c deletion in osteocytes results in increased bone mass. *J Bone Miner Res*. 2012;27(2):360–373.

112. Nakashima T, Hayashi M, Fukunaga T, et al. Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nat Med*. 2011;17(10):1231–1234.

113. Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. *Nat Med*. 2011;17(10):1235–1241.

114. van der Plas A, Nijweide PJ. Isolation and purification of osteocytes. *J Bone Miner Res*. 1992;7(4):389–396.

115. Mikuni-Takagaki Y, Suzuki Y, Kawase T, Saito S. Distinct responses of different populations of bone cells to mechanical stress. *Endocrinology*. 1996;137(5):2028–2035.

116. Zhao S, Zhang YK, Harris S, Ahuja SS, Bonewald LF. MLO-Y4 osteocyte-like cells support osteoclast formation and activation. *J Bone Miner Res*. 2002;17(11):2068–2079.

117. Stern AR, Stern MM, Van Dyke ME, Jahn K, Prideaux M, Bonewald LF. Isolation and culture of primary osteocytes from the long bones of skeletally mature and aged mice. *Biotechniques*. 2012;52(6):361–373.

118. Bonewald LF. Establishment and characterization of an osteocyte-like cell line, MLO-Y4. *J Bone Miner Metab*. 1999;17(1):61–65.

119. Rosser J, Bonewald LF. Studying osteocyte function using the cell lines MLO-Y4 and MLO-A5. *Methods Mol Biol*. 2012;816:67–81.

120. Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF. Establishment of an osteocyte-like cell line, MLO-Y4. *J Bone Miner Res*. 1997;12(12):2014–2023.

121. Bellows CG, Aubin JE. Determination of numbers of osteoprogenitors present in isolated fetal rat calvaria cells in vitro. *Dev Biol*. 1989;133(1):8–13.

122. Aubin JE. Advances in the osteoblast lineage. *Biochem Cell Biol*. 1998;76(6):899–910.

123. Bodine PV, Vernon SK, Komm BS. Establishment and hormonal regulation of a conditionally transformed preosteocytic cell line from adult human bone. *Endocrinology*. 1996;137(11):4592–4604.

124. Kato Y, Boskey A, Spevak L, Dallas M, Hori M, Bonewald LF. Establishment of an osteoid preosteocyte-like cell MLO-A5 that spontaneously mineralizes in culture. *J Bone Miner Res*. 2001;16(9):1622–1633.

125. Woo SM, Rosser J, Dusevich V, Kalajzic I, Bonewald LF. Cell line IDG-SW3 replicates osteoblast-to-late-osteocyte differentiation in vitro and accelerates bone formation in vivo. *J Bone Miner Res*. 2011;26(11):2634–2646.

126. Wolff J. *Das Gesetz der Transformation der Knochen*. Berlin: A Hirschwald; 1892.

127. Weinbaum S, Cowin SC, Zeng Y. A model for the excitation of osteocytes by mechanical loading-induced bone fluid shear stresses. *J Biomech*. 1994;27(3):339–360.

128. Klein-Nulend J, van der Plas A, Semeins CM, et al. Sensitivity of osteocytes to biomechanical stress in vitro. *FASEB J*. 1995;9(5):441–445.

129. Klein-Nulend J, Semeins CM, Ajubi NE, Nijweide PJ, Burger EH. Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts—correlation with prostaglandin upregulation. *Biochem Biophys Res Commun*. 1995;217(2):640–648.

130. Price C, Zhou X, Li W, Wang L. Real-time measurement of solute transport within the lacunar-canalicular system of mechanically loaded bone: direct evidence for load-induced fluid flow. *J Bone Miner Res*. 2011;26(2):277–285.

131. Kamel MA, Picconi JL, Lara-Castillo N, Johnson ML. Activation of β-catenin signaling in MLO-Y4 osteocytic cells versus 2T3 osteoblastic cells by fluid flow shear stress and PGE(2): implications for the study of mechanosensation in bone. *Bone*. 2010;47(5):872–881.

132. Kalogeropoulos M, Varanasi SS, Olstad OK, et al. Zic1 transcription factor in bone: neural developmental protein regulates mechanotransduction in osteocytes. *FASEB J*. 2010;24(8):2893–2903.

133. Lu XL, Huo B, Chiang V, Guo XE. Osteocytic network is more responsive in calcium signaling than osteoblastic network under fluid flow. *J Bone Miner Res*. 2012;27(3):563–574.

134. Han Y, Cowin SC, Schaffler MB, Weinbaum S. Mechanotransduction and strain amplification in osteocyte cell processes. *Proc Natl Acad Sci USA*. 2004;101(47):16689–16694.

135. Adachi T, Aonuma Y, Tanaka M, Hojo M, Takano-Yamamoto T, Kamioka H. Calcium response in single osteocytes to locally applied mechanical stimulus: differences in cell process and cell body. *J Biomech*. 2009;42(12):1989–1995.

136. Nicolella DP, Feng JQ, Moravits DE, et al. Effects of nanomechanical bone tissue properties on bone tissue strain: implications for osteocyte mechanotransduction. *J Musculoskelet Neuronal Interact*. 2008;8(4):330–331.

137. Xiao Z, Zhang S, Mahlios J, et al. Cilia-like structures and polycystin-1 in osteoblasts/osteocytes and associated abnormalities in skeletogenesis and Runx2 expression. *J Biol Chem*. 2006;281(41):30884–30895.

138. Malone AM, Anderson CT, Tummala P, et al. Primary cilia mediate mechanosensing in bone cells by a calcium-independent mechanism. *Proc Natl Acad Sci USA*. 2007;104(33):13325–13330.

139. Burra S, Nicolella DP, Francis WL, et al. Dendritic processes of osteocytes are mechanotransducers that induce the opening of hemichannels. *Proc Natl Acad Sci USA*. 2010;107(31):13648–13653.

140. Kwon RY, Temiyasathit S, Tummala P, Quah CC, Jacobs CR. Primary cilium-dependent mechanosensing is mediated by adenylyl cyclase 6 and cyclic AMP in bone cells. *FASEB J*. 2010;24(8):2859–2868.

141. Hoey DA, Kelly DJ, Jacobs CR. A role for the primary cilium in paracrine signaling between mechanically stim-

ulated osteocytes and mesenchymal stem cells. *Biochem Biophys Res Commun.* 2011;412(1):182–187.

