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Do osteocytes contribute to phosphate homeostasis?

Jian Q. Feng^a, Ling Ye^b, and Susan Schiavi^c

^aDepartment of Biomedical Sciences, Texas A&M Health Science Center, Baylor College of Dentistry, Dallas, Texas

^bOral Biology, University of Missouri-Kansas City, Kansas City, Missouri

^cEndocrine and Renal Sciences, Genzyme Corporation, Framingham, Massachusetts, USA

Abstract

Osteocytes, the terminally differentiated cell of the osteoblast lineage, account for over 90% of all bone cells. Due to their relative inaccessibility within mineralized matrix, little is known regarding their specific functions in comparison to the well studied surface bone cells, osteoblasts and osteoclasts. Furthermore, bone is often viewed as a mineral reservoir that passively releases calcium and phosphate in response to hormones secreted from remote organs. Noncollagenous matrix proteins produced in osteocytes, such as dentin matrix protein 1 (DMP1), have also been viewed as inert scaffolds for calcium–phosphate deposition. Recent discoveries of new genetic mutations in human diseases and development of genetically engineered animal models challenge these classic paradigms, suggesting that the osteocyte plays an active role in both mineralization and total systemic phosphate regulation. In this review, we will focus on roles of osteocytes in mineralization and particularly in phosphate regulation via the DMP1-FGF23 pathway.

Keywords

DMP1; FGF23; osteocyte; phosphate

Introduction

Confidence in our current understanding of phosphate homeostasis has been challenged by the recent discovery of a novel hormonal system originating from osteocytes; cells previously considered to be silent by virtue of their hibernation within bony caves. It is now recognized that elevated levels of FGF23, a phosphaturic hormone produced by late stage osteoblasts and early osteocytes, can exert a profound influence on phosphate homeostasis. FGF23 is elevated in chronic kidney disease and genetic diseases associated with phosphate dysregulation. Of even greater surprise is the finding that a presumably inert bone matrix protein, DMP1, may participate in the regulation of systemic phosphate homeostasis through its direct or indirect effects on FGF23 expression. Cumulatively, these findings have led to the speculation that cells of the osteoblast/osteocyte lineage may play a prominent role in

maintenance of systemic phosphate homeostasis through the local control of bone mineral, the major reservoir for calcium and phosphate.

Osteoblast/osteocyte lineage

Mineralized bone makes up over 90% of total calcium and phosphate content within the body. It is therefore easy to conceptualize how altered bone remodeling may contribute to the mineral dysregulation associated with pathological conditions such as chronic kidney disease–mineral bone disorder (CKD-MBD). Bone remodeling typically occurs on bone surfaces through the concerted actions of osteoclasts and osteoblasts. New evidence now suggests that the third bone cell type, the osteocyte, may also play an active role in bone remodeling and mineral maintenance. To begin the discussion it is relevant to consider the osteocyte as a highly differentiated cell within the osteoblast lineage.

Osteoblasts are cuboidal shaped cells with well developed Golgi complexes. They exclusively reside side by side on surfaces of existing bone tissue or calcified cartilage where they play a critical role in the formation and growth of new bone through their sequential deposition of osteoid followed by calcium and phosphorus. The life span of a typical osteoblast is a few months, after which it can undergo one of three potential fates: cell death, transition to a bone lining cell, or further differentiation into an osteocyte.

Our current knowledge of how osteocytes are formed and how they may specifically influence the remodeling process in response to mechanical loading has been limited by the difficulties associated with isolating live cells from a rock-like matrix and the inability to recapitulate the environment necessary to fully maintain differentiated primary or established osteocyte derived cells lines *in vitro*. Despite this limitation, microscopy studies suggest that there are several phenotypically distinct cell stages representing cell transitions between the mature osteoblast and osteocyte including osteoblast, osteoblastic-osteocyte, osteoid osteocyte and mature osteocyte [1–3]. During the process where osteoblasts are engulfed within mineralizing matrix and undergo further differentiation toward a mature osteocyte, cell organelles and cytoplasm are reduced and the cell acquires exceptionally long dendritic cell processes. The slender processes with numerous branches of the osteocytes occupy the many minute canals, named canaliculi, which radiate in all directions from the cell body encased within the core referred to as a lacunae. Within the bone, individual osteocyte lacunocanicular units form a large network allowing osteocytes to communicate with each other, surface osteoblast cells, and blood vessels. This system of osteocyte-filled caves and channels, allows osteocytes the freedom to respond to mechanical strain and coordinate adaptive bone remodeling responses [4]. It also provides a route in which released paracrine and endocrine factors may reach their local or systemic destination including osteoblast and osteocyte targets.

