Published in final edited form as:

Adv Exp Med Biol. 2012 ; 728: 65–83. doi:10.1007/978-1-4614-0887-1_4.

EVIDENCE FOR FGF23 INVOLVEMENT IN A BONE-KIDNEY AXIS REGULATING BONE MINERALIZATION AND SYSTEMIC PHOSPHATE AND VITAMIN D HOMEOSTASIS

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Abstract

Bone is involved in the maintenance of phosphate and vitamin D homeostasis via its production and secretion of FGF23 and serves as a reservoir for the storage and release of calcium and phosphate into the circulation. Alterations in mineralization of extracellular matrix and the remodeling activities of the skeleton are coupled to the kidney conservation of phosphate and production of 1,25(OH)₂D via the regulation of FGF23 production by osteocytes through yet-to-be defined locally derived factors. In addition, FGF23 production is regulated by 1,25(OH)₂D in a feedback loop where FGF23 stimulate Cyp24 mediated degradation of 1,25(OH)₂D that serves to protect the organism from the toxic effects of vitamin D excess. In this chapter, we will review the regulation and function of FGF23.

INTRODUCTION

Coordination of calcium and phosphate homeostasis involves absorption of these minerals by the intestine, influx and efflux of calcium and phosphate from bone and their kidney excretion or reabsorption. Traditionally, the regulation of calcium and phosphate homeostasis has been viewed from the perspective of the PTH/vitamin D axis, where 1,25(OH)₂D predominate effect is to increase gastrointestinal calcium and phosphate absorption, and PTH principal function is to maintain serum calcium levels by increasing 1,25(OH)₂D production and calcium reabsorption by the kidney and calcium and phosphate efflux from bone by stimulating bone remodeling. The discovery of FGF23, which suppresses 1,25(OH)₂D levels via effects on the kidney to stimulate Cyp24 mediated degradation and suppress Cyp27b1-mediated production, and inhibits renal phosphate reabsorption, has lead to an expansion of the PTH/Vitamin D axis to include another hormonal axis that functions as a counter regulatory pathway to suppress 1,25(OH)₂D production and enhance renal phosphate secretion. FGF23 is predominately produced by osteocytes in bone, which places it in an optimal place to coordinate renal phosphate handling with bone mineralization and turnover (Fig. 1). Our understanding of FGF23 biology is derived from comparative analyses of hereditary diseases involving mutations of essential bone factors such as Phex, a transmembrane endopeptidase and Dmp1, an

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extracellular matrix SIBLING protein. Most of these diseases are characterized by bone mineralization disorders, rickets, increased bone production of FGF23 and subsequent hypophosphatemia and abnormal vitamin D metabolism (XLH, ARHR). However, other disorders (McCune Albright syndrome, CKD) affecting the degree of bone remodeling and leading to different bone phenotypes are also associated with an elevation of FGF23 levels, suggesting that the status of bone mineralization and remodeling actually regulates FGF23 production. The precise mechanism in bone that regulate FGF23 are currently being elucidated. To date, it appears that Phex and Dmp1 are both essential and interconnected players in the local regulation of FGF23 production. Recent studies also propose the FGFs/FGF receptor pathway as an important step in the signalization cascade. This chapter reviews the well-known and emerging functions of FGF23 and its regulation in response to bone modification signals.

EVOLVING INSIGHTS INTO SKELETAL FUNCTIONS

Bone Remodeling

Bone is a dynamic load bearing organ that creates the framework of the human body. Bone structural integrity is maintained due to a constant remodeling of the bone matrix, which consists of an iterative cycle of removal of mineralized tissue by the osteoclast-mediated resorption followed by its replacement with new collagenous matrix (osteoid) by osteoblast-mediated bone formation. After a delay of several days, osteoid undergoes mineralization. During the mineralization process, a subset of the osteoblasts become embedded in the matrix forming osteocytes that have dendrite-like cytoplasmic extensions forming a canalicular (neural-like) network inside the mineralized matrix. Osteocytes function as sensors and effectors maintaining skeletal homeostasis. Mineralization of osteoid is a regulated process representing a balance between inorganic factors, such as the local phosphate and pyrophosphates, which respectively promote and inhibit hydroxyapatite formation as well as extracellular matrix proteins that either facilitate or impede the mineralization process. Autocrine, paracrine and hormonal factors as well as physical and chemical processes regulate the overall remodeling process.

Bone, a Target for Systemic and Local Factors Regulating Mineral Metabolism

In addition to its scaffolding function, bone is a metabolically active organ that participates in mineral metabolism. The traditional view is that bone is a mineral reservoir, where calcium and phosphate move in and out bone from a poorly described exhalable pool, from the area surrounding osteocytes (called osteolysis) and from osteoclast-mediated resorptive sites. In this conceptualization, calcium and phosphate are in equilibrium with the systemic milieu under steady state and the influx and efflux of calcium and phosphate from bone is under control of both passive physiochemical forces and active cellular processes, such as systemic hormones and mechanical/local factors. An example of physiochemical effects is metabolic acidosis, which leads to a net loss of bone mineral. The best example an endocrine network involving bone is the PTH-Vitamin D axis. In response to hypocalcemia, the Parathyroid Gland (PTG) increases the production and secretion of PTH, which targets the renal distal tubule to decrease renal calcium excretion and the proximal tubule to inhibit phosphate reabsorption and to stimulate 1,25(OH)₂D production. Action of 1,25(OH)₂D on

