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REGULATION AND FUNCTION OF THE FGF23/KLOTHO ENDOCRINE PATHWAYS

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Abstract

Calcium (Ca^{2+}) and phosphate (PO_4^{3-}) homeostasis are coordinated by systemic and local factors that regulate intestinal absorption, influx and efflux from bone, and kidney excretion and reabsorption of these ions through a complex hormonal network. Traditionally, the parathyroid hormone (PTH)/vitamin D axis provided the conceptual framework to understand mineral metabolism. PTH secreted by the parathyroid gland in response to hypocalcemia functions to maintain serum Ca^{2+} levels by increasing Ca^{2+} reabsorption and 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] production by the kidney, enhancing Ca^{2+} and PO_4^{3-} intestinal absorption and increasing Ca^{2+} and PO_4^{3-} efflux from bone, while maintaining neutral phosphate balance through phosphaturic effects. FGF23 is a recently discovered hormone, predominately produced by osteoblasts/osteocytes, whose major functions are to inhibit renal tubular phosphate reabsorption and suppress circulating $1,25(OH)_2D$ levels by decreasing Cyp27b1-mediated formation and stimulating Cyp24-mediated catabolism of $1,25(OH)_2D$. FGF23 participates in a new bone/kidney axis that protects the organism from excess vitamin D and coordinates renal PO_4^{3-} handling with bone mineralization/turnover. Abnormalities of FGF23 production underlie many inherited and acquired disorders of phosphate homeostasis. This review discusses the known and emerging functions of FGF23, its regulation in response to systemic and local signals, as well as the implications of FGF23 in different pathological and physiological contexts.

I. INTRODUCTION

The parathyroid hormone (PTH)-vitamin D axis has provided the basis for our conceptualization of bone and mineral homeostasis, but recent discovery of the fibroblast growth factor (FGF)23 bone-kidney axis regulating vitamin D metabolism and renal phosphate handling have led to new insights into physiology and pathophysiology of mineral metabolism.

Comprehensive reviews of vitamin D metabolism and PTH functions have been published previously in this journal (83). Briefly, the principal function of the PTH-vitamin D axis is to maintain serum calcium levels in a narrow range by stimulating 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] production and decreasing urinary calcium excretion by the kidney. PTH also increases calcium efflux from bone. PTH secretion is predominately regulated by the calcium-sensing receptor (CASR) located in the parathyroid gland, which responds to decrements in serum ionized calcium by increasing the secretion of PTH, an 84-amino acid peptide that targets PTHR1 G protein-coupled receptors that are highly expressed in the renal tubules and osteoblasts/osteocytes in bone. PTH stimulates the production of

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$1,25(OH)_2D$ in the proximal tubule by increasing CYP27b1 and increases calcium reabsorption in the distal tubule through regulation of TRPV5 (31). In bone, PTH increases calcium and phosphate efflux through stimulation of RANKL by osteoblasts, which in turn stimulates osteoclast-mediated bone resorption. In addition, increased $1,25(OH)_2D$ production by the kidney targets the small intestines to increase absorption of both calcium and phosphate. The combined effects of efflux of calcium from bone, conservation of calcium by the kidney, and increased dietary absorption of calcium restores serum calcium to normal. The increased phosphate efflux from bone and influx from the gastrointestinal track is balanced by PTH effects to decrease renal tubular phosphate reabsorption to maintain neutral phosphate balance.

In contrast, the FGF23-bone-kidney axis is part of newly discovered biological systems linking bone to other organ functions through a complex endocrine network that is integrated with the PTH/vitamin D axis and which plays an equally important role in health and disease. The discovery that osteoblasts and osteocytes are the principal site for FGF23 production and secretion identified bone, not only as the major reservoir for calcium and phosphate, but as an endocrine organ that communicates with other organs involved in mineral homeostasis. FGF23 secreted by bone targets the kidney to regulate renal phosphate handing and vitamin D metabolism (225). The FGF23 bone/kidney axis has at least two physiological functions: *1*) to provide a phosphaturic signal emanating from bone to coordinate bone phosphate flux due to alterations in bone turnover and mineralization with renal conservation of phosphate and *2*) to provide a counterregulatory hormone to protect the organism from adverse effects of excessive vitamin D exposure by FGF23-mediated suppression of $1,25(OH)_2D$ production and increased catabolism by the kidney. FGF23 may also have other functions to regulate phosphate and parathyroid gland functions, but additional knowledge is needed to fully understand the inconsistent data regarding regulation of FGF23 by phosphate and the physiological importance of a possible PTG-bone axis involving reciprocal regulation of PTH and FGF23.

II. FGF23 ORIGIN AND STRUCTURE

A. The FGF Family

Although FGFs are involved in diverse functions including development, repair, or metabolism, all derive from an *Fgf13-like* ancestral gene and share a conserved ~120-residue core structural domain with ~30–60% identity which provides the basis for their classification. The human/mouse *Fgf* gene family comprises 22 members from Fgf1 to Fgf23. Fgf15 and Fgf19 are orthologs in vertebrates (80), with Fgf15 being absent in human and Fgf19 absent in mouse. The FGF family can be divided into seven phylogenetic subfamilies composing three groups according to their action mechanisms (81): the intracellular, the canonical, and the hormone-like *Fgf* genes (FIGURE 1).

The intracellular FGF group includes the Fgf11/12/13/14 subfamily. These FGFs act as intracellular signaling molecules in an FGF receptor (FGFR)-independent manner (52, 223). The canonical FGF group includes the Fgf1/2/5, Fgf3/4/6, Fgf7/10/22, Fgf8/17/18, and Fgf9/16/20 subfamilies. Canonical FGFs mediate their biological responses as extracellular proteins by binding to and activating cell surface tyrosine kinase FGFR with heparin/heparan sulfate as a cofactor. They act as local signaling molecules in an autocrine/paracrine manner (80, 81, 201). The hormone-like or endocrine FGF group includes the Fgf19/21/23 subfamily. In contrast to the canonical FGF group, hormone-like FGFs act systemically as endocrine factors. However, they also mediate their response through FGFR-dependent mechanisms.

B. Hormone-like FGFs: The Endocrine Subfamily

In contrast to intracellular and canonical FGFs identified in invertebrates and vertebrates, hormone-like FGFs, *Fgf15/19*, *Fgf21*, and *Fgf23*, are vertebrate specific. The ancestral *Fgf15-like* gene of hormone-like FGFs was generated from the ancestral *Fgf4-like* gene of canonical FGFs by gene duplication early in vertebrate evolution. Later, *Fgf15/19*, *Fgf21*, and *Fgf23* were generated from the ancestral *Fgf15-like* gene by genome duplication events. Canonical FGFs have a heparin-binding site necessary for the stable binding of FGFRs and local signaling. In contrast, during their evolution, hormone-like FGFs acquired endocrine functions by reduction of the heparin-binding affinity and presence of a novel COOH terminus that permits activation of FGF receptors in the absence of heparin (79). Specifically, a conformational change occurs mainly in the β10–β12 region containing the residues accounting for the heparin affinity (51). On the one hand, the weak heparin binding affinity of the endocrine FGFs prevents them from being captured in the extracellular matrix and thus allows them to function as circulating endocrine factors. On the other hand, the reduced affinity for heparin/heparin sulfate also prevents direct interaction between endocrine FGFs and FGFRs (232). Instead of heparin, endocrine FGFs require alternate cofactors to mediate their effects through FGFRs. Many tissues express one or more FGFR isoforms that potentially function as receptors for FGFs. Thereby, the expression of the cofactor in a tissue determines the target organ of any endocrine FGF to control metabolism and ensure hormonal specificity (44).

FGF15/19 requires βKlotho as a cofactor (97, 111). Fgf15/19 regulates bile acid metabolism in the liver. There is still controversy regarding the role of βKlotho as the FGF21 cofactor. In vitro data suggested that βKlotho is essential for its activity (153). But this was not demonstrated in vivo: FGF21 signals are still transduced in the absence of βKlotho, βKlotho does not precipitate FGF21, and the FGF21 knockout mice do not reproduce the βKlotho knockout phenotype (203). Fgf21 regulates lipid metabolism in the white adipose tissue. Finally, FGF23 requires the αKlotho cofactor (207). FGF23 regulates serum phosphate and active vitamin D levels. The function of FGF23 and its regulation by local and systemic factors are the focus of the present review.

C. FGF23

FGF23 is a member of the FGF19 subfamily of endocrine FGFs, which also includes FGF15/19 and FGF21. FGF23 shows the highest degree of homology with FGF21. FGF23 was first identified in the ventrolateral thalamic nucleus of the mouse brain (225), and its importance was revealed in patients with autosomal dominant hypophosphatemic rickets (ADHR) (2). The missense mutation of the *Fgf23* gene resulting in ADHR confers resistance to the proteolytic cleavage of the FGF23 protein and consequently increased serum levels of full-length active FGF23 and disease development. Fgf23 is mainly expressed by osteocytes and osteoblasts in bone, but it is also expressed by salivary glands, stomach, and at much lower concentrations by other tissues, including skeletal muscle, brain, mammary gland, liver, and heart.

The *Fgf23* gene is located on the human chromosome 12 and mouse chromosome 6 and extends over 8.5 kb. It is composed by 3 exons separated by 2 introns and encodes a 32-kDa glycoprotein containing 251 amino acid residues. The protein comprises a 24 amino acids hydrophobic signal sequence, an NH_2 terminal of 154 amino acids containing the FGF core homology region, and a characteristic 73 amino acids COOH-terminal domain. After cleavage of the 24 amino acids signal sequence, and *O*-glycosylation by UDP-*N*-acetyl-α-D-galactosamine:polypeptide *N*-acetylgalactosaminyl-transferase 3 (GALNT3), the mature protein ^{25}-FGF23-^{251} is secreted into the circulation. In the bloodstream, the FGF23 protein circulates in two distinct forms: a full-length mature form (^{25}-FGF23-^{251}) and a shorter form

(^{25}-FGF23-^{179}) lacking the unique 73-amino acid COOH-terminal tail (7, 226). The shorter form arises from proteolytic cleavage at the ^{176}RXXR^{179} site, which follows the β10–β12 region of the FGF core homology region of FGF23 (51, 180, 217). Only the full-length form of FGF23 is active, since the COOH-terminal domain is essential for interaction with the cofactor αKlotho and downstream activation of FGFR signaling (51). *O*-glycosylation of FGF23 occurs in the 162–228 region (180) overlapping the ^{176}RXXR^{179} cleavage site, and this posttranslational modification appears to protect FGF23 from cleavage by subtilisin-like proprotein convertases when using recombinant peptides in vitro (85).

Fgf23 mutant mice have been created to determine the function of FGF23. Overexpression in transgenic animals or the parenteral administration of FGF23 to rodents suppresses phosphate reabsorption and inhibits the synthesis of 1,25$(OH)_2$D in the proximal renal tubules (9, 102, 182). Conversely, FGF23-null mice or humans with homozygous missense mutations in FGF23 develop severe hyperphosphatemia, elevated 1,25$(OH)_2$D levels, and soft-tissue calcifications (120, 178, 188).

III. FGF23 FUNCTION

A. Klotho and FGF Receptor Complexes

1. Klotho and Tissue Specificity—Klotho genes encode cofactor proteins imparting the tissue-specific action of heparin-independent endocrine FGFs. βKlotho determines the organ targets for FGF15/19. The βklotho gene encodes a single-pass transmembrane protein expressed in adipose tissue, liver, and pancreas. Mice deficient in βklotho have overlapping phenotypes with mice lacking FGF15 or FGFR4.

αKlotho was discovered while studying the phenotype of transgenic mice overexpressing the rabbit type I sodium-proton exchanger (95, 96). Indeed, one animal displayed a particular phenotype resembling aging, caused by the insertional mutation of the transgene in the promoter region of what will be later identified as the Klotho gene. The homozygous animals for the inserted transgene had a shorter life span and precociously developed pathologies related to aging including osteoporosis (86), hypogonadotropic hypogonadism, arteriosclerosis, skin atrophy, pulmonary emphysema (78, 169), neurodegenerative (5, 146), and auditory syndromes (84, 197).

The five exons of αklotho gene encode a 1,014-amino acid single pass, type I transmembrane, and β-glycosidase-like protein with β-glucuronidase activity. The αklotho protein shares 41% amino acid identity with βklotho. αKlotho is predominantly expressed in the kidney and the epithelium of the choroid plexus in the brain (108). In addition to these tissues, low expression of αklotho is also reported in the pituitary gland, placenta, skeletal muscle, urinary bladder, aorta, pancreas, testis, ovary, and colon (13, 96). Knock-in of the lacZ gene downstream of the translational initiation codon of αklotho also shows expression of αklotho in the parathyroid gland and sinoatrial cells of the heart (181, 196).

The target organs for FGF23 are defined by the coexpression of the membrane form of αKlotho and FGFRs (98, 207). The importance of the membrane protein αKlotho in FGF23 signaling is illustrated by both human and mouse genetic disorders where loss of αKlotho results in end-organ resistance to Fgf23, but abnormalities resemble Fgf23 deficiency (71, 75, 96, 173). Subsequent studies have identified αKlotho as being the necessary cofactor for FGF23, forming complexes with FGFRs and increasing their affinity for FGF23 (98, 207).

2. Soluble klotho—Although the exact functions of soluble Klotho are yet to be defined, circulating Klotho can arise from increased gene transcription of the alternatively spliced secreted isoform or from ectodomain shedding of membrane extracellular domain of full-

length αKlotho. The extracellular domain of the αKlotho protein is cleaved and secreted into the blood, cerebrospinal fluid (CSF), and urine by ADAM10 and ADAM17, two members of the A Desintegrin and Metalloproteinase (ADAM) family (26). In addition, αKlotho gene encodes a truncated, secreted form derived from alternative RNA splicing (133). Compared with the transmembrane form, this truncated gene product does not have the second internal repeat of the extracellular domain, the transmembrane domain, or the intracellular domain (133, 185). It only encodes the NH_2-terminal half of αKlotho with its extracellular domain. The relative contribution of ectodomain shedding and alternative splicing to the circulating Klotho levels remains uncertain.

Soluble Klotho acts as a humoral factor (72), potentially through an unknown cell-surface Klotho receptor, and as an enzyme (25, 202) regulating several cell surface glycoproteins. Indeed, secreted αKlotho inhibits insulin- and insulin-like growth factor I (IGF-I)-induced autophosphorylation of insulin and IGF-I receptor in vitro (99, 218). This effect is consistent with an anti-aging role for klotho since downregulation of IGF-I signaling pathway extends lifespan (34, 67). A well-established role of the secreted protein is to inhibit internalization of cell-surface calcium channel “transient receptor potential cation channel, subfamily V, member 5” (TRPV5), primarily responsible for the Ca^{2+} entry in transepithelial Ca^{2+} reabsorption in the kidney (24, 25). Klotho is indeed coexpressed with TRPV5, the Na^+/Ca^{2+} exchanger 1 (NCX1) and calbindin-D28K (a vitamin D-sensitive intracellular Ca^{2+} transporting protein) in a specialized region of the distal convoluted tubules where transepithelial Ca^{2+} reabsorption is actively regulated. This colocalization is important for the homeostatic control of Ca^{2+}. Indeed, mice lacking TRPV5 display diminished renal Ca^{2+} reabsorption, which causes severe hypercalciuria. The secreted form of Klotho also potentiates the action of $1,25(OH)_2D$ on renal reabsorption, as Klotho stabilizes TRPV5 on the membrane by hydrolyzing the extracellular sugar moieties of TRPV5. Similarly, soluble klotho also stabilizes calcium channel “transient receptor potential cation channel, subfamily V, member 6” (TRPV6), which is expressed in the intestinal and mammary epithelial cells (124). Soluble Klotho also regulates Ca^{2+} homeostasis by controlling PTH secretion (75), but these findings have not yet been confirmed. Moreover, intravenous infusion of soluble Klotho caused hypophosphatemia and phosphaturia, suggesting a phosphaturic role for the circulating form (69) independent of FGF23 signaling. Finally, Klotho is reportedly regulating “renal outer medullary potassium channel” (ROMK1) in the kidney (24).

Finally, emerging evidence reveals that Klotho deficiency is an early biomarker for chronic kidney disease (CKD) (70). Indeed, Klotho deficiency is associated with progression and chronic complications in CKD including vascular calcification and cardiac hypertrophy (3, 88, 227) in animal and human studies, and replacement of soluble Klotho and/or forced overexpression (59, 141, 210) protects the kidney from renal injuries, preserves kidney function, and suppresses renal fibrosis.

3. FGF receptors—The mammalian FGFRs are encoded by four distinct genes (*Fgfr1–Fgfr4*). The ectodomain of prototypical FGFRs consists of three Ig-like domains (D1–D3). A major alternative mRNA splicing event within the D3 of FGFR 1 to 3 generates “b” and “c” isoforms, which have distinct FGF-binding specificities. An additional splicing event generates shorter FGFR1–3 isoforms lacking D1 and/or D1–D2 linker.

FGF23 binds to multiple FGFRc isoforms in vitro, but has low affinity to the receptors (Kd 200–700 nM) and cofactors such as heparin/heparan sulfate do not function as with classical FGF/FGFR interactions to activate FGF signaling (12, 228). Rather, the unique feature of FGF19 family of FGF is that they require Klotho gene for FGFR activation. The COOH terminus of FGF23 forms a trimeric complex with αKlotho and the c isoforms of FGFR 1, 3, and 4. The ability of αKlotho to bind to FGFR2 is lower than with the other FGFRs. Binding

of αKlotho to FGFRs changes their affinity to FGFs. FGF23 binds to an FGFR-αKlotho complex with much higher affinity than FGFR alone (98, 207), and FGF23 signaling is maximum when binding to FGFR1c-αKlotho complexes (207).

The hypothesized mechanism for FGF23 bioactivity with αKlotho is the recruitment of FGFRs to form heteromeric complexes that signal through the mitogen-activated protein kinase (MAPK) cascade (94, 207). By interacting with FGFR-αKlotho complexes, FGF23 initiates downstream signaling events through a variety of intracellular signaling proteins including FRS2, Gab1, Shc, PLC, or STAT1. These are phosphorylated in response to FGFR activation leading to a possible mechanism for differential and/or additive gene expression and suggest differential responses according to the additive involvement of multiple FGFRs (201).

