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Review Article

Emerging roles of osteocytes in skeletal homeostasis and mineral metabolism

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Abstract: Osteocytes constitute 90-95% of total bone cells and are derived from mesenchymal stem cell lineage through osteoblast differentiation. Osteocytes are dispersed throughout the bone matrix and display a dendritic morphology. The cell body of the osteocyte resides within a lacuna whereas the dendritic processes travel through the bone (called canalicular system) to reach the bone marrow. Osteocytes form a network of cells that is thought to mediate the effects of mechanical loading on bone through this extensive lacunocanalicular network via the participation of gap junctions/transmembrane channels that connect the cytoplasm of two adjacent osteocytes. The impact of mechanical strains on osteocytes is translated to the generation of signals from osteocytes that are considered critical in the regulation of bone resorption and bone formation, the two key fundamental processes that maintain skeletal homeostasis by bone modeling and remodeling. Activation of wingless tail (Wnt)/β-catenin canonical pathway in osteocytes induces osteogenic effect. On the other hand, osteocytes produce a SOST protein, sclerostin that antagonizes LDL-receptor-related protein-5/6 (LRP5/6)-mediated Wnt signaling and mediates an anti-osteogenic effect. Discovery of sclerostin has led to a neutralization approach wherein humanized monoclonal antibody against sclerostin has been developed to induce osteogenic effect and positioned as a therapy in postmenopausal osteoporosis. Osteocytes also produce fibroblast growth factor-23 (FGF-23) that regulates mineralization of bone extracellular matrix and mineral metabolism via the regulation of phosphate and vitamin D metabolism. Inactivating mutations of several osteocyte-specific genes cause hereditary hypophosphatemic disorders and mineralization defects. This review aims to provide recent advances on the functionsof osteocytesin the regulation of bone remodeling and endocrine regulation of phosphate metabolism.

Keywords: Mechanosensing, wingless-tail pathway, sclerostin, FGF-23, phosphate metabolism, postmenopausal osteoporosis

Introduction

Osteocytes are derived from osteoblasts which have been trapped in the calcified bone matrix produced by them. Once trapped in the bone matrix, osteocytes develop cytoplasmic processes which run through canaliculi and form a connection network with neighboring osteocytes. Osteocytes account for approximately 95% of cells in the mature bone tissue and form an extensive network with each other as well as with osteoblasts and surface lining cells [1, 2]. These are the most abundant cells of the skeleton, with a life span of nearly 25 years [3, 4]. Until recently, difficulties in isolating osteocytes from bone matrix led to the mistaken impression that osteocytes would be passive cells, and their functions were mostly unknown. The discovery of osteocyte-specific markers, availability of new animal models, development of optimized techniques for osteocyte isolation and culture, and the establishment of phenotypically stable cell lines have contributed to improved understanding of osteocyte biology [2]. According to an estimate based on mathematical modeling of available literature on various osteocyte measurements, average adult human skeleton contains ~42 billion osteocytes and the total number of dendritic projections from these cells is ~3.7 trillion, with total of 23 trillion connections with each other and with bone surface cells [5]. This estimate suggests the existence of a highly complex and vast network of osteocytes in human skeleton which so far remained under-appreciated. Osteocytes have numerous important func-

tions in bone biology and mineral metabolism. Here, we would comprehensively review recent findings on osteocyte biology, advances in our understanding of osteocyte signaling, therapeutic advances in postmenopausal osteoporosis targeting osteocyte produced protein and regulation of bone-kidney axis through osteocytes.

Cell biology

Because osteocytes are derived from osteoblast differentiation, the origin of these cells can be traced to mesenchymal stem cell (MSC). Osteoblast to osteocyte transition involves the following four stages: osteoid-osteocyte, preosteocyte, young osteocyte, and mature osteocyte [4]. At the end of a bone formation cycle, a subpopulation of osteoblasts is embedded into the newly formed bone matrix to become osteocytes. This process is accompanied by morphological changes, most distinct of which is the reduction of the round osteoblast size. In addition, ultrastructural changes including the number of organelles such as Golgi apparatus and rough endoplasmic reticulum decreases, and the nucleus-to-cytoplasm ratio increases occur during osteocyte transition from osteoblast. These ultrastructural changes correspond to a decrease in the protein synthesis and secretion [6].