the small intestines increases active calcium and phosphate transport.² PTH also has direct effects on bone via PTH receptors in osteoblasts, resulting in increased calcium and phosphate efflux from the exchangeable bone fluid compartment³ and through RANKL-dependent, osteoclast-mediated bone resorption of mineralized bone.⁴ The direct kidney and bone effects of PTH, along with the concomitant actions of 1,25(OH)₂D, restore serum calcium levels to normal (Fig. 2A). The phosphaturic actions of PTH offset vitamin D-mediated gastrointestinal phosphate absorption² and PTH-dependent phosphate efflux from bone,⁵ thereby preventing the development of hyperphosphatemia. A dramatic clinical example of the contribution of bone remodelling to systemic calcium and phosphate homeostasis is “hungry bone syndrome”, where an acute lowering of PTH by parathyroidectomy in patients with hyperparathyroidism leads to hypocalcemia and hypophosphatemia due to rapid uptake of the calcium and phosphate into bone matrix as remodelling shifts from a bone resorption to a formation phase. A more recent example is that fat through leptin controls bone mass by a hypothalamic relay and sympathetic nervous system.⁶ Other hormones such as estrogens and androgens, thyroid hormone and glucocorticoids, as well as the sympathetic nervous system target receptors in bone to coordinate calcium and phosphate flux in response to systemic alterations in mineral homeostasis. Finally, malignant processes such as multiple myeloma or metastatic diseases that cause increased bone resorption can lead to hypercalcemia through release of calcium from bone.

Bone as an Endocrine Organ

A new concept that has emerged is that bone is an endocrine organ that releases hormones that communicate with other organs to control systemic homeostasis. At present, FGF23 and osteocalcin are two known hormones secreted by cells in the osteoblast lineage that act systemically to respectively coordinate mineral homeostasis¹ and energy metabolism.⁶

FGF23, the Prototypic Bone Derived Hormone

FGF23 is a ~32 kDa protein with an N-terminal FGF-homology domain and a novel 71 amino acid C-terminus.⁷ FGF23 is grouped with FGF19 (mouse FGF15) and FGF21^{7,8} subfamily that, in contrast to the paracrine and heparin-dependent functions of canonical FGFs,⁹ act as hormones.¹⁰ FGF23 is produced in cells in the osteoblast lineage, principally the osteocyte in some diseases of FGF23 excess, such as hereditary hypophosphatemic disorders and mainly in peritrabecular osteoblasts in other disorders, such as McCune Albrights Hereditary Osteodystrophy and osteitis fibrosa in chronic kidney disease. FGF23 targets organs that co-express FGFR/Klotho complexes,^{11,12} including the kidney, parathyroid gland, pituitary gland and choroid plexus.¹² α -Klotho is a Type I membrane, β -glycosidase-like protein, which binds to FGFRs and the C-terminus of FGF23^{12–15} to permit heparin independent activation of FGFRs by FGF23. The principal target for FGF23 is the kidney, where this hormone has phosphaturic effects that are mediated by inhibition of renal phosphate reabsorption and serves as a counter-regulatory factor that inhibits 1,25(OH)₂D production.^{11,12,16} Excess FGF23 causes hypophosphatemia via inhibition of solute carrier family 34, member 1-dependent (SLC34A1-dependent) and SLC34A2-dependent phosphate transport (also known as sodium phosphate cotransporter 2 [NPT2a] or NaPi-IIa and NPT2c or NaPi-IIc, respectively). Excess FGF23 also suppresses 1,25(OH)₂D via inhibition of 25-

hydroxyvitamin D-1 α -hydroxylase (CYP27B1) that is converted to 1,25(OH) $_2$ D and stimulation of 24-hydroxylase (CYP24) that inactivates 1,25(OH) $_2$ D in the proximal tubule of the kidney.^{17–20} Although the biological actions of FGF23 are in the proximal tubule, the precise segments of the kidney and receptors that mediate the renal response to FGF23 are not entirely clear. Ex vivo studies of proximal tubular segments or cell lines derived from proximal tubules have produced variable results of FGF23-mediated inhibition of sodium-dependent phosphate transport.^{19,21} The highest expression of Klotho-FGF receptor complexes are in the distal tubule,¹⁴ suggesting a distal to proximal feedback mechanism. Although initial studies that excluded individual effects of FGFR3 and FGFR4 and by default implicated FGFR1 and the proximal tubule as the principal target in kidney for FGF23 {Liu, 2008 #4165}, we have found that combined loss of FGFR 3 and 4 partially correct the Hyp phenotype. Thus, FGFR 1,3 and 4 may act in concert to mediate the effects of FGF23 on the kidney.

FGF23 also targets FGFR-Klotho complexes in the PTG,²² but the physiological effects of FGF23 remains controversial.^{14,23} On the one hand, there is clinical evidence that excess FGF23 stimulates PTH secretion,¹² as evidence by the strong association between elevated FGF23 levels and the severity of hyperparathyroidism in CKD. On the other hand, FGF23 has been shown to suppress PTH mRNA expression in vitro and decrease serum PTH in vivo.²⁴