B. Renal Targets

The major consequences of FGF23 excess are hypophosphatemia, aberrant vitamin D metabolism, impaired growth, and rickets/osteomalacia (9, 45, 102). Inversely, ablation of FGF23 in mice results in hyperphosphatemia, excess $1,25(OH)_2D$, and soft tissue calcifications (120, 178). Thus most of the known physiological function of FGF23 to regulate mineral metabolism can be accounted for by actions of this hormone on the kidney. On the one hand, the reduction of circulating $1,25(OH)_2D$ levels in response to FGF23 excess is due to the regulation of the anabolic and catabolic events through inhibition of 25-hydroxyvitamin D 1-α-hydroxylase (*Cyp27b1*) and stimulation of 1,25-dihydroxyvitamin D 24-hydroxylase (*Cyp24a1*) in the proximal tubules (9, 102, 114, 179, 183). Cyp27b1 and Cyp24a1 are the renal enzymes respectively responsible for the synthesis of the bioactive form of vitamin D and the initiation of the degradation of the bioactive form of vitamin D into calcitroic acid. On the other hand, the hypophosphatemia induced by FGF23 excess is mediated by the inhibition of the solute carrier family 34 member 1 (*SLC34a1, Npt2a, NaPi2a*) and member 3 (*SLC34a3, Npt2c, NaPi2c*) sodium-dependent phosphate cotransporters in the renal proximal tubules.

1. Vitamin D metabolism—The best characterized physiological function of FGF23 is to act as a vitamin D counterregulatory hormone (117). Prior to the discovery of FGF23, it was assumed that phosphate regulation occurred as a secondary action of PTH and $1,25(OH)_2D$. In hypophosphatemic disorders caused by FGF23 excess, $1,25(OH)_2D$ levels are abnormally suppressed for the degree of hypophosphatemia, which should increase $1,25(OH)_2D$ levels. FGF23 reduces $1,25(OH)_2D$ levels due to complex effects on Cyp27b1 and Cyp24a1 to decrease the production and increase the catabolism of $1,25(OH)_2D$ (76). Studies demonstrate that increased FGF23 inhibits Cyp27b1 and stimulates Cyp24a1 expressions, which is opposite from what one would anticipate in the presence of high circulating PTH levels and concomitant hypophosphatemia (9, 177, 178).

Interestingly, the stimulatory effect of FGF23 on Cyp24a1 expression (177) and the reduction of serum $1,25(OH)_2D$ levels are vitamin D receptor (VDR)-dependent mechanisms (76). The effects of FGF23 on Cyp27b1 are not consistent throughout the available literature. Injection of recombinant FGF23 to wild-type mice results in a dose-dependent decrease in renal abundance of Cyp27b1 and exerts a direct action on Cyp27b1 gene expression in human and mouse renal proximal tubule cells via an ERK1/2-dependent mechanism (159). Paradoxically, increased Cyp27b1 mRNA expression has been reported in association with FGF23 excess (43, 231), however, with a message to functional protein translational defect. In the Hyp mouse, murine homolog of human X-linked hypophosphatemic rickets (XLH) which displays excess FGF23, defects in translation of Cyp27b1 message have also been reported (28, 199). Taken together, these studies suggest

that the mechanism of action of FGF23 on the enzymes that regulate $1,25(OH)_2D$ production and degradation may differ from acute to chronic FGF23 excess (9, 136, 177, 178). Finally, FGF23 action on vitamin D metabolism is not dependent on a functional $1,25(OH)_2D$-VDR system, as treatment of VDR null mice with FGF23 further decreased hypophosphatemia due to reduced renal and intestinal phosphate absorption, accompanied by decreased NaPi2a, NaPi2b, and Cyp27b1 expression (142, 183).

2. Phosphate reabsorption—The kidney plays a central role in phosphate homeostasis. It adjusts urinary excretion of phosphate according to phosphate intake and maintains the serum phosphate concentration within a narrow range. Phosphate is reabsorbed almost exclusively in the renal proximal tubule through a transcellular pathway. The limiting step of this transepithelial transport system is the entry of phosphate at the apical domain of proximal tubular cells. This process requires sodium- dependent phosphate cotransporters that use the inward sodium gradient established and maintained by the activity of the Na^+-K^+-ATPase pump. There are three types of sodium-dependent phosphate cotransporters in renal proximal tubular cells: the type 1 cotransporter (NPT1) is an anion carrier that does not specifically mediate phosphate transport; the type 2 cotransporter family includes three carriers, NPT2a (encoded by the SLC34a1 gene), NPT2b (encoded by the SLC34a2 gene), and NPT2c (encoded by the SLC34a3 gene); and the type 3 comprises two transporters, PiT1 (encoded by the SLC20a1 gene) and PiT2 (encoded by the SLC20A2 gene).

It has been shown that FGF23 suppresses the expression of NPT2a and NPT2c and consequently can increase urinary phosphate excretion (47, 177). NPT2a is central to renal phosphate reabsorption and phosphate balance, and found almost exclusively in the apical membrane of renal proximal tubular cells. NPT2c is the isoform expressed at the brush-border membrane of the renal proximal tubular cells. Due to FGF23 effects on vitamin D metabolism (see above), and PTH (see below), it has been hypothesized that FGF23 regulation of NPT2 may be due to the reduction of serum $1,25(OH)_2D$ levels, and/or increased PTH. However, these actions are vitamin D independent (76, 183), and effects of FGF23 on serum phosphate can be observed in parathyroidectomized rats (177).

Whereas FGF23 effects on the Npt2c isoform appear to be variable, continuous exposure to recombinant FGF23 was shown to cause increased renal phosphate clearance resulting from decreased renal expression of NPT2a (9, 10, 102, 173, 177, 179, 183). The phosphaturic effect of FGF23 is further convincingly demonstrated in vivo. For instance, transgenic mice overexpressing Fgf23 have severe urinary phosphate wasting due to the suppression of renal Npt2 expression and/or activity (9, 182). Inversely, Fgf23 null mice characterized by severe hyperphosphatemia and ectopic soft tissue calcification display increased renal expression of Npt2a (101, 187). Genetically restoring the systemic actions of FGF23 in Fgf23 null mice reversed hyperphosphatemia to hypophosphatemia and prevented ectopic calcification (32).

C. Mechanisms for FGF23 Function in the Kidney

Interactions between αKlotho and FGFR are necessary to mediate FGF23 signaling, but the mechanisms underlying FGF23 bioactivity in vivo are unclear. First, at least three different FGFR have shown increased affinity for FGF23 when complexed with αKlotho (see sect. III*A2*). Second, the limiting cofactor αKlotho predominately localizes to the renal distal convoluted tubule (DCT), and FGF23 biological responses on NPT2 isoforms and vitamin D metabolizing enzymes are observed within the proximal tubules (PT) (114, 117, 179). Thus uncertainty exists regarding the physiologically relevant FGFR for FGF23 in the kidney and the precise tubular segments that are targeted by FGF23.

1. Relevant FGF receptor(s) for FGF23 signaling—Although FGF23 binds to FGFR1c, FGFR3c, FGFR4, and but not FGFR2c in vitro (96–98), there is strong support for FGFR1c:αKlotho being the relevant target for FGF23 in the kidney (119, 207). First, FGFR1:Klotho complexes have been identified as the principal binding partner for FGF23 (207). Second, neither loss of Fgfr3 nor Fgfr4 rescues the effects of FGF23 excess, hypophosphatemia, and aberrant vitamin D metabolism in the Hyp mice (119). Third, the conditional deletion of Fgfr1 in the kidney abolishes the phosphaturic effects of recombinant FGF23 administration (47). However, administration of recombinant FGF23 to FGFR4$^{-/-}$ caused a smaller decrease in serum phosphorus levels compared with wild-type or FGFR3$^{-/-}$ control mice (47). Additionally, in our studies, FGF23 levels were further elevated in Hyp/Fgfr3$^{-/-}$ and Hyp/Fgfr4$^{-/-}$ mice (119), consistent with end-organ resistance to FGF23 caused by loss of FGFR3 or FGFR4. Moreover, combined loss of FGFR3 and FGFR4 in Hyp mice partially corrected the hypophosphatemia and Npt2-dependent transport defect in the proximal tubule, and increased the production of 1,25$(OH)_2$D in wild-type mice (107).

Taken together, these data suggest that *1*) FGFR1 may be the principal FGF23 receptor mediating FGF23 phosphaturic effects; *2*) renal FGF23 effects are mediated through multiple FGF receptors and FGFR1, FGFR3, and FGFR4 may have redundant roles; and *3*) FGFR3 and -4, rather than FGFR1, mediate FGF23 effects on vitamin D metabolism.

2. Distal to proximal tubular paracrine feedback mechanism—The mechanisms underlying the differential effects of FGFRs to regulate proximal tubular phosphate and vitamin D metabolism are not clear, since both FGF23 binding and signaling pathways do not differ between FGFR1, -3, and -4 (98). FGFR3 is expressed in the PT (21, 119), whereas FGFR1, -3, and -4 are expressed in the distal tubules (119). However, the limiting cofactor αKlotho, as mentioned earlier, is predominately expressed in the distal tubule (108), and exogenous FGF23 administration in mice induces phospho-ERK1/2 only within the DCTs (38). Recent studies show weak presence of αKlotho protein and mRNA in the proximal tubule (69); however, these studies did not determine if the detected transcripts encode for the membrane and/or secreted forms. Ex vivo studies of proximal tubular segments or cell lines have demonstrated variable effects of exogenously added FGF23 to inhibit NPT2 (165, 179). Interpretation of these findings is confounded by the use of nonphysiological amounts of FGF23 and the authenticity of the proximal tubular phenotype in cell culture models, which may be contaminated with distal tubular cells and/or undergo dedifferentiation (15, 165, 166, 224, 228).

Independently of these findings, the highest levels of the FGFR:αKlotho complexes are in the distal tubules, whereas the biological actions of FGF23 are observed in the proximal tubules (108), which theoretically excludes a major direct effect of FGF23 on proximal tubules. Alternatively, FGF23 actions on the proximal tubule may be indirect, possibly through FGF23 stimulation of the distal tubule and release of paracrine factors that regulate proximal tubule function (i.e., a “distal-to-proximal tubular feedback mechanism”) among which soluble Klotho protein might be the ideal candidate. This mechanism is rendered possible in vivo, by the close proximity of proximal and distal cells, and is supported by studies showing that αKlotho can be released into the circulation from the distal tubule by either ectodomain shedding or secretion of an αKlotho isoform lacking the transmembrane domain. Accordingly, FGF23 decreases the expression of αKlotho by the kidney, thereby creating complex feedback pathways for regulating phosphate and calcium metabolism (75, 128). Overexpression of αKlotho causes phosphaturia (69, 145), and forced expression of αKlotho in the proximal tubule cells or cell-free membrane vesicles decreases the insertion of Npt2a into the membrane (69). This supports the hypothesis of secreted Klotho mediating

some of the effects of FGF23. Distinguishing between direct and indirect effects of FGF23 on renal tubular function requires additional studies.

Indeed, hereditary hypophosphatemia and hyperparathyroidism (HHH) is caused by a promoter region translocation that increases αKlotho expression and its circulating levels (145). However, this results in both elevated FGF23 and PTH (17), and it is hypothesized that αKlotho might directly regulate PTH secretion through its maintenance of cell surface Na^+-K^+-ATPase activity (75). This makes the interpretation of the animal models findings more complex and suggests that αKlotho phosphaturic effects are indirectly mediated through stimulation of PTH and FGF23. Moreover, the hypothesis that soluble αKlotho sequestrates Npt2a cotransporter independently of FGF23 (69) does not explain the FGF23-mediated suppression of Npt2a mRNA expression observed in states of excess FGF23, and is inconsistent with recent data showing that serum αKlotho levels are normal in patients with XLH (22). Furthermore, the Fgf23 and αKlotho double-knockout ($Fgf23^{-/-}$/ $\alpha Klotho^{-/-}$) mouse model provide compelling evidence that αKlotho does not have an FGF23-independent role in the regulation of systemic phosphate and vitamin D homeostasis, as their phenotype is similar to the single, $Fgf23^{-/-}$ or $\alpha Klotho^{-/-}$, mutant mice with regard to phosphate and vitamin D metabolism (148). If the distal to proximal feedback mechanism appears to be the only plausible explanation for FGF23 renal effects (FIGURE 2*A*), αKlotho does not seem to be the paracrine factor, responsible for conveying FGF23 signaling from the distal to the proximal tubules.

D. Extrarenal Targets

Rescue experiments of $FGF23^{-/-}$ mice by transgenic expression of human FGF23 in osteoblasts tend to demonstrate that FGF23 acts in a systemic manner (32), rather than as an autocrine/paracrine factor. As previously discussed, it appears that FGF23 primarily serves as a systemic factor to regulate phosphate homeostasis and vitamin D metabolism, but FGF23 may also directly signal through extrarenal FGFR/Klotho complexes (FIGURE 2*B*). Although less described, FGF23 also may regulate other genes in both the proximal and distal tubule, in addition to renal effects on phosphate cotransporters and vitamin D metabolism enzymes. Among them, some are likely to mediate FGF23 effects on other organs that do not express Klotho. Human or mouse genetic disorders where loss of Klotho results in end-organ resistance to Fgf23 and abnormalities resembling Fgf23 deficiency (71, 75, 96, 173) tend to plead against Klotho-independent effects. However, as FGF23 is capable of binding FGF receptors in absence of membrane Klotho, although with low affinity, it is plausible that activation of FGF receptors also occurs in presence of high FGF23 levels in organs where Klotho is absent (off-target effects). Finally, it is also plausible that another cofactor, yet undiscovered, may bind and activate FGF receptors.

1. Effects on parathyroid gland—The parathyroid glands express FGF receptors and Klotho, and the acute administration of recombinant FGF23 results in increments in Egr1 expression in parathyroid tissue in mice (207), but the role of FGF23 on the parathyroid gland in normal physiology is not clear (20, 96, 108). On the one hand, recent studies indicate that FGF23 negatively regulates PTH mRNA expression and protein secretion in vitro (92), and that FGF23 suppresses PTH secretion in vivo (13). On the other hand, patients with CKD typically exhibit secondary hyperparathyroidism associated with high serum FGF23 levels, which contradicts the ability of FG23 to suppress PTH secretion. In addition, overexpression of FGF23 in transgenic mice causes secondary hyperparathyroidism (9, 102). These contradictory results may be attributed to local and systemic confounding factors. For instance, Klotho/Fgfr1 expression is decreased in the parathyroid glands of patients and rats with advanced CKD (46, 91), and the functionality of the complexes is also impaired (46). Moreover, Klotho may also have an FGF23-

independent role by facilitating PTH secretion through maintenance of membrane Na^+-K^+-ATPase activity in the setting of hypocalcemia (75), although this mechanism has been recently disputed (131). Nevertheless, given that FGF23 suppresses Klotho expression, whereas 1,25$(OH)_2$D positively regulates it (206), increased FGF23 and hypovitaminosis D should impair Klotho-driven PTH secretion, in addition to considerably reducing FGF23 signaling, which may explain why the PTH resist the putative inhibitory effects of FGF23 in CKD. Furthermore, low 1,25$(OH)_2$D and subsequent hypocalcemia could also be responsible for secondary hyperparathyroidism. Nonetheless, serum FGF23 correlates to PTH in predialysis CKD patients (176, 213) and in patients with early CKD (213) when levels of phosphate and calcium are maintained within normal range. Moreover, a strong positive correlation between FGF23 and PTH is also found in XLH subjects (22) who are normocalcemic and display low to normal levels of 1,25$(OH)_2$D. On the contrary, Fgf23 null mice display low PTH levels (178) consistent with a stimulatory effect of FGF23 of PTH secretion.

Finally, Fgf23 null mice overexpressing human recombinant FGF23 display increased PTH (32) despite normal calcium and 1,25$(OH)_2$D, and hypophosphatemia, consistent with the hypothesis that FGF23 acts directly on the parathyroid gland to induce PTH production. Taken together, these results suggest that FGF23 and PTH may form a regulatory loop (refer to sect. IV*A*) similar to FGF23-vitamin D loop, but some of the FGF23-PTH associations could be attributed to alterations of other systemic and local factors.

2. Effects on skeleton—Physiological phosphate balance is of crucial biological importance to skeletal mineralization, and as a master regulator of phosphate homeostasis, FGF23 is bound to affect bone metabolism, cellular function, and mineralization. Similarly, by regulating 1,25$(OH)_2$D and PTH, the most described hormonal regulators of bone, and subsequent calcium changes due to 1,25$(OH)_2$D and PTH, FGF23 affects bone development and function. Although FGF23 does not act independently of Klotho in the systemic regulation of vitamin D and phosphate homeostasis (148), and despite reported Klotho absence from mineralized tissues, there is a debate regarding whether FGF23 has direct effects or the bone changes are to be attributed to phosphate, 1,25$(OH)_2$D, and PTH. Studies of FGF23 in human on genetic and acquired diseases and those using animal models have demonstrated that both under- and overexpression (2, 120, 177, 187) of FGF23 result in impairments in bone metabolism. Overexpression of FGF23 in mice causes hypophosphatemia, reduced 1,25$(OH)_2$D levels, and rickets/osteomalacia (182). The bones exhibit widened and disorganized growth plates and reduced bone mineral density. Although the defective skeletal mineralization observed in patients and animal models with FGF23 excess is likely a consequence of low phosphorus and vitamin D values, FGF23 treatment of primary calvarial osteoblasts from wild-type mice (186) and osteoblast-like cells (175) leads to an inhibition of mineralization. Consistent with these findings, studies in animal models and in cell culture suggest that FGF23 has also a direct effect on bone (208, 209). Overall, these results suggest that excess of FGF23 can negatively affect bone mineralization. On the opposite, FGF23 deficiency results in severe hyperphosphatemia, hypervitaminosis D, and increased circulating calcium (178, 187). These animals also display a severe bone phenotype, characterized by a disorganized growth plate lacking hypertrophic chondrocytes and decreased mineralized bone mass with increased osteoid (178, 193). Ablation of vitamin D or vitamin D signaling (62, 188) and decreasing the circulating levels of phosphate by dietary (193) or genetic approaches (186) significantly improved the abnormal phenotype associated with lack of Fgf23 activities. However, skeletal abnormalities observed in $Fgf23^{-/-}$ mice including the decrease in hypertrophic chondrocytes in the growth plate, the increased mineral deposition adjacent to the growth plate, and the osteomalacic phenotype were found to be similar to $Fgf23^{-/-}$/$NaPi2a^{-/-}$ compound mutants, despite the significantly reduced serum phosphate levels. These findings (186) suggest that Fgf23 may be an

essential factor necessary for bone development, although the increased $1,25(OH)_2D$ serum levels alone may be responsible for osteomalacia in $Fgf23^{-/-}$ mice. Indeed, studies have convincingly demonstrated that rats treated with high doses of $1,25(OH)_2D$ have impaired bone mineralization (35, 220). Since deletion of VDR partially rescues the bone phenotype of FGF23 null mice (62, 188, 191), the direct effects of FGF23 on bone may represent nonspecific effects, comparable to other FGFs. However, rescue of FGF23 null mice by transgenic overexpression of human recombinant FGF23 does not rescue the bone phenotype, despite normalization of $1,25(OH)_2D$ and calcium levels, which suggest that FGF23 has a cell autonomous effect (32). Alternatively, as both FGF receptors (100, 212), specifically FGFR1 and FGFR4, and Klotho (96) are expressed during myogenesis, skeletal FGF23 effects could be mediated through the muscular sites. Despite reported FGF23 expression in the skeletal muscle (2), none, to our knowledge, has studied FGF23 implication in myogenesis.

