

## REVIEW

# The emerging role of the fibroblast growth factor-23–klotho axis in renal regulation of phosphate homeostasis

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### Abstract

Normal mineral ion homeostasis is tightly controlled by numerous endocrine factors that coordinately exert effects on intestine, kidney, and bone to maintain physiological balance. The importance of the fibroblast growth factor (FGF)-23–klotho axis in regulating mineral ion homeostasis has been proposed from recent research observations. Experimental studies suggest that 1) FGF23 is an important *in vivo* regulator of phosphate homeostasis, 2) FGF23 acts as a counter regulatory hormone to modulate the renal  $1\alpha$ -hydroxylase and sodium–phosphate cotransporter activities, 3) there is a trend of interrelationship between FGF23 and parathyroid

hormone activities, 4) most of the FGF23 functions are conducted through the activation of FGF receptors, and 5) such receptor activation needs klotho, as a cofactor to generate downstream signaling events. These observations clearly suggest the emerging roles of the FGF23–klotho axis in maintaining mineral ion homeostasis. In this brief article, we will summarize how the FGF23–klotho axis might coordinately regulate normal mineral ion homeostasis, and how their abnormal regulation could severely disrupt such homeostasis to induce disease pathology.

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### Introduction

Maintaining physiological phosphate balance is of crucial biological importance, as it regulates various biological processes, as well as skeletal mineralization; it is an important component of nucleic acids, signaling proteins, coenzymes, and lipid bilayer of the cell membranes. Acute hypophosphatemia can cause myopathy, cardiac dysfunction, and hematological abnormalities; while chronic hypophosphatemia mostly affects bone mineralization to develop rickets and osteomalacia. On the other hand, hyperphosphatemia is associated with secondary hyperparathyroidism, commonly present in patients with chronic kidney diseases (CKDs). Understanding the molecular regulation of phosphate homeostasis is, therefore, of enormous clinical and biological importance. In an average healthy 70 kg adult individual, the total body phosphorus content is around 700 g, almost 80% of which is present in the skeleton as crystalline hydroxyapatite ( $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ), about 9% in the skeletal muscle, roughly 10·9% in the viscera, and the remaining 0·1% in the extracellular fluid (Gaasbeek & Meinders 2005). Intracellular phosphates are mostly present as organic phosphate compounds, such as creatine phosphate and ATPs; intracellular phosphate stores are also found in red cells as

2,3-diphosphoglycerate, which partly controls the  $\text{O}_2$  release to tissues by hemoglobin in red cells. Roughly 70% of the phosphate is absorbed in the duodenum and jejunum, through a sodium-dependent active transport, a process stimulated by 1,25-dihydroxyvitamin  $\text{D}_3$  ( $1,25(\text{OH})_2\text{D}$ ); moreover, parathyroid hormone (PTH) and low-phosphate diets can stimulate intestinal absorption of phosphate by exerting effects on vitamin D. For instance, hypophosphatemia can stimulate activity of the renal  $1\alpha$ -hydroxylase ( $1\alpha(\text{OH})\text{ase}$ ) to increase the synthesis of  $1,25(\text{OH})_2\text{D}$ .

The kidney is one of the most important sites for physiologic regulation of serum phosphate; it controls urinary phosphate excretion to balance the intestinal phosphate absorption. About 85% of renal reabsorption of phosphate occurs in the proximal tubules. Phosphate from the tubular lumen moves across the proximal tubular epithelial cells and effluxes at the basolateral membrane. Phosphate transport across the proximal tubular epithelial cells is a sodium-dependent process, and driven by a higher extracellular sodium gradient compared with inside the cell, and such a gradient is believed to be maintained by the basolateral membrane-associated Na,K-ATPase. Sodium/phosphate ( $\text{Na}/\text{Pi}$ ) cotransporter-mediated phosphate uptake across the

brush-border membrane is an electrogenic process and has pH sensitivity (Tenenhouse 2005). Although the precise mechanisms involved in the translocation of phosphate across the proximal tubular epithelial cells, and the efflux of phosphate across the basolateral membrane to the blood vessels are not yet clear, our understanding of the Na/Pi cotransporter system has significantly enhanced our knowledge of renal regulation of phosphate homeostasis.

### Renal regulation of phosphate homeostasis

Renal regulation of phosphate homeostasis is a complex process and is influenced by numerous intrinsic and extrinsic factors. For instance, factors that increase renal phosphate reabsorption include phosphate depletion, parathyroidectomy, 1,25(OH)<sub>2</sub>D, hypocalcemia, and hypocapnia; in contrast, factors that inhibit renal phosphate reabsorption include phosphate loading, PTH, volume expansion, hypercalcemia, carbonic anhydrase inhibitors, fibroblast growth factor (FGF)-7, FGF23, frizzled-related protein-4, and matrix extracellular phosphoglycoprotein. Many of these factors exert either stimulatory or inhibitory effects on renal Na/Pi cotransporters to positively or negatively regulate phosphate balance.