Importance of osteocytes in mineralization

Historically, static pictures from transmission electron microscopy (TEM) were the only basis for our limited understanding of the osteocyte's role in matrix production and mineralization [1]. More recently, however, live imaging techniques have been used to correlate differentiation, marked by GFP expression driven by the promoter of the

osteocytic-specific gene DMP1, and mineralization, marked by the calcium binding stain alizarin red [5]. These results suggest that early stage osteocytes play an active rather than passive role in the process of mineralization.

An additional approach used to define osteocyte function is the use of genetic models in which expression of specific genes are altered. The choice of models to study phosphate/calcium regulation has been largely based on identification of genes associated with human disorders of phosphate dysregulation. Interestingly, these studies have identified a group of proteins with apparently distinct functions which appear to be linked within a pathway by virtue of their relationships in human pathological conditions and include the secreted phosphaturic hormone, FGF23; the wnt inhibitor, sFRP4; bone matrix proteins of the SIBLINGs (Small Integrin-Binding Ligand, N-linked Glycoprotein) family, DMP1 and MEPE (matrix extracellular phosphoglycoprotein); and the membrane bound endopeptidase, PHEX [5–7].

The SIBLINGs proteins, DMP1, MEPE and osteopontin have now been shown to play a role in the regulation of bone mineral content. DMP1 and MEPE are expressed by late stage osteoblasts and osteocytes and are localized to the canaliculi and lacunae walls. In contrast to DMP1, MEPE appears to be a negative regulator of mineralization as MEPE null mice have increased bone density [8]. The ASARM peptide, a cleavage product of MEPE can directly block mineralization both *in vitro* [9] and *in vivo* [10]. Current data support a hypothesis that these molecules may be transducers of strain and participate in local mineral content of the perilacunar space. One model proposes that the function of a MEPE-derived fragment (ASARM) is to reduce local mineral content in lacunae whereas DMP1 has been proposed to be a positive regulator of mineralization at the same site [11–13].

The *Dmp1* null mouse model provides a good example of how genetic approaches have increased our understanding of genes associated with the mineralization process. DMP1 is a serine-rich acidic protein that has numerous potential phosphorylation sites that promotes mineral formation at the perilacunar surfaces [14,15]. Full length DMP1 is susceptible to proteolytic cleavage resulting in the generation of specific N-terminal and C-terminal fragments. However, the biological functions of intact versus these fragments are not clear. Although these mice clearly show that DMP1 is not essential for mineralization in early development [7], they display striking defects in osteocyte morphology, bone remodeling and mineralization during postnatal development when the skeleton bears mechanical loading [12–14,15]. Utilizing a combination of sensitive microscopy techniques we have examined local changes in osteocyte cell morphology and mineral deposition. The coordinate use of fluorescent dyes provided a strategy to examine the relationship of osteocytes (DAPI nuclear stain), active mineralization (calcein), total mineral content (alizarin red) and lacunocanicular space (procian red) in the *Dmp1* null mice relative to wild-type mice [16]. Backscattered scanning electron microscopy (SEM) was also used to view the lacunae and density of the surrounding mineral. These studies have confirmed that loss of DMP1 results in profound changes in both osteocyte structure and total bone mineralization [16–20] and are in agreement with previous studies in adult wild-type animals, where DMP1 osteocyte expression is dramatically induced in response to mechanical loading induced bone remodeling [21,22].

Taken together, the emerging data suggest that the phosphate regulated SIBLINGs proteins control the degree of mineralization at the interface of bone surfaces lining the lacunocanalicular space. It is further hypothesized that the degree of mineralization dictates the flexibility of the osteocyte thereby altering how it responds to mechanical induction and how it transmits signals that regulate active remodeling on the bone surface.