In addition, FGF23 is produced in the ventrolateral thalamic nucleus and may target the choroid plexus, which expresses FGFR-Klotho complexes, as well as sodium-dependent phosphate transporters. Since there is a gradient for phosphate between the Cerebral Spinal Fluid (CSF) and serum (with the phosphate concentration being significantly lower in the CSF), it is tempting to speculate that FGF23 may also regulate CSF phosphate concentrations. Another potential target of FGF23 is the pituitary gland, where upregulation of early response gene expression has been observed in response to acute FGF23 administration to mice. FGF23 null mice have severe growth retardation, suggesting that FGF23 may regulate pituitary function. There is controversy as to whether bone itself is a target for FGF23. Klotho, which is required for FGF23 actions, is not expressed in bone and the observed changes in bone in states of high and low FGF23 appear to be secondary to changes in serum phosphate and 1,25(OH) $_2$ D levels. However, recent studies may have identified that FGF23 suppresses differentiation and matrix production in osteoblasts cultures.²⁵ FGF23 may also impact upon glucose homeostasis, thymus function, growth and aging.^{26–28} At present, however, it appears that many of these abnormalities associated with FGF23 deficiency in tissues lacking Klotho represent secondary effects of elevated 1,25(OH) $_2$ D and hyperphosphatemia, rather than direct effects of FGF23 on these organs.^{29–31} In addition to these canonical effects mediated by FGFR-Klotho complexes, it has been suggested that high concentrations of FGF23, such as that observed in CKD, may have broader, nonspecific actions to activate FGFRs independent of membrane bound Klotho. Currently, there is no direct evidence for such off-target effects of FGF23.

GENETIC DISORDERS DEFINE FGF23 FUNCTION

Excess of FGF23

Several hereditary and acquired disorders are mediated by FGF23 leading to a common phenotype characterized by hypophosphatemia due to renal phosphate wasting and inappropriately normal 1,25(OH)₂D levels for the degree of hypophosphatemia, rickets/osteomalacia and the absence of hypercalciuria.

Hereditary hypophosphatemic disorders caused by a primary increase in circulating levels of FGF23 include:

- autosomal dominant hypophosphatemic rickets (ADHR; MIM 193100),
- autosomal recessive hypophosphatemic rickets (ARHR; MIM 241520),
- X-linked hypophosphatemic rickets (XLH; MIM 307800),
- osteoglophonic dysplasia (OGD; MIM 166250).

There is a single report of a patient with hypophosphatemic rickets and hyperparathyroidism (HRH; MIM pending).³² HRH differs from the others disorders described here in that hyperparathyroidism is a predominant feature.

Whereas the clinical features of ADHR, ARHR, XLH, OGD and HRH are caused by excess FGF23, the mechanisms whereby FGF23 levels are increased differ between these disorders. ADHR is caused by mutations (R176Q and R179W) in the RXXR furinlike cleavage domain of FGF23 that impairs proteolytic inactivation of FGF23.^{33,34} Interestingly, some small integrin-binding ligand N-linked glycoproteins (SIBLINGS) can participate or even be the cause of these pathological disorders. ARHR is caused by inactivating mutations in *DMP1*,^{35,36} a member of the SIBLING family of extracellular matrix protein that augments mineralization.³⁷ Loss of *DMP1* results in increased transcription of FGF23 by osteocytes.³⁵ Tumor-induced osteomalacia, or oncogenic osteomalacia, is a paraneoplastic syndrome of renal phosphate wasting, aberrant vitamin D metabolism and osteomalacia that is associated with elevated FGF23 levels.^{38,39} The proximate cause of increased FGF23 production is not known, but this disorder is associated with increased levels of MEPE, another member of the SIBLING family and sFRP4, which may regulate PHEX and *DMP1* metabolism, respectively.⁴⁰ XLH is caused by inactivating mutations in *PHEX*^{41–43} on the X chromosome. Loss of *PHEX* also leads to increased expression of FGF23 by osteocytes.⁴⁴ Mutant Phex in Hyp mice (murine homologous for X-Linked Hypophosphatemic rickets) induces an intrinsic bone mineralization defect associated with the increased production of FGF23 by the bone and hypophosphatemia. OGD is an autosomal dominant bone dysplastic disorder caused by activating mutations in the *FGFR1* gene, suggesting FGFR1 may regulate FGF23 expression in bone and/or the renal handling of phosphate.⁴⁵ HRH also has elevated FGF23 levels, but the primary genetic abnormality is a translocation causing elevated circulating levels of Klotho.³²

Deficiency in FGF23

In contrast, deficiency of FGF23 results in the opposite renal phenotype, consisting of hyperphosphatemia and elevated production of 1,25(OH)₂D.^{26–28,30,46,47} Hyperphosphatemic familial tumoral calcinosis (HFTC; MIM 211900) is a rare autosomal recessive disorder characterized by hyperphosphatemia, normal or elevated 1,25(OH)₂D levels, soft-tissue calcifications and typically massive lobulated periarticular calcifications levels.⁴⁸ In addition, *Fgf23*-null mice have soft tissue calcifications, severe growth retardation, abnormalities of bone mineralization and a shortened lifespan.^{27,28,30} To date there have been three different mutations identified as leading to either decreased bioactive circulating FGF23 levels or end-organ resistance to FGF23. These include mutations of the genes encoding FGF23,⁴⁶ Klotho⁴⁹ and GalNAc transferase 3 (GALNT3),⁵⁰ a Golgi-associated enzyme that *O*-glycosylates a furin-like convertase recognition sequence in FGF23.⁵¹ Missense mutations of FGF23 impair its secretion, leading to inadequate circulating levels of this phosphaturic factor.⁴⁶ Mutations of GALNT3 destabilize FGF23, resulting in a low-intact serum FGF23 levels but high levels of biologically inactive C-terminal FGF23 fragments. A mutation in the gene coding for Klotho (H 193R) results in decreased Klotho expression and reduced FGF23-Klotho-FGFR complex formation and to end-organ resistance to FGF23.⁴⁹ In mice, inactivating mutations or deletion of *Kl* results in end-organ insensitivity to FGF23, which is characterized by hyperphosphatemia, elevated 1,25(OH)₂D levels, early mortality and soft-tissue calcifications,^{23,49,52,53} a phenotype that resembles that of *Fgf23*-null mice.⁵⁴

