FIBROBLAST GROWTH FACTOR 23: MECHANISMS OF ACTION AND REGULATION

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ABSTRACT

Fibroblast growth factor 23 (FGF23) is a central regulator in mineral metabolism. It is produced mainly by osteocytes in the bone and exerts its effects on distant organs in an endocrine manner. FGF23 generally requires a transmembrane protein named αKlotho to evoke its signal via an FGF receptor (FGFR). In the kidney, FGF23 increases the renal phosphate excretion and decreases the production of 1, 25-dihydroxyvitamin D [1, 25$(OH_2)_2$ D]. In the parathyroid gland, it suppresses the secretion of parathyroid hormone (PTH). The placenta also expresses both FGFR1 and αKlotho, and an elevated level of maternal FGF23induces the placental expression of 25-hydroxyvitamin D-24-hydroxylase, affecting fetal vitamin D metabolism. Pathologically elevated levels of FGF23 may exert its effects even on the tissues without αKlotho expression, such as myocardium. Excessive action of FGF23 of various causes leads to hypophosphatemic rickets/osteomalacia, while its impaired action results in hyperphosphatemia and ectopic calcification. Some of the molecules responsible for hereditary hypophosphatemic rickets/osteomalacia reside in the osteocytes and function as local regulators of the production and/or activity of FGF23. The FGF23 expression is controlled by systemic factors also, among which 1, 25$(OH_2)_2$D appears to be a principal regulator. In chronic kidney disease (CKD), FGF23 levels begin to increase from the early stages, although the underlying mechanism still remains unclear. The elevated FGF23 levels in CKD have been shown to be associated with poor outcomes. Elucidation of the mechanism for action and regulation of FGF23 will contribute to the development of new strategies for diagnosis and treatment of the diseases with impaired mineral metabolism.

Keywords: FGF23, αKlotho, osteocytes, hypophosphatemic rickets, chronic kidney diseases

INTRODUCTION

Phosphorus is an essential nutrient involved in various biological processes including skeletal mineralization in vertebrates. In human adults, approximately 85% of the total phosphorus is distributed in the bone as hydroxyapatite (calcium-phosphate) crystals, while 15% is present in the soft tissues. Extracellular fluid contains only 0.1% of phosphorus, and phosphorus in the serum exists mostly as inorganic phosphate (Michigami 2013). Chronic hypophosphatemia leads to impaired skeletal mineralization, which is called rickets in children and osteomalacia in adults. Since the identification of fibroblast growth factor 23 (FGF23) as the responsible molecule for autosomal dominant hypophosphatemic rickets (ADHR) and tumor-induced hypophosphatemic osteomalacia (TIO) at the beginning of this century, accumulating studies have deepened our understanding on the molecular basis for the control of phosphate homeostasis (Razzaque 2009; Kovesdy & Quarles 2013a; White, et al. 2014). FGF23 is produced mainly by osteocytes in the bone and exerts its effects on the distant organs such as the kidney in an endocrine fashion, and it usually requires a transmembrane protein αKlotho to exert its signal through an FGF receptor (FGFR) (Kurosu, et al. 2006; Urakawa, et al. 2006). Genetic studies have revealed that loss-of-function mutations of several osteocytic molecules such as PHEX, DMP1, and FAM20C cause overproduction of FGF23 and hypophosphatemic rickets/osteomalacia (White, et al. 2014; Fukumoto, et al. 2015), suggesting these molecules locally regulate the production of FGF23. In addition, the expression of FGF23 is also regulated by systemic factors, among which 1,25-dihydroxyvitamin D [1,25$(OH_2)_2$ D] has been extensively studied (Kovesdy & Quarles 2013b). This review provides an overview on our current knowledge on the regulation and action of FGF23 in mineral metabolism as well as its association with various diseases.

1. Physiological Roles of FGF23

FGF23 is a 32-kDa protein that consists of 251 amino acids including a 24-amino acid signal peptide (Razzaque 2009; Kovesdy & Quarles 2013a; White, et al. 2014). It is produced mostly by the bone and exerts its effects on distant organs including the kidney. In the bone, osteocytes, which differentiate from osteoblasts and are embedded within the bone matrix, are the main source of FGF23 (Ubaidus, et al. 2009; Miyagawa, et al. 2014). FGF23 belongs to FGF19 subfamily together with FGF19 and FGF21, since they are unique among FGFs and act in an endocrine fashion to regulate diverse physiological processes. FGF19 plays a role in energy and bile acid homeostasis (Tomlinson, et al. 2002; Holt, et al. 2003), and FGF21 controls glucose and lipid metabolism (Kharitonenkov, et al. 2005). It has been suggested that the endocrine function of the FGF19 subfamily member is conferred by their low binding affinity to heparin/heparan sulfate, which allows them to enter the circulation with escaping the heparan sulfate surrounding their producing cells (Goetz, et al. 2007; Goetz, et al. 2012). Another feature shared by the members of FGF19 family is the requirement of Klotho protein for their signal transduction (Goetz, et al. 2007). FGF23 binds to the FGFR and αKlotho to form a complex, and induces the phosphorylation of the FGFR substrate 2α (FRS2α) and ERK1/2 downstream (Kurosu, et al. 2006; Urakawa, et