The Na/Pi cotransporter family mainly consists of three different types. Type I Na/Pi cotransporters are mostly present in the brush-border membrane of proximal tubular cells (Biber *et al.* 1993); subsequent studies, however, suggest that type I Na/Pi is not a typical cotransporter, rather influences intrinsic cellular phosphate transport (Soumounou *et al.* 2001). Type II Na/Pi cotransporters have three highly homologous isoforms; type IIa (Na/Pi-2a) and type IIc (Na/Pi-2c), which are almost exclusively present in the brush-border membrane of the renal proximal tubular epithelial cells (Custer *et al.* 1994, Segawa *et al.* 2002). In contrast, type IIb (Na/Pi-2b) cotransporters are present in the small intestinal lining epithelial cells, and are thought to be involved in intestinal phosphate absorption; of relevance, Na/Pi-2b cotransporters are not expressed in the kidney (Hilfiker *et al.* 1998). The third type of cotransporters, type III Na/Pi, are ubiquitously expressed (Kavanaugh *et al.* 1994), and present in the basolateral membrane of the renal tubules, where they are thought to serve a housekeeping function. Both Na/Pi-2a and Na/Pi-2c regulate renal phosphate transport in a sodium-dependent process; Na/Pi-2a-mediated transport is an electrogenic process, where membrane voltage acts as a kinetic determinant of inward fluxing of three sodium ions along with one divalent phosphorus anion (Forster *et al.* 2006). The intestinal Na/Pi-2b cotransporters are also electrogenic, while the Na/Pi-2c isoform mediates the electroneutral transport of two sodium ions with one divalent phosphorus anion. Since phosphate transport through the Na/Pi cotransporter system is the main regulatory mechanism of maintaining phosphate balance, the molecules that directly or indirectly affect the Na/Pi

cotransporter system could, therefore, influence phosphate homeostasis.

There are numerous factors that affect phosphate transport by exerting effects on the Na/Pi cotransporter system, one of the molecules that is widely studied is PTH. PTH could inhibit the Na/Pi cotransporter system across the brush-border membrane by endocytic retrieval of Na/Pi-2a and Na/Pi-2c proteins from the brush-border membrane to the subapical compartment; the internalized Na/Pi proteins are eventually degraded in lysosomes (Tenenhouse 2005, Forster *et al.* 2006). In contrast, phosphate deprivation is associated with microtubule-dependent recruitment of Na/Pi-2a and Na/Pi-2c proteins to the apical membrane (Tenenhouse 2005, Forster *et al.* 2006). The retrieval and recruitment of Na/Pi-2a and Na/Pi-2c proteins are a complex multi-step multifactorial process, and needs additional study for comprehensive understanding.

The essential role of Na/Pi-2a in regulating renal phosphate homeostasis was demonstrated in genetically altered mice in which the *Na/Pi-2a* gene was ablated by homologous recombination (Beck *et al.* 1998). Na/Pi-2a<sup>-/-</sup> mice have increased urinary phosphate excretion, resulting in hypophosphatemia. Furthermore, Na/Pi-2a<sup>-/-</sup> mice also develop hypercalcemia, hypercalciuria, and have low serum level of PTH (Chau *et al.* 2003). Perhaps as a compensatory effect, the Na/Pi-2c protein is markedly increased in the brush-border membrane of Na/Pi-2a<sup>-/-</sup> mice (Tenenhouse *et al.* 2003). Known regulators of renal phosphate reabsorption, including dietary phosphate intake or PTH administration have no significant effect on renal phosphate transport in Na/Pi-2a<sup>-/-</sup> mice, suggesting that Na/Pi-2a is the main regulatory molecule which handles renal phosphate transport activities in the mice (Tenenhouse 2005). It is predicted that in mice, in addition to Na/Pi-2a, about 30% of renal reabsorption could be mediated through Na/Pi-2c. In humans, however, Na/Pi-2c cotransporters appear to have a more important role in renal phosphate handling.

Hereditary hypophosphatemic rickets with hypercalciuria (HHRH), an autosomal recessive disorder was first identified in a large Bedouin kindred, and is characterized by hypophosphatemia secondary to renal phosphate wasting, with increased serum levels of 1,25(OH)<sub>2</sub>D that is associated with intestinal calcium hyperabsorption and hypercalciuria, and rickets and osteomalacia (Tieder *et al.* 1985). Recently, a genome-wide scan combined with homozygosity mapping has found a single nucleotide deletion in the *Na/Pi-2c* cotransporter gene in all affected individuals with HHRH (Bergwitz *et al.* 2006, Lorenz-Depiereux *et al.* 2006a). Interestingly, genomic deletion of Na/Pi-2c from mice has produced minor phenotypes (Segawa *et al.* 2006) when compared with Na/Pi-2a-ablated mice, again suggesting that there might be differences in the regulation of phosphate homeostasis in humans and mice, and that the Na/Pi-2c cotransporter may have more important regulatory functions in maintaining phosphate homeostasis in humans. Identification of FGF23, as ‘phosphatonin’ from tumor-induced osteomalacia (TIO) and patients with autosomal

dominant hypophosphatemic rickets (ADHR), which could induce urinary phosphate wasting by inhibiting the Na/Pi cotransporter system in the kidney has further enhanced our understanding of the renal regulation of phosphate homeostasis.

## FGF23

Human FGF23 was identified by positional cloning as a responsible gene for inducing phosphate wasting in patients with ADHR; gain-of-function mutations in the *FGF23* gene were identified in ADHR, that are believed to enhance biological activities of FGF23 to produce distinct clinical symptoms in these patients (ADHR\_Consortium 2000). FGF23 was also identified as a causative humoral factor that induces clinical symptoms in patients with TIO; tumors causing TIO have been shown to produce excessive amounts of FGF23 (Shimada *et al.* 2001). In accordance with human diseases, functional *in vivo* experimental studies have identified FGF23 as one of the most potent phosphatonins that induce renal phosphate wasting (Bai *et al.* 2004, Larsson *et al.* 2004, Shimada *et al.* 2004a,b, Sitara *et al.* 2004).