142. **Qiu N, Cao L, David V, Quarles LD, Xiao Z.** Kif3a deficiency reverses the skeletal abnormalities in Pkd1 deficient mice by restoring the balance between osteogenesis and adipogenesis. *PLoS One.* 2010;5(12):e15240.

143. **Salter DM, Robb JE, Wright MO.** Electrophysiological responses of human bone cells to mechanical stimulation: evidence for specific integrin function in mechanotransduction. *J Bone Miner Res.* 1997;12(7):1133–1141.

144. **You LD, Weinbaum S, Cowin SC, Schaffler MB.** Ultrastructure of the osteocyte process and its pericellular matrix. *Anat Rec A Discov Mol Cell Evol Biol.* 2004;278(2):505–513.

145. **Batra N, Burra S, Siller-Jackson AJ, et al.** Mechanical stress-activated integrin $\alpha 5\beta 1$ induces opening of connexin 43 hemichannels. *Proc Natl Acad Sci USA.* 2012;109(9):3359–3364.

146. **Nicolella DP, Moravits DE, Gale AM, Bonewald LF, Lankford J.** Osteocyte lacunae tissue strain in cortical bone. *J Biomech.* 2006;39(9):1735–1743.

147. **Bonivtch AR, Bonewald LF, Nicolella DP.** Tissue strain amplification at the osteocyte lacuna: a microstructural finite element analysis. *J Biomech.* 2007;40(10):2199–2206.

148. **Gu Y, Preston MR, El Haj AJ, Howl JD, Publicover SJ.** Three types of K(+) currents in murine osteocyte-like cells (MLO-Y4). *Bone.* 2001;28(1):29–37.

149. **Shao Y, Alicknavitch M, Farach-Carson MC.** Expression of voltage sensitive calcium channel (VSCC) L-type Cav1.2 (α1C) and T-type Cav3.2 (α1H) subunits during mouse bone development. *Dev Dyn.* 2005;234(1):54–62.

150. **Gu Y, Preston MR, Magnay J, El Haj AJ, Publicover SJ.** Hormonally-regulated expression of voltage-operated Ca(2+) channels in osteocytic (MLO-Y4) cells. *Biochem Biophys Res Commun.* 2001;282(2):536–542.

151. **Thompson WR, Majid AS, Czymmek KJ, et al.** Association of the $\alpha(2)\delta(1)$ subunit with Ca (v) 3.2 enhances membrane expression and regulates mechanically induced ATP release in MLO-Y4 osteocytes. *J Bone Miner Res.* 2011;26(9):2125–2139.

152. **Forwood MR.** Inducible cyclo-oxygenase (COX-2) mediates the induction of bone formation by mechanical loading in vivo. *J Bone Miner Res.* 1996;11(11):1688–1693.

153. **Watanuki M, Sakai A, Sakata T, et al.** Role of inducible nitric oxide synthase in skeletal adaptation to acute increases in mechanical loading. *J Bone Miner Res.* 2002;17(6):1015–1025.

154. **Kunnel JG, Igarashi K, Gilbert JL, Stern PH.** Bone anabolic responses to mechanical load in vitro involve COX-2 and constitutive NOS. *Connect Tissue Res.* 2004;45(1):40–49.

155. **Li J, Liu D, Ke HZ, Duncan RL, Turner CH.** The P2X7 nucleotide receptor mediates skeletal mechanotransduction. *J Biol Chem.* 2005;280(52):42952–42959.

156. **Wang N, Rumney RM, Yang L, et al.** The P2Y13 receptor regulates extracellular ATP metabolism and the osteogenic response to mechanical loading [published online ahead of print January 29, 2013]. *J Bone Miner Res.* doi:10.1002/jbmr.1877.

157. **Bakker AD, Soejima K, Klein-Nulend J, Burger EH.** The production of nitric oxide and prostaglandin E(2) by primary bone cells is shear stress dependent. *J Biomech.* 2001;34(5):671–677.

158. **Tan SD, Bakker AD, Semeins CM, Kuijpers-Jagtman AM, Klein-Nulend J.** Inhibition of osteocyte apoptosis by fluid flow is mediated by nitric oxide. *Biochem Biophys Res Commun.* 2008;369(4):1150–1154.

159. **Bakker AD, Silva VC, Krishnan R, et al.** Tumor necrosis factor α and interleukin-1β modulate calcium and nitric oxide signaling in mechanically stimulated osteocytes. *Arthritis Rheum.* 2009;60(11):3336–3345.

160. **Kamioka H, Miki Y, Sumitani K, et al.** Extracellular calcium causes the release of calcium from intracellular stores in chick osteocytes. *Biochem Biophys Res Commun.* 1995;212(2):692–696.

161. **Genetos DC, Geist DJ, Liu D, Donahue HJ, Duncan RL.** Fluid shear-induced ATP secretion mediates prostaglandin release in MC3T3–E1 osteoblasts. *J Bone Miner Res.* 2005;20(1):41–49.

162. **Cherian PP, Cheng B, Gu S, Sprague E, Bonewald LF, Jiang JX.** Effects of mechanical strain on the function of Gap junctions in osteocytes are mediated through the prostaglandin EP2 receptor. *J Biol Chem.* 2003;278(44):43146–43156.

163. **Cherian PP, Siller-Jackson AJ, Gu S, et al.** Mechanical strain opens connexin 43 hemichannels in osteocytes: a novel mechanism for the release of prostaglandin. *Mol Biol Cell.* 2005;16(7):3100–3106.

164. **Qiang YW, Chen Y, Brown N, et al.** Characterization of Wnt/β-catenin signalling in osteoclasts in multiple myeloma. *Br J Haematol.* 2010;148(5):726–738.

165. **Tang LY, Cullen DM, Yee JA, Jee WS, Kimmel DB.** Prostaglandin E2 increases the skeletal response to mechanical loading. *J Bone Miner Res.* 1997;12(2):276–282.

166. **Pead MJ, Lanyon LE.** Indomethacin modulation of load-related stimulation of new bone formation in vivo. *Calcif Tissue Int.* 1989;45(1):34–40.

167. **Grimston SK, Brodt MD, Silva MJ, Civitelli R.** Attenuated response to in vivo mechanical loading in mice with conditional osteoblast ablation of the connexin43 gene (Gja1). *J Bone Miner Res.* 2008;23(6):879–886.

168. **Zhang Y, Paul EM, Sathyendra V, et al.** Enhanced osteoclastic resorption and responsiveness to mechanical load in gap junction deficient bone. *PLoS One.* 2011;6(8):e23516.