al. 2006). The organs expressing both FGFR and αKlotho can be the targets for the physiological effects of FGF23, which include the kidney, parathyroid gland, pituitary gland, and choroid plexus (Kuro-o, et al. 1997; Stubbs, et al. 2007). The N-terminal domain of FGF23 binds to FGFR, while its C-terminus binds to αKlotho (Goetz, et al. 2007). FGF23 is inactivated by proteolytic cleavage between Arg179 and Ser180 by subtilisin-like enzymes that recognize the Arg176-X-X-Arg179/Ser180 (RXXR/S) motif (White, et al. 2014). It was reported that isolated C-terminal fragments of FGF23 inhibited the formation of FGF23/FGFR/αKlotho complex to alleviate FGF23-induced hypophosphatemia (Goetz, et al. 2010).

The kidney plays the central role in phosphate balance in mammals. FGF23 increases renal phosphate excretion by reducing the expression of type 2a and 2c sodium/phosphate (Na^+ /Pi) co-transporters (NPT2a and NPT2c) in the proximal tubules (Shimada, et al. 2001). In addition, FGF23 suppresses the renal expression of 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase) and induces that of 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase), leading to the reduced levels of 1,25(OH_2) D (Shimada, et al. 2001). Interestingly, the majority of αKlotho localizes to the renal distal tubules, although its small amount is expressed in proximal tubules (Farrow, et al. 2012). A mutant mouse model with conditional deletion of αKlotho in the renal distal tubules exhibited hyperphosphatemia and the elevated FGF23 levels, suggesting the importance of FGF23-mediated signaling in the distal tubules in phosphate homeostasis (Olauson, et al. 2012). Recently, it was also reported that FGF23 promoted calcium reabsorption in the distal tubules via transient receptor potential vanilloid-5 (TRPV5) channels (Andrukhova,et al. 2014).

In the parathyroid gland which also co-expresses FGFR and αKlotho, it has been shown that FGF23 suppresses the secretion and gene expression of PTH (Ben-Dov, et al. 2007). Interestingly, a recent study using parathyroid-specific conditional deletion of αKlotho has unraveled that FGF23 still suppresses PTH secretion through an NFAT pathway even in the absence of αKlotho (Olauson, et al. 2013). Thus, FGF23 appears to suppress the secretion of PTH in both αKlotho-dependent and -independent manners.

We have recently reported that the placenta also expresses both αKlotho and FGFR1 in the feto-maternal interface (Ohata, et al. 2011; Ohata, et al. 2014). Immunostaining detected the co-localization of αKlotho and FGFR1 in the syncytiotrophoblasts and mononuclear trophoblasts in mice and the syncytiotrophoblasts in human. These cells are originated from fetuses and face the maternal blood, providing the feto-maternal interface. We further investigated whether the placenta could be a target for FGF23 action and found that elevated level of FGF23 in maternal blood induced the placental expression of Cyp24a1 encoding vitamin D-24-hydroxylase, which affected the fetal vitamin D metabolism (Ohata, et al. 2014). The placental expression of Na^+ /Pi co-transporters, among which type 2b co-transporter was dominant, was not altered by the elevated FGF23.

In chronic kidney disease (CKD), higher FGF23 levels have been related to the increased risk of cardiovascular disease and mortality (Kovesdy & Quarles 2013b). Although the myocardium does not express α-Klotho, it has been suggested that FGF23 exerts its direct effects on the myocardium to induce left ventricular hypertrophy by FGFR-mediated activation of calcineurin/NFAT signaling pathway, which is an effect independent of αKlotho (Faul, et al. 2011).

Thus, in pathological conditions with extremely high levels of FGF23, it might exert its effects on the tissues that do not express αKlotho. The transmembrane protein αKlotho contains a large extracellular domain that interacts with FGF23 and a very short intracellular region (Kuro-o, et al. 1997; Imura, et al. 2004). The membrane-bound αKlotho can be cleaved near the transmembrane domain, producing a soluble form of αKlotho that is detectable in serum, cerebrospinal fluid, and urine (Imura, et al. 2004; Yamazaki, et al. 2010). Although the physiological role of soluble αKlotho is still largely unknown, it might mediate the FGF23 action in the tissues that do not express membrane-bound αKlotho (Kawai, et al. 2013).

