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Does Fgf23-klotho activity influence vascular and soft tissue calcification through regulating phosphate homeostasis?

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Abstract

Recent studies describe a novel role of fibroblast growth factor 23 (Fgf23)-klotho activity in the systemic regulation of calcium and phosphate homeostasis. Both *Fgf23* and *klotho* ablated mice develop extensive vascular and soft tissue calcification. Inability to clear the required amount of phosphate by the kidney, due to the absence of Fgf23-klotho activity, leads to increased serum accumulation of phosphate in these genetically modified mice, causing extensive calcification. Serum calcium levels are also elevated in both *Fgf23* and *klotho* ablated mice. Moreover, increased sodium-phosphate co-transporter activity in both *Fgf23* and *klotho* ablated mice increases renal phosphate reabsorption which in turn can facilitate calcification. Collectively, these observations bring new insights into our understanding of the roles of the Fgf23-klotho axis in the development of vascular and soft tissue calcification.

Keywords

Fgf23; klotho; calcification; hyperphosphatemia; NaPi2a

Fibroblast growth factor 23

FGF23 is an approximately 30 kDa secreted protein that is mostly synthesized by the osteocytes in the bone^{1,2}. FGF23 is a master *in vivo* regulator of phosphate homeostasis. Under physiological conditions, it controls renal phosphate excretion according to the need of the body through the regulation of the renal sodium-dependent phosphate cotransporter NaPi2a and NaPi2c^{3,4}. Genetic defects in FGF23 gene can produce distinct human diseases. For instance, gain-of-function mutations of FGF23 are responsible for the clinical symptoms observed in patients suffering from autosomal dominant hypophosphatemic rickets (ADHR)⁵. These mutations prevent the proteolytic cleavage of the FGF23 protein, leading to its increased biological activity and resulting in severe renal phosphate wasting. Similarly, increased serum levels of FGF23 in the patients with oncogenic osteomalacia (OOM) are found to be the causative factor for tumor-induced renal phosphate wasting⁶. Patients affected by X-linked hypophosphatemia (XLH), a dominant disorder caused by inactivating mutations of the gene encoding PHEX (the phosphate-regulating gene with homologies to endopeptidases on the X chromosome), exhibit increased serum FGF23 levels, phosphaturia and osteomalacia

7. A similar phosphate wasting effect, due to increased FGF23 serum level, has been detected in patients with autosomal recessive hypophosphatemia (ARHP) – a rare genetic disorder with essentially similar clinical features as those seen in the patients with OOM, XLH and ADHR^{8,9}. Recent studies using wild-type and ADHR mutant proteins have identified key FGF23-specific receptor-mediated signaling^{10,11}.

FGF-23 signaling

FGF23 exerts its bioactivity on selected target tissues by interacting with its cognate FGF receptors (FGFRs) in the presence of the cofactor klotho^{10–12}. The *klotho* gene encodes a single-pass transmembrane protein with an extracellular domain consisting of two homologous domains that share sequence homology with the [beta]-glucosidase of bacteria and plants. Klotho facilitates the binding of FGF23 to FGFR1c, -3c, and -4^{11,12}. FGFRs contain a signal-transducing extracellular ligand-binding domain and an intracellular tyrosine kinase domain. The restricted expression of klotho determines the tissue specificity of FGF23 function^{12,13}. Klotho is mostly expressed in the renal distal tubular epithelial cells, the parathyroid gland, and the pituitary gland^{13,14}.

FGF23, in the presence of klotho can activate downstream signaling molecules, as determined by activation or phosphorylation of FGFR substrate-2a, extracellular signal-regulated kinase (ERK), and early growth response element-1 (Egr-1)^{10,11}. Only in presence of klotho, cells exposed to FGF23 underwent ERK phosphorylation and increased the expression of Egr-1 protein. Klotho also enhances FGF23 binding to its receptor since FGF23 has a greater affinity to the Klotho/FGFR complex with than to the FGFR alone, underscoring the important role of klotho as a cofactor in the FGF23 FGFR interaction and subsequent signaling¹¹. Our understanding of FGF23 and its receptor interactions, along with the downstream signaling events helps us focus on its biological functions. Recent animal genetic studies, generating *Fgf23* and *klotho* ablated mice, have shown that altered mineral ion metabolism in the mutant mice is associated with extensive vascular and soft tissue calcification^{15–17}.