PHYSIOLOGICAL FUNCTIONS OF FGF23

Since both extremes of hypophosphatemia and hyperphosphatemia have negative biological effects,^{55,56} it is not surprising that adaptive mechanisms have evolved to protect organisms from hypophosphatemia and hyperphosphatemia. The PTH-Vitamin D axis has long held the position of a phosphate regulating pathway, but the regulation and function of PTH are more consistent with its principal biological role in maintaining calcium homeostasis (Fig. 2A). In the kidney there is threshold for phosphate reabsorption in the tubules, such that phosphate above a specific concentration is excreted in the urine. While PTH regulates the tubular threshold for phosphate, other circulating factors, called phosphatonins, have been postulated to regulate phosphate homeostasis under conditions not explained by alteration in PTH or other regulators of renal tubular phosphate handling. FGF23 is the first bone fide phosphatonin to be characterized. Three major physiological functions of FGF23 have been recognized thus far.

A Counter Regulatory Hormone for 1,25(OH)₂D

Surprisingly, of the potential systemic factors that might be expected to regulate FGF23 expression, such as calcium, phosphate, PTH and 1,25(OH)₂D, only 1,25(OH)₂D has been shown to regulate FGF23 production (Fig. 2B). In this regard, 1,25(OH)₂D directly stimulates FGF23 expression in osteocytes via a vitamin D response element (VDRE) in the *Fgf23* promoter.¹⁶ Since FGF23 targets the kidney to suppress 1,25(OH)₂D production, 1,25(OH)₂D stimulation of FGF23 closes a feedback loop.¹⁶ In the setting of excess 1,25(OH)₂D, which also reduces PTH levels, FGF23-mediated phosphaturia helps prevent

potential hyperphosphatemia from enhanced 1,25(OH)₂D-dependent gastrointestinal phosphate absorption and diminished PTH-mediated phosphaturia. 1,25(OH)₂D may also indirectly regulate FGF23 expression. In this regard, selective deletion of the vitamin D receptor (VDR) in cartilage produces an unidentified chondrocyte-derived inhibitor of *FGF23* transcription.⁵⁷ Thus, FGF23's ability to enhance phosphate excretion and suppress 1,25(OH)₂D production may have evolved to protect an organism from vitamin D intoxication.¹⁶

Regulation of Parathyroid Gland Function?

FGF23 targets FGFR-Klotho complexes in the PTG,²² but the physiological effects of FGF23 remains controversial.^{14,23} On the one hand, there is clinical evidence that excess FGF23 stimulates PTH secretion,¹² as evidence by the strong association between elevated FGF23 levels and the severity of hyperparathyroidism in chronic kidney disease (CKD). On the other hand, FGF23 has been shown to suppresses PTH mRNA expression in vitro and decreases serum PTH in vivo.²⁴ There is also controversy about how or if PTH regulates the circulating level of FGF23. PTH does not appear to directly stimulate FGF23 production, since PTH does not stimulates FGF23 in osteoblasts in vitro¹⁶ or in mice⁵⁸ in the setting of normal renal function. Also, FGF23 is not elevated in patients with primary hyperparathyroidism.⁵⁹ However, the PTH-cyclinD1 transgenic mouse model of primary hyperparathyroidism has elevated FGF23 levels that decreased after parathyroidectomy.⁶⁰ There is also potential cross-talk between PTH and FGF23 through the indirect mechanism of PTH stimulation of 1,25(OH)₂D.⁶¹

Given that FGF23 is a “phosphaturic hormone”, it is expected to be regulated by serum phosphate. Extracellular phosphate, however, does not appear to directly stimulate *FGF23* mRNA levels or *FGF23* gene promoter activity in osteoblastic cultures.¹⁶ Although phosphate loading in mice increases FGF23 levels,⁶² evidence of the importance of dietary phosphate in regulating FGF23 levels in humans is also conflicting.^{63,64} Phosphate may regulate FGF23 certain pathological circumstances such as CKD, where elevations in FGF23 compensate for the reduced renal clearance of phosphate and the degree of FGF23 elevation correlates with the severity of hyperphosphatemia.³⁹ The stimulus for increased FGF23 in CKD is not known, but the pattern of FGF23 expression in bone is distinct from hereditary disorders of FGF23 excess, suggesting other regulatory pathways, possibly from the kidney to close the bone-kidney axis loop. In addition, metabolic acidosis is another setting where phosphate appears to regulate FGF23. In this regard, low levels of FGF23 can be restored to normal after oral phosphate therapy in chronic metabolic acidosis.⁶⁵

Pathways controlling serum calcium may also regulate FGF23. In this regard, elevated FGF23 level is associated with both low calcium intake in the absence of vitamin D deficiency;⁶⁶ whereas suppression of FGF23 is observed in response to the hypocalcemic hormone calcitonin in patients with tumor-induced osteomalacia.^{60,67} However, neither extracellular calcium nor PTH directly stimulate FGF23 promoter activity in osteoblasts,¹⁶ suggesting that their effects may also be indirect or that the response to PTH might be different according to the short vs long term exposure to PTH. We also know that the bone remodelling status varies according to the intermittent vs continuous exposure to PTH. This

reinforces the hypothesis that FGF23 levels are regulated by the bone remodelling status and that PTH might indirectly regulate FGF23 through local bone remodelling factors.