During osteoblast to osteocyte transition, cytoplasmic processes (up to 50 per cell) begin to appear before the osteocytes are cloaked into the bone matrix [7]. Osteocyte cell body is located inside lacunae whereas the processes that originate from the lacunar space form tiny tunnels called canaliculi, forming the osteocyte lacunocanalicular system, and interstitial fluid flows between the osteocytes processes and canaliculi enables the intercellular transport of signaling molecules such as prostaglandins and nitric oxide between these cells [8]. In addition, the osteocyte lacunocanalicular system is juxtaposed to the vascular supply for oxygen and nutrients supply to osteocytes [9]. The lacunocanalicular system also provides microporosity to mineralized bone.

The underlying mechanisms in the development of osteocyte cytoplasmic processes are not well understood. E11/gp38, also called podoplanin has been suggested to have an important role in the process as this protein is highly expressed in osteocytes in the process of being embedded or those that are recently embedded, similarly to other cell types with dendritic morphology including type II lung alveolar cells, endothelial cells of lymphatic vessels, podocytes and cells of the choroid plexus [10-12]. Inhibition of E11/gp38 expression in MLO-Y4 osteocytic cells has been shown to abrogate dendrite elongation, suggesting that E11/gp38 is involved in dendrite formation in osteocytes [12].

Mature osteocytes (cells that are completely embedded in the bone matrix) display downregulation of osteoblastic genes including osteocalcin, alkaline phosphatase, type 1 collagen and bone sialoprotein-II, and upregulation of dentine matrix protein 1 (DMP-1), phosphate regulating endopeptidase homolog, X-linked (PHEX), Matrix extracellular phosphoglycoprotein/osteoblast factor 45 (MEPE/OF45), fibroblast growth factor-23 (FGF23) and sclerostin, and these upregulated genes serve as osteocyte markers [13-15]. Osteocyte apoptosis is considered as a chemotactic signal to bone resorption by osteoclasts and has a critical role in the initiation of bone remodeling process [16-19]. Attesting to this finding, reports have shown that osteoclasts engulf apoptotic osteocytes during bone resorption [20-22]. Resorption is the initial stage of bone remodelingprocess which is key to the maintenance of skeletal homeostasis. Mechanical loading is an important determinant of initiation of bone remodeling and osteocytes transduce mechanical signal into biochemical signals.

Osteocytes and mechanical loading

Osteocytes have stellate/dendritic shape (although not uniform) and are housed in small pores in the bone called lacunae. Osteocytes from trabecular bone are more rounded than osteocytes from cortical bone which exhibit an elongated morphology [23]. Osteocyte lacunae have ellipsoid shape although with significant variation. These lacunar systems create an extensive network through the skeleton. All these shape variations could influence lacunar deformation subsequently resulting in sensing of local mechanical strains on the bone matrix, osteocytemechanosensation. Recent studies showed a link between osteocyte lacunar and collagen orientation [24-26]. These studies have