Roles of osteocytes in control of phosphate homeostasis

Phosphate is essential not only for skeletal health and integrity, but also required for a diverse array of biological processes, including numerous cellular mechanisms involved in energy metabolism, cell signaling, nucleic acid synthesis, and membrane function [23]. Importantly, 90% of total body phosphorus is stored in bone, with only 1% in circulation. The relationship between control of phosphorus homeostasis by the kidney and bone mineralization is clearly illustrated by the known functions of parathyroid hormone (PTH). PTH directly induces phosphorus release from bone via activation of osteoclast-induced resorption and simultaneously promotes renal phosphate excretion thereby providing a mechanism by which the body can handle excess phosphorus during net catabolic states of bone remodeling.

The newly discovered hormone FGF23 also promotes phosphorus excretion but its local actions at its main site of expression within bone are not entirely understood [16,24,25]. Despite earlier reports failing to identify FGF23 expression in bone, it is now fully recognized that FGF23 is expressed in osteoblasts and/or osteocytes depending on the model [24,26]. Liu *et al.* [24] generated a combined *Fgf23*-deficient GFP reporter (where GFP is used to reflect the endogenous *Fgf23* expression) and the *Phex*-deficient *Hyp* mouse model of the human disease, X-linked hypophosphatemic rickets (XLH) and demonstrated that FGF23 was expressed in osteocytes but not in *Phex*-deficient osteoblast cells. Furthermore, in bone marrow stromal cells derived from *Fgf23*-null/*Hyp* mice, GFP expression was selectively increased in osteocyte-like cells within mineralization nodules consistent with osteocyte-specific expression [27,28]. In contrast, expression was localized to osteoblasts in calvaria sections of *Fgf23*-lacZ knock-in mice (where the LacZ reporter is used to reflect the endogenous *Fgf23* expression) [26]. It is likely that these differences may reflect relative sensitivity of detection methods, abnormal expression of related pathway genes, and/or animal age. As discussed below, detection using the more sensitive method of in-situ hybridization suggests FGF23 is expressed in both osteoblasts (high level) and osteocytes (low level). A clear understanding of the timing, localization and relative level of FGF23 expression in bone during normal states of remodeling will be required to define its physiological function.

Our lack of understanding of the role of FGF23 on bone has also been limited by the fact that FGF23 overexpression induces hypophosphatemia leading to rickets and osteomalacia. As phosphate is needed for normal mineralization, FGF23 expression can impact mineralization simply by removing a critical substrate needed for mineralization. Recent studies do support specific effects of FGF23 on mineralization. Although FGF23 null are hyperphosphatemic and have a general increase in bone mineral density, there is a localized increase in osteoid with corresponding decrease in mineralization. Furthermore, mice

deficient in both FGF23 and the renal phosphate transporter, Npt2a exhibit reversal of hyperphosphatemia while retaining skeletal defects [29]. Finally, high levels of FGF23 can inhibit nodule formation and mineralization of an osteoblast cell line, as well as bone formation in the parietal bone organ culture model, supporting a direct role of FGF23 on bone *in vitro* [30]. Despite these lines of evidence, the apparent absence in bone of detectable KLOTHO, a FGF23 coreceptor, raises mechanistic questions regarding how FGF23 may direct specific effects on bone mineralization.

Additional data supporting the concept that osteocytes control phosphate homeostasis in association with the anabolic mineralization process are mainly from studies of two hypophosphatemic animal models: *Dmp1*-null mice and *Hyp* mice, where the membrane bound endo-peptidase PHEX is mutated. PHEX is predominantly located in osteoblasts and osteocytes. Inactive Phex mutations lead to increased production of FGF23, principally by osteocytes due to an unknown matrix-derived FGF23 stimulatory factor [24,27,28]. These two animal models share essentially the same phenotype: hypophosphatemia rickets/osteomalacia and high FGF23 levels [16,24,31]. Humans harboring loss of function mutations in either the PHEX or DMP1 genes also have elevated FGF23 levels [16,32–34]. Although the similarities between these phenotypes suggest that DMP1 and PHEX might be in the same pathway, current evidence suggests that intact DMP1 is not a substrate for PHEX.