FGF23 could suppress the expression of Na/Pi-2a and Na/Pi-2c cotransporters that mediate physiological phosphate uptake in proximal tubular epithelial cells (Shimada *et al.* 2004c); by suppressing the Na/Pi cotransporter system, FGF23 could reduce renal phosphate reabsorption, and thereby increase urinary phosphate wasting. In addition, FGF23, not only suppresses the expression of the 1 $\alpha$ (OH)ase, the essential enzyme that mediates the production of the active vitamin D metabolite, 1,25(OH)<sub>2</sub>D, but could also enhance the expression of 24-hydroxylase, an enzyme that converts 1,25(OH)<sub>2</sub>D into more hydrophilic metabolites with lesser biological activity (Shimada *et al.* 2004c). Since 1,25(OH)<sub>2</sub>D could also enhance intestinal phosphate absorption, FGF23, by reducing vitamin D activities, could reduce serum phosphate levels by inhibiting phosphate absorption in the intestine, in addition to its renal phosphate wasting effects.

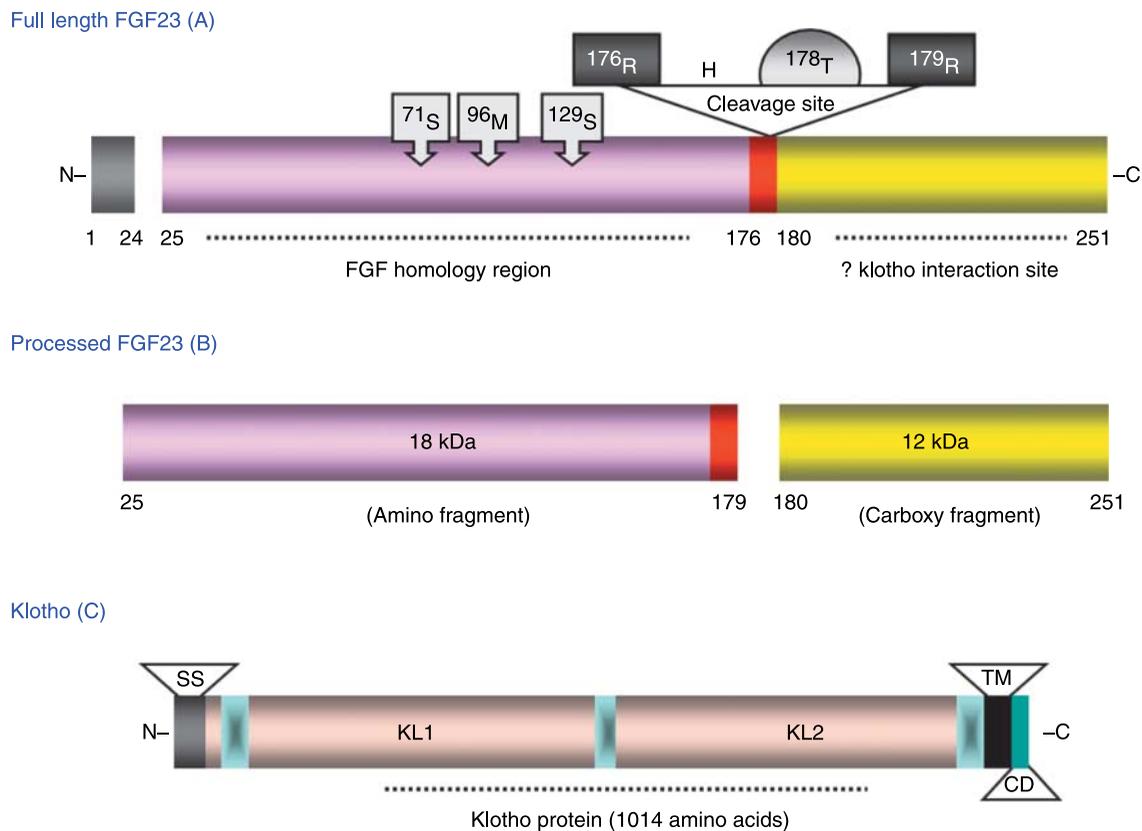
FGF23, a 30 kDa protein, is proteolytically processed between arginine179 and serine180 to generate smaller N-terminal (18 kDa) and C-terminal (12 kDa) fragments (Fig. 1). The N-terminal fragment of FGF23 contains the FGF receptor (FGFR)-binding domain; just adjacent to the proteolytic processing site, this amino acid sequence arginine176-X-X-arginine179 is recognized by furin, a subtilisin-like proprotein convertase. Studies have shown that inhibitors for proprotein convertase could also inhibit the processing of the FGF23 protein (Benet-Pages *et al.* 2004). Although *in vivo* studies using synthetic peptides have claimed that neither processed N-terminal nor C-terminal fragments of FGF23 have biological activity, in terms of reducing serum phosphate levels (Shimada *et al.* 2002), the functionality of synthetic peptides might be different than naturally processed fragments. Of relevance, a recent study has shown phosphaturic activities of C-terminal fragments, and peptides

corresponding to amino acids 180–205 could induce urinary phosphate wasting (Berndt *et al.* 2007); despite the absence of the FGFR-binding domain, how the C-terminal could induce urinary phosphate wasting needs to be investigated by carefully designed studies. The C-terminal fragment is also proposed to be necessary for interaction with klotho, which is believed to be a cofactor in FGF23–FGFR interactions (Goetz *et al.* 2007). Whether a complex generated through FGF23 C-terminal and klotho interaction could activate FGFR to exert phosphaturic effects needs additional study.