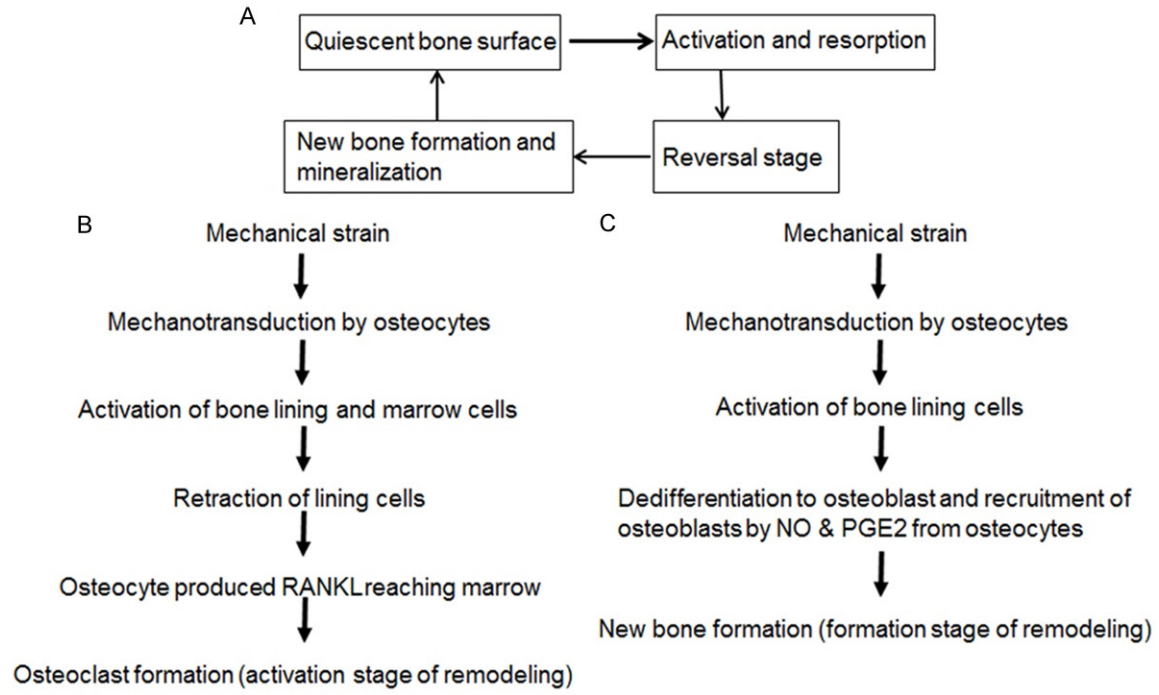

Figure 1. Schematic diagram showing the roles of osteocytes in bone remodeling process. A. Various stages of bone remodeling cycle. Tissue deformation and microcracks on quiescent bone surface stimulates osteoclast formation and bone resorption. After the formation of resorptive pits, osteoclasts die via apoptosis and osteoblast precursor cells being to populate the pit (reversal stage). Finally, pits are filled up with newly formed mineralized matrix (bone formation) and the bone surface becomes quiescent. B. Mechanical strain generated signals cause osteocytes to activate bone lining cells which are retracted to make way for osteocyte processes to reach bone marrow and release RANKL and thus triggering osteoclast differentiation (the activation stage). C. Osteocytes activated by mechanical strain stimulate bon lining cells which are dedifferentiated to osteoblasts and secrete nitric oxide (NO) and prostaglandin E2 (PGE2). Production of NO and PGE2 results in the recruitment of more osteoblasts thus leading to new bone formation.

found that the main axis of lacunae was parallel to the collagen in both longitudinally oriented osteons (collagen parallel to osteon axis) and transversely oriented osteons (collagen perpendicular to osteon axis). The link between lacunar and collagen orientations suggests that the shape of osteocyte lacuna is determined during the bone formation phase. Indeed, in periosteum, osteoblasts were shown to be aligned with the collagen they produce [25]. Further, when primary cultures of osteoblasts were placed on fabricated collagen substrates, the cells aligned to the substrate, and the newly synthesized matrix was oriented along the cells which suggested that osteoblasts and collagen interact in determining the orientation of each other [27]. The connection between osteocyte and collagen orientation could help to understand how mechanical loading affects osteocyte shape.

Conversely, varying shapes of osteocyte cell bodies may have a different mechano-response to the same mechanical signal. For example, round osteocytes have lesser stiffness but more sensitive mechano-response than flat osteocytes [28, 29]. Flat cells have more stress fibers and focal adhesion centers compared to loosely adherent cells that have round morphology. The functional differences between round and flat osteocytes therefore suggest that cytoarchitecture is necessary to mechanosensation. Furthermore, the effect of the lacunar shape would regulate the transfer of stress information to the cell. It is thus surmised that osteocyte mechanosensation is dependent on both the cellular and lacunar shape.