2. FGF23 Associated Diseases

2.1 Hyperphosphatemic disease caused by impaired actions of FGF23

Hyperphosphatemic familial tumoral calcinosis (HFTC, OMIM #211900) is a rare autosomal recessive disease, which is characterized by hyperphosphatemia, normal or elevated levels of serum $1,25(OH_2)_2D$, and ectopic calcification. These manifestations are similar to the phenotype of *Fgf23* knockout mice. To date, loss-of-function mutations in three genes, *FGF23*, *GALNT3*, and *Klotho*, have been identified to be responsible for HFTC (Hori, et al. 2011; Araya, et al. 2005; Benet-Pages, et al. 2005; Topaz, et al. 2004; Frishberg, et al. 2007; Ichikawa, et al. 2007). The *GALNT3* gene encodes an enzyme UDP-N-acetyl-α-D-galacosamine:polypeptide N-acetylgalactosaminyltransferase 3(GalNAc-T3), which mediates the attachment of O-glycans to Thr^{178} of FGF23. This O-glycosylation at Thr^{178} has been suggested to prevent the proteolytic cleavage between Arg^{179} and Ser^{180} (Kato, et al. 2006).

Currently, two kinds of assays are used for measurement of circulating FGF23. An ELISA using two antibodies that recognize the N-terminal and C-terminal portions of the FGF23 cleavage site measures only the bioactive, intact FGF23 proteins (Yamazaki, et al. 2002). On the other hand, C-terminal assays use two antibodies against the C-terminal region and detect both the intact and the cleaved C-terminal fragments of FGF23 (Jonsson, et al. 2003). In patients with HFTC caused by GALNT3 inactivating mutations, FGF23 levels measured by a C-terminal assay are high, while those detected by an intact assay are low to normal range (Topaz, et al. 2004; Frishberg, et al. 2007). The O-glycosylation at Thr178 is impaired in these patients, resulting in the susceptibility of FGF23 protein to the inactivation by cleavage. It was reported that ablation of the Galnt3 gene in mice lead to low level of intact FGF23 in the serum and hyperphosphatemia, despite the increased Fgf23 expression in the bone (Ichikawa, et al. 2009).

In patients with HFTC caused by loss-of-function mutations in FGF23 as well, FGF23 levels determined by a C-terminal assay are elevated, while the levels of intact FGF23 are low (Araya, et al. 2005). When the mutant FGF23 causing HFTC was expressed in cultured cells, only the C-terminal fragment was secreted into the media, and the full-length protein was retained in the Golgi complex (Araya, et al. 2005; Benet-Pages, et al. 2005). These results suggest that the mutations causing HFTC may impair the secretion of full-length FGF23, although the precise mechanism still remains to be elucidated. The inappropriately low level of bioactive intact FGF23 leads to hyperphosphatemia, elevated $1,25(OH_2)_2$ D and ectopic calcification.

In 2007, a case of HFTC was reported where a homozygous missense mutation in the *KLOTHO* gene encoding

αKlotho was identified to be responsible. The identified mutation H193R occurred in the extracellular FGF23-binding domain of αKlotho protein. In this patient, the FGF23 levels were quite high by both full-length and C-terminal assays, reflecting the resistance to FGF23 in the target organs (Ichikawa, et al. 2007).

2.2 Hypophosphatemic rickets/osteomalacia caused by excessive actions of FGF23

Various kinds of hypophosphatemic rickets/osteomalacia including hereditary diseases are caused by excessive actions of FGF23. These conditions are characterized by urinary phosphate wasting, hypophosphatemia, inappropriately low level of serum $1,25(OH)_2$ D, and impaired skeletal mineralization (Hori, et al. 2011; White, et al. 2014; Fukumoto, et al. 2015).

Autosomal Dominant Hypophosphatemic Rickets (ADHR, OMIM #193100) is caused by missense mutations in the *FGF23* gene, which occur at Arg^{176} or Arg^{179} within the RXXR/S motif recognized by subtilisin-like proprotein convertase, making the protein resistant to cleavage (ADHR-CONSORTIUM, 2000). ADHR may manifest as either early or delayed onset with variable expressivity, and the serum levels of intact FGF23 are not always high in ADHR patients (Imel, et al. 2007). Clinical and translational studies have revealed an association between FGF23 levels and iron metabolism in ADHR. The late-onset ADHR occurs primarily in post-pubertal females, who are prone to be iron-deficient (Imel, et al. 2007; Econs & McEnery 1997). Low serum iron concentration was associated with the elevation in both C-terminal and intact FGF23 levels in ADHR patients (Imel, et al. 2007). On the other hand, in healthy controls, low levels of serum iron were associated with only elevated C-terminal FGF23, but not intact FGF23. Thus, iron deficiency leads to the elevation in intact FGF23 levels only in ADHR patients but not in healthy subjects (Imel, et al. 2011). Contribution of iron deficiency to the manifestation of ADHR was confirmed by translational studies using FGF23-knockin mice carrying the R176Q ADHR point mutation (Farrow, et al. 2011).