169. **Loiselle AE, Jiang JX, Donahue HJ.** Gap junction and hemichannel functions in osteocytes. *Bone.* 2013;54(2):205–212.

170. **Xia X, Batra N, Shi Q, Bonewald LF, Sprague E, Jiang JX.** Prostaglandin promotion of osteocyte gap junction function through transcriptional regulation of connexin 43 by glycogen synthase kinase 3/β-catenin signaling. *Mol Cell Biol.* 2010;30(1):206–219.

171. **Kitase Y, Barragan L, Qing H, et al.** Mechanical induction of PGE2 in osteocytes blocks glucocorticoid-induced apoptosis through both the β-catenin and PKA pathways. *J Bone Miner Res.* 2010;25(12):2657–2668.

172. **Sawakami K, Robling AG, Ali M, et al.** The Wnt co-receptor LRP5 is essential for skeletal mechanotransduction but not for anabolic bone response to parathyroid hor-

mone treatment. *J Biol Chem.* 2006;281(33):23698–23711.

173. **Lin C, Jiang X, Dai Z, et al.** Sclerostin mediates bone response to mechanical unloading through antagonizing Wnt/β-catenin signaling. *J Bone Miner Res.* 2009;24(10):1651–1661.

174. **Javaheri B, Dallas M, Zhao H, Bonewald L, Johnson M.** b-Catenin haploinsufficiency in osteocytes abolishes the osteogenic effect of mechanical loading in vivo. *J Bone Miner Res.* 2011;26(suppl 1):S24.

175. **Lee K, Jessop H, Suswillo R, Zaman G, Lanyon L.** Endocrinology: bone adaptation requires oestrogen receptor-α. *Nature.* 2003;424(6947):389.

176. **Zaman G, Jessop HL, Muzylak M, et al.** Osteocytes use estrogen receptor α to respond to strain but their ERα content is regulated by estrogen. *J Bone Miner Res.* 2006; 21(8):1297–1306.

177. **Yang W, Lu Y, Kalajzic I, et al.** Dentin matrix protein 1 gene cis-regulation: use in osteocytes to characterize local responses to mechanical loading in vitro and in vivo. *J Biol Chem.* 2005;280(21):20680–20690.

178. **Gluhak-Heinrich J, Ye L, Bonewald LF, et al.** Mechanical loading stimulates dentin matrix protein 1 (DMP1) expression in osteocytes in vivo. *J Bone Miner Res.* 2003;18(5):807–817.

179. **Gluhak-Heinrich J, Pavlin D, Yang W, Macdougall M, Harris SE.** MEPE expression in osteocytes during orthodontic tooth movement. *Arch Oral Biol.* 2007;52(7):684–690.

180. **Vezeridis PS, Semeins CM, Chen Q, Klein-Nulend J.** Osteocytes subjected to pulsating fluid flow regulate osteoblast proliferation and differentiation. *Biochem Biophys Res Commun.* 2006;348(3):1082–1088.

181. **Heino TJ, Hentunen TA, Vaananen HK.** Conditioned medium from osteocytes stimulates the proliferation of bone marrow mesenchymal stem cells and their differentiation into osteoblasts. *Exp Cell Res.* 2004;294(2):458–468.

182. **Raheja LF, Genetos DC, Yellowley CE.** Hypoxic osteocytes recruit human MSCs through an OPN/CD44-mediated pathway. *Biochem Biophys Res Commun.* 2008; 366(4):1061–1066.

183. **Zaman G, Pitsillides AA, Rawlinson SC, et al.** Mechanical strain stimulates nitric oxide production by rapid activation of endothelial nitric oxide synthase in osteocytes. *J Bone Miner Res.* 1999;14(7):1123–1131.

184. **Papanicolaou SE, Phipps RJ, Fyhrie DP, Genetos DC.** Modulation of sclerostin expression by mechanical loading and bone morphogenetic proteins in osteogenic cells. *Biorheology.* 2009;46(5):389–399.

185. **Basso N, Heersche JN.** Effects of hind limb unloading and reloading on nitric oxide synthase expression and apoptosis of osteocytes and chondrocytes. *Bone.* 2006;39(4):807–814.

186. **Caballero-Alias AM, Loveridge N, Lyon A, Das-Gupta V, Pitsillides A, Reeve J.** NOS isoforms in adult human osteocytes: multiple pathways of NO regulation? *Calcif Tissue Int.* 2004;75(1):78–84.

187. **Blackwell KA, Raisz LG, Pilbeam CC.** Prostaglandins in bone: bad cop, good cop? *Trends Endocrinol Metab.* 2010; 21(5):294–301.

188. **Raisz LG, Fall PM, Gabbitas BY, McCarthy TL, Kream BE, Canalis E.** Effects of prostaglandin E2 on bone formation in cultured fetal rat calvariae: role of insulin-like growth factor-I. *Endocrinology.* 1993;133(4):1504–1510.

189. **Nagata T, Kaho K, Nishikawa S, Shinohara H, Wakano Y, Ishida H.** Effect of prostaglandin E2 on mineralization of bone nodules formed by fetal rat calvarial cells. *Calcif Tissue Int.* 1994;55(6):451–457.

190. **Raisz LG, Kream BE.** Regulation of bone formation (second of two parts). *N Engl J Med.* 1983;309(2):83–89.

191. **Jee WS, Ueno K, Deng YP, Woodbury DM.** The effects of prostaglandin E2 in growing rats: increased metaphyseal hard tissue and cortico-endosteal bone formation. *Calcif Tissue Int.* 1985;37(2):148–157.

192. **Gong Y, Slee RB, Fukai N, et al.** LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell.* 2001;107(4):513–523.

193. **Kato M, Patel MS, Levasseur R, et al.** Cbfa 1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J Cell Biol.* 2002;157:303–314.

194. **Zhang Y, Wang Y, Li X, et al.** The LRP5 high-bone-mass G171V mutation disrupts LRP5 interaction with Mesd. *Mol Cell Biol.* 2004;24(11):4677–4684.

195. **Li J, Sarosi I, Cattley RC, et al.** Dkk1-mediated inhibition of Wnt signaling in bone results in osteopenia. *Bone.* 2006; 39(4):754–766.

196. **Heiland GR, Zwerina K, Baum W, et al.** Neutralisation of Dkk-1 protects from systemic bone loss during inflammation and reduces sclerostin expression. *Ann Rheum Dis.* 2010;69(12):2152–2159.