Altered lacunar morphology and size have been suggested to be associated with bone loss. For example, one study reported that in humans, the osteocytes in osteopenic individual was relatively round and large compared with osteoarthritic and osteopetrotic individuals [30]. However, another study failed to find a significant difference in lacunar size or shape between wo-

men with and without osteoporotic fracture, despite the presence of a large range of sizes and shapes in both groups [31]. Although, a significant correlation exists between osteocyte shape and osteoporosis, caution must be exercised before inferring the causality from correlation.

Regulation of bone remodeling by osteocytes

Bone remodeling consists of five stages: a) activation: preosteoclasts are differentiated by the stimulation of RANKL to become mature osteoclasts, b) resorption: osteoclasts digest matrix and mineral and forms resorption pits, c) reversal: termination of resorption, d) formation: osteoblasts synthesize new bone to fill the pits and e) quiescence: osteoblasts become resting bone lining cells on the newly formed bone surface or trapped in the matrix to become osteocytes. Micro-damages/cracks in the bone matrix cause osteocyte apoptosis. Dying osteocytes are targeted by osteoclasts for removal. In addition, osteocytes also produce copious amounts of RANKL and regulate osteoclast formation [32], and this mechanism could trigger the process of bone remodeling (activation stage). Osteocytes produce nitric oxide and prostaglandin E2 which could stimulate osteoblast function [3] (formation stage). In addition, osteocytes produce sclerostin, an antagonist of canonical Wnt signaling andinhibitsosteoblast function. Schematic diagram of regulation of bone remodeling by osteocytes is shown in **Figure 1**.

Role of Wnt signaling in mechanosensing by osteocytes

Wnt, Wingless-related integration, are a group of cysteine-rich, glycosylated and palmitoylated secretory proteins [33]. They are involved in transducing extracellular signals via frizzled surface and other transmembrane co-receptors (LRP5/6) to nucleus with transcriptional co-activator (β-catenin) to modulate cell survival, proliferation and differentiation in autocrine or paracrine mode [33]. Wnt ligands are involved in regulation of embryonic bone remodeling and chondro-osteoprogenitor differentiation. Overall, Wnt signaling has three major functions in osteoblasts: to promote osteoblast-specific differentiation of MSC; to stimulate osteoblast proliferation; and to enhance survivability of osteoblasts and osteocytes [34].

Mutations that render the function of LRP5 protein inactive were linked to the low bone mass observed in osteoporosis-pseudoglioma syndrome [35]. Conversely, a syndrome of high bone mass was found to be caused by G171V mutation in LRP5 gene due to increase in wnt activity in osteoblasts [36]. Later, six other amino acid substitutions were also shown to cause high bone mass phenotype 5 [37]. Animal data were in agreement with human disease, as $LRP5^{-/-}$ mice displayed low bone mass and diminished osteoblast proliferation, whereas transgenic mice generated to express human G171V LRP5 mutation had an increased bone mass [38, 39].

Dickkopfs (Dkk) are vertebrate Wnt antagonists as they compete for the LRP5/6 receptors to inhibit canonical signaling. Deletion of Dkk1 gene in mice displays increased bone mass phenotype whereas overexpressing of Dkk1 gene resulted in severe osteopenia and reduced osteoblast population. Increased production of Dkk1 appears to be associated with osteolytic lesions in malignancy as observed in myeloma patients with bone lesions having enhanced levels of Dkk1 in plasma cells compared with myeloma patients without bony lesions. Attesting to these observations, myeloma-bearing SCID-rab mice treated with anti-Dkk1 antibody showed decrease in osteolytic lesions [40].