FGF23 Coordinates Bone Mineralization/Turnover and Renal Phosphate Handling

The other function of FGF23 appears to be to serve as the “primary” phosphaturic hormone in a bone-kidney axis to coordinate renal phosphate handling with bone mineralization and possibly bone remodeling activity (Fig. 2C). The possibility that bone is an endocrine organ that secretes FGF23 to coordinate renal phosphate handling to match bone mineralization and turnover has arisen from the study of XLH and ARHR and metabolic bone diseases associated with elevated FGF23.^{26,35,68,69}

PHEX Regulation of FGF23

XLH is caused by mutations of PHEX, or Phosphate regulating gene with homologies to endopeptidases on the X chromosome, a member of the endothelin-converting enzyme family that leads to increased FGF23 gene transcription. Conditional deletion of Phex in the osteoblast lineage in vivo is sufficient to reproduce the Hyp phenotype, suggesting bone is the main source of FGF23.⁷⁰ How Phex mutations lead to increased Fgf23 gene transcription in bone is not clear and the relative importance of Fgf23 production by osteoblasts and osteocytes is not known.

Although an initial study suggested that Phex processes FGF23,⁷¹ subsequent studies failed to establish Phex-dependent cleavage of FGF23.^{34,44,72} Screening of substrate phage libraries by us and others have identified that Phex cleaves small peptides,⁷³ but failed to identify a physiologically relevant substrate for Phex.⁷² The ASARM peptide, a motif in MEPE and Dmp1, is substrate for Phex in vitro⁷⁴ and additional data suggest that accumulation of ASARM as a consequence of inactivation of Phex can impair mineralization.⁷⁵ Although we have shown that ASARM binds to and inhibits Phex activity against a synthetic substrate in vitro,⁶⁹ it is unlikely that ASARM from MEPE is responsible for stimulating FGF23 in Hyp, since ablation of MEPE fails to alter FGF23 expression in Hyp mice.⁷⁶

In the osteoblastic lineage Phex and FGF23 expression are not fully concordant. FGF23 is markedly increased in terminally differentiation osteoblasts embedded in mineralization nodules, equivalent of an osteocyte embedded in bone,^{41,44} whereas Phex is expressed in osteoblasts and osteocytes. Thus, Phex deficiency is necessary, but not sufficient to stimulate FGF23 (a functional Phex is missing in osteoblasts of Hyp mice, but osteoblasts do not upregulate FGF23 expression until they differentiate into osteocytes).²⁶ The importance of additional Fgf23 stimulating factors is illustrated by the paradox that restoration of Phex in transgenic mice using heterologous promoters fails to rescue the elevated FGF23 expression.^{77–79}

DIMP1 Regulation of FGF23

The breakthrough in understanding FGF23 transcriptional regulation in bone came through the comparative analysis of the Hyp mouse homologue of XLH and dentin matrix acidic phosphoprotein 1 (Dmp1) null mice. Ablation of Dmp1, an extracellular matrix SIBLING

protein that regulates mineralization, markedly stimulates the transcription of FGF23 by osteocytes in mice, leading to the discovery that ARHR is caused by inactivating mutations of DMP1.⁸⁰ DMP1 is a multifunctional protein localized to the dendrites of osteocytes and its main function is to act as a nucleator of mineralization. Dmp1 is also reported translocate to the nucleus to regulate gene transcription,⁸¹ however, we have excluded this mechanism for increased Fgf23 transcription in Dmp1 null mice, since overexpression of Dmp1 fails to regulate the Fgf23 promoter. DMP1 contains an RGD domain for integrin binding, an ASARM peptide (which might allow DMP1 to bind to PHEX), a large number of acidic domains, an N-terminal site for binding to MMP9, sites for casein kinase-2-mediated phosphorylation and conserved cleavage sites for bone morphogenetic protein I/Tolloid-like MMPs (BMP1/Tolloid-like MMPs) and cathepsin B. DMP1 exists as a latent protein that is cleaved into 37-kDa and 57-kDa fragments by BMP1 or cathepsin B. The highly phosphorylated C-terminal 57-kDa fragment functions as a nucleator for mineralization. The NH₂-terminal fragment from DMP1 is a proteoglycan with a chondroitin sulfate chain attached through Ser74 that binds to proMMP-9 and may sequester growth factors.⁸² Recent studies indicate that FGF23 is increased in callus during fracture healing, consistent with local matrix derived FGF23 stimulating factors.⁸³

We have excluded several potential mechanisms whereby loss of Dmp1 or Phex leads to increased FGF23 expression in osteocytes. For example, PHEX does not cleave DMP1, indicating that other enzymes are required for DMP1 processing.⁸⁴ Similarly, AS ARM in Dmp1 cannot be responsible for elevations of FGF23 since ablation of Dmp1 leads to increased FGF23 in Dmp1 null mice. Impaired differentiation of osteocytes, an explanation for Dmp1-dependent regulation of Fgf23 proposed by others, cannot be the reason for increased Fgf23 expression, since Fgf23 is not upregulated in earlier stages of the osteoblast lineage. Rather, assessment of bone marrow stromal cultures and explantation of either Hyp⁸⁵ or Dmp1 null bone⁸⁵ into wild-type mice demonstrates that the high expression of FGF23 in osteocytes is an intrinsic/local effect caused by the loss of either Phex or Dmp1. Moreover, there is strong evidence that the proximate cause of increased FGF23 expression in osteocytes in Hyp and Dmp1 null mice is intrinsic to the bone milieu and is mediated by the presence of unknown matrix-derived FGF23 stimulatory factors that are increased as a consequence of either Phex or Dmp1 mutations. In addition, FGFR1-dependent signaling pathways have emerged as important regulator of FGF23 expression in osteocytes. In this regard, OsteoGlophonic Dysplasia (OGD), an autosomal dominant bone dysplastic disorder caused by activating mutations in FGFR1, also has hypophosphatemia and elevated FGF23 levels.⁴⁵