Vascular calcification

Vascular calcification is a complex, regulated process that involves the molecular interplay between calcification stimulators and inhibitors. Although numerous individual molecules and/or factors have been identified as stimulators of calcification, including inorganic phosphate, calcium, sodium-phosphate cotransporters, Runx2, tissue non-specific alkaline phosphatase (TNAP), glucose, acetylated LDL, tumor necrosis factor-alpha (TNF- α), and bone morphogenetic protein 2 (BMP-2)^{18–20}, their exact mechanism to induce vascular calcification and their interaction with the calcification inhibitors is not yet clearly understood.

Recent studies have shed some light on vascular calcification and how a disrupted balance between calcification inhibiting and promoting factors can lead to calcification. As mentioned, there are several key factors that have been shown to directly regulate the induction and progression of vascular calcification; these include but are not limited to circulating factors (i.e., phosphate, calcium, pyrophosphate, parathyroid hormone: PTH) and their signaling components, matrix molecules (i.e., Matrix Gla Protein: MGP), and catalyzing enzymes (i.e., TNAP).

Serum phosphate and calcium levels are important determinants of vascular calcification, as inadequate regulation of these minerals can lead to spontaneous deposition of calcium-phosphate in the blood vessels and soft tissues. Hyperphosphatemia in dialysis patient correlates with vascular calcification and effective phosphate control with noncalcium phosphate binders is correlated with attenuated progression of vascular calcification in these patients²¹. In addition, *in vitro* studies have shown that smooth muscle cells grown in the in

the presence of elevated inorganic phosphorus undergo a dramatic phenotypic change characterized by the downregulation of smooth muscle cell lineage genes and the upregulation of the osteochondrogenic lineage genes²². Similar to phosphorus, a positive calcium balance is linked to vascular calcification in humans²³. *In vitro*, calcium promotes mineralization in vascular smooth muscle cell and the calcium-induced mineralization upregulates the expression of the major sodium-dependent phosphate cotransporters in these cells²⁴.

Inorganic pyrophosphate inhibits vascular calcification by restricting hydroxyapatite formation and propagation through its biophysical chelator-like role, as well as stabilizing the aortic phenotype by acting as a paracrine regulator¹⁹. Reduced plasma pyrophosphate levels are reported in hemodialysis patients and are exacerbated as a result of pyrophosphate clearance²⁵. It is therefore likely that restoring pyrophosphate levels may help in limiting vascular calcification. Another factor, TNAP, an enzyme produced in several tissues including bones, serves as a functional phenotypic marker of osteoblasts and is often used as a molecular marker for vascular calcification. Since pyrophosphate is a substrate for TNAP and phosphorus is the product of its catalytic activity, one can theoretically anticipate that upregulated TNAP expression acts as a precursor to vascular calcification²⁶. PTH can also influence vascular calcification. Uncontrolled secretion of PTH can release excessive amount of calcium from bone, which can precipitate as calcifying foci in blood vessels and soft tissue²⁷.

BMP-2 plays a role in calcification by exerting osteogenic effects on blood vessels and soft tissues²⁸. Studies have shown a positive correlation between BMP2 and vascular calcification. Furthermore, matrix proteins, like MGP can inhibit vascular calcification. A positive correlation exists between the local expression of MGP and calcification in arteries. In MGP knockout mice and human Keutel Syndrome, the deficiency of MGP, are associated with ectopic calcification²⁹. MGP is able to control vascular calcification partly through its Gla residues, which have a calcium/hydroxyl apatite chelating capacity.