To accomplish bone anabolism, wnt/β-catenin signaling in osteocytes appears to hold the key. In a recent study, researchers generated a mouse model in which a dominant active β-catenin gene ($da\beta cat^{Ot}$) was expressed specifically in osteocytes, resulting in activation of canonical Wnt signalling in these cell [41]. Bone mineral density and trabecular bone volume in the $da\beta cat^{Ot}$ mice at both axial and the appendicular sites was higher than in wild type littermates. Moreover, $da\beta cat^{Ot}$ mice displayed increased periosteal bone formation and increased number of active osteoblasts over wild type littermates. In addition, both osteoclast number and the levels of bone resorption at the trabecular sites were higher in the $da\beta cat^{Ot}$ mice compared with wild type littermates. Finally, the genes that are downstream of Notch and Wnt signalling pathways were upregulated in $d\alpha\beta cat^{Ot}$ mice compared to wild type littermates. Mice expressing the same dominant active β-catenin specifically in osteoblasts showed reduced resorption thus suggesting

that the activation of β-catenin signaling in osteocytes versus in less mature cells of the osteoblastic lineage leads to opposite effects on resorption. This difference is due to increased production of SOST protein in $d\alpha\beta cat^{Ot}$ mice skeleton, which in turn resulted in increased secretion of the osteoclastogenic cytokines, RANKL. Although, $d\alpha\beta cat^{Ot}$ mice displayed increased bone accrual, there is no data on bone strength to eliminate the possibility that the radiological features of the cortical and trabecular bones were not sclerotic. Also, these mice had a very high cortical porosity which is a feature of hyperparathyroidism. Since osteocytes are intricately involved in phosphate metabolism, FGF-23, vitamin D3 and PTH levels should have been assessed.

Sclerostin, an osteocyte produced SOST protein, also antagonizes LRP5/6-mediated Wnt signaling. Osteoblastic transcription factors such as osterix and cbfa1 (Runx2) stimulate SOST expression. Several bone morphogenetic proteins (BMP-2, -4 and -6) also upregulate SOST expression, thus making SOST a protective protein that prevents excessive bone formation by osteoblasts. Loss of sclerostin function gives rise to a rare skeletal pathology known as sclerosteosis, which is characterized by generalized progressive osteosclerosis. By contrast, high bone mass in Van Buchem disease is caused by the inactivating mutation in the SOST gene. Mice lacking SOST gene have high bone mass state and transgenic mice over-expressing sclerostin exhibit severe bone loss, thus confirming that sclerostin is a strong negative regulator of osteoblast function. These studies establish sclerostin as a key negative regulator of bone formation. Intermittent PTH, the only FDA recommended bone anabolic therapy, has been shown to decrease sclerostin levels in osteocytes. In addition, osteocyte-specific over-expression of PTH receptor (PTHR1) in mice had resulted in increased osteoblast number and bone mass due to reduced sclerostin expression. Taken together, these data suggested that inhibiting sclerostin could be an effective bone anabolic approach to treat post-menopausal osteoporosis.

Neutralizing scelorstin as a therapeutic strategy for post-menopausal osteoporosis

Effect of sclerostin inhibition was studied in ovariectomized (OVX) rats that were left untreated after surgery to develop osteopenia. Administration of a sclerostin neutralizing monoclonal antibody (produced in mouse) for 5 weeks at the frequency of two times per week completely reversed bone mass and biomechanical strength loss caused by prolonged estrogen deficiency [42]. The skeletal restorative effect by sclerostin neutralization was generalized as it was observed at both axial and appendicular sites as well as in cancellous and cortical bones. Bone formation was also increased at both cancellous and cortical sites in OVX rats treated with sclerostin neutralizing antibody, which suggested that the skeletal restorative effect of this treatment involved bone anabolic mode [42]. However, lack of data on resorption parameters in this study failed to demonstrate unequivocally that the restorative effect by sclerostin neutralizing antibody was due to only osteogenic effect. In gonad-intact, aged male rats, sclerostin neutralizing antibody increased bone mass, bone formation and bone strength [43]. In a rat immobilization/disuse model which is characterized by a reduced bone formation and an enhanced bone resorption, sclerostin neutralizing antibody increased both trabecular and cortical bones in the underloaded limb compared to control by not only stimulating bone formation but also reducing bone resorption [44]. Indeed, one study reported that sclerostin neutralizing antibody given to OVX rats acutely increased bone formation and decreased bone resorption. Bone formation response of sclerostin neutralizing antibody was observed at the shorter duration (6 weeks) of the study whereas the anti-resorptive response was observed for a longer duration (26 weeks). mRNA levels of osteocytic genes including SOST and Dkk-1 were decreased in the bones of rats treated with sclerostin neutralizing antibody for 26 weeks which suggested that long term suppression of sclerostin resulted in increased synthesis of anti-osteoblastogenic genes to prevent excessive bone accrual [45].