3. Effects on other organs—FGF23 action on organs other than kidney and parathyroid remain unstudied, with the exception of bone, mainly because current data support that FGF23, even at supraphysiological concentrations, likely has no effect without the presence of Klotho. However, little is known about FGF23 effects on organs that do express Klotho such as the brain (108) and the cardiovascular system (13, 96, 181, 196). The lack of studies in these areas is likely due to the masking of toxic effects of hyperphosphatemia and elevated $1,25(OH)_2D$ levels that are admitted to be primarily responsible for increased mortality in absence of FGF23 signaling (62, 186, 191).

Theoretically FGF23 may also have central effects on FGFR/Klotho complexes in the choroid plexus and pituitary where FGF23 is also expressed. Despite the fact that in stages with very high FGF23 such as end-stage renal disease (ESRD), cognitive dysfunction is highly prevalent in patients when compared with the general population (39, 174), no direct relationship was established between FGF23 and cognitive impairement.

Understanding the extrarenal functions of FGF23 is of particular importance in patients with renal failure (103, 211) for whom FGF23 is a marker of early cardiovascular changes (139). Despite established Klotho expression in heart and aorta (181, 196), FGF23 effects were believed to be indirect or off-target effects only occurring at supraphysiological concentrations. Nonetheless, FGF23 is positively associated with left ventricular mass index and increased risk of having left ventricular hypertrophy (57, 68, 104, 138, 140).

Additional abnormalities are associated with FGF23, such as abnormalities in glucose homeostasis, growth retardation, abnormalities in thymic function, and ageing phenotypes (62, 120, 178, 187), consistent with a broader role for FGF23. However, many of the toxic effects of Fgf23 deficiency are indirect consequences of the concurrent elevated $1,25(OH)_2D$ actions, since generating compound FGF23 null and either Cyp27B1 or VDR null mice, results in the disappearance of abnormal findings and soft tissue calcifications (62). However, both $Fgf23^{-/-}$ and $Klotho^{-/-}$ (148, 155) showed thymic atrophy and a reduced number of splenocytes, indicating that FGF23 signaling could influence hematopoiesis. Given that FGF23 is physiologically expressed in the thymus and that FGF23 is elevated in plasma cell dyscrasias (190), this suggests that FGF23 may well play a role in lymphopoiesis and hematopoiesis. Moreover, high doses of FGF23 can induce proliferation of murine bone marrow-derived pro-B cell lines (228), since Klotho is reported absent in lymphatic organs including bone marrow, thymus, and spleen (148).

IV. FGF23 REGULATION

A. Systemic Regulators of FGF23

1. Promoter activation by 1,25(OH)$_2$D—1,25(OH)$_2$D is the most important systemic factor regulating FGF23. The administration of 1,25(OH)$_2$D increases FGF23 levels, while the disruption of 1,25(OH)$_2$D pathways reduces circulating FGF23 in mice (117, 166). Increased 1,25(OH)$_2$D targets the gastrointestinal tract to increase calcium and phosphate absorption. Increments in calcium along with 1,25(OH)$_2$D target the parathyroid gland to suppress PTH, which in turn targets the kidney to increase urinary calcium excretion to maintain neutral calcium balance. However, lowering of PTH levels decreases phosphate excretion and would potentially result in positive phosphate balance from vitamin D-mediated increase in gastrointestinal phosphate absorption if not for compensatory elevations of FGF23, which also suppresses 1,25(OH)$_2$D to counter the increase in vitamin D (117). This constitutes a classical hormonal loop: increased 1,25(OH)$_2$D→increased FGF23→decreased 1,25(OH)$_2$D. The expression of FGF23 is regulated by both VDR-dependent and VDR-independent signaling. Stimulation of the 1,25(OH)$_2$D-VDR pathway induces the expression of FGF23, as evidenced by increased FGF23 levels after 1,25(OH)$_2$D administration. In line with these findings, VDR null mice showed undetectable FGF23 levels (183, 229). In addition, normalization of serum calcium and phosphate levels by dietary means increased FGF23 levels in VDR null mice, indicating that FGF23 expression is also regulated by a VDR-independent pathway (102, 128, 177, 182).

2. Regulation by Phosphate—As of today, the effects of phosphate on FGF23 remain unclear. Unlike calcium, which has a calcium sensing receptor (CaSR) that permits the sensing and tight control of calcium levels, a phosphate sensor has not been identified and the regulation of serum phosphate levels is not so tightly controlled. Phosphate loading in mice increases FGF23 levels (158), but the magnitude of the phosphate regulation of FGF23 is small compared with the effects of 1,25(OH)$_2$D and the importance of dietary phosphate in regulating FGF23 in humans is conflicting (41, 152). In humans, it was shown that serum FGF23 was regulated by dietary phosphate (41), whereas a subsequent study has shown that humans consuming diets containing increasing amounts of phosphate displayed decreased FGF23 concentration. Overall, in humans, the changes of secreted FGF23 are quite variable and modest when measured after high- or low-phosphate diets of long duration (18, 103, 158). In rodents, high-phosphate diet increases circulating FGF23, but this seems to be a 1,25(OH)$_2$D-VDR dependent mechanism, as dietary phosphate failed to increase FGF23 expression in absence of VDR (183). This might indicate that in case of low or high vitamin D, the determinant factor for FGF23 is 1,25(OH)$_2$D (152). FGF23 levels are also elevated in renal failure, and the degree of elevation correlates with the degree of hyperphosphatemia (211). In these settings, phosphate restriction failed to lower elevated FGF23 levels in patients with CKD (77). Use of phosphate binders in combination with dietary phosphate restriction sufficient to lower urinary phosphate excretion were less effective than expected in reducing FGF23 levels. Among the phosphate binders, only sevelamer hydrochloride, a binder known to provide an acidic load which may alter bone phosphate flux, mildly lowered FGF23 levels without significant changes in serum calcium or phosphate levels in humans (156). Similar studies involving uremic rats showed that the administration of sevelamer required 2 wk to achieve reductions in PTH and FGF23, whereas correction in hyperphosphatemia occurred rapidly (147). A high phosphorus diet was shown to enhance, and a low phosphorus diet to inhibit, the elevation of serum FGF23 levels in nephrectomized rats, but this result was obtained after 4 wk of dietary treatment (166). Altogether these data show that FGF23 may vary in animals and patients without any changes in serum phosphate levels, suggesting that phosphate load, rather than plasma levels, should be considered for regulation of FGF23. If true, this also suggests that circulating phosphorus levels do not

adequately reflect phosphorus balance and that serum phosphate is not the major regulator of FGF23, at least in CKD. Additionally, low calcium intake is also associated with hypophosphatemia and elevated FGF23 levels in the absence of vitamin D deficiency in African and Asian populations (160). However, neither extracellular calcium nor phosphate directly stimulates FGF23 expression in osteoblast cultures (117).

3. Regulation by PTH—As mentioned in section III*D1*, a controversy is emerging regarding the interactions between PTH and FGF23. A PTH-FGF23 feedback loop (increased PTH → increased FGF23 → decreased PTH) challenges the simply "FGF23 counterregulatory hormone for $1,25(OH)_2D$" hypothesis. The effect of PTH to increase FGF23 expression is now well established. Indeed, FGF23 is increased in primary hyperparathyroidism (87), in Jansens' metaphyseal chondrodysplasia caused by activating PTH/PTHrp receptor mutations (16), and parathyroidectomy results in decrease in FGF23 in CKD (170). Moreover, PTH directly stimulates FGF23 gene expression in vitro (105). The ability of PTH to stimulate FGF23, however, is context dependent. For example, PTH failed to directly stimulate FGF23 production or FGF23 promoter activity in ROS17/2.8 osteoblast-like cells (117), or in calvarial culture (167). Furthermore, PTH null mice on Hyp background (8) and patients with hypoparathyroidism (54) display increased FGF23 levels, despite low levels of PTH. The mechanism underlying the variable effects of PTH on FGF23 gene expression is not known, but may involve VDR-dependent pathways. In this regard, vitamin D- and vitamin D receptor-deficient mice show abnormally low levels of FGF23 despite severe hyperparathyroidism (76, 168), and injection of vitamin D into PTH-deficient mice restores FGF23 production (117). Altogether, this indicates that PTH effects on FGF23 may be dependent on vitamin D and mineral status. Indirect effects to stimulate FGF23 could be mediated through PTH-mediated increases in $1,25(OH)_2D$ or the presence of cofactors modulating PTH effects. The apparent ability of PTH to increase or decrease FGF23 might reflect the differential anabolic and catabolic effects of PTH on bone remodeling. In this regard, intermittent administration of PTH leading to net increments in bone formation results in reduced FGF23 levels (168), consistent with the need to conserve phosphate. In contrast, continuous administration of PTH that leads to catabolic effects on bone might be predicted to stimulate FGF23, which would help eliminate the increased phosphate efflux from bone.

4. Other systemic regulators—Elevated $1,25(OH)_2D$ is closely correlated to increased fat mass in humans (49, 93) and mice (135, 205) and accompanied by a sustained decrease in 25(OH)D, which will in turn deepen the hyperparathyroidism and amplify its effects on the skeleton. Recently, it has been shown that leptin, a hormone secreted by white adipose tissue that acts centrally on hypothalamus and peripherally on other organs to control energy intake, expenditure, and bone homeostasis, stimulates FGF23 production in bone (205). Consistent with this, administration of intraperitoneal leptin in leptin-deficient *ob*/*ob* mice corrected abnormally elevated $1,25(OH)_2D$, calcium, and phosphate (134, 135). Furthermore, injection of FGF23 in leptin-deficient *ob*/*ob* mice corrected the over-production of $1,25(OH)_2D$, whereas addition of leptin to renal tubular cells did not modify CYP27b1 activity. Leptin as a marker of lipid metabolism should be found elevated together with FGF23 in pathologies with increased cardiovascular risks. This hypothesis is strengthened by the fact that in a recent study in humans (137), FGF23 has been associated with fat mass and dyslipidemia. In this context, bone appears by all standards as an endocrine organ (161) in a more complex model of multiorgan interaction, in which bone plays a central role to integrate both its endocrine functions in controlling energy and phosphate metabolism. As FGF23 is believed to be mainly a skeletal hormone, many of the factors regulating bone metabolism would impact upon FGF23 synthesis. Estrogen deficiency is a major cause of osteoporosis as estrogens play a key role in balancing bone

remodeling directly (127, 163) and indirectly through modulation of PTH, calcium, and vitamin D signaling (110, 151, 198). Recent studies have found that estrogen treatment of ovariectomized rats caused hyperphosphaturia and hypophosphatemia due to the downregulation of the NaPi2a cotransporter in the renal proximal tubule and independently of changes in serum PTH levels (37). Consistently, estrogens correlated in a dose-dependent manner with the magnitude of the FGF23 increments, suggesting that estrogens may be a potent stimulator for FGF23 (23). Similarly, recent studies, although unpublished, reference glucocorticoid-regulated FGF23 production, in the context of pathogenesis of glucocorticoid-induced bone disease.

There is also evidence that the kidney may secrete factors or lead to other systemic effects that regulate FGF23, thereby closing a feedback loop. For example, FGF23 levels were slightly elevated in Hyp/Fgfr3$^{-/-}$ and Hyp/Fgfr4$^{-/-}$ mice (119) and more so in Hyp/Fgfr3$^{-/-}$/Fgfr4$^{-/-}$ (107). This is consistent with end-organ resistance to FGF23 caused by loss of FGFR3 and FGFR4 and a feedback mechanism linking end-organ resistance to compensatory increments in FGF23 production by bone. Additionally, in CKD, also progressive increments in FGF23 occur in proportion to the degree of loss of renal function, again suggesting a compensatory mechanism. This factor is not phosphate, per se, since phosphate restriction fails to prevent the increments in FGF23 in CKD.

B. Bone Local Regulators of FGF23 Transcription

1. Regulation by PHEX and DMP1—Phosphate regulating gene with homologies to endopeptidases on the X chromosome (PHEX) is a 106-kDa protein member of the endothelin-converting enzyme family expressed by osteoblasts and osteocytes in bone. Inactivating mutation of *Phex* leads to increased *Fgf23* gene transcription by bone (120). The conditional deletion of *Phex* in the osteoblast lineage in vivo is also sufficient to increase *Fgf23* expression, suggesting that PHEX participates in the local mechanisms regulating *Fgf23* (230). To date, the mechanisms whereby PHEX regulates Fgf23 gene transcription in bone are still unclear, and the relevance for Fgf23 production by osteoblasts and osteocytes is not known. Although an initial study suggested that PHEX processes FGF23 (15), subsequent studies failed to establish Phex-dependent cleavage of FGF23 (13, 53, 113). Screening of substrate phage libraries by us and others have identified that PHEX cleaves small peptides (19), but failed to identify a physiologically relevant substrate for PHEX (53). The ASARM peptide, a motif in MEPE and DMP1, is a substrate for PHEX in vitro (1). Additional data suggest that accumulation of ASARM as a consequence of inactivation of Phex can impair mineralization (129) and phosphate homeostasis (29). We have shown that ASARM binds to and inhibits PHEX activity against a synthetic substrate in vitro (115), but 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 (30, 112).

Dentin matrix protein 1 (DMP1) is a 94-kDa member of the SIBLINGs extracellular matrix proteins. Similar to PHEX, DMP1 is expressed by osteoblasts and osteocytes in bone. Inactivation of DMP1 also leads to increased Fgf23 expression in bone (40, 121, 122). The main function of DMP1 is to regulate the mineralization of the extracellular matrix (48, 61). DMP1 exists as a latent protein that is cleaved into 37- and 57-kDa fragments by BMP1 or cathepsin B (195). The highly phosphorylated COOH-terminal 57-kDa fragment of DMP1 contains an RGD domain for integrin binding and an ASARM peptide for binding to PHEX. The NH_2-terminal 37-kDa fragment of DMP1 is a proteoglycan with a chondroitin sulfate chain attached through Ser-74 that binds to proMMP-9 and may sequester growth factors (154).

The breakthrough in understanding Fgf23 transcriptional regulation in bone came through the comparative analysis of the Phex-mutant Hyp mouse and Dmp1 null mouse (130). The discovery that both mutations induce identical intrinsic abnormalities of mineralization and FGF23-dependent hypophosphatemia provided the initial insights into a bone kidney axis that coordinates bone mineralization and systemic phosphate homeostasis (162). Under physiological conditions, this coordination relies at least in part on the appropriate functions of PHEX and DMP1 to inhibit Fgf23 expression in bone and maintain bone mineralization status (FIGURE 3*A*). The loss of function of DMP1 or PHEX results in the intrinsic increase of Fgf23 by osteocytes (118). Several hypothetical mechanisms possibly leading to this increase have already been excluded. For example, PHEX does not cleave DMP1, indicating that other enzymes are required for DMP1 processing (125). Additionally, DMP1 does not regulate Fgf23 transcription directly by translocation to the nucleus (149), since the overexpression of DMP1 in mice does not decrease FGF23 levels below normal (126). Finally, the ASARM peptide resulting from the degradation of DMP1 (129) is not responsible for the elevation of FGF23, since the ablation of Dmp1 leads to increased FGF23 levels in Dmp1 null mice. Rather, there is stronger 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 this regard, recent studies indicate that FGF23 is increased in callus during fracture healing, consistent with local matrix-derived FGF23-stimulating factors (50).

2. Regulation by FGFs/FGFRs pathway—FGFR-dependent signaling pathways have also 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 (216). FGFs/FGFR pathways may regulate FGF23 expression in bone via canonical, noncanonical, or intracrine pathways.

a) canonical: FGFR activation is mediated by secreted low-molecular-weight FGFs through binding to FGFR in the presence of heparin sulfate. We have identified by microarray gene expression analysis increments in FGF1 mRNA expression in Hyp and Dmp1 bone and have shown the ability of canonical activation of FGFR pathways by addition of recombinant FGF1 and FGF2 to osteoblast cultures stimulates Fgf23 promoter activity (221). These findings are consistent with the effects of long-term administration of FGF2 in vivo to induce hypophosphatemia and impair matrix mineralization (109, 150). FGFs are also stored in the extracellular matrix of bone through binding to heparin sulfate proteoglycans, and increased release of stored FGFs might also stimulate FGFRs. We have also shown that selective inhibition of FGFR blocks increments in FGF23 in Hyp-derived bone marrow stromal cell cultures (unpublished data), consistent with the loss of Phex function imparting FGFR-mediated regulation of FGF23 production. Collectively, these findings suggest that FGFs/FGFR activation is a central pathway regulating FGF23 expression in bone (FIGURE 3*B*).

b) noncanonical: FGFR-protein interactions facilitate FGFR activation. Noncanonical FGFR may serve to either sensitize or desensitize FGFR signaling. Klotho interaction with FGFRs is an example of noncanonical FGFR activation. The extracellular domain of Klotho is expressed by alternative splicing or secreted into the blood and urine by ectodomain shedding, making it theoretically possible that the extracellular domain of Klotho may activate FGFRs in the presence of FGF23, and creating a positive feedback loop. Klotho-dependent noncanonical activation of FGFR1 may explain the unexpected increase in serum FGF23 caused by translocations in humans that increase circulating Klotho levels and

FGF23-mediated hypophosphatemic rickets (17). The ability of $\alpha_v\beta_3$ integrins to bind to FGF1 leading to FGFR activation is another example of noncanonical FGFR activation (144). We believe that this noncanonical FGFR activation is a strong candidate to explain the molecular mechanism whereby PHEX and DMP1 mutations regulate FGF23 expression, since DMP1 comprises the RGD and ASARM sequences allowing the binding with integrins and PHEX, respectively. This association might explain why some FGFR1 mutations lead to increased FGF23 and mutations at other sites in the receptor do not.

c) intracrine: The effects of FGFs that are independent of cell surface FGFR activation have also been described. FGF2 is produced as a high-molecular-weight isoform (HMW) and a low-molecular-weight isoform (LMW) by means of alternative usage of translation start sites in a single Fgf2 mRNA. Both HMW and LMW FGF2 isoforms are synthesized by osteoblasts. While the LMW forms are deposited in extracellular matrix where they are released to activate canonical FGFR pathways (36), HMW-FGFs localize to the nucleus to activate poorly defined “intracrine” pathways. Col1a1 (3.6 kb) promoter-driven HMW-FGF2 overexpression in mice causes hypophosphatemia and increases FGF23 expression (222), suggesting that intracrine functions of FGFs may also play a role in FGF23 regulation (FIGURE 3*B*). In contrast, overexpression of LMW-FGF2 under the control of the same promoter had increased bone mass with normal calcium/phosphate homeostasis (221).