197. **Bodine PV, Zhao W, Kharode YP, et al.** The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. *Mol Endocrinol.* 2004;18(5):1222–1237.

198. **Bodine PV, Billiard J, Moran RA, et al.** The Wnt antagonist secreted frizzled-related protein-1 controls osteoblast and osteocyte apoptosis. *J Cell Biochem.* 2005;96(6):1212–1230.

199. **Tanaka K, Yamaguchi Y, Hakeda Y.** Isolated chick osteocytes stimulate formation and bone-resorbing activity of osteoclast-like cells. *J Bone Miner Metab.* 1995;13:61–70.

200. **Kartsogiannis V, Zhou H, Horwood NJ, et al.** Localization of RANKL (receptor activator of NF κB ligand) mRNA and protein in skeletal and extraskeletal tissues. *Bone.* 1999;25(5):525–534.

201. **Silvestrini G, Ballanti P, Patacchioli F, et al.** Detection of osteoprotegerin (OPG) and its ligand (RANKL) mRNA and protein in femur and tibia of the rat. *J Mol Histol.* 2005;36(1–2):59–67.

202. **Kurata K, Heino TJ, Higaki H, Vaananen HK.** Bone marrow cell differentiation induced by mechanically damaged osteocytes in 3D gel-embedded culture. *J Bone Miner Res.* 2006;21(4):616–625.

203. **Kulkarni RN, Bakker AD, Everts V, Klein-Nulend J.** Inhibition of osteoclastogenesis by mechanically loaded osteocytes: involvement of MEPE. *Calcif Tissue Int.* 2010; 87(5):461–468.

204. **Ikeda T, Utsuyama M, Hirokawa K.** Expression profiles of receptor activator of nuclear factor κB ligand, receptor

activator of nuclear factor κB, and osteoprotegerin messenger RNA in aged and ovariectomized rat bones. *J Bone Miner Res.* 2001;16(8):1416–1425.

205. **Gu G, Mulari M, Peng Z, Hentunen TA, Vaananen HK.** Death of osteocytes turns off the inhibition of osteoclasts and triggers local bone resorption. *Biochem Biophys Res Commun.* 2005;335(4):1095–1101.

206. **Heino TJ, Kurata K, Higaki H, Vaananen HK.** Evidence for the role of osteocytes in the initiation of targeted remodeling. *Technol Health Care.* 2009;17(1):49–56.

207. **Yang J, Shah R, Robling AG, et al.** HMGB1 is a bone-active cytokine. *J Cell Physiol.* 2008;214(3):730–739.

208. **Kogianni G, Mann V, Noble BS.** Apoptotic bodies convey activity capable of initiating osteoclastogenesis and localized bone destruction. *J Bone Miner Res.* 2008;23(6):915–927.

209. **Tan SD, de Vries TJ, Kuijpers-Jagtman AM, Semeins CM, Everts V, Klein-Nulend J.** Osteocytes subjected to fluid flow inhibit osteoclast formation and bone resorption. *Bone.* 2007;41(5):745–751.

210. **Zhao Q, Brauer PR, Xiao L, McGuire MH, Yee JA.** Expression of parathyroid hormone-related peptide (PthrP) and its receptor (PTH1R) during the histogenesis of cartilage and bone in the chicken mandibular process. *J Anat.* 2002;201(2):137–151.

211. **Lupp A, Klenk C, Rocken C, Evert M, Mawrin C, Schulz S.** Immunohistochemical identification of the PTHR1 parathyroid hormone receptor in normal and neoplastic human tissues. *Eur J Endocrinol.* 2010;162(5):979–986.

212. **Bellido T, Ali AA, Gubrij I, et al.** Chronic elevation of parathyroid hormone in mice reduces expression of sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis. *Endocrinology.* 2005;146(11):4577–4583.

213. **Keller H, Kneissel M.** SOST is a target gene for PTH in bone. *Bone.* 2005;37(2):148–158.

214. **O'Brien CA, Plotkin LI, Galli C, et al.** Control of bone mass and remodeling by PTH receptor signaling in osteocytes. *PLoS One.* 2008;3(8):e2942.

215. **Qing H, Ardeshirpour L, Pajevic PD, et al.** Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation. *J Bone Miner Res.* 2012;27(5):1018–1029.

216. **Verborgt O, Gibson GJ, Schaffler MB.** Loss of osteocyte integrity in association with microdamage and bone remodeling after fatigue in vivo. *J Bone Miner Res.* 2000;15(1):60–67.

217. **Verborgt O, Tatton NA, Majeska RJ, Schaffler MB.** Spatial distribution of Bax and Bcl-2 in osteocytes after bone fatigue: complementary roles in bone remodeling regulation? *J Bone Miner Res.* 2002;17(5):907–914.

218. **Kennedy OD, Herman BC, Laudier DM, Majeska RJ, Sun HB, Schaffler MB.** Activation of resorption in fatigue-loaded bone involves both apoptosis and active pro-osteoclastogenic signaling by distinct osteocyte populations. *Bone.* 2012;50(5):1115–1122.

219. **Tomkinson A, Reeve J, Shaw RW, Noble BS.** The death of osteocytes via apoptosis accompanies estrogen withdrawal in human bone. *J Clin Endocrinol Metab.* 1997;82(9):3128–3135.

220. **Dodd JS, Raleigh JA, Gross TS.** Osteocyte hypoxia: a novel mechanotransduction pathway. *Am J Physiol.* 1999;277:C598–C602.

221. **Weinstein RS, Nicholas RW, Manolagas SC.** Apoptosis of osteocytes in glucocorticoid-induced osteonecrosis of the hip. *J Clin Endocrinol Metab.* 2000;85(8):2907–2912.

222. **Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC.** Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. *J Clin Invest.* 1998;102(2):274–282.

223. **O'Brien CA, Jia D, Plotkin LI, et al.** Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. *Endocrinology.* 2004;145(4):1835–1841.

224. **Plotkin LI, Manolagas SC, Bellido T.** Glucocorticoids induce osteocyte apoptosis by blocking focal adhesion kinase-mediated survival. Evidence for inside-out signaling leading to anoikis. *J Biol Chem.* 2007;282(33):24120–24130.

225. **Pacifici R, Brown C, Puscheck E, et al.** Effect of surgical menopause and estrogen replacement on cytokine release from human blood mononuclear cells. *Proc Natl Acad Sci USA.* 1991;88(12):5134–5138.