Unlike primates and humans, rodents lack Haversian system in skeleton and hence rodents display a significantly different bone remodeling features from humans. Therefore, preclinical therapeutic efficacy of a given agent (in this case sclerostin neutralizing antibody) is required to be assessed in primates before human trials are undertaken. When adult female cynomolgus monkeys were administered with a humanized sclerostin-neutralizing

monoclonal antibody once a month for two months, BMD was increased at the cancellous sites of long bones and compressive strength of lumbar vertebra was greater than the control group. Moreover, the biochemical surrogate of osteogenic marker, serum P1NP, was higher and histomophometric bone formation measures showed increased rate of mineralized bone deposition in the neutralizing antibody treated group over the control [46].

Romosozumab (AMG 785) is the first humanized anti-sclerostin monoclonal antibody to be tested in clinical trials. In a phase 2 multicentre, international, randomized placebo-controlled trial on postmenopausal osteoporotic women (age 55 to 85 years), romosozumab treatment after 12 months significantly increased BMD at lumbar spine, femur neck and total hip, and a transitory rise in bone formation markers and a sustained decreases in bone resorption markers [47]. Another investigational monoclonal anti-sclerostinantibody, blosozumab, has also been investigated for its osteoanabolic ability in postmenopausal osteoporosis [48]. Blosozumab increased BMD values of spine, total hip and femur neck over the placebo group. Serum biochemical makers of bone formation including P1NP, osteocalcin and bone-specific alkaline phosphatase were elevated in the treatment arm over the placebo arm. Serum CTX-1, a marker of bone resorption was also decreased in the treatment arm compared with placebo. Adverse event rates with both anti-sclerostin neutralizing antibodies were similar between treatment and placebo arms without any significant safety findings. A follow-up study after discontinuation of treatment with blosozumab showed that although spine and hip BMD values were higher than in placebo, the benefits were getting progressively lost [49] as is the case with teriparatide, the bone anabolic therapy. Results of phase 3 trials with anti-sclerostin antibodies in post-menopausal osteoporosis are awaited. Recently, cardio-vascular safety concerns with long-term systemic neutralization of sclerostin, as it is expected to be done in postmenopausal osteoporotic patients, has been raised as preclinical and clinical data suggest expression of SOST protein in calcifying vascular tissue [50-52].

Anti-sclerostin antibody could be effective in bone loss conditions other than post-menopausal osteoporosis and aging-induced bone loss. In humans, spinal cord injury (SCI) is a frequent cause of bone loss, and the resulting sublesional osteoporosis predisposes these individuals to an increased risk of low-trauma fracture. In preclinical studies, anti-sclerostin antibody has been shown to prevent cancellous loss in proximal femur and distal tibia caused by SCI and stimulated bone formation in rats [53].

Osteocytes are the critical regulators of phosphate and vitamin D metabolism

Both osteoblasts and osteocytes produce fibroblast growth factor-23 (FGF-23), a 32 kDa protein with an N-terminal FGF homology domain and a C-terminus containing 72 amino acids that are unique in FGF family. Because osteocytes constitute >90% of bone cells, FGF-23 secretion by these cells contributes to the circulatory levels of FGF-23. Circulating FGF-23 binds to and activates receptor complexes consisting of the receptors of FGF including FGFR-1, FGFR-3 or FGFR-4 and the transmembrane β-glucuoronidase α-Klotho [54-57], which is located in target tissues of FGF-23.