By in-situ hybridization and immunohistochemistry, we showed that FGF23 is mainly expressed in osteoblasts with relatively low expression in the wild type osteocytes (Fig. 1, right panel). In contrast, relative to wild type mice, *DMP1* null mice had a dramatic increase of FGF23 in osteocytes with expression unchanged in osteoblast cells (Fig. 1, left panel) (also see [16]). On the basis of these observations we speculate that DMP1, a protein highly expressed in osteocytes and associated with mineralization negatively regulates FGF23 expression, a protein associated with reduced mineralization. This hypothesis is consistent with the fact that expression of multiple genes is typically repressed during osteocyte differentiation and the mineralization process. Many markers of osteoblasts, such as alkaline phosphatase activity, and collagen type 1 mRNA, as well as osteoid-osteocyte markers such as E11/gp38 protein [16], are greatly elevated in *Dmp1*-null osteocytes regardless of whether they are newly formed or deeply embedded. These observations not only explain the abnormal skeletal phenotype of *Dmp1*-null mice, but also reveal a failure to repress osteoblastic gene expression in the *Dmp1*-null osteocytes.

The most striking change in the *Dmp1*-null osteocyte is the switch to an abnormal endocrine cell, which releases large amounts of FGF23 into blood circulation, leading to a hypophosphatemia [16]. By definition, an endocrine cell should release hormones into the blood stream easily and quickly. Unlike the osteoblast lining cells, which are directly exposed to the blood circulation, osteocytes are embedded in the mineralized matrix. On the basis of the molecule weight of FGF23, it is too large to pass through the gap junctions connecting osteocytes and osteoblasts. However, the lacunocanalicular transportation system containing proteoglycans and extravascular fluid surrounding the osteocyte [35] provides an environment for transportation of fluid, signals, and both a flexible medium and space for cellular movement. It is also known that there is a close connection between large blood

vessels and osteocytes (Fig. 2a). Recently, we studied the relationship between a capillary blood vessel (10 μ m diameter) and neighboring osteocytes using a resin casted SEM method with acid etching of the sample surface (Ye *et al.*, in preparation). Figure 2b depicts direct contacts between an osteocyte cell body or dendrites and the capillary wall. Another relevant observation is the finding that the *Dmp1*-null bone appears more immature with increased blood supply to both the cortical and trabecular bone relative to wild type mouse bone (Fig. 2c). Thus, there may be an enhanced accessibility of *Dmp1*-null osteocytes to the vascular system in these mice.

Although the above studies clearly demonstrate a role for FGF23 in pathological conditions, evidence also demonstrates that it has a physiological role in normal phosphate homeostasis. Yamazaki *et al.* [36^{*}] showed that a single injection of neutralizing FGF23 antibodies into wild type mice nearly doubled the serum phosphate level, supporting a PTH-independent function in regulation of phosphate homeostasis. Serum FGF23 levels are enhanced within hours in response to increases in serum phosphate or vitamin D. It is also known that FGF23 inhibits 1 α -hydroxylase, the rate-limiting enzyme for 1,25-hydroxyvitamin D₃ production and inhibits PTH synthesis via KLOTHO/FGFR1 complexes on the parathyroid system [37,38]. These findings suggest that FGF23 is an important component of a hormonal feedback system. Evidence suggests that FGF23's role in this potential feedback system is to manage phosphate changes over hours as changes in FGF23 serum levels respond more slowly to challenges relative to PTH. Recently, Tatsumi *et al.* [39^{**}] showed that mice with ablated osteocytes display no changes in phosphate homeostasis or serum FGF23 levels suggesting that FGF23 actions might be redundant or secondary to the actions of PTH. However, caution in the over-interpretation of these experiments is warranted as increased FGF23 from osteoblasts or residual osteocytes may account for the apparent lack of effects on systemic phosphate levels.

Taken together, these suggest that FGF23 and its associated osteocyte proteins may coordinate mineralization with systemic regulation of phosphorus levels. One possibility is that these proteins directly regulate the differentiation of osteoblasts into osteocytes with either direct or indirect regulation of mineral deposition in active remodeling units. Another scenario is that these molecules regulate the mineral density along the perilacunar surfaces adjacent to embedded osteocytes. Released mineral from these sites could be a potential source of serum calcium and phosphorus. It is also proposed that changes in perilacunar mineralization might alter the ability of the osteocyte to respond to mechanical load with resulting changes in released signals such as sclerostin that regulate normal bone remodeling at the bone surface. Regardless of the mechanism, the release of FGF23 appears to be correlated with decreased mineralization states in the presence of a functional kidney.