PHEX and DMP1-Dependent Defect in Mineralization: A Comparative Analysis

PHEX and DMP1 are both produced by osteoblasts and osteocytes,^{26,41,44} where they control the mineralization of extracellular matrix, thereby providing signaling pathways for coordinating bone phosphate accretion with renal phosphate conservation through the regulation of Fgf23. Inactivation of Phex leads to two fundamental abnormalities in osteoblasts/osteocytes, 1) elevations of FGF23, which leads to hypophosphatemia and decreased 1,25(OH)₂D production and increments in a putative mineralization inhibitor called Minhibin, that leads to an intrinsic defect in the mineralization of extracellular matrix

that is independent of phosphate.⁸⁶ Inactivation of either Phex or Dmp1 in mice results in nearly identical increments in FGF23 production by osteocytes and overlapping phenotypes characterized by hypophosphatemia, aberrant vitamin D metabolism and rickets/osteomalacia.^{26,80,87,88} Ablation of FGF23 corrects these abnormalities in both Phex- and Dmp1-deficient mice.^{26,87} However, restoring phosphate and 1,25(OH)₂D serum levels in Hyp does not completely correct the mineralization defect.⁸⁹

FGF23-dependent hypophosphatemia contributes to impaired mineralization in Hyp and Dmp1 null mice, but we know that the increase in FGF23 levels is not the only cause for bone mineralization defects even if recent studies suggest that FGF23 may also have direct effects on bone to inhibit mineralization.⁹⁰ Indeed, we failed to detect Klotho expression in bone on microarray analysis, consistent with the failure to detect Klotho transcripts in bone by RT-PCR.⁹¹ FGF23 also failed to stimulate FGF23 promoter activity in cultured osteoblasts. Consequently, it is unlikely that putative direct actions of FGF23 are mediated by FGFR-Klotho-dependent mechanisms. Rather, bone explantation studies indicate that an intrinsic defect in mineralization of extracellular matrix persists in Phex mutant mice, even when phosphate is normalized.⁸⁵ In Dmp1-null mice, the absence of the nucleation and mineral propagation functions of DMP1 likely contribute to the defective mineralization.^{37,92} Indeed, Col 1 a-3.6 promoter-mediated overexpression of Dmp1 in osteoblast lineage is sufficient to rescue the increased Fgf23 expression and bone phenotype in Dmp1 null mice.⁸⁴ The mechanism whereby Phex mutations lead to impaired mineralization is less clear. Phex gene restoration in Hyp mice only partially corrects the bone phenotype and does not have any impact on the hypophosphatemia.^{77,78} PHEX does not cleave DMP1, indicating that other enzymes are required for DMP1 processing.⁸⁴ Another possibility is that the ASARM peptides derived from Mepe regulate mineralization in Hyp, since the ASARM peptides accumulate in Hyp bone⁷⁵ and deletion of MEPE in Hyp leads to increased mineralization of osteoblasts ex vivo.⁷⁶ One of the reason for ASARM accumulation in Hyp bone is that Phex is able to bind ASARM⁷⁵ and to cleave it⁷⁴ in normal condition. Moreover, Hyp bone is characterized by elevated proteolytic activity that is associated with the increased production of an ASARM peptide from MEPE and DMP1 and the administration of cathepsin inhibitors improves bone mineralization in Hyp mice, without correcting FGF23 expression or hypophosphatemia, indicating the separate regulation of hypophosphatemia and mineralization by PHEX.⁹³ However, deletion of MEPE from Hyp mice fail to rescues the rickets or osteomalacia, suggest that other factors may be important in mediating the intrinsic mineralization defect resulting from PHEX mutations.⁷⁶

Gene expression profiling in Hyp bone also identified several previously unrecognized abnormalities in factors regulating mineralization in Hyp bone. For example, we found that the expression of matrix Gla-protein (Mgp), a inhibitor of mineralization,^{94,96} was increased in Hyp bone. Thrombospondin 4 (Thbs4) is also markedly increased in Hyp bone as well as Hyp-derived cultured osteoblasts. Thbs4 is a member of the group B thrombospondin family of extracellular matrix glycoproteins containing heparin- and calcium-binding sites, as well as binding sites for collagens I, II, III and V, laminin-1, fibronectin and matrilin-2. Thbs4 is known to be expressed in osteogenic tissues,^{97,98} but its physiological function in bone has not been established. However, the observations that ablation of the related Thbs3 results in accelerated endochondrial ossification⁹⁹ and over expression of Thbs1 inhibits

mineralization in osteoblast cultures,¹⁰⁰ suggest that increased *Thbs4* in Hyp osteoblasts may play a role in the intrinsic mineralization defect. We found that carbonic anhydrase 12 (*Car 12*) and 3 (*Car3*) as well as the sodium-dependent citrate transporter (*Slc13a5*), are decreased in Hyp bone. *Car12* and *Car 3* are members of the family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and thereby have the potential to influence mineralization and osteoclast function by regulating the pH in the local milieu. While little is known about the membrane bound *Car 12* in bone, *Car2*, a cytosolic member of this family causes autosomal recessive osteopetrosis via disruption of osteoclast function.¹⁰¹ The reduction of *Slc 13a5* might lead to local increases in citrate levels, which could impair mineralization, as well as deprive the cell of citrate necessary for energy metabolism in Hyp bone. With regards to known regulators of mineralization, we found no significant changes in *Dmp1*, *Enpp1* and *Tnap* in Hyp bone. *Ank (1.7)* and *Enpp6 (1.7)* were respectively increased and decreased in Hyp bone.