A pathological role of FGF23 has also been speculated in patients with McCune–Albright syndrome, a disease usually characterized by skin pigmentation, endocrine disorders, polyostotic fibrous dysplasia of bone, with hypophosphatemia; FGF23 levels have suggested a correlation with disease burden of bone, and hypophosphatemia in these patients (Riminucci *et al.* 2003). Dysregulation of FGF23 is also involved in a number of other human diseases with abnormal phosphate homeostasis (Table 1). One such disease is X-linked hypophosphatemia (XLH).

FGF23 has been shown to be involved in contributing to XLH, a common cause of vitamin D-resistant rickets/osteomalacia. Positional cloning identified inactivating mutations in *PHEX* (a phosphate-regulating gene with homologies to endopeptidases on the X-chromosome) in patients with XLH (HYP\_Consortium 1995). It is, however, not yet clear how mutations in the *PHEX* gene induce renal phosphate wasting leading to hypophosphatemia; the high circulatory levels of FGF23 in most of the patients with XLH (Jonsson *et al.* 2003) suggest that renal phosphate wasting might be the consequence of enhanced activity of FGF23. *Hyp* mice that carry various inactivating deletions in the *Phex* gene show comparable phenotypes to patients with XLH (Beck *et al.* 1997); in accordance with XLH patients, circulatory Fgf23 levels are high in *Hyp* mice (Liu *et al.* 2006, Sitara *et al.* 2006), may be because of increased production of Fgf23 in the bone (Sitara *et al.* 2006). The pathological significance of high levels of Fgf23 in *Hyp* mice became even more evident from the studies showing that genetic ablation of the *Fgf23* gene from *Hyp* mice reversed the hypophosphatemia to hyperphosphatemia (Sitara *et al.* 2004). It is, therefore, likely that mutations in the *PHEX* gene, in a yet unknown mechanism, could enhance the expression of *FGF23* in bone that is subsequently released into the circulation to exert its phosphaturic effects in patients with XLH and in *Hyp* mice.

Recently, mutations in the dentin matrix protein-1 (*DMP-1*) gene have been shown to be associated with autosomal recessive hypophosphatemic rickets/osteomalacia (ARHR; Feng *et al.* 2006, Lorenz-Depiereux *et al.* 2006b). *DMP-1*, as apparent from the name, is a matrix protein that is mostly produced by osteocytes and odontoblasts. Although the exact mechanism is yet to be determined, the clinical symptoms of hypophosphatemia in patients with ARHR are believed to be due to high circulatory levels of FGF23. *Dmp-1* null mice have also high circulatory levels of Fgf23, and showed phenotypes resembling ARHR patients (Feng *et al.* 2006).



**Figure 1** Schematic diagram showing the structure of full-length FGF23 protein (A) with its putative signal peptide (aa 1–24), the amino terminal of FGF23 (aa 25–179) has the homologous region to other known FGFs; the carboxy terminal of FGF23 (aa 180–251) might have possible klotho-interacting site. Moreover, aa 176–179 represents the subtilisin-like cleavage site, where full-length FGF23 protein is cleaved into the smaller 18 kDa amino-terminal and the 12 kDa carboxy-terminal fragments (B). Mutations in <sup>176</sup>R and <sup>179</sup>R have been reported in patients with ADHR, whereas mutations in <sup>71</sup>S, <sup>96</sup>M, and <sup>129</sup>S have been associated with patients affected by FTC. FGF23 has been shown to be O-glycosylated by the enzyme GALNT3 and the major glycosylation site is believed to be <sup>178</sup>T, as shown in the diagram. Klotho protein (C) is composed of 1014 amino acids, and possesses a putative signal sequence (SS) at its N-terminus and a putative transmembrane domain (TM) with a short cytoplasmic domain (CD) at the C-terminus. The extracellular domain of the klotho protein consists of two internal repeats (KL1 and KL2) that share sequence homology to the  $\beta$ -glucosidase.

In contrast to patients with several hypophosphatemic diseases as mentioned earlier, there are contrasting human diseases, including familial tumoral calcinosis (FTC), that are thought to be due to loss of function mutations in the *FGF23* gene (Benet-Pages *et al.* 2005). FTC is characterized by ectopic calcification that is usually associated with hyperphosphatemia. Patients with FTC have enhanced renal tubular phosphate uptake with higher serum levels of 1,25(OH)<sub>2</sub>D, possibly leading to the hyperphosphatemia. Interestingly, *Fgf23* null mice also develop hyperphosphatemia with high serum levels of 1,25(OH)<sub>2</sub>D (Shimada *et al.* 2004a, Sitara *et al.* 2004).

In addition to FGF23, recently GALNT3 has been identified by positional cloning as another responsible gene causing FTC (Topaz *et al.* 2004). This gene encodes a protein called UDP-N-acetyl- $\alpha$ -D-galactosamine: polypeptide N-acetylgalactosaminyltransferase-3 (ppGalNTase-T3) and is involved in post-translational modifications of serine or threonine residues by O-glycation; patients with FTC that

**Table 1** Serum parameters in human phosphate wasting diseases. Note that low serum phosphate level is always accompanied by high fibroblast growth factor (FGF)-23 level, irrespective of disease types