The three mechanisms whereby FGFs/FGFR pathways might regulate FGF23 are not mutually exclusive. These observations implicate either canonical FGFR1 and/or the recently characterized integrative nuclear FGF receptor signaling in regulating FGF23 promoter activity in bone (33). We have proposed a hypothetical model to explain how mutations in Phex and Dmp1 lead to activation of FGFR through both canonical and noncanonical pathways.

3. Novel hypothetical concept linking transcriptional regulation of FGF23 by mineralization-dependent intrinsic matrix factors—

There is growing evidence that another physiological function of FGF23 is to respond to changes in bone mineralization and turnover to adjust renal phosphate handling and balance the phosphate flux from bone. Bone is a buffer for minerals and can release calcium and phosphate into the circulation. Impaired mineralization would impair bone buffering capacity, leading to adaptive changes to excrete greater amounts of phosphate. Consistent with this possibility, there is an inverse relationship between FGF23 production by osteocytes and impaired mineralization.

a) primary defects in bone mineralization can regulate FGF23 production: Inactivating mutations of Enpp1, which more typically causes hereditary generalized arterial calcification of infancy (GACI), can also cause a variant of autosomal recessive hypophosphatemic rickets, characterized by hypophosphatemia and elevated FGF23 levels in some patients (106, 123). Enpp1 generates inorganic pyrophosphate (PP_i), an essential physiological inhibitor of calcification, and substrate for alkaline phosphatase which converts it to P_i necessary for mineralization of bone. The inactivation of Enpp1 reduces the ratio of PP_i to P_i, leading to increased mineralization of soft tissues. Treatment of GACI with bisphosphonates, however, causes hypocalcemia and elevated PTH in these patients, suggesting an unusual sensitivity of bone. Although there is no information on bone histology from GACI patients, studies of Enpp1 null mice indicate defective mineralization in long bones, which is postulated to occur because the PP_i limits the availability of free P_i for mineralization of cortical bone (6). These findings suggest that primary physiochemical-mediated impairment of mineralization can somehow stimulate FGF23 expression in bone. Alternatively, the finding of hypophosphatemia and no evidence of aberrant vascular calcifications GACI patients with elevated FGF23 suggest a systemic pathway whereby ectopic soft tissue calcifications per se, or additional functions for Enpp1 are not yet

discovered (106, 123). The regulation of FGF23 in Enpp1 null mice has not yet been evaluated, but might be informative regarding effects of impaired mineralization on FGF23 expression.

b) bone turnover can regulate FGF23 production: Low turnover bone disease, such as adynamic bone, leads to decreased phosphate buffering by bone, which could lead to increased production of FGF23, similar to defective mineralization. Consistent with this possibility, antiresorptive agent osteoprotegerin produced a profound reduction in bone resorption and formation in male and oophorectomized female mice, accompanied by an increase in serum levels of FGF23 (168). Theoretically, high rates of bone turnover would release calcium and phosphate from bone, leading to calcium-mediated suppression of PTH and elevated FGF23 preventing hyperphosphatemia. There is an association of increased FGF23 and plasma cell dyscrasias (190), but a formal assessment of the effect of increased osteoclastic mediated bone resorption of FGF23 expression in bone has not been performed. It is also possible that the discrepancies between PTH lack of a direct effect on FGF23 promoter activity and the apparent ability of intermittent versus continuous administration of PTH to respectively inhibit and stimulate FGF23 may be due to primary effects of PTH to affect bone remodeling. Also, the recent observation that leptin stimulates FGF23 might also be mediated through effects on bone remodeling and also points to the complex interplay between nutrition, fat, bone, and energy metabolism (205).

Altogether, the concept that bone mineralization and turnover might regulate the local production of FGF23 is an interesting hypothesis that explains the existing observations but requires experimental validation.

C. Posttranslational Regulation of FGF23: Circulating levels of biologically active full-length FGF23 are also regulated by furin-like proteases and the GalNAc transferase 3 (GALNT3), respectively, responsible for the proteolytic processing and initiation of *O*-glycosylation of FGF23. FGF23 contains an RXXR subtilisin-like proprotein convertase recognition sequence motif. The cleavage of the active full-length FGF23 protein at this site generates two inactive 180-amino acid NH2-terminal and 71-amino acid COOH-terminal fragments. This processing can be blocked by selective *O*-glycosylation of the cleavage site by GALNT3 (85). Indeed, mutations of GALNT3 or serine 71/glycine (S71G) and serine 129/phenylalanine (S129F) mutations of FGF23 at additional glycosylation sites result in hyperphosphatemic familial tumoral calcinosis (HFTC) (11, 189, 204). The defective *O*-glycosylation observed in HFTC is associated with hyperphosphatemia and massive ectopic calcifications and leads to low intact FGF23 levels with marked increase of processed COOH-terminal fragments in the circulation. The discovery of these mutations highly contributed to our understanding of the posttranslational regulation of FGF23. On the one hand, the discovery of the mutation of GALNT3 revealed the importance of *O*-glycosylation in preventing FGF23 from degradation by furin-like proteases (132), and on the other hand, the S71G and S129F mutations have shown evidence that *O*-glycosylation is essential and required for externalization of FGF23 (14, 85).

V. PHYSIOLOGICAL AND PATHOLOGICAL IMPLICATIONS OF FGF23

The discovery of FGF23 and its regulation and function, in addition to defining new physiological pathways and networks, has led to a new pathophysiological framework for classifying hereditary and acquired hypo- and hyperphosphatemic disorders, reconsideration of the treatment approaches to manage hereditary hypophosphatemic disorders, new insights into the pathogenesis of disordered mineral metabolism in chronic kidney disease, a mechanism for understanding the mechanism of hyperphosphatemia caused by various

drugs, and identification of possible links between disordered mineral metabolism and cardiovascular mortality.

A. Physiological Implications of FGF23

Over 85% of the total body phosphate is stored in bone in both mineralized matrix and exchangeable pools. Phosphate moves in and out of bone (typically with calcium) in a coordinate fashion with bone mineralization and bone remodeling, the renal handling of phosphate, dietary absorption of phosphate, and acid-base status. In addition to extracellular phosphate being critical for mineralization of bone matrix, intracellular phosphate is critical for energy metabolism and intracellular signaling. Metabolic functions related to intracellular phosphate are most sensitive to hypophosphatemia, whereas excess phosphate overcomes the inhibitory effects of matrix Gla proteins and other factors to induce extraskeletal calcifications and untoward effects on the cardiovascular system and soft tissues leading to increased morbidity and mortality. The ability of excess phosphate to promote soft tissue calcifications is related to bone turnover, such that low bone turnover states are associated with increased vascular calcifications. The bone buffering capacity for phosphate is a difficult entity to quantify, but its effects on systemic phosphate homeostasis are indirectly observed in clinical disorders, such as severe hyperparathyroidism in chronic kidney disease, where excess efflux of phosphate from bone contributes to both hyperphosphatemia and serum calcium levels, and rapid reductions of PTH as occurs with parathyroidectomy results in profound hypophosphatemia and hypocalcemia due to increased uptake of calcium and phosphate in the “hungry bone syndrome.” Given the reservoir and buffering function of bone in phosphate homeostasis, and the toxicity of excess circulating phosphate, regulatory signals arising from bone to coordinate bone turnover and mineralization with systemic phosphate homeostasis would appear to be essential.

Excessive $1,25(OH)_2D$ can exhibit toxicity that is mediated through its effects to increase the gastrointestinal absorption of calcium and phosphate and to stimulate RANKL in bone leading to increased osteoclastogenesis and efflux of calcium and phosphate from the skeleton. The increased calcium will suppress PTH through activation of the CASR in parathyroid glands. Reductions in PTH will reduce the renal production of $1,25(OH)_2D$ through loss of PTH stimulation of CYP27b1. Reductions in PTH also result in loss of the tonic effects of PTH to increase renal phosphate restriction. To prevent hyperphosphatemia in the setting of increased $1,25(OH)_2D$ and reduced PTH requires an additional regulator of renal phosphate handling.

There is much that remains to be discovered about FGF23 regulation and function. We have advanced the hypothesis that the principal physiological functions of FGF23 and the reason for its predominant expression in bone is that this hormone functions to protect the organism from the toxic effects of excess phosphate and $1,25(OH)_2D$. We propose that $1,25(OH)_2D$ directly regulates FGF23 transcription in osteoblasts/osteocytes, but that phosphate, rather than directly modulating FGF23, has indirect effects that are mediated through the effects of phosphate on extracellular matrix mineralization. We also propose that bone formation and the bone buffering capacity of bone provides the mechanistic link between phosphate and regulation of FGF23 through the regulation of extracellular matrix factors involving the endopeptidase PHEX and the SIBLING protein dentin matrix protein 1 (DMP1). Finally, regulation of FGF23 is integrated with mineral and energy metabolism through cross-talk between PTH, $1,25(OH)_2D$, leptin, and secreted Klotho.

B. Hypophosphatemic Disorders Caused by Excess FGF23

There are four hereditary disorders caused by single gene mutations that are associated with increased FGF23 levels by bone in cells of the osteoblast lineage (typically osteocytes). These include *1*) mutations of the RXXR site of FGF23 cleavage in autosomal dominant hypophosphatemic rickets (ADHR; OMIM no. 193100); *2*) inactivating mutations of Phex, a cell surface endopeptidase, in XLH (OMIM no. 307800); *3*) inactivation mutations of DMP1 in autosomal recessive hypophosphatemic rickets 1 (ARHR1; OMIM no. 241520); and *4*) inactivation of ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1), an enzyme that generates pyrophosphate, in autosomal recessive hypophosphatemic rickets 2 (ARHR2; OMIM no. 613312) (TABLE 1).

The biochemical hallmarks of excess FGF23 in these disorders are hypophoshatemia, inappropriately low or normal $1,25(OH)_2D$, and rickets and/or osteomalacia that mostly reflect the skeletal effects of hyphosphatemia. Hyperparathyroidism is often present in states of excess FGF23, likely due to FGF23-mediated reductions in $1,25(OH)_2D$. Inactivation of ENPP1 in ARHR2 differs from the one of infancy, presumably due to the failure to generate the mineralization inhibitor pyrophosphate. PP_i is a substrate for alkaline phosphatase, which increases local P_i in bone. The paradoxical impaired mineralization of bone is likely due to a decreased local production of P_i by alkaline phosphatase due to reductions in the PP_i substrate.

These four mutations are also providing insights into additional regulation mechanisms and physiological functions of FGF23. For example, the mutation in RXXR site points to metabolism of FGF23 as an important point of control of FGF23 levels; however, to date, the specific enzymes and physiological relevance of FGF23 degradation are not known. The mutations in XLH, ARHR1, and ARHR2 point to an important role of the extracellular matrix milieu in regulation of FGF23 expression in osteocytes. Studies of the Hyp and Dmp1 null mouse homologs of XHL and ARHR1 are uncovering pathways whereby PHEX and DMP1 interact to regulate both FGF23 expression and bone mineralization, whereas the alterations in the PP_i/P_i ratio that controls bone mineralization provides additional support for a physiological role of FGF23 to coordinate bone phosphate buffering capacity with renal phosphate handling. The precise mechanisms whereby PHEX, DMP1, and ENPP1 regulate FGF23 expression in osteocytes are not known.

Another single gene missense mutation on Fgfr1 leads to constitutive activation in osteoglophonic dysplasia (OGD; OMIM no. 166250) (TABLE 1) and results in elevated FGF23 and hypophosphatemia, but with different skeletal abnormalities and additional clinical features. Additional evidence links local FGF pathway activation in osteoblasts with FGF23 production (refer to sect. IV*B2*). How Phex and Dmp1 mutations are linked to FGFR1 signaling in bone is an area of current investigations. However, these pathological abnormalities may provide a clue to a physiological function of FGF23, and a possible reason this phosphaturic hormone is predominantly produced in bone, which is to provide a pathway to coordinate bone phosphate buffering capacity related to bone mineralization and turnover with the renal handling of phosphate to maintain systemic phosphate homeostasis.

The hereditary disorder caused by activating mutation in Gnas1, which encodes the alpha subunit of stimulatory G protein, in McCune-Albright syndrome (OMIM no. 174800) and two sporadic/acquired disorders, including tumor-induced osteomalacia (TIO) and hypophosphatemic linear nevus sebaceous syndrome (OMIM no. 163200) (65), also result in elevated FGF23 and hypophosphatemia. GNAS1 leads to variable increase in FGF23 that is limited to fibrotic lesions of bone, whereas TIO is produced from mesenchymal derived tumors and linear nevus sebaceous syndrome (66) appears to have increased bone remodeling as a potential source of FGF23 (TABLE 1). These data suggest that high bone

remodeling states may lead to increased FGF23 expression. However, activating mutations of PTH receptor 1 (PTHR1) observed in Jansen-type metaphyseal chondrodysplasia (OMIM no. 156400) are not reported to result in increased FGF23. Moreover, conditions associated with elevated PTH levels, such as secondary hyperparathyroidism in renal failure and pharmacological stimulation of bone remodeling with exogenous PTH, have variable effects on FGF23 expression. Parathyroidectomy suppresses FGF23 in uremic hyperparathyroidism, suggesting that high PTH in this setting stimulates FGF23 production. In addition, continuous PTH administration stimulates FGF23, whereas intermittent PTH administration does not. Since continuous PTH is catabolic to bone, and intermittent PTH is anabolic, this suggests that bone turnover can regulate FGF23. However, it is difficult to dissociate direct effects of PTH from indirect effects mediated by alterations in $1,25(OH)_2D$ production. Indeed, Cyp27b1 null mice have low FGF23 levels in spite of very high PTH levels.

Finally, a translocation adjacent to αKlotho gene has been reported in a single individual leading to increased circulating αKlotho and FGF23 levels, hyperparathyroidism, and the metabolic bone disease hypophosphatemic rickets and hyperparathyroidism (OMIM no. 612089) (17). As noted above, FGF23 targets FGFR:Klotho complexes in cell membranes. The translocation leading to increased circulating levels of αKlotho suggests that circulating Klotho also may regulate pathways that stimulate FGF23 as well as PTH production.

Further research is needed to understand the interrelationships between bone remodeling, PTH, vitamin D, Klotho, and FGF23. Understanding the molecular mechanisms linking bone mineralization, vitamin D metabolism, and FGF23 production offers the potential to develop more effective and safe therapies for hereditary and acquired hypophosphatemic disorders. FGF23 levels are also proving to be important in diagnosing hereditary and acquired hypophosphatemic and hyperphosphatemic disorders. In addition, the link between $1,25(OH)_2D$ and FGF23 has raised questions about the current use of active vitamin D analogs to treat XLH, which has recently been shown to further increase FGF23 in XLH patients (4, 74).

C. Hyperphosphatemic DDisorders DDue to Disruption of FGF23

In contrast to the FGF23-mediated hypophosphatemic disorders, there are also several hyperphosphatemic diseases caused by FGF23 deficiency including inactivating mutations of GALNT3 (HFTC), FGF23 (HFTC, refer to sect. IV*C*), and Klotho. All of these mutations lead to the clinical syndrome of hyperphosphatemic tumoral calcinosis (OMIM no. 211900), which is characterized by soft tissue and periarticular calcifications, hyperphosphatemia, and elevated serum $1,25(OH)_2D$ levels, caused by the loss of functional FGF23 (71). Mutations of GALNT3 decrease the stability of FGF23, whereas Klotho mutations prevent complex formation with FGF23 and lead to decreased FGF23 signaling.

D. FGF23 in Nonhereditary Disorders

FGF23 may provide a new biological framework to understand the pathogenesis of metabolic bone and mineral disorders in chronic kidney diseases and how disorders of phosphate homeostasis may affect diverse extraskeletal functions, including cardiovascular disease, vascular calcification, and energy metabolism.

1. CKD—CKD leads to increased PTH and FGF23 and decreased circulating $1,25(OH)_2D$ levels, hypocalcemia, hyperphosphatemia, bone disease, vascular calcifications, and cardiovascular morbidities, collectively referred to as chronic kidney disease-mineral and bone disorder (CKD-MBD) (143). The pathogenesis of CKD-MBD is traditionally viewed from the perspective of the PTH-vitamin D axis, and current treatments focus on

suppressing PTH with active vitamin D analogs (215). Prior to the discovery of FGF23, secondary HPT during progression of CKD was thought to be due to a decline in Cyp27b1-mediated production of $1,25(OH)_2D$ by the proximal tubule due to loss of renal mass (27). There is emerging evidence, however, that increased FGF23 is the initial event leading to reductions in $1,25(OH)_2D$ and elevations of PTH in response to loss of renal function. Cross-sectional studies in humans show early FGF23 elevations in CKD in proportion to reduced GFR (56) and greater elevations in ESRD (56, 73, 211). FGF23 levels correlate with the degree of hyperphosphatemia (73, 211) and predict refractory hyperparathyroidism in patients with ESRD (90). Since FGF23 inhibits the production of and stimulates the catabolism of $1,25(OH)_2D$ through its respective inhibition of Cyp27b1 and stimulation of Cyp24, the reduced levels of $1,25(OH)_2D$ in CKD may not represent a true "vitamin D-deficient" state. Rather, FGF23-mediated suppression of circulating $1,25(OH)_2D$ levels is an adaptive response, which protects against hyperphosphatemia through reduction of $1,25(OH)_2D$'s effects on gastrointestinal phosphate absorption. This active mechanism is supported by studies showing that treatment with FGF23 neutralizing antibodies prevents the decrease in serum calcitriol in rats with progressive CKD(60). Additionally, the decline in $1,25(OH)_2D$ levels increases PTH production which acts in concert with FGF23 to stimulate phosphaturia. Treatment approaches to prevent the elevations of FGF23 may become the initial therapeutic focus. Treatment with paracalcitol further elevates FGF23 in ESRD (192). Since calcitriol increases FGF23, calcitriol sparing therapies may be warranted, such as combined low-dose paracalcitol and calcimimetics, which we show lowers FGF23 levels in ESRD patients (214, 215).