Integrative Model Explaining PHEX and DMP1 Regulation of FGF23

Based on our current understanding of PHEX and DMP1 structure and function, we proposed a model that outlines a possible mechanism for local regulation of FGF23 expression in osteocytes by PHEX and DMP1. The model predicts that in wild-type bone DMP1 and PHEX suppress FGF23 transcription, possibly through sequestration of putative FGF23 stimulating factors. The model also predicts that *Dmp1* binds to *Phex* via its ASARM motif and integrins via its RGD sequence located in the 57 kDa C-terminal fragment to form a PHEX-DMP1-integrin “ternary complex”, required for suppression of FGF23 transcription. Recent studies found that $\alpha v \beta 3$ integrins bind to FGF1 leading to FGFR activation.¹⁰² The ASARM motif in SIBLING protein MEPE binds to PHEX and inhibit its activity^{103,104} and DMP1, which also contains the ASARM motif, inhibits PHEX activity, providing indirect evidence for DMP1-PHEX interactions. The C terminus of DMP1, which contains RGD integrin-binding and ASARM domains, is sufficient to rescue the phenotype of *Dmp1*-null mice,¹⁰⁵ suggesting that this region holds the key to understanding FGF23 regulation.

In contrast, inactivating mutations of PHEX and DMP1 increase FGF23 expression in this model via indirect effects mediated by the production and accumulation in the bone matrix of an unidentified PHEX substrate or interacting protein that acts as an FGF23-stimulating factor. We know that DMP1 is not a PHEX substrate. Impaired processing of DMP1 by other enzymes, such as BMP 1, would also be predicted stimulate FGF23, by creating a functional DMP1-deficient state equivalent to *Dmp1*-null mice. Regardless, loss of PHEX or DMP1 result in the production or increased bioavailability of an unknown, local, bone-derived “FGF23 stimulating factor” or less likely an intrinsic cellular defect, leading to increased FGF23.

FGFR 1 and possible paracrine/autocrine effects of other members of the FGF family may be a proximal signal mediating the effects of PHEX and DMP1 mutations. First, activating mutations of FGFR 1 are associated with increased FGF23,⁴⁵ Second, microarray analysis of Hyp bone and *Fgf23* promoter analysis have identified FGF-dependent regulation of *Fgf23* transcription. Third, FGF have phosphaturic effects. For example, FGF2 is synthesized by

osteoblasts, deposited in extracellular matrix and exerts autocrine or paracrine effects to inhibit osteoblastic differentiation,¹⁰⁶ also causes hypophosphatemia,¹⁰⁷ possibly through stimulation of FGF23. The phosphaturic effects of FGF7 might also be mediated via FGFR stimulation of FGF23.¹⁰⁸ Other putative phosphatonins, such as MEPE and secreted frizzled-related protein 4 (sFRP4) are also incorporated in this model. For example, MEPE actions may be mediated by binding of the ASARM peptide to PHEX and inhibition of its activity.^{68,69} sFRP4 might inhibit BMP1-mediated processing of DMP1, leading to elevated FGF23 levels.

Evidence That Bone Turnover Regulates FGF23 Production

Several studies report elevation of FGF23 levels in association with primary alterations in bone remodelling. In case of low bone turnover, the hypothesis would predict that when bone resorption and formation activities are so reduced that there is almost no replacement of the existing mineralized matrix and therefore limited capacity for bone to buffer phosphate. Under these circumstances the continuous intake of phosphate creates a need for increasing phosphate excretion to avoid hyperphosphatemia that is met by increased FGF23 production by bone. A recent study supports the idea that the degree of bone turnover regulates FGF23 production by bone. In this study, low bone remodelling induced by antiresorptive agents, was associated with increased FGF23 circulating levels.⁵⁸ On the other hand, low bone turnover caused by low PTH levels in Gcm2 knockout mice are associated with low serum FGF23 levels. This might be explained by the dual effect of loss of PTH to inhibit bone remodeling and decrease 1,25(OH)₂D levels, the later which are necessary for maintaining FGF23 levels.¹⁶ Idiopathic Phosphate Diabetes (IPD) leading to male osteoporosis have normal serum phosphate but low FGF23 serum levels.¹⁰⁹ Thus, the relationship between low bone remodelling and FGF23 levels is not always concordant.