	XLH	ADHR	TIO	ARHR
<b>Normal range<sup>a</sup></b>				
Phosphorus (2.4–4.1 mg/dl)	Low	Low	Low	Low
Calcium (8.5–10.2 mg/dl)	Normal	Normal	Normal	Normal
PTH (10–55 pg/ml)	Normal	Normal	Normal	Normal
1,25(OH) <sub>2</sub> D (39–102 pmol/l)	Low/ normal	Low	Low/ normal	Normal
FGF23 (220–540 RU/ml)	High	High	High	High

<sup>a</sup>Normal value ranges may vary among different laboratories.

is due to mutations in the *GALNT3* gene have low circulatory levels of intact FGF23 (Garringer *et al.* 2006), but exhibit high circulatory levels of the processed C-terminal FGF23 fragment. How mutations in the *GALNT3* gene cause enhanced processing of FGF23 needs further study. Recently, ppGaNase-T3 has been shown to specifically induce O-glycosylation of the threonine178 residue located in the processing site of FGF23 (Kato *et al.* 2006). This O-glycosylation appears to prevent cleavage of the intact FGF23 protein, by either modifying the protein structure or interfering with the proprotein convertases to access the processing site of FGF23. Of relevance, the FGF23 protein has three O-glycan chains. From the studies of Frishberg *et al.* (2007), it appears likely that mutations in the *GALNT3* gene cause specific impairment of O-glycosylation of threonine178 in the FGF23 protein (Frishberg *et al.* 2007), and defects in glycosylation may facilitate rapid processing of the protein, while the fully glycosylated FGF23 protein is resistant to processing. Such a phenomenon might explain why patients with FTC due to *GALNT3* mutation have low circulatory intact FGF23 but high processed C-terminal FGF23 (Garringer *et al.* 2006).

Circulatory FGF23 levels are also extremely high in patients with CKD (Larsson *et al.* 2003, Imanishi *et al.* 2004). The exact role of elevated circulatory levels of FGF23 in CKD patients is not clear, and whether low levels of vitamin D, and secondary hyperparathyroidism that are noted in CKD patients are influenced by increased circulatory levels of FGF23 is a complex issue; since FGF23 is a counter regulatory hormone for vitamin D, it is likely that elevated circulatory levels of FGF23 might contribute to the hypovitaminosis D in CKD patients, and that might eventually facilitate the development of compensatory secondary hyperparathyroidism. Of relevance, the administration of FGF23 reduces the expression of the  $1\alpha(\text{OH})\text{ase}$  mRNA and protein in experimental animals, while genetic ablation of the *Fgf23* gene from mice is associated with upregulation of the renal  $1\alpha(\text{OH})\text{ase}$  that is associated with an increase in serum levels of  $1,25(\text{OH})_2\text{D}$  (Shimada *et al.* 2001, Razzaque *et al.* 2006). The etiological diversity, along with multi-stage, multifactorial molecular events of different stages of CKD (Eddy & Neilson 2006, Taguchi & Razzaque 2007) make it clinically difficult to pinpoint and target one single risk factor

to minimize or delay the progression of the disease; the conventional treatment of CKD patients, either with vitamin D analogs or PTH-lowering drugs, could theoretically be more effective, if FGF23 levels are also therapeutically reduced to eliminate its counter regulatory effects on vitamin D.

### In vivo functional studies on genetically altered Fgf23 mouse models

The understanding of the *in vivo* effects of FGF23 on the regulation of phosphate homeostasis has been significantly enhanced by the generation of *Fgf23* knockout and transgenic models. In 2004, two separate groups have reported the effects of genetic ablation of *Fgf23* on phosphate homeostasis (Shimada *et al.* 2004a, Sitara *et al.* 2004). Both studies have found that *Fgf23* null mice develop severe hyperphosphatemia starting in early life, due to increased renal phosphate uptake by the Na/Pi-2a cotransporter; such abnormal phosphate homeostasis in the mutant mice affects skeletal mineralization and produces extensive soft tissue calcifications (Fig. 2; Razzaque *et al.* 2005, Lanske & Razzaque 2007a), and the resultant effect being shortened lifespan. To determine the exact physiological role of the increased renal expression of sodium-phosphate cotransporter (NaPi-2a) protein in *Fgf23* null mice, we have ablated of *NaPi-2a* gene from *Fgf23*<sup>-/-</sup> mice, and found that hypersphosphatemia of *Fgf23*<sup>-/-</sup> mice reversed to hypophosphatemia in *NaPi-2a*<sup>-/-</sup>/*Fgf23*<sup>-/-</sup> mice by 6 weeks of age, providing the genetic evidence of the importance of NaPi-2a in regulating renal phosphate homeostasis in *Fgf23*<sup>-/-</sup> mice (Sitara *et al.* 2006).