Among the hereditary hypophosphatemic rickets, X-linked hypophosphatemic rickets (XLH, OMIM #307800) is the most common form. XLH is caused by inactivating mutations in the *phosphate -regulating gene homologous to endopeptidase on X chromosome (PHEX)*, whose product is a member of the M13 family of type II cell-surface zinc-dependent proteases (HYP-CONSORTIUM, 1995). PHEX is expressed in the osteoblast/osteocyte lineage cells with higher expression in osteocytes, which is similar to FGF23 (Beck, et al. 1997; Miyagawa, et al. 2014). Patients with XLH have elevated levels of intact FGF23 and biochemical features of excessive FGF23 actions, namely, urinary phosphate wasting, hypophosphatemia and inappropriately low levels of $1,25(OH)_2$ D (Carpenter, et al. 2011). In *Phex-deficient Hypmouse*, a widely used model for human XLH, the *Fgf23* expression in the osteocytes has been shown to be elevated (Liu, et al. 2006; Miyagawa, et al. 2014), suggesting that PHEX negatively regulates the FGF23 expression. However, this regulation of FGF23 by PHEX might be indirect, since it has been shown that FGF23 does not serve as a substrate for PHEX (Benet-Pages, et al. 2004). We previously investigated the detailed gene expression profile in the isolated osteocytes and found that the expression of *dentin matrix protein 1 (Dmp1), family with sequence similarity 20, member C (Fam20C), and solute carrier family 20a1 (Slc20a1)* encoding type III Na^+/Pi co-transporter Pit1 was also elevated *in*

Hyposteocytes compared to wild-type osteocytes. The up-regulation of *Fgf23, Dmp1 and Fam20C in Hypbones* began before birth, when serum Pi levels were comparable between *Hyp* and wild-type mice. On the other hand, the expression of *Slc20a1* in *Hypbone* was similar to that in wild-type bone in fetal stage and was increased after birth, probably in response to a decrease in serum phosphate level. Interestingly, the genes for FGF1, FGF2, their receptors, and Egr-1 that is a target of FGF signaling were also up-regulated in *Hyposteocytes*, suggesting the activation of FGF/FGFR signaling (Miyagawa, et al. 2014). Increased FGF/FGFR signaling in Hyp bone has been reported by other groups as well (Martin, et al. 2011; Martin, et al. 2012). Moreover, Xiao, et al. recently reported that osteocyte-specific deletion of *Fgfr1* in *Hyp* mice suppressed the expression of Fgf23 in the bone and partially rescued the hypophosphatemia and rickets (Xiao, et al. 2014).

Autosomal recessive hypophosphatemica rickets Type 1 (ARHR1, OMIM #241520) is caused by loss-of-function of *DMP1*, which is highly expressed in the osteocytes as well as dentin (Feng, et al. 2006; Lorenz-Depiereux, et al. 2006). DMP1 is an extracellular matrix protein belonging to the SIBLING (small integrin-binding ligand, N-linked glycoproteins) family. ARHR1 patients and *Dmp1*-null mice are featured by elevated serum FGF23, hypophosphatemia, inappropriately low 1,25(OH) $_2$D and skeletal hypomineralization, as are XLH patients and *Phex*-deficient *Hyp* mice (Feng, et al. 2006; Lorenz-Depiereux, et al. 2006). As described earlier, Martin, et al. have suggested that both PHEX and DMP1 regulates FGF23 expression in osteocytes through FGFR signaling pathway, based on the finding in compound *Phex* and *Dmp1* mutant mice (*Hyp/Dmp1*$^{-/-}$) (Martin, et al. 2011). Regulation of FGF23 by FGFR signaling is also suggested by osteoglophonic dysplasia, which is caused by an activating mutation of FGFR1 and is associated with increased FGF23 levels and hypophosphatemia (White, et al. 2005).

ARHR Type 2 (ARHR2, OMIM #613312) is also associated with excessive actions of FGF23and is caused by loss of function mutations in *ectonucleotide pyrophosphatase phosphodiesterase-1 (ENPP1)* (Lorenz-Depiereux, et al. 2010; Levy-Litan, et al. 2010). This gene encodes an enzyme involved in the production of pyrophosphate, an inhibitor of mineralization. Inactivating mutations of this gene are also known to be responsible for hypermineralization disorders such as generalized arterial calcification in infancy (GACI, OMIM #208000) (Ruf, et al. 2005; Rutsch, et al. 2003). *Enpp1*-null mice exhibited elevated FGF23 and decreased serum phosphate, but not rickets/osteomalacia (Mackenzie, et al. 2012).