226. **Al-Dujaili SA, Lau E, Al-Dujaili H, Tsang K, Guenther A, You L.** Apoptotic osteocytes regulate osteoclast precursor recruitment and differentiation in vitro. *J Cell Biochem.* 2011;112(9):2412–2423.

227. **Mulcahy LE, Taylor D, Lee TC, Duffy GP.** RANKL and OPG activity is regulated by injury size in networks of osteocyte-like cells. *Bone.* 2011;48(2):182–188.

228. **Mabilleau G, Mieczkowska A, Edmonds ME.** Thiazolidinediones induce osteocyte apoptosis and increase sclerostin expression. *Diabet Med.* 2010;27(8):925–932.

229. **Maurel DB, Jaffre C, Rochefort GY, et al.** Low bone accrual is associated with osteocyte apoptosis in alcohol-induced osteopenia. *Bone.* 2011;49(3):543–552.

230. **Shandala T, Shen Ng Y, Hopwood B, Yip YC, Foster BK, Xian CJ.** The role of osteocyte apoptosis in cancer chemotherapy-induced bone loss. *J Cell Physiol.* 2012;227(7):2889–2897.

231. **Bonewald L.** Osteocytes. In: Marcus R, Nelson D, Rosen C, eds. *Osteoporosis.* Vol 1. 3rd ed. Burlington, MA: Elsevier; 2008:169–190.

232. **Weinstein RS, Jilka RL, Almeida M, Roberson PK, Manolagas SC.** Intermittent parathyroid hormone administration counteracts the adverse effects of glucocorticoids on osteoblast and osteocyte viability, bone formation, and strength in mice. *Endocrinology.* 2010;151(6):2641–2649.

233. **Yajima A, Inaba M, Tominaga Y, Nishizawa Y, Ikeda K, Ito A.** Increased osteocyte death and mineralization inside bone after parathyroidectomy in patients with secondary hyperparathyroidism. *J Bone Miner Res.* 2010;25(11):2374–2381.

234. **Kousteni S, Bellido T, Plotkin LI, et al.** Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell.* 2001;104(5):719–730.

235. **Aguirre JI, Plotkin LI, Gortazar AR, et al.** A novel ligand-independent function of the estrogen receptor is essential

for osteocyte and osteoblast mechanotransduction. *J Biol Chem*. 2007;282(35):25501–25508.

236. **Weinstein RS, O'Brien CA, Almeida M, et al.** Osteoprotegerin prevents glucocorticoid-induced osteocyte apoptosis in mice. *Endocrinology*. 2011;152(9):3323–3331.

237. **Bellido T.** Antagonistic interplay between mechanical forces and glucocorticoids in bone: a tale of kinases. *J Cell Biochem*. 2010;111(1):1–6.

238. **Plotkin LI, Lezcano V, Thostenson J, Weinstein RS, Manolagas SC, Bellido T.** Connexin 43 is required for the anti-apoptotic effect of bisphosphonates on osteocytes and osteoblasts in vivo. *J Bone Miner Res*. 2008;23(11):1712–1721.

239. **Bivi N, Condon KW, Allen MR, et al.** Cell autonomous requirement of connexin 43 for osteocyte survival: consequences for endocortical resorption and periosteal bone formation. *J Bone Miner Res*. 2012;27(2):374–389.

240. **Follet H, Li J, Phipps RJ, Hui S, Condon K, Burr DB.** Risedronate and alendronate suppress osteocyte apoptosis following cyclic fatigue loading. *Bone*. 2007;40(4):1172–1177.

241. **Plotkin LI, Weinstein RS, Parfitt AM, Roberson PK, Manolagas SC, Bellido T.** Prevention of osteocyte and osteoblast apoptosis by bisphosphonates and calcitonin. *J Clin Invest*. 1999;104(10):1363–1374.

242. **Plotkin LI, Mathov I, Aguirre JI, Parfitt AM, Manolagas SC, Bellido T.** Mechanical stimulation prevents osteocyte apoptosis: requirement of integrins, Src kinases, and ERKs. *Am J Physiol Cell Physiol*. 2005;289(3):C633–C643.

243. **Emerton KB, Hu B, Woo AA, et al.** Osteocyte apoptosis and control of bone resorption following ovariectomy in mice. *Bone*. 2010;46(3):577–583.

244. **Xia X, Kar R, Gluhak-Heinrich J, et al.** Glucocorticoid-induced autophagy in osteocytes. *J Bone Miner Res*. 2010;25(11):2479–2488.

245. **Jia J, Yao W, Guan M, et al.** Glucocorticoid dose determines osteocyte cell fate. *FASEB J*. 2011;25(10):3366–3376.

246. **Zhao H, Xiong J, Onal M, et al.** Osteocyte autophagy declines with age in mice and suppression of autophagy decreases bone mass. *J Bone Miner Res*. 2011;27(Suppl1):S13.

247. **Marotti G, Ferretti M, Remaggi F, Palumbo C.** Quantitative evaluation on osteocyte canalicular density in human secondary osteons. *Bone*. 1995;16(1):125–128.

248. **Belanger LF, Belanger C, Semba T.** Technical approaches leading to the concept of osteocytic osteolysis. *Clin Orthop Relat Res*. 1967;54:187–196.

249. **Recklinghausen FV.** *Untersuchungen uber Rachitis und Osteomalacia*. Jena, Germany: Gustav Fischer; 1910.

250. **Bonucci E, Gherardi G, Mioni G, et al.** Clinico-morphological correlations in uremic osteodystrophy of patients with conservative and hemodialytic treatment with special regard to the ultrastructure [in Italian]. *Minerva Nefrol*. 1975;22(2–3):99–108.

251. **Bonucci E, Gherardi G.** Osteocyte ultrastructure in renal osteodystrophy. *Virchows Arch A Pathol Anat Histol*. 1977;373(3):213–231.

252. **Baud CA.** Structure and functions of osteocytes in normal conditions and under the influence of parathyroid extract [in French]. *Schweiz Med Wochenschr*. 1968;98(19):717–720.

253. **Baud CA.** Submicroscopic structure and functional aspects of the osteocyte. *Clin Orthop Relat Res*. 1968;56:227–236.

254. **Belanger LF, Robichon J.** Parathormone-induced osteolysis in dogs. A microradiographic and alpharadiographic survey. *J Bone Joint Surg Am*. 1964;46:1008–1012.