Kidney is the major target for FGF-23. Increases in FGF-23 inhibit renal phosphate reabsorption by decreasing the expression and membrane localization of Na^+-dependent phosphate co-transporters. In addition, excess FGF-23 also suppresses the renal enzyme CYP27B1 (which converts 25-hydroxyvitamin D, the pro-hormone to 1,25-dihydroxyvitamin D, the active hormone) and thus suppressing circulating levels of 1,25-dihydroxyvitamin D by inhibiting the enzyme CYP27B1 and also possibly by stimulating the catabolism of 1,25-dihydroxyvitamin D by activating 24-hydroxylase (CYP24) present in the proximal tubule [58-64]. Klotho is a type 1 membrane protein, with a single transmembrane domain near its C-terminus and Klotho-FGF receptor complex binds to FGF-23 at much higher affinity than either the FGF receptor or Klotho alone. Elevated FGF-23 is associated with reduced Klothoexpression [65], which suggests the existence of a feedback mechanism to prevent FGF-23 signaling from becoming uncontrolled.

FGF-23 level in osteoblasts and osteocytes is re-gulated by locally supplied bone-derived factors including PHEX, DMP-1 and E-NPP1

through activation of FGFR-1 pathways and by systemic factors, including 1,25-dihydroxyvitamin D and PTH [66-73]. Excess FGF-23 in both humans and mouse models gives rise to hypophosphatemia, suppression of 1,25-dihydroxyvitamin D levels and rickets (in childhood) or osteomalacia (during adulthood) [58, 59, 74, 75]. Study of autosomal dominant hypophosphatemic rickets, which is caused by mutations in the RXXR site in FGF-23 protein that inhibits its cleavage, helped in explaining the functional role of FGF-23 as an essential factor that regulates phosphate and vitamin D metabolism [76]. Elevated levels of FGF-23 also cause acquired hypophosphatemic disorders, such as in tumor-induced osteomalacia, and is an adaptive response in chronic kidney disease (CKD) leading to preservation of phosphate balance and development of secondary hyperparathyroidism due to reductions in 1,25-dihydroxyvitamin D levels [77]. The function of FGF-23 beyond the realms of bone and kidney needs investigation as there is a strong association between increased serum FGF-23 levels and mortality not only in renal failure patients but also in the general population [78].

In summary, osteoblast/osteocyte produced hormone FGF-23 participates in several endocrine axes involving 1,25-dihydroxyvitamin D or PTH and critically coordinates systemic phosphate and vitamin D homeostasis with bone mineralization.

Summary and conclusion

From being considered as senescent osteoblasts trapped in the mineralized matrix with unknown function, osteocytes presently have emerged as the key regulators of skeletal functions that promote the translation of mechanical stimulus to biochemical signals. Regulation of bone remodeling by osteocytes seems to be mediated by sclerostin, encoded by *SOST* gene. Sclerostin inhibits Wnt signaling in osteoblast lineage cells, which promotes osteoblast proliferation and differentiation. Osteocytes also secrete RANKL and OPG, contributing to the regulation of bone resorption and thereby linking mechanical forces in bone to regulate bone mass and shape through remodeling. Given the negative impact of sclerostin in bone formation, neutralizing antibody against this protein has reached the advanced stages of development for the treatment of postmenopausal osteoporosis. In addition, osteocytes are involved in regulation of bone matrix mineralization and phosphate metabolism. Modulation of osteocyte specific genes including PHEX, MEPE, DMP1 and FGF-23 are primarily involved in systemic phosphate homeostasis and matrix mineralization. In this regard, other possible FGF-23-endocrine axes could be physiologically important.

Disclosure of conflict of interest

None.

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