FAM20C, also known as Golgi Casein Kinase (G-CK) or dentine matrix protein-4 (DMP4), is a kinase that phosphorylates various secreted proteins including the members of the SIBLING family such as DMP1, osteopontin, and MEPE (matrix extracellular phosphoglycoprotein) (Tagliabracci, et al. 2012). In addition, FAM20C directly phosphorylates FGF23 also and regulates its glycosylation and proteolysis (Tagliabracci, et al. 2014). It was reported that *Fam20c*-null mice exhibited increased levels of full-length FGF23 in serum, hypophosphatemia, skeletal hypomineralization, decreased expression of *Dmp1* in osteocytes, and dental defects (Wang, et al. 2012a; Wang, et al. 2012b). Detailed analysis of dental defects in mice with constitutive and conditional deletion of *Fam20c* suggested its profound roles in both amelogenesis and dentinogenesis (Wang, et al. 2013). In human,

inactivating mutations in *FAM20C* are known to be responsible for Raine syndrome (RNS, OMIM #259775), which is an autosomal recessive, neonatal osteosclerotic bone dysplasia (Simpson, et al. 2007). Although RNS usually results in death within the first few weeks of life, some patients survive into childhood. In 2013, an exome sequencing study identified the *FAM20C* mutations in the brothers who manifested increased FGF23, hypophosphatemia and dental anomaly, although they did not show skeletal hypomineralization (Rafaelsen, et al. 2013). We also recently reported a case with a mild variant of RNS, where a homozygous inactivating mutation caused increased level of FGF23, hypophosphatemic osteomalacia and bone sclerosis. This patient had lost all his teeth before 17 years of age (Takeyari, et al. 2014).

Tumor induced osteomalacia (TIO) is a paraneoplastic disease usually caused by benign phosphaturic mesenchymal tumors, and FGF23 has been identified to be a responsible molecule (Folpe, et al. 2004; Shimada, et al. 2001). Overproduction of FGF23 from the tumors leads to hypophosphatemia, low $1,25(OH)_2$ D levels and osteomalacia. Removal of the responsible tumors rapidly decreases the serum FGF23 level and cures the disease (Takeuchi, et al. 2004).

McCune-Albright syndrome (MAS, OMIM #174800) is characterized by polyostotic fibrous dysplasia, café-au-lait skin pigmentation, and precocious puberty, although it is clinically heterogeneous and may include various other endocrinological abnormalities. MAS is caused by a somatic activating mutation in *GNAS1* encoding the subunit of stimulatory G protein. Some patients manifest hypophosphatemia associated with excessive FGF23 action (Riminucci, et al. 2003). Although the production of FGF23 from the skeletal lesions of fibrous dysplasia has been demonstrated, it is still unclear how the GNAS1 mutation results in the increased FGF23.

There are several case reports of FGF23-associated hypophosphatemia caused by intravenous administration of saccharated ferric oxide (Schouten, et al. 2009; Shimizu, et al. 2009). This drug is composed of iron and maltose and is widely used to treat iron-deficient patients with anemia. Although the mechanism for the elevation in FGF23 levels after the administration of saccharated ferric oxide is unclear, the discontinuance of the drug rapidly restores high FGF23 levels and hypophosphatemia.

3. Regulation of FGF23

3.1 Local regulators

As described above, the levels of FGF23 are altered in various genetic disorders. Some of the molecules responsible for these conditions locally regulate the production of FGF23 in the osteoblasts and osteocytes. PHEX, DMP1, FAM20C, and ENPP1 function as negative regulators for the *FGF23* expression, as suggested by the associated disorders and the mouse models. It is interesting that these molecules play critical roles in biomineralization as well as phosphate homeostasis. Although the physiological substrates for PHEX remains uncertain, it cleaves and inactivates acidic serine-and aspartate-rich motif (ASARM) peptides derived from MEPE, osteopontin, and probably other proteins of SIBLING family that act as inhibitors of mineralization (Rowe 2012; Martin, et al. 2008). DMP1 has been shown to regulate mineralization of dentin through the induction of dentine sialophosphoprotein (DSPP) (Sreenath, et al. 2003; Gibson, et al. 2013). Inactivating mutations in *FAM20C* and *ENPP1* are responsible for the conditions characterized by the ectopic calcification,

as described earlier (Simpson, et al. 2007; Ruf, et al. 2005; Rutsch, et al. 2003). These finding suggest a link between the regulation of FGF23 and biomineralization, although the underlying molecular basis remains to be elucidated.