There are important gaps in our knowledge of FGF23 regulation and function in CKD. It is unknown how end-organ resistance to FGF23 due to loss of FGFR function and/or the presence of CKD lead to increased FGF23 production in bone and whether this upregulation of FGF23 represents a feedback loop between kidney and bone. New pathways for selective regulation of gene products by FGFR3, -4, and -1 (i.e., selective control of gene products by different FGFRs that may dissociate the regulation of phosphate transport from vitamin D metabolism as well as other functions) are yet to be fully explored.

2. Clarifying the Association between Elevated FGF23 and Mortality—As CKD progresses to ESRD, the progressive increase in FGF23 becomes maladaptive, possibly contributing to more rapid progress of renal failure (42), cardiac dysfunction (68), and vascular calcifications (82) and contributing to excessive morbidity and mortality (58). Analysis of cohorts with ESRD show that increased FGF23 is also associated with increased mortality, independent of serum phosphate levels (58). Elevated FGF23 may have untoward effects on mortality and cardiovascular outcomes in patients without end-stage renal disease (58, 139, 157). Increased FGF23 is associated with cardiovascular disease/mortality in elderly patients with "normal renal function" (139) and in patients with advanced CKD (219). FGF23 may also provide an explanation for the association between low bone remodeling and cardiovascular calcifications, since low osteocalcin expression, as a marker of bone turnover, is associated with high FGF23 and cardiovascular mortality (157). In addition, there is an association between elevations of FGF23 with hypertension and cardiac hypertrophy in patients with XLH (194). There is also an association between increased FGF23 with progression of renal fibrosis in CKD (42).

The mechanisms of the toxic effects of FGF23 are not clear. The association between elevated FGF23 and increased mortality is independent of phosphate levels in ESRD (58), suggesting other factors are involved. At this point, it is not clear whether the untoward effects of FGF23 are mediated by its suppression of Klotho expression by the kidney or other FGF23-regulated kidney-derived factors, or due to off-target effects of the very high levels of FGF23 found in CKD. There is emerging evidence that FGF23 suppresses kidney

expression of ACE2, which unlike ACE, generates peptides, such as angiotensin-(1–7), that have vasodilatation and natriuretic effects to reduce blood pressure (55, 206). FGF23 upregulates midkine, a factor that is linked to increased ACE activity and hypertension in CKD (63). Thus FGF23 excess could impact on the cardiovascular system by suppressing Klotho or by enhancing the renin-angiotensin system (RAS) through multiple mechanisms.

3. TIO—TIO (also called oncogenic osteomalacia) is an acquired paraneoplastic disorder characterized by hypophosphatemia, osteomalacia, and aberrant vitamin D metabolism, similar to the hereditary hypophosphatemic disorders, but caused by increased production of FGF23 by benign tumors. FGF23 was cloned and characterized from tumors causing hypophosphatemic osteomalacia (179), and removal of the tumors results in decreased FGF23 levels (211). Serum FGF23 levels are useful for diagnosing TIO in patients with unexplained hyposphosphatemia, and octreotide sestabmibi scans can be helpful in identifying the site of occult benign mesenchymal neoplasms that secrete FGF23 (64).

4. Drug Treatment Effects—FGF23 bone kidney axis offers new explanations for hypophosphatemia and hyperphosphatemia observed in several other clinical settings. There are several drugs that cause hyper- or hypophosphatemia through modulating FGF23 expression. These include inhibitors of FGFR kinases being developed for cancer therapies that are reported to lead to hyperphosphatemia as well as intravenously injected iron preparations used to treat anemia (171, 172, 184) and glucocorticoids which cause hypophosphatemia. The mechanism of these effects is not entirely clear, but the effects of parenteral iron administration to cause hypophosphatemia through increased FGF23 might be mediated by inhibition of Phex (171) and/or bone mineralization. The effects of receptor tyrosine kinase inhibitors might be due to their effect to block the end-organ effects of FGF23. In addition, prednisone is associated with increased FGF23 as well as osteocytic osteolysis, possibly providing additional evidence for a link between bone mineralization and FGF23 expression (200).

VI. CONCLUSION

The discovery of FGF23 has changed our understanding of mineral metabolism by indentifying more complex cross-talk and endocrine feedback loops between the parathyroid gland, intestines, bone, and kidney to maintain systemic mineral homeostasis, energy metabolism, and bone health. A full understanding of the integrative functions of these hormonal networks remains to be elucidated, but the importance of FGF23 is revealed by the profound effects of its excess or deficiency on mineral homeostasis and the integration of many local and systemic factors to regulate FGF23 expression at the level of the osteocyte in bone. The main functions of FGF23 are its systemic effects to *1*) act as a counterregulatory hormone for $1,25(OH)_2D$ (117) and *2*) coordinate renal phosphate handling to match bone mineralization (40, 115, 120, 164). Unequivocal evidence supporting a principal role in regulating vitamin D metabolism consists of $1,25(OH)_2D$ regulation of FGF23 expression in bone and FGF23 dominant effects to regulate $1,25(OH)_2D$ levels in states of FGF23 excess and deficiency. Evidence for a coupling between mineralization and FGF23 is more indirect, but is suggested by the effect of Dmp1 or Phex deficiency to increased FGF23 production by osteocytes in ARHR and XLH through local matrix factors (116) as well as other data linking alterations in bone turnover and phosphate flux with changes in FGF23. This coupling may regulate phosphate excretion by the kidneys to match the differing rates of phosphate deposition into bone (89, 117, 162). It is likely that many factors that regulate bone as well as energy metabolism will be discovered to also regulate FGF23 expression. Further studies are required to understand the molecular mechanisms whereby local alterations in the bone milieu and systemic factors regulate FGF23 expression in bone.

FGF23 likely has additional functions to regulate PTG function, but it is uncertain whether FGF23 inhibits or stimulates PTG function through the FGFR/Klotho complexes in the PTG. Also, whether the putative direct effects of PTH to regulate FGF23 expression in bone via transcriptional control of FGF23 expression or indirectly due to PTH-induced alterations in bone remodeling/phosphate flux remains to be determined. A proposed PTG-bone axis where PTH stimulates FGF23 and FGF23 inhibits PTH is paradoxical to the findings that PTH does not regulate FGF23 in the absence of $1,25(OH)_2D$ (168) and the effects of FGF23 to suppress $1,25(OH)_2D$ and stimulate PTH. An integrative hypothesis to explain these disparate findings needs to be developed. Similarly, FGF23 is clearly involved in the regulation of phosphate homeostasis, but unlike the tight coupling of changes in serum calcium with PTH secretion that is mediated through the calcium receptor CasR, the relationship between changes in phosphate and FGF23 is more variable and perhaps indirect. The molecular mechanism of phosphate regulation of FGF23 is not known and may involve phosphate effects on bone mineralization. Moreover, FGF23 is likely to have additional functions that are yet to be discovered. Indeed, the autocrine/paracrine effects of FGF23 on bone metabolism and other tissues where FGF23 is expressed in low abundance are poorly understood. Finally, the functions of FGF23 in the brain and pituitary are unknown, despite the fact that it was the first tissue where FGF23 was identified. There are also significant gaps in our understanding of FGF23 effects on the kidney. The apparent functional heterogeneity of FGFR isoform activation by FGF23, the tubular segments targeted by FGF23, and specific gene products regulated by FGF23 remain to be fully defined. Finally, the discovery of FGF23 has had a significant clinical impact, including a better understanding of how to diagnose and treat hereditary and acquired hypophosphatemic disorders, new insights into the pathogenesis of disordered mineral metabolism in chronic kidney disease, a new framework for understanding the mechanism of hyperphosphatemia caused by various drugs, and a molecular mechanism to link disordered mineral metabolism with increased cardiovascular mortality. However, much also remains to be discovered regarding the pathological significance of FGF23 in various diseases states, whether therapeutic strategies should consider the potential biological effects of altered FGF23 expression and if FGF23 itself will become a relevant therapeutic target for enhancing and/or inhibiting FGF23 function in various clinical settings.

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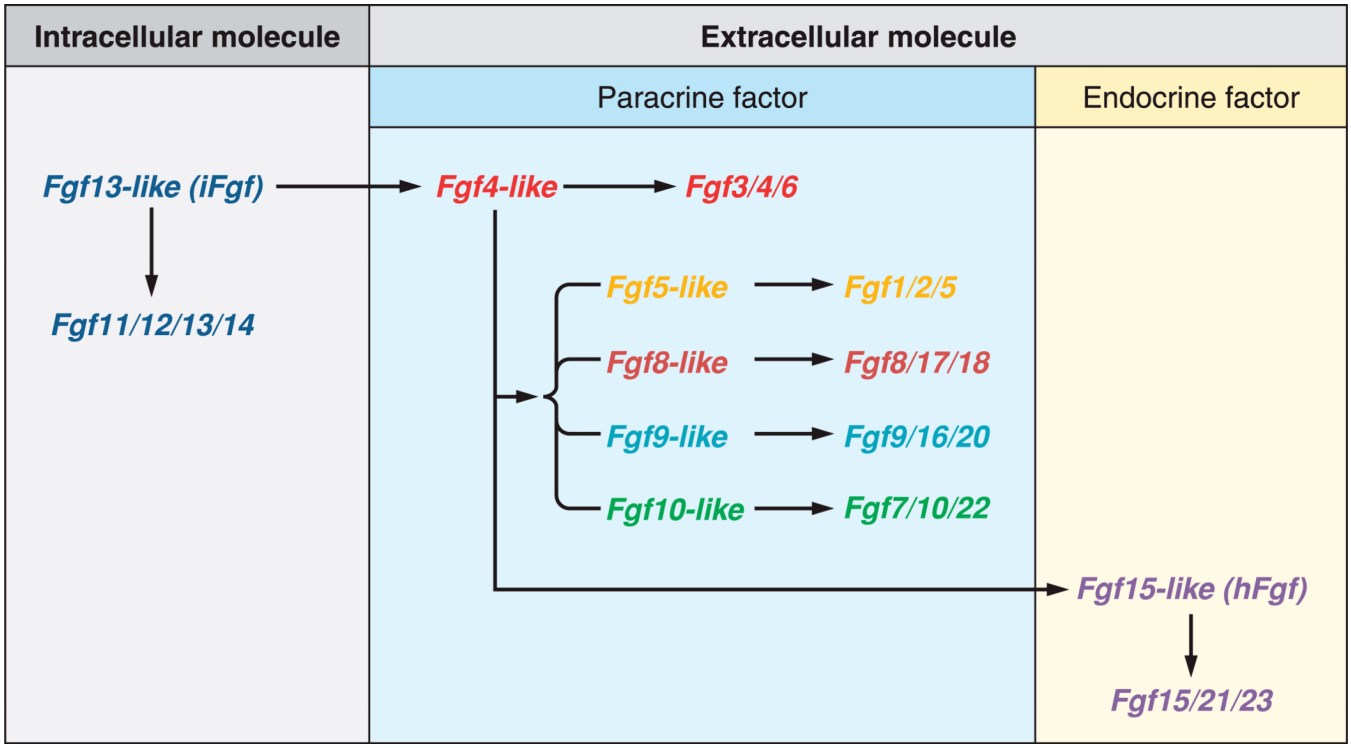

FIGURE. 1.
Functional evolutionary history of ancestors of the mouse Fgf gene family. [From Itoh et al. (75), with permission from John Wiley and Sons.]

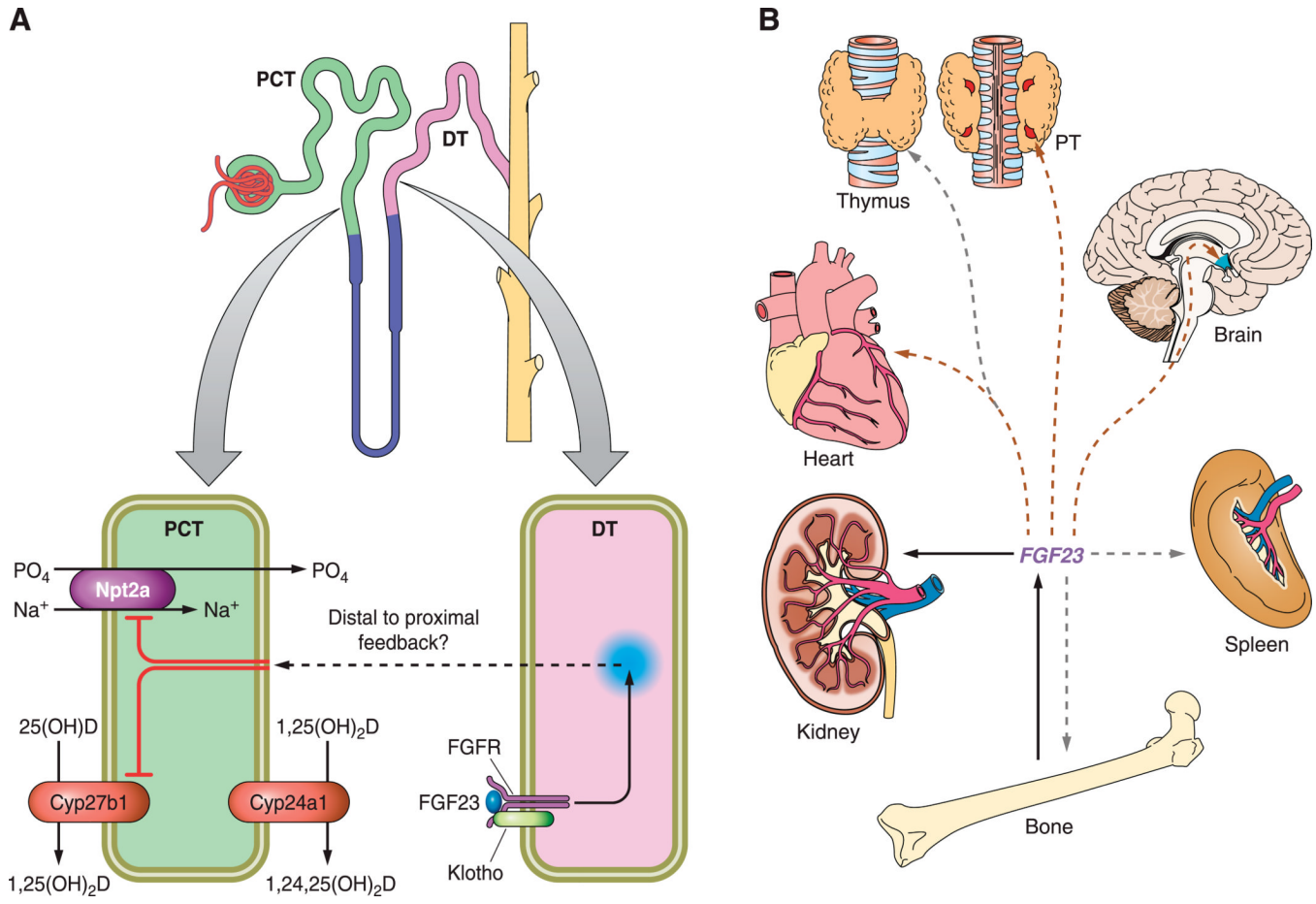

FIGURE. 2.

Renal and extrarenal functions of FGF23. *A*: hypothetical distal to proximal feedback mechanism: FGF23 activates FGFR/Klotho complexes in the renal distal tubules (DT) leading to two predominant events in the proximal convoluted tubules (PCT): the inhibition of expression of Npt2a and the inhibition of Cyp27b1. As a consequence, phosphate reabsorption and $1,25(OH)_2D$ production are respectively decreased. Additionally, the increase in Cyp24a1 expression contributes to lowering $1,25(OH)_2D$ levels due to increased catabolism of $1,25(OH)_2D$. *B*: extrarenal targets of FGF23 are tissues that express FGFR and Klotho, including kidney, but also parathyroid gland (PTG), heart, and brain (brown arrows) or tissues that express the FGFR alone such as thymus, spleen, or bone (gray arrows), indicating possible paracrine effects of FGF23. Possible hormonal regulation loops are yet to be discovered.