In case of high bone turnover, the replacement rate of the existing matrix is such that no time is allowed to the resulting phosphate release to be buffered by bone and that existing reserves in phosphate are being released in excess.¹¹⁰ Two different high bone turnover bone disease are associated with elevated FGF23 levels. The McCune-Albright syndrome (MIM 174800), also called Polyostotic Fibrous Dysplasia (PFD), is caused by a mosaicism for postzygotic activating mutations in the guanine nucleotide binding protein, alpha stimulating gene (*GNAS1*). This syndrome is characterized by the replacement of the medullary bone by fibrodysplastic tissue, together with hypophosphatemia and elevated circulating FGF23 levels in some patients.¹¹⁰ Also, in Chronic Kidney Diseases (CKD), FGF23 levels are markedly increased and its actions to decrease CYP27B1 and increase CYP24 lead to diminish calcitriol levels and contributes to the development of secondary hyperparathyroidism.¹¹¹ In addition, parathyroidectomy is associated with reduced FGF23 levels in patients with renal failure, suggesting that PTH-mediated high bone turnover may lead to increased FGF23 production. The pattern of FGF23 expression in CKD, unlike the osteocytic predominance observed in hereditary hypophosphatemic disorders is located in peritrabecular osteoblasts. The common feature between McCune Albright syndrome and CKD is that both have excessive bone turnover mediated by activation of Gas. FGF23 producing mesenchymal tumours causing Tumour-Induced Osteomalacia (TIO) may represent an benign neoplasia that produces FGF23 in a mesenchymal cell lineage.¹¹² Also,

fracture healing is associated with an increase in FGF23 mRNA expression, particularly in osteoblasts and granulation tissue in the fracture callus. Whether increased FGF23 expression in these “high turnover” disorders is through a common pathway is not known. Osteoblasts express PTH receptors and addition of PTH or PTHrP to primary osteoblast cultures increases FGF23 expression.¹¹³ In vivo, PTH(1-34) treatment increases FGF23 levels as well as bone turnover in some studies.⁵⁸ Other studies, however, found that administration of PTH to mice in doses resulting in increased bone remodeling resulted in a reduction of FGF23.⁵⁸ In addition, 1,25(OH)₂D deficient mice (1 alpha hydroxylase knock-out) display increased PTH, increased bone formation but undetectable FGF23 which revokes the hypothesis that PTH directly regulates FGF23 production.⁵⁸ Since the direct effects of PTH on FGF23 remain controversial, the origin of FGF23 in mesenchymal lineages and the diversity of clinical states leading to increased FGF23, suggests that special cells emerge during high turnover states that produce FGF23. Multiple myeloma which leads to increased bone resorption is associated with increased FGF23 levels may be an example of this mechanism. Chronic metabolic acidosis which causes increased bone resorption and impaired mineralization is also associated with increased circulating FGF23 levels and fractional excretion of phosphate.⁶⁵ FGF23 serum levels in senile or postmenopausal osteoporosis, in which bone resorption is also enhanced, has not yet been evaluated.

CONCLUSION

Bone metabolism is integrated with the gastrointestinal tract and kidney to maintain mineral homeostasis. Bone is both responsive to physiochemical and hormones, such as PTH and 1,25(OH)₂D, that regulate mineral metabolism and is also a source of FGF23, which targets the kidney and parathyroid gland to modulate phosphate 1,25(OH)₂D and PTH levels. Thus, in addition to its function as a storage compartment of minerals in equilibrium with systemic milieu, bone, through its hormonal actions of FGF23, actually regulates the systemic milieu to adjust serum phosphate and 1,25(OH)₂D levels in response to the bone remodelling/mineralization state and vitamin D status. In normal physiological conditions, a healthy skeleton with normal buffering capacity and adequate remodelling activity, basal physiological levels of FGF23 would contribute to the equilibrium between bone phosphate flux, renal phosphate reabsorption and 1,25(OH)₂D-mediated gastrointestinal phosphate absorption. However, when there is a primary defect in bone mineralization, in which extracellular phosphate is not deposited into the collagenous matrix, FGF23 would be increased to increase the loss of phosphate in the urine to re-establish systemic phosphate homeostasis. Similarly, in states of primary reductions in bone remodelling status, in which bone resorption and formation activities are so reduced that the phosphate buffering capacity of bone is impaired, FGF23 would be increased to shunt the phosphate from bone deposition to kidney excretion.¹¹⁴ The other extreme of bone remodelling, characterized by large release of phosphate from bone resorption in excess of that which can be redeposited into existing matrix, might also lead to increased FGF23-mediated phosphate wasting.¹¹⁴ FGF23-mediated coordination of bone metabolism and systemic phosphate homeostasis provides an important physiological pathway to prevent the potential adverse effects of hyperphosphatemia and also to protect the organism from the potential toxic effects of excess vitamin D. Many questions remain regarding FGF23 regulation and function. Given

the severity of the phenotypes of having too little or too much FGF23 in animal models, it is likely that alterations of FGF23 are clinically relevant in a variety of human diseases. If so, FGF23 levels may be an important biomarkers and agents that either stimulate or inhibit FGF23-mediated effects might be therapeutically useful in respectively managing disorders caused by FGF23 deficiency and excess.

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Figure 1.

Regulation and function of FGF23. Local and systemic factors regulate FGF23 expression principally in osteocytes in bone. Circulating FGF23 targets koltho/FGFR complexes in a limited number of tissues, including kidney, PTG, choroid plexus and pituitary. The main physiological functions of FGF23 are to inhibit renal phosphate reabsorption and 1,25(OH)₂D production by the kidney.

Figure 2. Comparison of PTH and FGF23 functions. A) PTH-Vitamin D axis. B) FGF23 as a Vitamin D counter regulatory hormone. C) FGF23 in a Bone-Kidney axis coordinating bone remodeling and mineralization with renal phosphate handling. Adapted with permission from Quarles LD. J Clin Invest 2008; 118(12):3820-8.¹