As to PHEX and DMP1, aforementioned mouse studies have demonstrated that they both suppress the expression of FGF23 through the inactivation of FGFR signaling in the osteocytes (Martin, et al. 2011). Interestingly, FGF ligands including FGF1, low molecular weight (LMW)-FGF2, FGF7 as well as high molecular weight (HMW)-FGF2 were shown to be increased in Hyp and/or Dmp1-null mice with the increased osteocytic expression of Fgf23 (Martin, et al. 2011; Miyagawa, et al. 2014; Xiao, et al. 2010; Xiao, et al. 2013; Liu, et al. 2009). These findings, together with the identification of activating mutations in FGFR1 in osteoglophonic dysplasia associated with elevated FGF23 levels and hypophosphatemia (White, et al. 2005), indicate the involvement of FGF/FGFR signaling in the regulation of FGF23 expression.

The FGF2 gene produces LMW and HMW FGF2 isoforms (18 KDa and 22–34 KDa, respectively, in human) generated by alternative initiation codons. Osteoblast lineage cells produce both LMW- and HMW-FGF2 isoforms (Arnaud, et al. 2009). LMW-FGF2 secreted out of cells and forms complexes with cell surface FGFRs and heparan-sulfate proteoglycans to evoke signals through PI3K/Akt, RAS/MAPK, and PLCγ intracellular pathways (Eswarakumar, et al. 2005). On the other hand, HMW-FGF2 isoforms have a nuclear localization sequence (NLS) that confers their nuclear localization and activation of intracellular FGFR1/CBP/CREB pathway (Stachowiak, et al. 2003). Hurley and her colleagues demonstrated that overexpression of HMW-FGF2 isoforms in the osteoblastic lineage cells in mice resulted in hypophosphatemic rickets/osteomalacia associated with elevated FGF23 levels (Xiao, et al. 2010). Furtherore, Han, et al. recently reported that LMW-FGF2 transactivated FGF23 promoter in osteoblasts through membranous FGFR-mediated PLCγ/calcineurin/NFAT and MAPK pathways, while HMW-FGF2 isoforms stimulated the FG23 promoter activity via the intracellular FGFR1/CBP/CREB pathway (Han, et al. 2015). Thus, both LMW- and HMW-FGF2 derived from osteoblast-lineage cells appear to induce the expression of FGF23 locally.

FAM20C may regulate FGF23 levels through several ways. It phosphorylates a broad range of secreted proteins including FGF23 itself and SIBLINGs family of proteins such as DMP1 and MEPE (Tagliabracci, et al. 2012; Tagliabracci, et al. 2014; Tagliabracci, et al. 2015). In *Fam20c*-null mice with elevated FGF23 levels, the expression of Dmp1 was decreased (Wang, et al. 2012a). In addition, knockdown of FAM20C in osteoblastic cell lines led to a remarkable down-regulation of DMP1 along with the increased expression of FGF23 (Wang, et al. 2012a). These observations suggest that inactivation of FAM20C might increase the FGF23 expression through the down-regulation of DMP1. However, the authors also found that overexpression of Dmp1 did not decrease the serum FGF23 levels and failed to rescue the bone defects in *Fam20c*-null mice (Wang, et al. 2014). Therefore, the down-regulation of DMP1 may not significantly contribute to the elevated FGF23 levels in *Fam20c*-null mice, and the direct effect of FAM20C on FGF23 is more likely to be the case. It is reported that FAM20C phosphorylates FGF23 on Ser^{180} within the $R^{176}XXR^{179}/S^{180}$ subtilisin-like proprotein convertase

motif and that this phosphorylation inhibits O-glycosylation of FGF23 by GalNAc-T3 and promotes FGF23 cleavage (Tagliabracci, et al. 2014). Indeed, it has been recently confirmed that FGF23 in the bone is phosphorylated (Lindberg, et al. 2015).

Bone remodeling also appears to regulate serum levels of FGF23. It was previously reported that FGF23 levels were rapidly decreased after the intravenous administration of anti-bone resorptive agent bisphosphonate to patients with osteogenesis imperfecta (Kitaoka, et al. 2011). The decrease in the FGF23 levels in these patients preceded the decline in serum phosphate. In addition, we recently demonstrated that acute local bone resorption induced by calvarial injection of interleukin-1 in mice resulted in the elevation in serum FGF23 levels without the increase of its mRNA expression, and this effect was abolished by the pre-treatment with a bisphosphonate pamidronate (Yamazaki, et al. 2015).These findings suggest that osteoclatic bone resorption also might modulate the serum levels of FGF23.