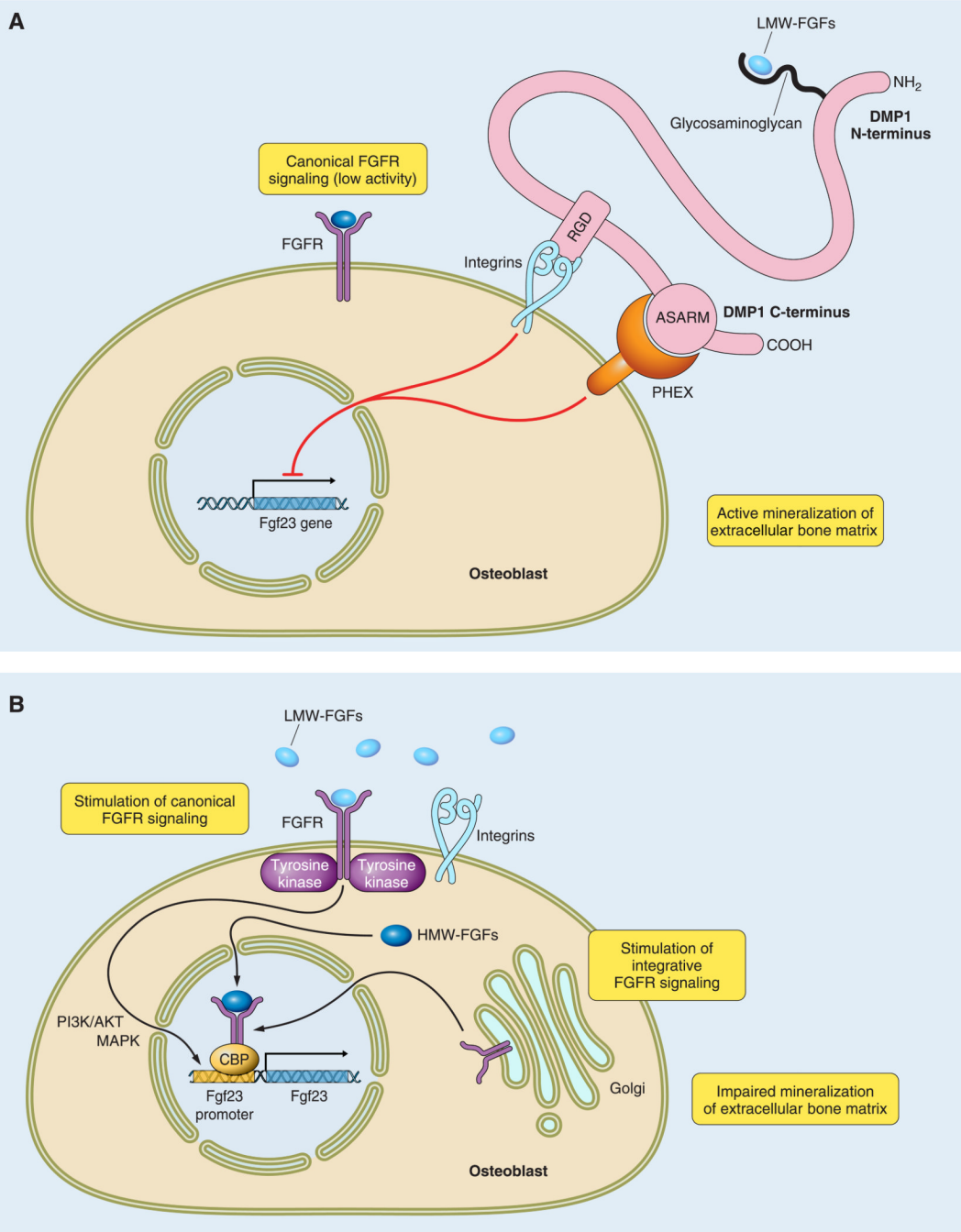

FIGURE. 3.

Predictive model for local regulation of Fgf23 transcription in bone. The COOH-terminal fragment of DMP1 binds to PHEX and integrins via the ASARM and the RGD motifs, respectively. *A*: in physiological conditions, this binding participates in maintaining low circulating FGF23 levels by inhibiting its transcription. *B*: absence of DMP1 or PHEX, due to inactivating mutations, leads to a pathological increase in Fgf23 transcription by stimulation of the FGFs/FGFRs pathway. Activation of the FGFs/FGRs pathway occurs when integrins are release from COOH-terminal DMP1 binding and low-molecular-weight FGFs (LMW-FGFs) are released from NH_2-terminal DMP1 binding, thus allowing the formation of a complex between LMW-FGFs/integrins/FGFR. Alternatively, internalization

of intracytoplasmic FGFR and high-molecular-weight FGFs (HMW-FGFs) also lead to increased Fgf23 transcription in absence of DMP1 and PHEX.

Table 1

Comparative analysis of gene mutations leading to increased FGF23 expression in bone

Factor for Comparison							
Hereditary hypophosphatemic disorders	ARHR2	ARHR1	XLH	OGD	None	MAS	ADHR
OMIM no.	613312	241520	307800	166250	None	174800	193100
Mutated gene	Enpp1	Dmp1	Phex	Fgfr1	Fgf2-HMW	Gnas2	Fgf23
Type of mutation	Loss of function	Loss of function	Loss of function	Gain of function	Gain of function	Gain of function	Gain of function
Mouse homolog	Enpp1−/−	Dmp1−/−	Hyp	None	Tg-Fgf2-HMW	None	Tg-Fgf23

ARHR, autosomal recessive hypophosphatemic rickets; XLH, *X*-linked hypophosphatemic rickets; OGD, osteoglophonic dysplasia; MAS, McCune-Albright syndrome; ADHR, autosomal dominant hypophosphatemic rickets.

Molecular Regulation of Phosphate Metabolism by Fibroblast Growth Factor-23–Klotho System

Chung-Yi Cheng, Makoto Kuro-o, and Mohammed S. Razzaque

Phosphorus is an essential nutrient and is routinely assimilated through consumption of food. The body's need of phosphate is usually fulfilled by intestinal absorption of this element from the consumed food, whereas its serum level is tightly regulated by renal excretion or reabsorption. Sodium-dependent phosphate transporters, located in the luminal side of the proximal tubular epithelial cells, have a molecular control on renal phosphate excretion and reabsorption. The systemic regulation of phosphate metabolism is a complex multiorgan process, and the identification of fibroblast growth factor-23 (FGF23)–Klotho system as a potent phosphatonin has provided new mechanistic insights into the homeostatic control of phosphate. Hypophosphatemia as a result of an increase in urinary phosphate wasting after activation of the FGF23–Klotho system is a common phenomenon, observed in both animal and human studies, whereas suppression of the FGF23–Klotho system leads to the development of hyperphosphatemia. This article will briefly summarize how delicate interactions of the FGF23–klotho system can regulate systemic phosphate homeostasis.

Key Words: Klotho, FGF23, Vitamin D, Calcium, NaPi, PTH

Phosphate is a widely distributed element, and more than 80% of the total phosphate in the body is present in bones and teeth, whereas the rest is present in the viscera and skeletal muscle, with a very small amount in the extracellular fluids. About 20% of the intracellular phosphate is present in the mitochondria, and influences various essential cellular functions, including oxidative phosphorylation, whereas about 30% of total cellular phosphate is stored in the endoplasmic reticulum and is used for phosphorylation of various proteins.[1-3] The remaining cellular phosphate is distributed in other cellular components, including nucleus, Golgi complex, and lysosomes. Maintaining phosphate balance is not only important for structural and functional activities of bones and teeth, but also essential for normal cellular function and survival. Despite clinical importance of maintaining phosphate balance, the molecular events related to its regulation are not yet clearly understood. Recent identification of bone-derived factors in regulating numerous systemic functions has helped us to realize the importance of endocrine regulation of mineral ion homeostasis.[4-8]

Traditionally, it was a well accepted fact that parathyroid hormone (PTH) and the active form of vitamin D, 1, 25-dihydroxyvitamin D3 [1α,25$(OH)_2D_3$] were 2 major endocrine factors that regulated calcium homeostasis. When calcium-sensing receptors in the parathyroid gland detect a decrease in serum calcium levels, PTH is secreted and acts to restore the serum calcium level to normal. PTH stimulates 1α-hydroxylase enzyme expression in the kidneys, and thus increases synthesis of 1α,25$(OH)_2D_3$.[9] The function of 1α,25$(OH)_2D_3$ is to increase the efficiency of calcium and phosphate absorption in the intestine and kidneys (Fig 1). PTH also acts on bones to stimulate osteoclastogenesis and to promote mobilization of calcium and phosphate from the reservoir, thereby increasing blood calcium levels. However, it does not cause phosphate retention as a result of its phosphaturic effect in the kidneys. Therefore, maintenance of phosphate balance was considered to be passively regulated by PTH and 1α,25$(OH)_2D_3$.

Recent studies have added new dimensions to this traditional view on the endocrine regulation of phosphate balance. Phosphatonins are the most recently discovered players in phosphate homeostasis. The term "phosphatonin" was originally described as a circulating factor that induced renal inorganic phosphate wasting in patients with tumor-induced osteomalacia (TIO).[10] Patients with TIO have mesenchymal tumors and exhibit mineral metabolism derangements, including normal or slightly low serum calcium, normal PTH, inappropriately low serum 1α,25$(OH)_2D_3$, and low serum phosphate levels owing to renal phosphate wasting with defective bone mineralization.[11] Because removal of tumors restores these mineral metabolism derangements, it was speculated that the tumors secreted a putative endocrine factor (phosphatonin) that induced renal phosphate wasting and suppressed 1α,25$(OH)_2D_3$ synthesis. Search for phosphatonin in patients with TIO led to the identification of fibroblast growth factor-23 (FGF23). Many phosphatonin molecules, such as the FGF23, secreted frizzled-related protein-4 (sFRP-4), fibroblast growth factor-7 (FGF-7), and matrix extracellular phosphoglycoprotein (MEPE), have been described in recent studies.[12-17] Identification of these phosphatonins has significantly advanced our understanding of the molecular regulation of phosphate homeostasis, and in this brief article, we will present how the FGF23–Klotho system is involved in such regulation.

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Fibroblast Growth Factor-23

FGF23 is a bone-derived phosphatonin that has been most extensively studied. It was originally identified as a gene mutated in patients with autosomal dominant hypophosphatemic rickets (ADHR).[12] Patients with ADHR carry missense mutations in the FGF23 gene that confer resistance to proteolysis inactivation of the FGF23 protein. As a result, patients with ADHR have high serum FGF23 levels. FGF23 has an activity that reduces the number of sodium-dependent phosphate (NaPi) cotransporters, type-2a (NaPi-2a), on the brush border membrane of proximal tubules, thereby promoting renal phosphate excretion.[18] This reduction in the number of NaPi-2a by FGF23 seems to be independent of PTH.[19] When the serum phosphate level is high, FGF23 is secreted from bones and acts on the kidneys to induce phosphaturia and suppress active vitamin D synthesis, thereby inducing a negative phosphate balance to maintain phosphate homeostasis.[20] In an animal study, administration of FGF23 intravenously to rodents decreased serum $1\alpha,25(OH)_2D_3$ levels within hours.[21] FGF23 downregulates expression of the *Cyp27b1* gene, which encodes 1α-hydroxylase, the enzyme that synthesizes the active form of vitamin D [$1\alpha,25(OH)_2D_3$]. Furthermore, FGF23 upregulates *Cyp24* gene expression that encodes 24-hydroxylase, the enzyme that inactivates $1\alpha,25(OH)_2D_3$. Therefore, FGF23 functions as a counter-regulatory hormone for vitamin D.[22] In contrast, $1\alpha,25(OH)_2D_3$ upregulates FGF23 gene expression and forms a negative feedback loop (Fig 2). Activated $1\alpha,25(OH)_2D_3$ binds to nuclear vitamin D receptor and triggers the formation of a heterodimer with another nuclear receptor RXR. The vitamin D receptor-retinoid X receptor (RXR) heterodimer in turn transactivates the FGF23 gene expression.[23] Similarly, elevated serum $1\alpha,25(OH)_2D_3$ levels in *klotho* knockout mice is associated with extremely high serum FGF23 levels. More importantly, eliminating vitamin D activities from *klotho* knockout mice resulted in almost undetectable levels of FGF23 in *klotho/1α-hydroxylase* double knockout mice, clearly suggesting the *in vivo* role of vitamin D in the induction of FGF23.[24,25]

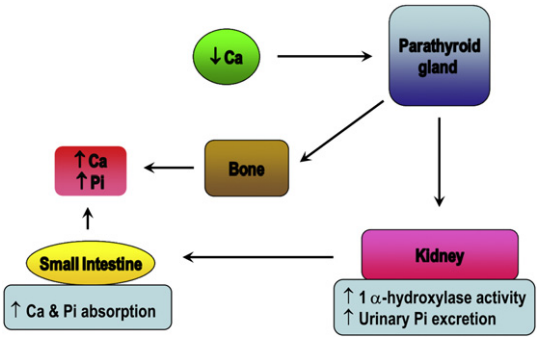

Figure 1. Classical pathway of passively regulated phosphate (Pi) balance. On decrease in serum calcium (Ca^{2+}) level, parathyroid hormone (PTH) is secreted and acts on its cognate G-protein-coupled receptor, PTH1R, at its target tissues (bones and kidneys) and restores the serum Ca^{2+} level. In kidneys, PTH induces phosphaturia and increases levels of circulating $1\alpha,25(OH)_2D_3$. $1\alpha,25(OH)_2D_3$ in turn acts on the small intestine to increase Ca^{2+} and Pi absorption. In bones, PTH causes an increase in bone resorption, and thus increases serum Ca^{2+} and Pi levels. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

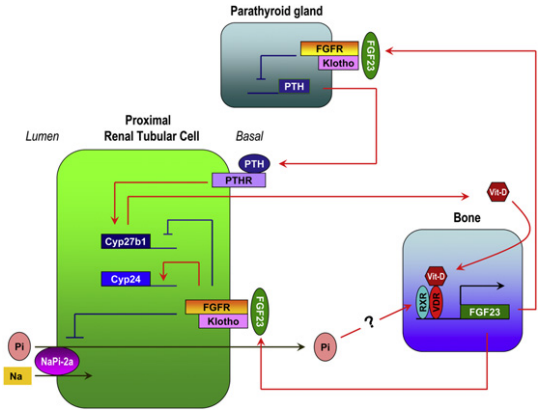

Figure 2. The bone–kidney–parathyroid endocrine axes mediated by fibroblast growth factor-23 (FGF23) and Klotho. High serum and/or dietary Pi increases FGF23 secretion from the bones. FGF23 acts on the Klotho–FGF receptor (FGFR) complex expressed in the kidneys and the parathyroid gland. In the kidneys, FGF23 downregulates expression of the *Cyp27b1* gene and upregulates *Cyp24* gene, thus suppresses synthesis of active vitamin D. Binding of FGF23 to Klotho–FGFR complex also suppresses Pi reabsorption by inhibiting NaPi-2a activity. In the parathyroid gland, FGF23 suppresses production and secretion of PTH. PTH binds to PTH receptor (PTHR) expressed on the renal tubular cells, in turn, upregulating *Cyp27b1* gene expression. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

The biological response to FGF23 in its target tissue (kidneys) requires binding and activation of its cognate fibroblast growth factor receptors (FGFRs) that belong to type 1 transmembrane phosphotyrosine kinase receptors. Although FGF23 belongs to the FGF ligand superfamily,[26] phylogenetic and sequence analyses have segregated FGF23 and 2 additional FGFs (FGF19 and FGF21) from the other FGF family members.[27] These 3 atypical FGFs are collectively called endocrine FGFs.[23] These FGFs have low affinity to heparan sulfate (HS). The unique structural feature of reduced affinity of endocrine FGFs to HS allows them to escape from HS-rich extracellular matrices and enter into systemic circulation. Although the low affinity of endocrine FGFs to HS may be advantageous for the endocrine mode of action, it may be disadvantageous for signal transduction through FGFRs. FGF23 cannot activate FGF signaling in most cultured cells, whereas classic FGFs, such as FGF2, can do so,[28] thus implicating that endocrine FGFs may require a cofactor(s) other than HS for FGFR activation. Identity of the putative cofactor(s) required for FGF23 to activate FGFRs was not clear until it was realized that the phenotypes of *Fgf23*-deficient mice are identical to those of *Klotho*-deficient mice. In fact, the physical, morphological, biochemical, and molecular phenotypes of *fgf23* or

klotho single knockout mice are indistinguishable from *fgf23–klotho* double knockout mice.[29]

Klotho

The *klotho* gene was originally identified as a gene mutated in the *klotho* mouse. A defect in *klotho* gene expression in mice leads to a complex phenotype resembling human premature-aging syndromes, including shorten life span, growth retardation, hypogonadism, skin atrophy, muscle atrophy, premature thymic involution, osteopenia, pulmonary emphysema, and vascular and soft-tissue calcification.[30] This gene encodes a 1014-aminoacid-long protein with a long extracellular domain, and a short cytoplasmic region that does not contain signaling capabilities. The extracellular domain is composed of 2 homologous regions, named KL1 and KL2. Klotho is expressed at the cell surface (membrane-bound KL, mKL) and is also present in the plasma as secreted forms (sKL). One form of sKL results from the shedding of mKL from the cell surface. This form of sKL comprises the KL1 and KL2 domains, referred to as "cut mKL."[31] The second sKL is formed by alternative splicing in exon 3 that results in a protein containing only the KL1 domain, but not the transmembrane domain. Both cut mKL and sKL found in the circulation have led to the interpretation that Klotho itself may act as a hormone. However, function of the Klotho protein was not clear until it was realized that phenotypes of Klotho-deficient mice were almost identical to those of mice lacking *Fgf23*. The *fgf23*-null mice develop phosphate–retention phenotypes characterized by hyperphosphatemia and extensive soft-tissue calcification.[32] Genetic restoration of the systemic actions of human FGF23 in *fgf23* knockout mice reversed the hyperphosphatemia to hypophosphatemia and prevented associated complications, including ectopic calcification.[33] In addition to these phenotypes, *fgf23*-null mice also exhibit complex phenotypes, including growth retardation, hypogonadism, premature thymic involution, osteopenia, skin atrophy, muscle atrophy, and pulmonary emphysema, which are reminiscent of the premature aging syndrome in *Klotho*-deficient mice. These observations revealed an unexpected link between FGF23 and Klotho.

FGF23–Klotho System

Klotho protein forms a binary complex with several FGFR isoforms and significantly enhances their affinity for FGF23.[28,34] Co-immunoprecipitation studies indicated that Klotho binds to FGFR1c, 3c, and 4 with higher affinity than to the other FGFR isoforms. Without Klotho, these FGFRs cannot bind to FGF23, indicating that FGF23 requires Klotho as a coreceptor. This explains why Klotho-deficient mice have extremely high levels of FGF23. Also, kidney-specific expression of Klotho explains why FGF23 can identify kidneys as its target organ among many other tissues that express multiple FGFR isoforms. Thus, Klotho is required for FGF23 to induce negative phosphate balance by increasing renal phosphate excretion as well as reduce intestinal phosphate absorption.

Recent mouse genetic studies have convincingly demonstrated the *in vivo* importance of Klotho in FGF23-mediated regulation of phosphate homeostasis.[35-37] For instance, functionally active FGF23 recombinant protein is unable to reduce serum phosphate levels in mice lacking Klotho activities (either *Klotho-deficient* mice or *fgf23/klotho* double knockout mice).[29,35] Similarly, *phex* mutant mice have hypophosphatemia because of high serum levels of FGF23, whereas inactivation of the *Klotho* function from the *phex* mutant mice resulted in hyperphosphatemia in *phex/klotho* double mutant mice, even though these mice have significantly higher serum levels of FGF23.[29,35,38] Likewise, genetically ablating *Klotho* function from *FGF23* transgenic mice reversed the hypophosphatemic phenotype in these mice to hyperphosphatemic.[39] In a similar study in human beings, despite high serum levels of FGF23, a homozygous loss-of-function mutation in the *KLOTHO* gene induced severe hyperphosphatemia in the affected patient with tumoral calcinosis.[40] Together, these human and experimental evidences clearly suggest that Klotho is indispensable for the FGF23-mediated regulation of systemic phosphate homeostasis, and that dysregulation of the FGF23–Klotho system, either alone or in combination, can induce phosphate imbalance.