255. **Iagodovskii VS, Triftanidi LA, Gorokhova GP.** Effect of space flight on rat skeletal bones (an optical light and electron microscopic study) [in Russian]. *Kosm Biol Aviakosm Med*. 1977;11(1):14–20.

256. **Haller AC, Zimny ML.** Effects of hibernation on interradicular alveolar bone. *J Dent Res*. 1977;56(12):1552–1557.

257. **Alcobendas M, Baud CA, Castanet J.** Structural changes of the periosteocytic area in *Vipera aspis* (L.) (Ophidia, Viperidae) bone tissue in various physiological conditions. *Calcif Tissue Int*. 1991;49(1):53–57.

258. **Heller-Steinberg M.** Ground substance, bone salts, and cellular activity in bone formation and destruction. *Am J Anat*. 1951;89(3):347–379.

259. **Ruth EB.** Basophilic islands in osseous tissue and their relation to resorption. *Anat Rec*. 1961;140:307–320.

260. **Wergedal JE, Baylink DJ.** Distribution of acid and alkaline phosphatase activity in undemineralized sections of the rat tibial diaphysis. *J Histochem Cytochem*. 1969;17(12):799–806.

261. **Nakano Y, Toyosawa S, Takano Y.** Eccentric localization of osteocytes expressing enzymatic activities, protein, and mRNA signals for type 5 tartrate-resistant acid phosphatase (TRAP). *J Histochem Cytochem*. 2004;52(11):1475–1482.

262. **Baylink DJ, Wergedal JE.** Bone formation by osteocytes. *Am J Physiol*. 1971;221(3):669–678.

263. **Zambonin-Zallone A, Teti A, Primavera MV, Pace G.** Mature osteocytes behaviour in a repletion period: the occurrence of osteoplastic activity. *Basic Appl Histochem*. 1983;27(3):191–204.

264. **Yamashita T, Yoshioka M, Itoh N.** Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain. *Biochem Biophys Res Commun*. 2000;277(2):494–498.

265. **Shimada T, Kakitani M, Yamazaki Y, et al.** Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *J Clin Invest*. 2004;113(4):561–568.

266. **Liu S, Lu Y, Xie Y, et al.** Elevated levels of FGF23 in dentin matrix protein 1 (DMP1) null mice potentially explain phenotypic similarities to Hyp mice. *J Bone Miner Res*. 2006;21(Suppl1):S51.

267. **Riminucci M, Collins MT, Fedarko NS, et al.** FGF-23 in fibrous dysplasia of bone and its relationship to renal phosphate wasting. *J Clin Invest*. 2003;112(5):683–692.

268. **Urakawa I, Yamazaki Y, Shimada T, et al.** Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature*. 2006;444(7120):770–774.

269. **Gattineni J, Bates C, Twombley K, et al.** FGF23 decreases renal NaPi-2a and NaPi-2c expression and induces hy-

pophosphatemia in vivo predominantly via FGF receptor 1. *Am J Physiol Renal Physiol*. 2009;297(2):F282–F291.

270. **Larsson T, Marsell R, Schipani E, et al.** Transgenic mice expressing fibroblast growth factor 23 under the control of the α1(I) collagen promoter exhibit growth retardation, osteomalacia, and disturbed phosphate homeostasis. *Endocrinology*. 2004;145(7):3087–3094.

271. **Miyamoto K, Ito M, Kuwahata M, Kato S, Segawa H.** Inhibition of intestinal sodium-dependent inorganic phosphate transport by fibroblast growth factor 23. *Ther Apher Dial*. 2005;9(4):331–335.

272. **Liu S, Zhou J, Tang W, Jiang X, Rowe DW, Quarles LD.** Pathogenic role of Fgf23 in Hyp mice. *Am J Physiol Endocrinol Metab*. 2006;291(1):E38–E49.

273. **Perwad F, Azam N, Zhang MY, Yamashita T, Tenenhouse HS, Portale AA.** Dietary and serum phosphorus regulate fibroblast growth factor 23 expression and 1,25-dihydroxyvitamin D metabolism in mice. *Endocrinology*. 2005;146(12):5358–5364.

274. **Burnett SM, Gunawardene SC, Bringhurst FR, Juppner H, Lee H, Finkelstein JS.** Regulation of C-terminal and intact FGF-23 by dietary phosphate in men and women. *J Bone Miner Res*. 2006;21(8):1187–1196.

275. **Lavi-Moshayoff V, Wasserman G, Meir T, Silver J, Naveh-Many T.** PTH increases FGF23 gene expression and mediates the high-FGF23 levels of experimental kidney failure: a bone parathyroid feedback loop. *Am J Physiol Renal Physiol*. 2010;299(4):F882–F889.

276. **Rhee Y, Bivi N, Farrow E, et al.** Parathyroid hormone receptor signaling in osteocytes increases the expression of fibroblast growth factor-23 in vitro and in vivo. *Bone*. 2011;49(4):636–643.

277. **Krajisnik T, Bjorklund P, Marsell R, et al.** Fibroblast growth factor-23 regulates parathyroid hormone and 1α-hydroxylase expression in cultured bovine parathyroid cells. *J Endocrinol*. 2007;195(1):125–131.

278. **Ben-Dov IZ, Galitzer H, Lavi-Moshayoff V, et al.** The parathyroid is a target organ for FGF23 in rats. *J Clin Invest*. 2007;117(12):4003–4008.

279. **Mirza MA, Larsson A, Lind L, Larsson TE.** Circulating fibroblast growth factor-23 is associated with vascular dysfunction in the community. *Atherosclerosis*. 2009;205(2):385–390.

280. **Faul C, Amaral AP, Oskouei B, et al.** FGF23 induces left ventricular hypertrophy. *J Clin Invest*. 2011;121(11):4393–4408.

281. **Desjardins L, Liabeuf S, Renard C, et al.** FGF23 is independently associated with vascular calcification but not bone mineral density in patients at various CKD stages. *Osteoporos Int*. 2012;23(7):2017–2025.

282. **Mirza MA, Alsio J, Hammarstedt A, et al.** Circulating fibroblast growth factor-23 is associated with fat mass and dyslipidemia in two independent cohorts of elderly individuals. *Arterioscler Thromb Vasc Biol*. 2011;31(1):219–227.

283. **Rowe PS, Oudet CL, Francis F, et al.** Distribution of mutations in the PEX gene in families with X-linked hypophosphataemic rickets (HYP). *Hum Mol Genet*. 1997;6(4):539–549.