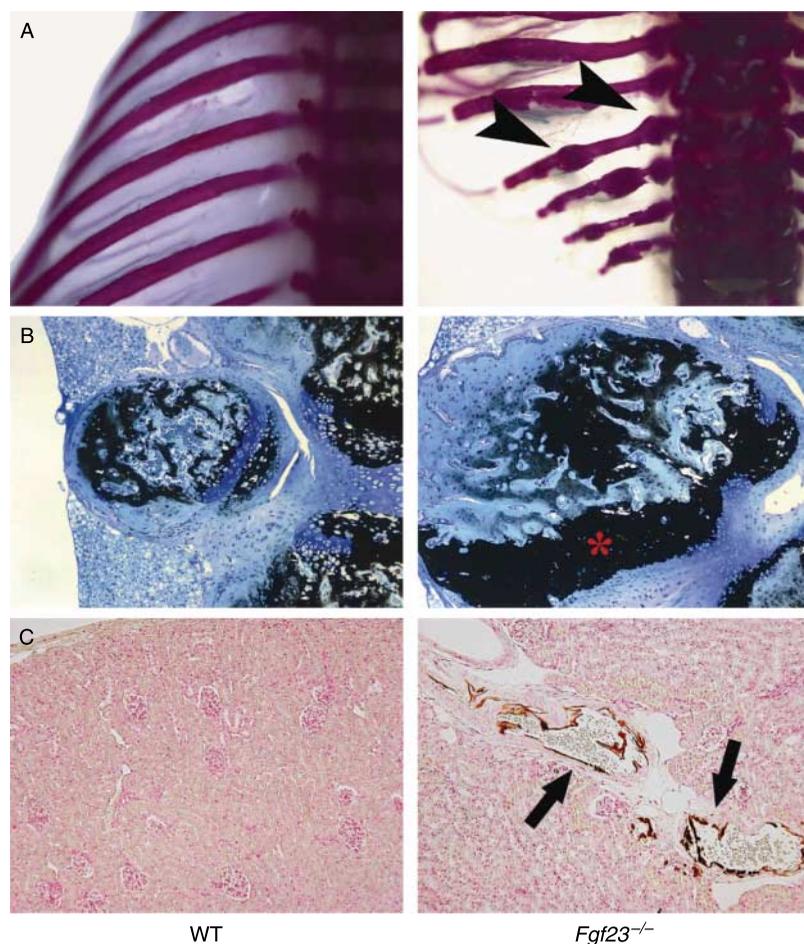
More importantly, the phenotype of *Fgf23* null animals resembles the one of the patients with FTC, an autosomal recessive disorder characterized by ectopic calcifications and elevated serum levels of phosphate due to inactivating mutations in the *FGF23* gene (Benet-Pages *et al.* 2005). Conversely, the phenotype of *FGF23* transgenic animals mimics the one of the patients with ADHR carrying mutations in the *FGF23* gene located within three nucleotides between residues 176 and 179 in the proprotein convertase cleavage site (ADHR\_Consortium 2000); these mutations change arginine residues at 176 and/or 179 to

**Table 2** Serum parameters in fibroblast growth factor-23 (*Fgf23*) knockout (*Fgf23*<sup>-/-</sup>) and *FGF23* transgenic (*FGF23Tg*) mice. Note the opposing effects in all measured serum parameters in *Fgf23*<sup>-/-</sup> (Shimada *et al.* 2004a, Sitara *et al.* 2004) and *FGF23Tg* mice (Bai *et al.* 2004, Larsson *et al.* 2004, Shimada *et al.* 2004b). The high and low serum values are determined in comparison with the values of control mice that are shown as normal

	Control	<i>Fgf23</i> <sup>-/-</sup>	<i>FGF23 Tg</i>
Phosphorus	Normal (8–10 mg/dl)	High (> 16 mg/dl)	Low (< 6 ml/dl)
Calcium	Normal (8–10 mg/dl)	High (> 11 mg/dl)	Low (~ 8 mg/dl)
PTH	Normal (20–80 pg/ml)	Low (not detectable)	High (> 200 pg/ml) <sup>a</sup>
1,25(OH) <sub>2</sub> D	Normal (~ 150 pg/ml) <sup>b</sup>	High (> 400 pg/ml)	Low (< 100 pg/ml)
FGF23	Normal (~ 100 RU/ml)	Absent (0 RU/ml)	High (> 7500 RU/ml)

<sup>a</sup>Shimada *et al.* in their transgenic mice reported no elevation of serum PTH level.

<sup>b</sup>Levels of 1,25(OH)<sub>2</sub>D can vary as much as 30%, depending on the method of measurements.



**Figure 2** Altered mineralization in bone and kidney of *Fgf23* knockout (*Fgf23*<sup>-/-</sup>) mice. Note abnormal nodular lesions (arrow) in the ribs of *Fgf23*<sup>-/-</sup> mice, compared with control (A). Histomorphometric analysis on methyl methacrylate sections, stained with toluidine blue and von Kossa's stains, shows increased mineral deposition (black) in the mutant ribs (asterisk; B). It is worth to mention that in *Fgf23*<sup>-/-</sup> mice, skeletal mineralization has heterogeneous distribution, where increased mineral deposition is also accompanied with unmineralized osteoid (light blue) formation (B). Extensive soft tissue mineralization is a characteristic feature in *Fgf23*<sup>-/-</sup> mice, as shown here in the kidney (C).

glutamine or other residues to prevent proteolytic cleavage of the FGF23 protein by furin to presumably inactive fragments; the net effect being phosphate wasting in the affected patients, perhaps due to enhanced biologic activities of FGF23 (White *et al.* 2001).

In 2004, three separate groups also reported the generation of transgenic mice over-expressing *Fgf23/FGF23* (Bai *et al.* 2004, Larsson *et al.* 2004, Shimada *et al.* 2004b); these mice have hypophosphatemia, due to suppression of the renal Na/Pi cotransporters; the long-term effect of hypophosphatemia in these mutant mice is evident from obvious skeletal mineral defects in the form of rickets/osteomalacia. Of relevance, the biochemical changes of *Fgf23/FGF23* transgenic mice are essentially opposite from those noted in *Fgf23* null mice (Table 2); these genetically modified animal models have not only provided insights into the role of FGF23 in regulating phosphate homeostasis, but also provided the *in vivo* tool to study in depth the biology of FGF23. Existing

information supports the notion that FGF23 is the master molecule which regulates renal phosphate metabolism (Berndt & Kumar 2007, Fukumoto & Yamashita 2007, Lanske & Razaque 2007b). However, how FGF23 exerts its bioactivities is an intense area of research, and preliminary observations suggest that it interacts with FGFRs in the presence of klotho to exert its functions (Fig. 3; Yu *et al.* 2005, Goetz *et al.* 2007).