3.2 Systemic regulators

In addition to the local factors, various systemic factors also appear to regulate the levels of FGF23, including 1,25(OH) $_2$ D, PTH, leptin, estrogen, calcium and phosphate (Kovesdy & Quarles 2013a; Liu, et al. 2006; Kawata, et al. 2007; Lavi-Moshayoff, et al. 2010; Rhee, et al. 2011; Tsuji, et al. 2010). However, the regulation of FGF23 by these factors is complex and context-dependent, and the underlying molecular mechanisms remain unclear. 1,25(OH) $_2$D appears to be the principal regulator of FGF23, and it increases the FGF23 expression in the osteoblast lineage cells (Liu, et al. 2006). Studies have demonstrated that 1, 25$(OH)_2$ D stimulates the FGF23 transcription via vitamin D receptor (VDR) (Haussler, et al. 2012). The critical role of 1,25$(OH)_2$ D in the regulation of FGF23 is also suggested by the decreased serum FGF23 levels in the patients with vitamin D deficiency (Kubota, et al. 2014). Furthermore, in FGF23-associated hypophosphatemic rickets such as XLH, patients are currently treated with active vitamin D and phosphorus to correct the impaired vitamin D metabolism and hypophosphatemia (Carpenter, et al. 2011). However, the administration of active vitamin D further increases the level of FGF23, which might worsen the disease (Imel, et al. 2010). Recently, a neutralizing antibody against FGF23 has been developed as a new therapy for XLH (Carpenter, et al. 2014; Imel, et al. 2015).

Regulation of FGF23 by other factors still remains controversial. PTH was reported to stimulate FGF23 expression in some studies (Kawata, et al. 2007; Lavi-Moshayoff, et al. 2010; Rhee, et al. 2011), but not in others (Liu, et al. 2006; Saji, et al. 2010). The effects of phosphate on FGF23 levels also seem inconsistent. Alteration in dietary intake of phosphate resulted in the changes in FGF23 levels in some studies (Antoniucci, et al. 2006; Burnett, et al. 2006; Ferrari, et al. 2005; Perwad, et al. 2005), but had no effects in others (Larsson, et al. 2003; Nishida, et al. 2006). It was also reported that acute changes in serum phosphate by infusion of dibasic potassium phosphate solution did not influence FGF23 levels in healthy humans (Ito, et al. 2007). In cell studies using the primary osteocytes isolated from mouse bones, we demonstrated that the 24-hour treatment with high extracellular phosphate resulted in a marked increase in the *Dmp1* expression, but had no effects on the *Fgf23* expression (Miyagawa, et al. 2014). On the other hand, Ito, et al. reported that extracellular phosphate modulated the effects of 1,25$(OH)_2$ D on the

FGF23 expression in an osteocytic cell line (Ito, et al. 2013). These inconsistent findings suggest that phosphate may affect the production of FGF23 indirectly, rather than directly, and may involve the vitamin D action, osteoblast differentiation and/or mineralization.

Recent studies suggest the regulation of FGF23 levels by calcium. It was reported that a diet low in both calcium and vitamin D resulted in hypocalcemia and low FGF23 levels in normal rats despite high PTH and high $1,25(OH)_2$ D levels. In addition, administration of calcium gluconate significantly increased the FGF23 level in parathyroidectomized rats (Rodriguez-Ortiz, et al. 2012). Interestingly, Quinn, et al. demonstrated that an increase in the FGF23 levels showed a stronger correlation with the calcium × phosphate products than those with individual mineral ions (Quinn, et al. 2013).

3.3 Regulation of FGF23 by circadian rhythm

Accumulating evidence has established the important roles of the circadian clock network for adaptation of living organisms to the environmental cues such as the nutrients (Green, et al. 2008; Bass, et al. 2010). Circadian control of metabolism occurs at both central and peripheral levels. Since sympathetic tone displays a circadian profile and is activated by food intake, we have recently investigated whether phosphate metabolism is regulated by circadian clock through the food intake-associated sympathetic activation (Kawai, et al. 2014). Skeletal expression of *Fgf23* showed a circadian rhythm with higher expression during the dark phase in mice fed standard chow ad libitum, which was associated with the increased levels of circulating FGF23 and enhanced phosphate excretion in urine. In the mice fed ad libitum, food consumption reaches highest at the beginning of dark phase. When the mice were fed only in the light phase, the peaks in the skeletal *Fgf23* expression and urinary phosphate excretion were shifted from dark phase to light phase. Sympathetic activation by administration of β-adrenergic agonist induced the skeletal *Fgf23* expression in a circadian-dependent manner, which was not observed in the mice deficient for a clock gene *Bmal1*. These results suggest that the skeletal expression of *Fgf23* is regulated by the time and food intake at least partly through the alteration in circadian profile of sympathetic activity in mice (Kawai, et al. 2014). Further studies are needed to clarify whether this is the case in humans as well. Various factors involved in the regulation of FGF23 and its physiological actions are summarized in Figure 1.