284. **Liu S, Guo R, Simpson LG, Xiao ZS, Burnham CE, Quarles LD.** Regulation of fibroblastic growth factor 23 expression

but not degradation by PHEX. *J Biol Chem*. 2003;278(39):37419–37426.

285. **Martin A, Liu S, David V, et al.** Bone proteins PHEX and DMP1 regulate fibroblastic growth factor Fgf23 expression in osteocytes through a common pathway involving FGF receptor (FGFR) signaling. *FASEB J*. 2011;25(8):2551–2562.

286. **Martin A, David V, Laurence JS, et al.** Degradation of MEPE, DMP1, and release of SIBLING ASARM-peptides (minhibins): ASARM-peptide (s) are directly responsible for defective mineralization in HYP. *Endocrinology*. 2008;149(4):1757–1772.

287. **Gowen LC, Petersen DN, Mansolf AL, et al.** Targeted disruption of the osteoblast/osteocyte factor 45 gene (OF45) results in increased bone formation and bone mass. *J Biol Chem*. 2003;278(3):1998–2007.

288. **Rowe PS, Garrett IR, Schwarz PM, et al.** Surface plasmon resonance (SPR) confirms that MEPE binds to PHEX via the MEPE-ASARM motif: a model for impaired mineralization in X-linked rickets (HYP). *Bone*. 2005;36(1):33–46.

289. **Guo R, Rowe PS, Liu S, Simpson LG, Xiao ZS, Quarles LD.** Inhibition of MEPE cleavage by Phex. *Biochem Biophys Res Commun*. 2002;297(1):38–45.

290. **Jain A, Fedarko NS, Collins MT, et al.** Serum levels of matrix extracellular phosphoglycoprotein (MEPE) in normal humans correlate with serum phosphorus, parathyroid hormone and bone mineral density. *J Clin Endocrinol Metab*. 2004;89(8):4158–4161.

291. **Dobbie H, Unwin RJ, Faria NJ, Shirley DG.** Matrix extracellular phosphoglycoprotein causes phosphaturia in rats by inhibiting tubular phosphate reabsorption. *Nephrol Dial Transplant*. 2008;23(2):730–733.

292. **Marks J, Churchill LJ, Debnam ES, Unwin RJ.** Matrix extracellular phosphoglycoprotein inhibits phosphate transport. *J Am Soc Nephrol*. 2008;19(12):2313–2320.

293. **David V, Martin A, Hedge AM, Drezner MK, Rowe PS.** ASARM peptides: PHEX-dependent and -independent regulation of serum phosphate. *Am J Physiol Renal Physiol*. 2011;300(3):F783–F791.

294. **Bresler D, Bruder J, Mohnike K, Fraser WD, Rowe PS.** Serum MEPE-ASARM-peptides are elevated in X-linked rickets (HYP): implications for phosphaturia and rickets. *J Endocrinol*. 2004;183(3):R1–R9.

295. **David V, Martin A, Hedge AM, Rowe PS.** Matrix extracellular phosphoglycoprotein (MEPE) is a new bone renal hormone and vascularization modulator. *Endocrinology*. 2009;150(9):4012–4023.

296. **Hamrick MW.** A role for myokines in muscle-bone interactions. *Exerc Sport Sci Rev*. 2011;39(1):43–47.

297. **Pedersen BK.** Muscles and their myokines. *J Exp Biol*. 2011;214:337–346.

298. **Broholm C, Laye MJ, Brandt C, et al.** LIF is a contraction-induced myokine stimulating human myocyte proliferation. *J Appl Physiol*. 2011;111(1):251–259.

299. **Hamrick MW, Shi X, Zhang W, et al.** Loss of myostatin (GDF8) function increases osteogenic differentiation of bone marrow-derived mesenchymal stem cells but the osteogenic effect is ablated with unloading. *Bone*. 2007;40(6):1544–1553.

300. **Hamrick MW, McNeil PL, Patterson SL.** Role of muscle-derived growth factors in bone formation. *J Musculoskelet Neuronal Interact*. 2010;10(1):64–70.

301. **Jähn K, Lara-Castillo N, Brotto L, et al.** Skeletal muscle secreted factors prevent glucocorticoid-induced osteocyte apoptosis through activation of β-catenin. *Eur Cell Mater*. 2012;24:197–209.

302. **Lara N, Jaehn K, Romero-Suarez S, et al.** A muscle derived soluble factor activates PI3K/β-catenin signaling in osteocytes. *J Bone Miner Res*. 2011;26(suppl 1):S242.

303. **Mo C, Romero-Suarez S, Bonewald L, Johnson M, Brotto M.** Prostaglandin E2: from clinical applications to its potential role in bone-muscle crosstalk and myogenic differentiation. *Recent Pat Biotechnol*. 2012;6(3):223–229.

304. **Yellowley CE, Li Z, Zhou Z, Jacobs CR, Donahue HJ.** Functional gap junctions between osteocytic and osteoblastic cells. *J Bone Miner Res*. 2000;15(2):209–217.

305. **Qiu S, Rao DS, Palnitkar S, Parfitt AM.** Reduced iliac cancellous osteocyte density in patients with osteoporotic vertebral fracture. *J Bone Miner Res*. 2003;18(9):1657–1663.

306. **Loveridge N, Fletcher S, Power J, et al.** Patterns of osteocytic endothelial nitric oxide synthase expression in the femoral neck cortex: differences between cases of intracapsular hip fracture and controls. *Bone*. 2002;30(6):866–871.

307. **Caballero-Alias AM, Loveridge N, Pitsillides A, et al.** Osteocytic expression of constitutive NO synthase isoforms in the femoral neck cortex: a case-control study of intracapsular hip fracture. *J Bone Miner Res*. 2005;20(2):268–273.

308. **Calder JD, Buttery L, Revell PA, Pearse M, Polak JM.** Apoptosis—a significant cause of bone cell death in osteonecrosis of the femoral head. *J Bone Joint Surg Br*. 2004;86(8):1209–1213.

309. **Humphreys S, Spencer JD, Tighe JR, Cumming RR.** The femoral head in osteonecrosis. A quantitative study of osteocyte population. *J Bone Joint Surg Br*. 1989;71(2):205–208.

310. **Perrotta I, Cristofaro MG, Amantea M, et al.** Jaw osteonecrosis in patients treated with bisphosphonates: an ultrastructural study. *Ultrastruct Pathol*. 2010;34(4):207–213.