### Klotho

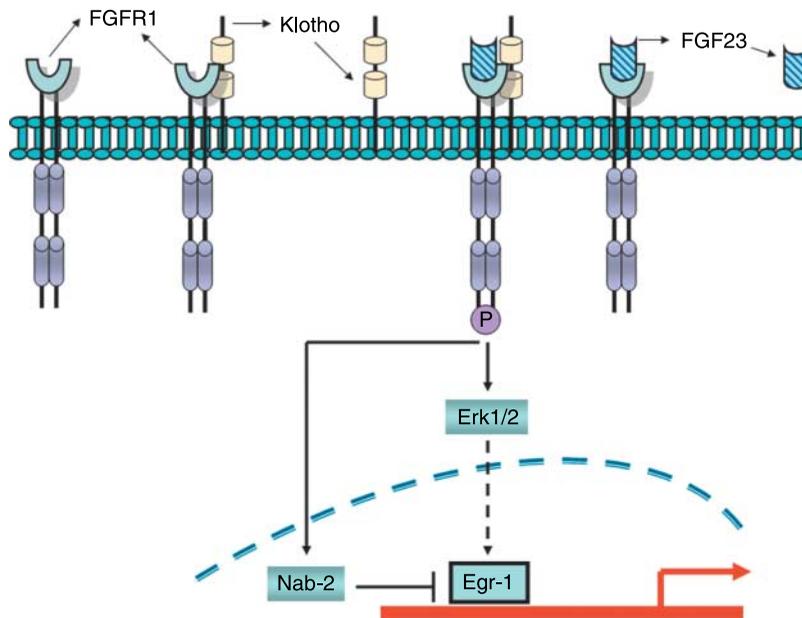
The Klotho protein contains 1014 amino acids with a putative signal sequence at its N-terminus and a single transmembrane domain near its C-terminus which is believed to anchor the protein to the membrane. Since the *klotho* gene encodes a type 1 membrane protein, it was predicted to be present on the cell surface of klotho-expressing cells. However, the

extracellular domain of klotho could be cleaved and detectable in the blood and cerebrospinal fluid. The klotho, therefore, could act as a circulating hormone. The extracellular domain of the klotho protein has two internal repeats, termed KL-1 and KL-2 (Fig. 1). KL-1 and KL-2 have sequence homology to  $\beta$ -glucosidase of bacteria and plants. Human Klotho has about 86% homology to mouse klotho and is mapped to chromosome 13q12 (Matsumura *et al.* 1998). Klotho expression is observed in restrictive tissues and is predominantly present in tissues that regulate calcium homeostasis, including the distal convoluted tubules in the kidney, parathyroid gland, and the epithelium of the choroid plexus in the brain (Matsumura *et al.* 1998). Inactivation of klotho activities could produce extensive premature aging-like features in the mouse (Kuro-o *et al.* 1997). Interestingly, demonstration of similar premature aging-like features in *Fgf23*-ablated mice, as *klotho*-ablated mice, has facilitated the identification of a common signaling cascade through which FGF23 exerts its bioactivities (Razzaque *et al.* 2006, Razzaque & Lanske 2006, Lanske & Razzaque 2007c).

### How FGF23 exerts its bioactivities?

Most of the FGF family members exert their functions through interacting with FGFRs. To date, four FGFRs with alternative spliced variants for each of them have been reported (Goldfarb 2005, Mohammadi *et al.* 2005). For

instance, alternative splicing of an exon coding for the third immunoglobulin-like loop produces three different subtypes of FGFR1, namely FGFR1a, FGFR1b, and FGFR1c. Similarly, alternative splicing produces two subtypes of FGFR2 and FGFR3, namely FGFR2b, FGFR2c, FGFR3b, and FGFR3c. Several studies, using the Biacore system, have suggested that FGF23 has the ability to interact with FGFR1c, FGFR2c, FGFR3c, and FGFR4 (Yamashita *et al.* 2002, Yu *et al.* 2005). Despite the ubiquitous presence of FGFRs, the induction of FGF23-responsive downstream molecules, such as activation of early growth response (Egr)-1 and phosphorylation of ERK protein were only detected in very restricted tissues that include kidney, parathyroid, and pituitary glands, when mice and rats were treated with recombinant FGF23 protein (Urakawa *et al.* 2006); such observations suggest that FGF23 might have selective effects on specific tissues that might be different from most of the other members of the FGF family. Considering the unique biological activities of FGF23, which is mostly produced in the bone (Sitara *et al.* 2006), but exerts most of its functions in the kidney, existence of a novel humoral signaling pathway is likely. A major breakthrough of how FGF23 exerts its bioactivities has been achieved by the recent demonstration of strikingly similar physical and biochemical phenotypes of *Fgf23* knockout with *klotho* hypomorph mice (Kuro-o *et al.* 1997, Razzaque *et al.* 2006, Razzaque & Lanske 2006). Such extremely high similarities in phenotypes (Table 3) suggest a functional relationship between these molecules, and this has



**Figure 3** Schematic outline of FGF23–FGFR interactions. Signaling of FGF23–FGFR involves klotho as a cofactor to induce such downstream signaling molecules as Erk1/2, which could influence the activation of Egr-1. FGF23 also induces Nab-2 (Fukuda *et al.* 2007), specific corepressor of Egr-1 that could suppress the transcriptional activity of Egr-1, and thereby establish a negative feedback loop to regulate physiological activities of FGF23.