Klotho and NaPi System

Regulation of phosphate homeostasis in the kidneys occurs primarily in the proximal convoluted tubule and is controlled mainly by PTH and the FGF23–Klotho endocrine axis. Approximately 80% of filtered phosphate is reabsorbed along with sodium through NaPi cotransporters, NaPi-2a and NaPi-2c, located in the proximal tubule. In fact, molecular and biochemical analyses suggest that increased renal activity of NaPi-2a leads to severe hyperphosphatemia in *Klotho* knockout mice.[41,42] More importantly, the extensive physical, morphological, and molecular phenotypes in *klotho* knockout mice can be suppressed by genetically reducing serum phosphate levels in *NaPi-2a/klotho* double knockout mice to extend their survival. For instance, both male and female hyperphosphatemic *klotho* knockout mice have hypogonadism that is associated with premature infertility in these mutant mice, whereas genetically reducing serum phosphate levels can regain fertility in the *NaPi2a/klotho* double knockout mice.[41,42] A similar trend is also noted in hyperphosphatemic *fgf23* knockout mice, where suppressing NaPi-2a activity can reduce serum phosphate levels in *NaPi2a/fgf23* double knockout mice, and reverse associated complications, including fertility.[43] These genetics studies in mice clearly suggest that the NaPi system is actively involved in FGF23–Klotho-mediated regulation of phosphate homeostasis.[41,42,44,45] Whether the FGF23–Klotho system directly reduces the renal NaPi cotransporter activity or whether such a response is mediated through other FGF23 target

molecules is an unsolved issue; however, recent studies have provided possible molecular insights.[46]

The bone–kidney endocrine axis mediated by FGF23 and Klotho reduces the number of NaPi-2a on the brush border membrane of the proximal tubules, thereby promoting renal phosphate excretion. It should be noted that Klotho protein is expressed much more abundantly in distal convoluted tubules than in proximal tubules. This has raised the question as to how Klotho protein expressed in distal tubules controls the function of proximal tubules. A recent animal study reported that activation of the FGF signaling pathway was detectable only in the distal convoluted tubules after injection of FGF23 into mice.[47] This study supported the possibility that FGF23 might first act on distal convoluted tubules and then generate a paracrine factor that acts on the adjacent proximal tubules to reduce phosphate reabsorption and vitamin D synthesis. A candidate of this putative paracrine factor might be sKL protein because it functions as a phosphaturic substance independent of FGF23. Injection of sKL protein induces phosphaturia in *fgf23* knockout mice. This study showed that sKL mediates deglycosylation of *N*-linked glycans on the NaPi2a protein through its putative glycosidase activity. Deglycosylation of *N*-linked glycans renders NaPi-2a protein more susceptible to proteases residing in the proximal tubular brush border membrane, resulting in reduction in the number and activity of NaPi-2a.[46] Therefore, Klotho can induce phosphaturia by 2 ways: by functioning as a coreceptor for FGF23 and by functioning as a phosphaturic substance independent of FGF23.

Phosphate-independent Effects of Klotho

Although FGF23-mediated functions are mostly Klotho-dependent, Klotho also has numerous FGF23-independent functions, including adipogenesis.[48] Studies have shown Klotho as a regulator of oxidative stress and senescence.[49] The other functional role of Klotho in the kidneys is its involvement in renal calcium handling. Klotho may regulate calcium homeostasis through direct regulation of renal ion transport in addition to modulation of PTH and 1α,25$(OH)_2D_3$ levels.[50,51] sKL activates the transient receptor potential v-5 (TRPV5) by entrapping the channel on the plasma membrane and inhibiting its endocytosis, leading to enhanced calcium reabsorption in the distal nephron.[52,53] A similar Klotho effect was also found on renal outer medullary potassium channel (ROMK1).[54] Treatment with sKL removes terminal sialic acids from *N*-linked glycans of TRPV5 and ROMK1. Removal of terminal sialic acids exposes underlying galactose residues, which are good ligands for galectin-1 in extracellular matrices. Therefore, Klotho-mediated removal of sialic acids triggers interaction between galectin-1 and glycans of these ion channels, preventing them from endocytosis. As a result, sKL promotes calcium absorption and potassium excretion. The *in vivo* ability of sKL to activate TRPV5 and ROMK1 to renal physiology and pathophysiology remains to be determined.

Other Phosphatonins

Several other phosphatonins, including MEPE, sFRP-4, dentin matrix protein-1 (DMP-1), and FGF-7, have not been studied as extensively as FGF23 (Table 1). Similar to FGF23, sFRP-4 has been shown to decrease renal phosphate reabsorption by reducing the number of NaPi cotransporters in the renal proximal tubules and by inhibiting synthesis of 1α,25$(OH)_2D_3$.[15] FGF-7 inhibits NaPi transport in opossum kidney cells and reportedly has a phosphaturic activity *in vivo*.[58] Similarly, MEPE has been shown to increase the fractional excretion of phosphate and induce hypophosphatemia *in vivo*.[17] In contrast to FGF23, MEPE does not inhibit 1α,25$(OH)_2D_3$ formation. As such, one phosphatonin can influence the functionality of the others. For instance, mutations in the DMP-1 gene, found in patients with autosomal recessive hypophosphatemic rickets/osteomalacia,[59] resulted in increased circulating levels of FGF23, leading to hypophosphatemia. Similarly, dmp-1 knockout mice developed hypophosphatemia because of increased bioactivities of FGF23.[57] One of the areas that will need additional studies includes determining how numerous phosphatonins molecularly interact with each other to regulate physiologic phosphate balance, and how these molecules facilitate cross-organ talks among bones,

Table 1. Phosphatonins Other than FGF23

Phosphatonins	Associated Diseases	References
MEPE	Increased expression in tumors associated with TIO	13
	Increased serum levels in XLH	55
	Induced phosphaturia when injected into rats	56
sFRP-4	Increased expression in tumors associated with TIO	13
	Induced phosphaturia when injected into rats	15
DMP-1	Increased expression in tumors associated with TIO	13
	Loss-of-function causes ARHR, increased serum FGF23	57
FGF-7	Increased expression in tumors associated with TIO	13
	Suppression of phosphate uptake in cultured cells	58

Abbreviations: MEPE, matrix extracellular phosphoglycoprotein; sFRP-4, secreted frizzled-related protein 4; DMP-1, dentin matrix protein 1; FGF-7, fibroblast growth factor 7; TIO, tumor-induced osteomalacia; XLH, X-linked hypophosphatemia; ARHR, autosomal recessive hypophosphatemic rickets.

kidneys, parathyroid gland, and intestine. Such understanding will help us to determine the cause of abnormal regulation of phosphate homeostasis in a wide range of diseases, including in patients with CKD.

FGF23–Klotho System and CKD

In patients with CKD, circulating levels of FGF23 are progressively increased as kidney function declines,[60] whereas Klotho expression in the kidneys is significantly decreased in such patients.[61] Therefore, CKD may be viewed as a state of FGF23 resistance caused by Klotho deficiency. Increases in FGF23 precede increases in serum phosphate (hyperphosphatemia) during CKD progression, suggesting that patients with early-stage CKD maintain normal serum phosphate levels by increasing FGF23 to compensate for increased renal resistance to FGF23. However, this is at the expense of $1\alpha,25(OH)_2D_3$, as FGF23 induces phosphaturia and suppresses $1\alpha,25(OH)_2D_3$ synthesis at the same time. Low serum levels of $1\alpha,25(OH)_2D_3$ not only cause secondary hyperparathyroidism but also reduce Klotho expression further, because $1\alpha,25(OH)_2D_3$ is a potent inducer of Klotho expression.[51] Therefore, low serum levels of $1\alpha,25(OH)_2D_3$ induced by compensatory increase in serum FGF23 levels can trigger deterioration spiral of Klotho expression. The benefit of calcitriol therapy may be partly attributed to interruption of this vicious cycle. A recent study showed that high serum FGF23 levels were associated with resistance to calcitriol treatment, and concluded that FGF23 could be a predictor for calcitriol therapy resistance.[62]

Dietary phosphorus load as well as serum phosphate levels can influence FGF23 levels in human beings. For instance, serum FGF23 levels increased during high dietary phosphate/calcium intake, whereas serum PTH levels declined; $1\alpha,25(OH)_2D_3$ levels showed an inverse relation with FGF23 levels.[63,64] In a similar study, effects of providing various amount of phosphate-containing food (400, 800, and 1200 mg) on serum FGF23 showed that its levels remained either similar or slightly decreased by 6 hours after consumption of a high phosphate meal. In contrast, a significantly increased serum FGF23 level was detected in approximately 8 hours after intake of 1200 mg of phosphate, suggesting that high amount of phosphate consumption may induce FGF23 to restore phosphate balance.[65]

Recently, an increased serum level of FGF23 has been shown to affect mortality of incident hemodialysis patients.[66] Furthermore, an association between increased serum levels of FGF23 and left ventricular hypertrophy has found in patients with CKD undergoing hemodialysis treatment.[67] If the serum FGF23 level is a surrogate marker for the renal Klotho expression level, high FGF23 may suggest low Klotho expression in kidneys and severe loss of functional nephron mass.[66] When functional renal mass is decreased to a level that fails to maintain phosphate excretion in response to increased FGF23 and PTH, hyperphosphatemia ensues.

The molecular interaction of FGF23–Klotho system and PTH is a complex process, and whether FGF23–Klotho system is involved in the genesis of secondary hyperparathyroidism in patients with CKD is still not clear. Studies have found decreased expression of Klotho and FGFR1 in the hyperplastic parathyroid glands of uremic patients. This observation implicates that intrinsic changes in the parathyroid gland may provoke FGF23 resistance or unresponsiveness toward the gland.[68] Contrary to human observation, the experimental studies found increased parathyroid expression of Klotho and FGFR1 in uremic animals.[69] Furthermore, exogenous FGF23 failed to inhibit development of hyperparathyroidism in experimental uremic animals.[70] It remains to be determined whether decline in renal Klotho expression precedes increase in serum FGF23 during CKD progression. Of relevance, experimental studies have shown that eliminating Klotho function from mice is associated with extremely high serum levels of FGF23.[14,35]

Conclusions

Our knowledge of the mechanisms that regulate phosphate homeostasis has greatly improved during the past few years.[19,20,37] Seminal discoveries on the FGF23–Klotho endocrine system have been a major driving force for this progress. It has become increasingly clear that hyperphosphatemia should be aggressively treated to improve life expectancy of patients with CKD. Within this context, the manipulation of the FGF23–Klotho axis is expected to be a novel target of therapeutic intervention in diseases associated with abnormal mineral ion metabolism. However, it is worth mentioning that, at present, there is no direct evidence that can establish a positive link between accelerated progression of CKD and FGF23 toxicity.[71] Any strategy of therapeutic lowering of FGF23 in patients with CKD needs additional studies. A controlled therapeutic suppression of FGF23 might be of therapeutic benefit for patients with excessive urinary phosphate wasting diseases, including ADHR, autosomal recessive hypophosphatemic rickets/osteomalacia, and X-linked hypophosphatemic rickets. As such, the current treatment for hypophosphatemic genetic diseases is mostly symptomatic, including oral phosphate replacement. The prolonged use of such replacement therapy can lead to the development of secondary hyperparathyroidism. Again, whether patients with CKD might also benefit from the therapeutic lowering of FGF23 is an area that needs additional studies. In contrast to anti-FGF23 therapy, providing exogenous bioactive FGF23 or Klotho protein might help restore the phosphate balance and reduce abnormal calcifications in patients with familial tumoral calcinosis (FTC), which is usually caused by the reduced activity of FGF23. Our challenge will be to use experimental observations to fine-tune the existing therapeutic options

by manipulating the FGF23–Klotho system to treat patients suffering from the complications of abnormal mineral ion metabolism, including CKD.[14,36,72]

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REVIEW

Overview of the FGF23-Klotho axis

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Abstract Recent studies have identified a novel bone–kidney endocrine axis that maintains phosphate homeostasis. When phosphate is in excess, fibroblast growth factor-23 (FGF23) is secreted from bone and acts on the kidney to promote phosphate excretion into urine and suppress vitamin D synthesis, thereby inducing negative phosphate balance. One critical feature of FGF23 is that it requires Klotho, a single-pass transmembrane protein expressed in renal tubules, as an obligate coreceptor to bind and activate FGF receptors. Several hereditary disorders that exhibit inappropriately high serum FGF23 levels are associated with phosphate wasting and impaired bone mineralization. In contrast, defects in either FGF23 or Klotho are associated with phosphate retention and a premature-aging syndrome. The aging-like phenotypes in Klotho-deficient or FGF23-deficient mice can be rescued by resolving hyperphosphatemia with dietary or genetic manipulation, suggesting a novel concept that phosphate retention accelerates aging. Phosphate retention is universally observed in patients with chronic kidney disease (CKD) and identified as a potent risk of death in epidemiological studies. Thus, the bone–kidney endocrine axis mediated by FGF23 and Klotho has emerged as a novel target of therapeutic interventions in CKD.

Keywords Klotho · FGF23 · Phosphate · Vitamin D · CKD

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Introduction

The blood phosphate level is determined by counterbalance between absorption of dietary phosphate from the intestine, mobilization from bone (the major reservoir of calcium and phosphate in the body), and excretion from the kidney into urine [1]. These processes are coordinately regulated by several endocrine factors. Vitamin D and parathyroid hormone (PTH), which have been extensively studied as hormones that regulate calcium metabolism [2], are also involved in phosphate metabolism. The active form of vitamin D (1,25-dihydroxyvitamin D_3) is synthesized in the kidney and acts on the intestine to increase absorption of dietary calcium and phosphate. It also acts on bone to stimulate osteoclastogenesis and promote mobilization of calcium and phosphate from the reservoir, thereby increasing blood levels of both calcium and phosphate. PTH acts on the kidney to promote both vitamin D synthesis and phosphaturia (phosphate excretion into urine). As a result, unlike vitamin D, PTH can selectively increase blood calcium levels without concomitant increase in blood phosphate levels [3].

Recent studies have identified fibroblast growth factor-23 (FGF23) as a novel hormone that lowers blood phosphate levels [4–6]. When phosphate is in excess, FGF23 is secreted from bone and acts on the kidney to induce phosphaturia and suppress vitamin D synthesis, thereby inducing a negative phosphate balance to maintain phosphate homeostasis [6–9]. FGF23 requires Klotho protein as a coreceptor for high affinity binding to cognate FGF receptors (FGFRs). The purpose of this review was to overview recent progress in our understanding of endocrine regulation of phosphate metabolism by FGF23 and Klotho and to discuss its potential role in the pathophysiology of chronic kidney disease (CKD).

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FGF23

FGF23 was originally identified as a gene mutated in patients with autosomal dominant hypophosphatemic rickets (ADHR) [5]. Patients with ADHR carry missense mutations in the *FGF23* gene that confer resistance to proteolytic degradation of the FGF23 protein [10]. As a result, ADHR patients exhibit high serum FGF23 levels. Because FGF23 has an activity that induces negative phosphate balance, ADHR patients exhibit phosphate-wasting phenotypes such as hypophosphatemia and rickets. Although the protease(s) that inactivates FGF23 remains to be identified, it cleaves FGF23 at the ^{176}RXXR^{179} motif and generates two inactive fragments [11]. Missense mutations in this critical motif (R176Q, R179Q/W) make the protein resistant to the protease and increase the half-life of FGF23 in the blood [12]. Several other phosphate-wasting syndromes associated with FGF23 excess have been identified, including tumor-induced osteomalacia (TIO), X-linked hypophosphatemia (XLH), and autosomal recessive hypophosphatemic rickets (ARHR). TIO is caused by FGF23-producing tumors [13]. XLH and ARHR are caused by mutations in the *PHEX* (a phosphate-regulating gene with homologies to endopeptidases on the X-chromosome) gene [14] and the *DMP-1* (dentin matrix protein-1) gene [15, 16], respectively. These two genes are expressed in osteocytes in the bone where FGF23 is primarily produced and secreted. Recent animal studies have demonstrated that PHEX and DMP-1 may be involved in the regulation of FGF23 gene expression. *Hyp* mice, which have deletions in the *Phex* gene, show high FGF23 expression in the bone, high serum levels of FGF23, hypophosphatemia, and impaired bone mineralization, as observed in XLH patients [17, 18]. DMP-1 knockout mice also exhibit high FGF23 expression and phosphate-wasting phenotypes, as observed in ARHR patients [15]. The precise mechanism by which PHEX and DMP-1 proteins suppress expression of the *FGF23* gene remains to be determined.

In contrast to patients with ADHR, ARHR, XLH, and TIO that exhibit phosphate-wasting phenotypes, patients with familial tumoral calcinosis (FTC) exhibit phosphate-retention phenotypes including hyperphosphatemia and ectopic calcification associated with low serum FGF23. FTC is caused by mutations in the *GALNT3* gene that encodes a glycosyl transferase called UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase-3 (ppGaNTase-T3) [19]. This enzyme is required for O-glycosylation of FGF23 at Thr^{178}, which resides within the cleavage motif of FGF23 [20]. It is likely that loss of O-glycosylation at Thr^{178} increases susceptibility of FGF23 to proteolytic degeneration, resulting in low serum levels of FGF23. Mice that completely lack FGF23 (*Fgf23*$^{-/-}$ mice) develop severe phosphate-retention phenotypes characterized by extensive soft tissue calcification and hyperphosphatemia [21]. These studies on phosphate-wasting and phosphate-retention syndromes in mice and humans have provided unequivocal evidence indicating that FGF23 is an essential hormone for maintaining phosphate homeostasis.