311. **Biasotto M, Chiandussi S, Zacchigna S, et al.** A novel animal model to study non-spontaneous bisphosphonates osteonecrosis of jaw. *J Oral Pathol Med*. 2010;39(5):390–396.

312. **Huja SS, Fernandez SA, Phillips C, Li Y.** Zoledronic acid decreases bone formation without causing osteocyte death in mice. *Arch Oral Biol*. 2009;54(9):851–856.

313. **Schulz A, Bressel M, Delling G.** Activity of osteoclastic bone resorption in primary hyperparathyroidism—a comparative electron microscopic and histomorphometric study. *Calcif Tissue Res*. 1977;22 Suppl:307–310.

314. **Padagas J, Colloton M, Shalhoub V, et al.** The receptor activator of nuclear factor-κB ligand inhibitor osteoprotegerin is a bone-protective agent in a rat model of chronic renal insufficiency and hyperparathyroidism. *Calcif Tissue Int*. 2006;78(1):35–44.

315. **Silvestrini G, Ballanti P, Leopizzi M, et al.** Effects of intermittent parathyroid hormone (PTH) administration on SOST mRNA and protein in rat bone. *J Mol Histol*. 2007;38(4):261–269.

316. **Kramer I, Loots GG, Studer A, Keller H, Kneissel M.** Parathyroid hormone (PTH)-induced bone gain is blunted in SOST overexpressing and deficient mice. *J Bone Miner Res*. 2010;25(2):178–189.

317. **Drake MT, Srinivasan B, Modder UI, et al.** Effects of parathyroid hormone treatment on circulating sclerostin levels in postmenopausal women. *J Clin Endocrinol Metab*. 2010;95(11):5056–5062.

318. **Mirza FS, Padhi ID, Raisz LG, Lorenzo JA.** Serum sclerostin levels negatively correlate with parathyroid hormone levels and free estrogen index in postmenopausal women. *J Clin Endocrinol Metab*. 2010;95(4):1991–1997.

319. **Ominsky MS, Vlasseros F, Jolette J, et al.** Two doses of sclerostin antibody in cynomolgus monkeys increases bone formation, bone mineral density, and bone strength. *J Bone Miner Res*. 2010;25(5):948–959.

320. **Tian X, Setterberg RB, Li X, Paszty C, Ke HZ, Jee WS.** Treatment with a sclerostin antibody increases cancellous bone formation and bone mass regardless of marrow composition in adult female rats. *Bone*. 2010;47(3):529–533.

321. **Li X, Warmington KS, Niu QT, et al.** Inhibition of sclerostin by monoclonal antibody increases bone formation, bone mass, and bone strength in aged male rats. *J Bone Miner Res*. 2010;25(12):2647–2656.

322. **Marenzana M, Greenslade K, Eddleston A, et al.** Sclerostin antibody treatment enhances bone strength but does not prevent growth retardation in young mice treated with dexamethasone. *Arthritis Rheum*. 2011;63(8):2385–2395.

323. **Rudmann DG, Page TJ, Vahle JL, et al.** Rat-specific decreases in platelet count caused by a humanized monoclonal antibody against sclerostin. *Toxicol Sci*. 2012;125(2):586–594.

324. **ADHR Consortium.** Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23. *Nat Genet*. 2000;26(3):345–348.

325. **Larsson T, Nisbeth U, Ljunggren O, Juppner H, Jonsson KB.** Circulating concentration of FGF-23 increases as renal function declines in patients with chronic kidney disease, but does not change in response to variation in phosphate intake in healthy volunteers. *Kidney Int*. 2003;64(6):2272–2279.

326. **Imanishi Y, Inaba M, Nakatsuka K, et al.** FGF-23 in patients with end-stage renal disease on hemodialysis. *Kidney Int*. 2004;65(5):1943–1946.

327. **Aono Y, Yamazaki Y, Yasutake J, et al.** Therapeutic effects of anti-FGF23 antibodies in hypophosphatemic rickets/osteomalacia. *J Bone Miner Res*. 2009;24(11):1879–1888.

328. **Mirza MA, Hansen T, Johansson L, et al.** Relationship between circulating FGF23 and total body atherosclerosis in the community. *Nephrol Dial Transplant*. 2009;24(10):3125–3131.

329. **Mirza MA, Larsson A, Melhus H, Lind L, Larsson TE.** Serum intact FGF23 associate with left ventricular mass, hypertrophy and geometry in an elderly population. *Atherosclerosis*. 2009;207(2):546–551.

330. **Hsu HJ, Wu MS.** Fibroblast growth factor 23: a possible cause of left ventricular hypertrophy in hemodialysis patients. *Am J Med Sci*. 2009;337(2):116–122.

331. **Dalal M, Sun K, Cappola AR, et al.** Relationship of serum fibroblast growth factor 23 with cardiovascular disease in older community-dwelling women. *Eur J Endocrinol*. 2011;165(5):797–803.

332. **Sylvester FA, Wyzga N, Hyams JS, et al.** Natural history of

bone metabolism and bone mineral density in children with inflammatory bowel disease. *Inflamm Bowel Dis*. 2007; 13(1):42–50.

333. **Pappa HM, Saslowsky TM, Filip-Dhima R, et al.** Efficacy and harms of nasal calcitonin in improving bone density in young patients with inflammatory bowel disease: a randomized, placebo-controlled, double-blind trial. *Am J Gastroenterol*. 2011;106(8):1527–1543.

334. **Oostlander AE, Bravenboer N, Sohl E, et al.** Histomorphometric analysis reveals reduced bone mass and bone formation in patients with quiescent Crohn's disease. *Gastroenterology*. 2011;140(1):116–123.

335. **Giuliani N, Ferretti M, Bolzoni M, et al.** Increased osteocyte death in multiple myeloma patients: role in myeloma-induced osteoclast formation. *Leukemia*. 2012;26(6): 1391–1401.

336. **Terpos E, Christoulas D, Katodritou E, et al.** Elevated circulating sclerostin correlates with advanced disease features and abnormal bone remodeling in symptomatic myeloma; reduction post-bortezomib monotherapy. *Int J Cancer*. 2012;131(6):1466–1471.

THE ENDOCRINE SOCIETY®

Save the Date for **Clinical Endocrinology Update (CEU)**, September 26–28, 2013, Hyatt Regency New Orleans New Orleans, LA

www.endo-society.org/CEU2013