**Table 3** Similarities between fibroblast growth factor 23 (*Fgf23*)- and *klotho*-ablated mice. Comparison of phenotype between *Fgf23*<sup>-/-</sup> and *klotho*-ablated mice (Kuro-o *et al.* 1997, Razzaque *et al.* 2006). Note remarkable physical, biochemical, and morphological similarities in the phenotypes of *Fgf23* and *klotho* mutants (Razzaque & Lanske 2006)

	<i>Fgf23</i> mutant	<i>Klotho</i> mutant
<b>Phenotypes</b>		
Gross appearances		
Body weight	Reduced	Reduced
Growth retardation	Present	Present
Kyphosis	Present	Present
Physical activity	Sluggish	Sluggish
Gait walk	Present	Present
Body hair	Sparse	Sparse
Generalized atrophy		
Thymus atrophy	Present	Present
Spleen atrophy	Present	Present
Muscle wasting	Present	Present
Skin atrophy	Present	Present
Intestinal atrophy	Present	Not known
Hypogonadism	Present	Present
Morphological changes		
Atherosclerosis/art- eriosclerosis	Present	Present
Ectopic calcifica- tions	Present	Present
Altered skeletal mineralization	Present	Present
Osteopenia/rickets	Present	Present
Emphysema	Present	Present
Renal NaPi-2a expression	Increased	Increased
Biochemical changes		
Serum 1,25(OH) <sub>2</sub> D <sub>3</sub>	High	High
Serum phosphate	High	High
Serum calcium	High	High
Serum PTH	Low	Low
Serum FGF23	Absent	High
Overall affect		
Infertility	Present	Present
Lifespan	Short	Short

led to the identification of *klotho* as a cofactor in FGF23 and its receptor interactions, and subsequent signaling (Fig. 3).

The *klotho* extracellular domain is believed to facilitate FGF23 binding to its receptor complex with much higher affinity than to FGFR alone, implicating *klotho* as a cofactor in FGF23–FGFR interaction and subsequent signaling (Kuro-o 2006). *In vitro* studies have shown that FGF23–non-responsive cells get responsive to FGF23 in the presence of *klotho*. Such responsiveness was determined by enhanced phosphorylation of ERK and induction of Egr-1 (Urakawa *et al.* 2006), implying that *klotho* is required for eliciting downstream FGF23 signaling, and thereby its functions. Using L6 cells that lack FGFRs, it has been shown that FGF23 could not induce downstream signaling events, even after transfection of a *klotho*-expressing vector (Urakawa *et al.* 2006), clearly suggesting that FGF23 exerts its

functions through interacting with FGFRs, in the presence of *klotho*. Further studies are needed to determine how and where *klotho* binds during FGF23 receptor interactions. *Klotho* has been suggested to bind to FGFRs, including FGFR1c, FGFR3c, and FGFR4, and that FGF23 could only bind to *klotho*–FGFR1c, *klotho*–FGFR3c, and *klotho*–FGFR4 complexes (Kuro-o 2006). On the other hand, Urakawa *et al.* (2006) in their recent study have shown that *klotho* could even bind to FGF23, and artificial induction of *klotho* protein could create a specific binding site for FGF23 in cultured cells (Urakawa *et al.* 2006). Further studies are needed to clarify the exact nature of the FGF23–*klotho* interaction; existing information, however, suggests that *klotho* converts canonical FGFR into a specific receptor for FGF23.

Now that we know the importance of the FGF23–*klotho* axis, in retrospect, it is, therefore, not very surprising to find extreme similarities in the phenotype of both *Fgf23* and *klotho*-ablated mice that include but are not limited to shortened lifespan, impaired sexual maturation leading to infertility, kyphosis, atherosclerosis, extensive soft tissue calcifications, skin atrophy, muscle wasting, T-cell dysregulation, pulmonary emphysema, osteopenia, abnormal mineral ion metabolism, and impaired vitamin D homeostasis (Razzaque & Lanske 2006).

### Concluding remarks

In this brief article, based on experimental observations, we have presented our views on how the newly discovered FGF23–*klotho* axis could regulate phosphate homeostasis by affecting the renal Na/Pi system, and how altered regulation of one of these molecules could adversely affect mineral ion metabolism to induce disease pathology in both soft tissues and bone. The renal Na/Pi cotransporters expressed in the brush-border membrane of proximal tubular epithelial cells are responsible for reabsorption of the bulk of the filtered phosphate. FGF23, by suppressing the Na/Pi cotransporter system in the kidney, could induce urinary phosphate wasting. Finally, in this article, we wanted to emphasize the importance of the FGF23–*klotho* axis in regulating mineral ion metabolism. At this stage, FGF23 that is produced in bone appears to exert its hormonal effects in the kidney, and perhaps in the parathyroid gland, in the presence of *klotho* to regulate mineral metabolism. In less than a decade after identification of FGF23, studying the human hypo- and hyperphosphatemic disorders that are associated with FGF23 dysregulation has significantly improved our overall understanding of hormonal regulation of mineral ion metabolism. A comprehensive understanding of the FGF23–*klotho* axis on the physiologic adaptation following dietary phosphate uptake, and their coordinated effects on PTH and 1,25(OH)<sub>2</sub>D will help us to explain how altered regulation of factors controlling mineral ion homeostasis could adversely affect disease pathology.

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