4. FGF23 in CKD

Hyperphosphatemia is often observed in patients with advanced CKD and those on dialysis. In CKD patients, serum levels of both C-terminal and intact FGF23 are elevated (Wolf 2010; Wahl & Wolf 2012). Interestingly, FGF23 levels begin to rise in the early stages of CKD with neutral phosphate balance and normal serum phosphate levels (Hill, et al. 2013), suggesting that phosphate is unlikely to contribute to the elevation of FGF23 in early CKD. Although $1,25(OH)_2$ D is a primary stimulator of FGF23 expression as described earlier, its levels in serum are usually decreased in CKD. Elevations in PTH might contribute to the increased FGF23 in established CKD, but it was reported that levels of C-terminal and intact FGF23 increased rapidly soon after the onset of acute kidney failure, independently of PTH as well as phosphate and $1,25(OH)_2$ D (Christov, et al. 2013). Thus, the mechanisms for the regulation

of FGF23 in CKD still remain unclear. Decreased expression of αKlotho in the diseased kidney might result in the resistance to FGF23 and its secondary increase (Koh, et al. 2001). Production of FGF23 from the tissues other than the bone also may be involved in the increased FGF23 levels in CKD.

Mounting evidence has indicated the association between elevated FGF23 levels and an increased mortality in patients on dialysis, non-dialysis CKD patients and post-kidney transplantation patients, as well as even in the subjects with normal kidney function (Gutierrez, et al. 2008; Isakova, et al. 2011; Jean, et al. 2009; Fliser, et al. 2007; Wolf, et al. 2011; Arnlov, et al. 2013). In addition, it has been demonstrated that elevated FGF23 levels are associated with increased progression of CKD (Isakova, et al. 2011; Jean, et al. 2009; Frishberg, et al. 2007; Wolf, et al. 2011; Kendrick, et al. 2011), cardiovascular events (Parker, et al. 2010), vascular calcification (Khan, et al. 2012), LVH (Gutierrez, et al. 2009; Faul, et al. 2011; Hsu & Wu 2009; Kirkpantur, et al. 2011), and arterial stiffness and endothelialdysfunction (Mirza, et al. 2009). As described earlier, animal studies have suggested αKlotho-independent direct effects of FGF23 on myocardium as pathogenesis of LVH in CKD (Faul, et al. 2011). Recently, an association between FGF23 levels and the levels of inflammatory markers was also shown in CKD patients (Munoz Mendoza, et al. 2012), and it was reported that FGF23 increased the production of inflammation-related molecules such as lipocalin-2, transforming growth factor-β and tumor necrosis factor-α in experimental studies (Dai, et al. 2012). These findings suggest that FGF23 may increase the adverse outcomes partially through its effects on inflammation.

CONCLUSION

Accumulating studies have provided important insights into the critical roles of FGF23 in mineral metabolism and its regulatory mechanisms. The production and/or activity of FGF23 are regulated by various local and systemic factors, and both excessive and impaired FGF23 actions contribute to the pathogenesis of disorders in mineral metabolism. In CKD, the levels of FGF23 begin to rise from the early stages and are associated with poor outcomes. Clarification of the basis for the action and regulation of FGF23 will lead to the development of new therapeutic approaches for the disorders in mineral metabolism.

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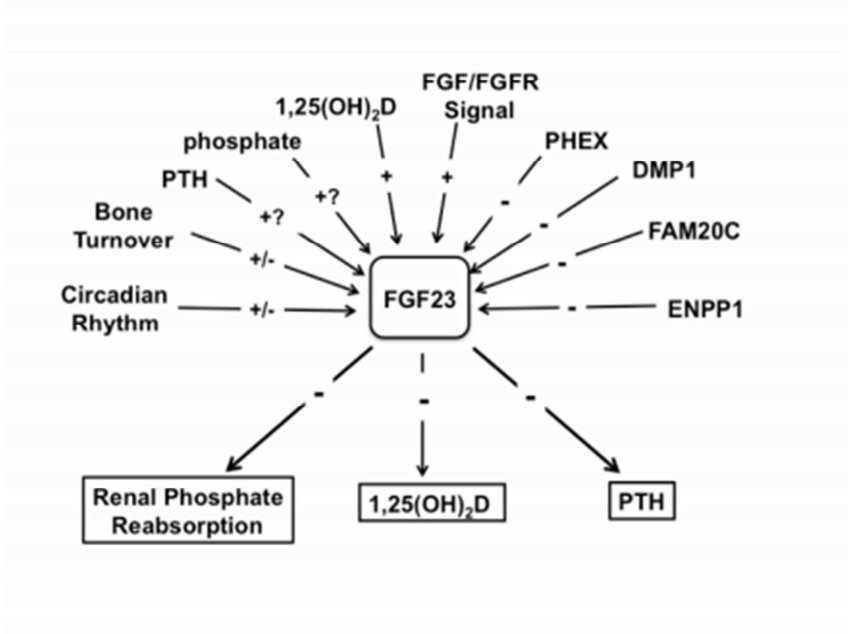
Figures

Figure 1. Physiological actions of FGF23 and the various local and systemic factors involve in its regulation.



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