Although FGF23 belongs to the FGF ligand superfamily [22], phylogenetic and sequence analyses have segregated FGF23 and two additional FGFs (FGF19 and FGF21) from the other FGF family members [23]. These three atypical FGFs—namely, FGF19, FGF21, and FGF23—are collectively called endocrine FGFs because they function as endocrine factors, unlike the other classic FGFs that basically function as paracrine and/or autocrine factors [24]. The molecular basis behind the endocrine mode of action may lie in the fact that these endocrine FGFs have low affinity to heparan sulfate (HS). In general, FGF ligands have a conserved core region with 12 antiparallel β strands, where the HS-binding domain resides [25–27]. However, the HS-binding domain of endocrine FGFs deviates from that of the other paracrine-acting FGFs and prohibits formation of hydrogen bonding between HS and amino acid residues in the HS-binding domain, which is the basis of affinity to HS [28, 29]. This unique structural feature reduces affinity of endocrine FGFs to HS and allows them to escape from HS-rich extracellular matrices and enter into systemic circulation. Although the low affinity of endocrine FGFs to HS may be advantageous for the endocrine mode of action, it may be disadvantageous for signal transduction through FGFRs because HS is required for high-affinity binding of FGFs to FGFRs. FGFRs are single-pass transmembrane receptor tyrosin kinases that dimerize upon binding FGFs. HS participates in the FGF-FGFR interaction and promotes formation of a 2:2:2 FGF-FGFR-HS signaling complex, which is essential for efficient activation of FGFR tyrosine kinase [30]. Thus, endocrine FGFs may require a cofactor(s) other than and/or in addition to HS to secure efficient dimerization and activation of FGFR. In fact, FGF23 cannot activate FGF signaling in most cultured cells, even when they express FGFRs endogenously, whereas classic FGFs such as FGF2 can activate FGF signaling in these cells [31]. Identity of the putative cofactor(s) required for FGF23 to activate FGFRs was not clear until it was realized that the phenotypes of FGF23-deficient mice are identical with those of Klotho-deficient mice.

Klotho

The *klotho* gene, named after a Greek goddess who spins the thread of life, was originally identified as a gene mutated in a mouse strain that inherits a premature-aging

syndrome in an autosomal recessive manner [32]. Mice defective in *klotho* gene expression develop multiple aging-like phenotypes around 3–4 weeks after birth, including growth retardation, hypogonadotropic hypogonadism, rapid thymus atrophy [33], skin atrophy, sarcopenia, vascular calcification, osteopenia [34], pulmonary emphysema [35–37], cognition impairment [38], hearing disturbance [39], and motor neuron degeneration [40], and die around 2 months of age. In contrast, transgenic mice that overexpress Klotho live longer than wild-type mice [41]. Thus, the *Klotho* gene may be an aging suppressor gene that extends life span when overexpressed and accelerates aging when disrupted [42]. Furthermore, polymorphisms in the human *KLOTHO* gene are associated with life span [43] as well as bone mineral density [44–46], high-density lipoprotein (HDL) cholesterol level, blood pressure, stroke [47], coronary artery disease [48], and cognitive function [49], suggesting that Klotho may be involved in the regulation of aging processes in humans. The *Klotho* gene encodes a single-pass transmembrane protein that belongs to a family 1 glycosidase [50] and is expressed primarily in renal tubules in the kidney and choroid plexus in the brain [32]. Although recombinant Klotho protein was reported to have weak β-glucuronidase activity in vitro [51], physiological relevance of the β-glucuronidase activity in vivo was not clear.

The clue to understanding Klotho protein function was the fact that FGF23-deficient mice and Klotho-deficient mice develop identical phenotypes. FGF23-deficient mice not only exhibit phosphate retention but also develop multiple aging-like phenotypes [21], which is reminiscent of Klotho-deficient mice. Conversely, Klotho-deficient mice not only develop a premature-aging syndrome but also exhibit hyperphosphatemia [52, 53], which is reminiscent of FGF23-deficient mice. These observations suggested that Klotho and FGF23 might function in a common signal transduction pathway. In fact, Klotho protein forms a constitutive binary complex with several FGF receptor isoforms (FGFR1c, 3c, 4) and significantly increases the affinity of these FGFRs specifically to FGF23 [31]. Thus, Klotho protein functions as an obligate coreceptor for FGF23. This finding was later confirmed in an independent study [54]. The fact that FGF23 requires Klotho protein as a coreceptor explains why Klotho-deficient mice, FGF23-deficient mice, and mice lacking both Klotho and FGF23 [55] develop identical phenotypes. It also explain why extremely high serum FGF23 levels of Klotho-deficient mice [54] do not cause any adverse effects in Klotho-deficient mice [55]. In addition, kidney-specific expression of Klotho explains why FGF23 can identify the kidney as its target organ among many other tissues that express multiple FGFR isoforms. Klotho protein function is to compensate for the low affinity of FGF23 to heparan sulfate and specifically support FGFR activation with FGF23, which represents a novel mechanism for confining target organs in redundant ligand-receptor interactions.

Endocrine regulation of phosphate metabolism

The bone−kidney endocrine axis mediated by FGF23 and Klotho has emerged as the major regulator of phosphate homeostasis [4, 9, 24, 56–58]. FGF23 has an activity that reduces the number of sodium-phosphate cotransporter type-2a (NaPi-2a) on the brush border membrane of proximal tubules, thereby promoting renal phosphate excretion [59–62]. Thus, FGF23 functions as a phosphaturic hormone. In addition, FGF23 suppresses synthesis and promotes degradation of 1,25-dihydroxyvitamin D_3 in proximal tubules [63]. FGF23 down-regulates expression of the *Cyp27b1* gene, which encodes 1α-hydroxylase, the enzyme that synthesizes the active form of vitamin D (1,25-dihydroxyvitamin D_3) from its inactive precursor (25-hydroxyvitamin D_3). Furthermore, FGF23 up-regulates expression of the *Cyp24* gene that encodes 24-hydroxylase, the enzyme that hydrolyzes and inactivates 1,25-dihydroxyvitamin D_3. Thus, FGF23 functions as a counterregulatory hormone for vitamin D [8]. The ability of FGF23 to reduce serum 1,25-dihydroxyvitamin D_3 levels also contributes to induction of negative phosphate balance through reducing phosphate absorption from the intestine. Importantly, 1,25-dihydroxyvitamin D_3 up-regulates expression of the FGF23 gene [63] and closes a negative feedback loop (Fig. 1). Disruption of this negative feedback loop results in high serum 1,25-dihydroxyvitamin D_3 levels, as observed in Klotho-deficient mice, FGF23-deficient mice, and FTC patients. Recently, a patient carrying a homozygous missense mutation in the *KLOTHO* gene (H193R) was reported [64]. The patient exhibited phosphate-retention phenotypes similar to FTC patients, indicating that the H193R mutation is a loss-of-function mutation. This is the first case in humans exhibiting phosphate retention due to a defect in Klotho protein.

It should be noted that Klotho protein is expressed much more abundantly in distal convoluted tubules than in proximal tubules, whereas both phosphate reabsorption and vitamin D synthesis take place in proximal tubules. This discrepancy has raised two possibilities that are not mutually exclusive. One possibility is that, although Klotho expression levels in proximal tubules are not as high as distal convoluted tubules, FGF23 may signal through the FGFR-Klotho complex on proximal tubules and directly regulate NaPi-2a expression and vitamin D synthesis. In this case, the function of Klotho protein abundantly expressed in distal convoluted tubules must be addressed. The other possibility is that FGF23 may act first on distal

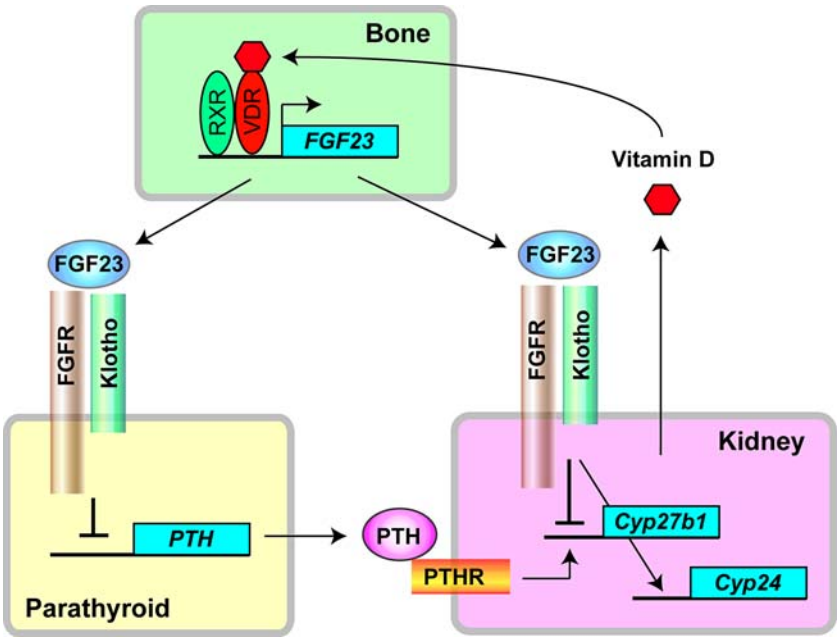

Fig. 1 The bone–kidney–parathyroid endocrine axes mediated by fibroblast growth factor-23 (FGF23) and Klotho. Active form of vitamin D (1,25-dihydroxyvitamin D_3) binds to vitamin D receptor (VDR) in the bone (osteocytes). The ligand-bound VDR forms a heterodimer with a nuclear receptor RXR and transactivates expression of the FGF23 gene. FGF23 secreted from bone acts on the Klotho-FGF receptor (FGFR) complex expressed in the kidney (the bone–kidney axis) and parathyroid gland (the bone–parathyroid axis). In the kidney, FGF23 suppresses synthesis of active vitamin D by down-regulating expression of the *Cyp27b1* gene and promotes its inactivation by up-regulating expression of the *Cyp24* gene, thereby closing a negative feedback loop for vitamin D homeostasis. In the parathyroid gland, FGF23 suppresses production and secretion of parathyroid hormone (PTH). PTH binds to the PTH receptor (PTHR) expressed on renal tubular cells, leading to up-regulation of the *Cyp27b1* gene expression. Thus, suppression of PTH by FGF23 reduces expression of the *Cyp27b1* gene and serum levels of 1,25-dihydroxyvitamin D_3. This closes another long negative feedback loop for vitamin D homeostasis

convoluted tubules and then generate a secondary signal that instructs proximal tubules to reduce phosphate reabsorption and vitamin D synthesis. Recent animal studies may support the latter possibility. Despite the fact that proximal tubules primarily express FGFR3, knockout of the *Fgfr3* gene in *Hyp* mice, which have elevated serum FGF23 levels, failed to rescue their phosphate-wasting phenotypes [65]. Furthermore, it was reported that activation of the FGF signaling pathway was detectable only in distal convoluted tubules after injection of FGF23 into mice [66]. These findings suggest that activity of FGF23 may be independent of FGF signaling activation in the proximal tubule.

PTH plays an important role not only in calcium metabolism but also in phosphate homeostasis. Like FGF23, PTH has an activity that induces phosphaturia [3]. However, in contrast to FGF23, PTH up-regulates expression of the *Cyp27b1* gene and increases serum 1,25-dihydroxyvitamin D_3 levels [2]. Recent studies showed that the parathyroid gland is one of the few organs that express a decent amount of Klotho protein endogenously, indicating that the parathyroid may be a target organ of FGF23. In fact, FGF23 down-regulates PTH expression and suppresses PTH secretion in vivo and in vitro [67, 68]. The ability of FGF23 to reduce serum PTH levels may further enhance the activity of FGF23 as a counterregulatory hormone for vitamin D and contribute to a long negative feedback loop involving bone, kidney, and parathyroid gland (Fig. 1). However, it should be noted that patients with CKD typically exhibit secondary hyperparathyroidism associated with high serum FGF23 levels, which seemingly contradicts the ability of FG23 to suppress PTH secretion and production. Considering that Klotho expression is positively regulated by vitamin D [53], one possible explanation is that low serum vitamin D levels in CKD patients may reduce Klotho expression not only in the kidney (discussed below) but also in the parathyroid glands and make these organs resistant to FGF23.

Phosphate toxicity

Defects in either Klotho or FGF23 disrupt the negative feedback loops that maintain phosphate and vitamin D homeostasis, resulting in high serum phosphate and vitamin D levels. High serum vitamin D promotes intestinal absorption of calcium and induces hypercalcemia as well. Importantly, this metabolic state characterized by high serum phosphate, calcium, and vitamin D levels is

associated with a premature aging syndrome, as observed in Klotho-deficient mice and FGF23-deficient mice. These observations imply that phosphate, calcium, and/or vitamin D may be toxic when retained and thus accelerate aging. Several animal studies have supported this notion. First, vitamin-D-deficient diet not only restored serum phosphate and calcium levels but also rescued several aging-like phenotypes in Klotho-deficient mice and FGF23-deficient mice [53, 69]. Second, ablation of vitamin D activity in Klotho-deficient mice and FGF23-deficient mice by disrupting the *Cyp27b1* gene [70, 71] or vitamin D receptor gene [72] also rescued hyperphosphatemia, hypercalcemia, and the premature aging syndrome. Lastly, low phosphate diet rescued shortened life span and vascular calcification in FGF23-deficient mice and Klotho-deficient mice [69, 73]. These studies provide evidence that the premature aging syndrome caused by defects in the bone−kidney endocrine axis is due to retention of phosphate, calcium, and/or vitamin D. It should be noted that low phosphate diet rescued FGF23-deficient mice despite the fact that it further increased already high serum calcium and vitamin D levels [69], suggesting that phosphate, but not calcium or vitamin D, is primarily responsible for the aging-like phenotypes. It is likely that low vitamin D diet and ablation of vitamin D activity rescued accelerated aging through reducing serum phosphate levels, although it remains to be determined whether high serum vitamin D and/or calcium levels are a prerequisite for phosphate to accelerate aging.

Chronic kidney disease

Phosphate retention is universally observed in patients with CKD. Hyperphosphatemia has been identified as a potent, independent risk of death [74]. Why does hyperphosphatemia increase mortality? One likely explanation is that high blood phosphate levels trigger vascular calcification and accelerate life-threatening complications such as cardiovascular events [75]. Vascular calcification is a very common complication in CKD and has been shown to contribute to the high morbidity and mortality in terms of cardiovascular events. The National Kidney Foundation task force indicated that the cardiovascular mortality of a 35-year-old patient on dialysis is equivalent to that of an 80-year-old healthy individual, rendering CKD to be one of the most potent accelerators of aging [76]. In addition, the American Heart Association announced that CKD should be included in the highest-risk group for cardiovascular disease and that patients with CKD should receive aggressive therapeutic measures to reduce morbidity and mortality [77]. Thus, lowering blood phosphate levels is expected to reduce vascular calcification and cardiovascular events, thereby improving prognosis of CKD patients. In fact, CKD patients with hyperphosphatemia (≥6.5 mg/dl) were reported to have higher risk for death resulting from cardiovascular disease than those with the lower serum phosphate levels (< 6.5 mg/dl) [78]. Based on these observations, control of blood phosphate levels <6.5 mg/dl has been proposed as one of the most important therapeutic goals for improving life expectancy of CKD patients.

It is likely that dysregulation of the FGF23-Klotho endocrine axis may be involved in the mechanism by which CKD patients fail to maintain phosphate homeostasis. In fact, serum FGF23 levels are increased with advancing stages of CKD [79], whereas Klotho expression in the kidney is significantly decreased in CKD patients [80] and in various animal models of chronic and acute renal damage [81, 82]. Thus, CKD may be viewed as a state of FGF23 resistance caused by Klotho deficiency. This viewpoint explains several observations on phosphate metabolism in CKD that lack mechanistic insights. For example, epidemiological studies have indicated that serum FGF23 levels increase long before serum phosphate levels increase during the progression of CKD [79]. In other words, patients with early stages of CKD require higher serum FGF23 levels than normal people to maintain normal serum phosphate levels. This may represent compensation for end-organ resistance to FGF23 due to decreased Klotho expression in the kidney. It has been also known that serum vitamin D levels decrease long before serum phosphate levels increase during CKD progression [79]. This may be a result of the secondary hyper-FGF23-emia caused by decreased renal Klotho expression, because FGF23 has an activity that lowers serum vitamin D levels. In addition, epidemiological studies have indicated that high serum FGF23 levels are associated with poor prognosis in patients undergoing dialysis [83]. This may be explained by assuming that high serum FGF23 indicates low renal Klotho expression associated with severe renal damage. It remains to be determined whether decrease in Klotho expression is one of the earliest changes in the progression of CKD.

Of note, recent animal studies have shown that Klotho functions as a renoprotective factor. Although the mechanism is yet to be determined, overexpression of Klotho ameliorated progressive renal injury in mouse models of glomerulonephritis [81] and acute kidney injury [82]. Thus, it may be of therapeutic value for CKD to preserve Klotho expression in the kidney. Klotho expression is down-regulated by angiotensin II [84, 85] and up-regulated by peroxisome proliferator-activated receptor-gamma (PPARγ) agonists such as thiazolidinediones [86]. These observations suggest that renoprotective effects of angiotensin-converting enzyme inhibitors and thiazolidinediones may be partly attributed to their potential for increasing or preserving Klotho expression in the kidney. Klotho expression is also up-regulated by 1,25-dihydroxyvitamin D_3 [53].

Thus, low serum vitamin D caused by secondary hyper-FGF23-emia further reduces Klotho expression, potentially leading to deterioration spiral of Klotho expression. The benefit of vitamin D replacement therapy may be partly attributed to interruption of this vicious cycle.

In addition to functioning as an obligate coreceptor for FGF23, Klotho protein functions as a humoral factor that regulates activity of several ion channels and growth-factor receptors [41], which represents a novel function of Klotho protein. The entire extracellular domain of Klotho protein is clipped by a membrane-anchored protease ADAM10/17 on the cell surface and released into the extracellular space [87]. In fact, Klotho ectodomain (secreted Klotho protein) is detectable in the blood, urine, and cerebrospinal fluid [41, 88]. The secreted Klotho protein in turn functions as a putative sialidase that removes terminal sialic acids in the glycans of several ion channels, including a calcium channel, transient receptor potential vanilloid type isoform 5 (TRPV5) [89, 90], and a potassium channel, renal outer medullary potassium channel-1 (ROMK1) [91]. Removal of sialic acids by secreted Klotho protein on the cell surface prevents internalization of these ion channels, resulting in increase in transepithelial calcium (Ca^{2+}) absorption and potassium (K^{+}) secretion in distal nephrons, respectively. Thus, Klotho protein not only regulates phosphate metabolism by functioning as a coreceptor for FGF23 but also regulates calcium and potassium metabolism by functioning as a humoral factor that modifies trafficking of TRPV5 and ROMK1. Significance of the secreted Klotho protein in the regulation of calcium and potassium homeostasis and in pathophysiology in CKD remains to be determined.

It has become increasingly clear that phosphate metabolism plays a critical role in the pathophysiology in CKD and that hyperphosphatemia should be aggressively treated to improve life expectancy of CKD patients. In this context, the Klotho and FGF23 axis is expected to be a novel target of therapeutic interventions in CKD.

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