Osteocytes' role in phosphate dysregulation associated with chronic kidney disease

In addition to the hypophosphatemic diseases associated with normal renal function, FGF23 levels are progressively increased during CKD-MBD. These elevations occur early in CKD before increases in PTH or serum phosphate and correlate strongly with cardiovascular calcification and mortality [40^{**},41,42]. Serum phosphate elevations even in the normal

range have been linked to increased mortality within both the general and the CKD population [43]. It is currently unclear whether FGF23 has direct effects on disease progression or whether the dramatic progressive increases in FGF23 are simply a consequence of uncontrolled increases in serum phosphate. Given the evidence described earlier, it is appealing to consider that FGF23 may be an important molecule signaling early changes in bone health. This possibility is consistent with new histological evidence demonstrating that renal osteodystrophy [44,45] and vascular calcification can be found early in CKD and are not limited to ESRD. In a pivotal radiolabel study performed in the early 90s it became clear that different forms of renal osteodystrophy with reduced or enhanced turnover can alter the ability of bones to 'buffer' excess calcium [46] and presumably by extension, phosphorus. This study demonstrated how alterations in bone remodeling could dramatically impact systemic calcium and phosphorus load. By extension, it is conceivable that alterations in proteins such as FGF23 that either directly regulate or sense changes in bone mineralization could contribute to the early and progressive dysregulation in mineral metabolism. Interestingly, a recent study in pediatric ESRD patients revealed a link between serum FGF23 levels and mineralization rates [47]. At the present time there are no clear mechanistic data to link these parallel observations. Nonetheless, they provide a basis for hypothesis generation and suggest future experimentation to explore the roles of FGF23, DMP1 and osteoblast/osteocytes in mineral homeostasis.

Conclusion

Although histological evidence has supported osteocyte roles in bone mineralization, until recently direct functional evidence was lacking. In the last decade this concept has been validated by studies utilizing genetically engineered animal models (like *Dmp1*-null model) that are based on genetic mutations (such as FGF23, PHEX and DMP1) associated with phosphate dysregulation in human diseases. These studies have also been supported by technical advances in live fluorescent and electron microscopy. These evidences now suggest that osteocytes participate in bone remodeling, mineralization, and phosphate regulation. Future studies are needed to increase our mechanistic understanding and address specific questions such as: how does DMP1 control maturation of osteocytes? Does phosphate contribute to bone maturation itself? Does FGF23 have direct roles in bone? Is high phosphate a harmful factor for osteocytes?

Acknowledgments

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Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 363–364).

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Figure 1. FGF23 level is sharply increased in Dmp1-null osteocytes

In-situ hybridization shows increased Fgf23 mRNA expression (red in color) in 3 week-old Dmp1-null osteocytes (left upper panel) in comparison to the control where Fgf23 is expressed in osteoblast (right upper panel). Immunohistochemistry shows the same pattern: FGF23 is increased in the Dmp1 null osteocytes (brown, high intensity, left lower panel), whereas FGF23 is mainly expressed in the control osteoblast (right lower panel). Ob, osteoblast (red arrows); Oyc, osteocyte (white arrows).

Figure 2. Connections between blood vessels and osteocyte-lacunocanalicular

(a) Parallel (100 $\times$, left panel) and transverse (250 $\times$, right panel) sections of the cortical bone (decalcified). Osteocyte-lacunocanalicular is arranged concentrically around the Haversian canal, which contains blood vessels and nerves, which suggest that there might be connections between blood vessels and osteocytes. (Adapted from Gray's Anatomy of the Human Body from the classic 1918 publication). (b) The image is an acid-etched resin embedded rabbit tibia and visualized by scanning electron microscopy, which document that there are direct connections of a capillary and osteocyte-lacunocanalicular either by cell bodies or by canaliculi. (c) The photograph of forelimbs where the blood vessels are infused with the red resin. This image shows sharp increases in blood vessels in the 14-day-old *Dmp1*-null forelimb (control, upper panel; *Dmp1*-null, lower panel).