Vascular calcification is histologically divided into four main types: 1) atherosclerotic intimal calcification, 2) medial artery calcification (Monckeberg sclerosis), 3) cardiac valve calcification, and 4) arteriole calcification in the form of calciphylaxis. Although systemic factors have great importance in inducing calcification, the interplay between the resident cells of the vasculature usually determines the extent of the damage; cross talks and phenotypic alteration of endothelial cells, smooth muscle cells, pericytes and perhaps mesenchymal stem cells, in response to systemic dysregulation of mineral balance can significantly influence the calcification process. In general, there are significant similarities between skeletal mineralization and vascular and soft tissue calcification²⁸. The reader is referred to recent reviews for an in-depth overview of the general aspects of bone and vascular calcification^{18, 28}. The purpose of this review is to briefly discuss the potential effects of FGF23-klotho activity on the development of vascular and soft tissue calcification.

Does FGF23-klotho activity influence calcification?

Both human and animal studies have shown that reduced FGF23 or klotho activities are closely associated with vascular and soft tissue calcification.

Animal studies

Extensive vascular and soft tissue calcification is observed in *Fgf23* knockout mice by 6 weeks of age; small and medium sized arteries and the proximal tubules in the kidneys are the most extensively affected sites, in addition to the aorta. Interestingly, the expression of the sodium-phosphate co-transporter, NaPi2a of the proximal tubular epithelial cells is also upregulated in *Fgf23* knockout mice¹. Increased tubular sodium-phosphate co-transporter expression (Figure

1) in these mice may translocate extracellular phosphate within the cells to facilitate calcification. In addition to kidney and blood vessels (Figure 2), *Fgf23* knockout mice also exhibit widespread soft tissue calcification in the lungs, skeletal muscle, skin, urinary bladder, testes, and cardiac muscle. Vascular and soft tissue calcification appear as early as 6 weeks postnatally and progress with age in *Fgf23* knockout mice^{15,16}. Such widespread calcification in *Fgf23* knockout mice is associated with osteopenia. Autoradiographic studies of both fore- and hind limbs show that the bone mineral density (BMD) is strikingly reduced in *Fgf23* knockout mice, compared to their control littermates¹. Interestingly, despite reduced BMD, the total body mineral content (BMC) in *Fgf23* knockout mice is higher due to their extensive vascular and soft tissue calcification^{1,15}. The *Fgf23* knockout mouse phenotype has clinical relevance, as human studies have also shown an association between reduced BMD and vascular calcification³⁰; low BMD is suggested to independently predict coronary artery disease in women, with a higher odds ratio than traditional risk factors³¹. Lower BMD, yet higher BMC in *Fgf23* knockout mice, therefore, provides a unique model to study molecular regulation of osteoporosis and vascular calcification^{1,15}.

Similar to the *Fgf23* knockout animals, mice homozygous for the hypomorphic alleles of the *klotho* gene show increased expression of NaPi2a and NaPi2c co-transporters in the proximal tubular epithelial cells (Figure 1). Furthermore, extensive calcification in both vascular and soft tissues, including lungs, skin, testis, and heart is noted in *klotho* ablated mice. Taking into consideration the phenotypes of both *Fgf23* and *klotho* ablated mice, it seems likely that *in vivo* dysregulation of the FGF23-*klotho* axis can lead to vascular calcification, possibly by affecting mineral ion metabolism^{4,32–34}. Needless to mention that extensive vascular and soft tissue calcification in both *Fgf23* and *klotho* ablated mice is associated with severe hyperphosphatemia, and increased serum level of 1,25 hydroxyvitamin D^{15–17}. The experimental relevance of both *Fgf23* and *klotho* ablated mice has significantly increased due to the fact that mutations in either human FGF23 or *klotho* genes are also associated with ectopic calcification.

Human studies

In accord with the animal studies, human diseases associated with inactivating mutations in either *FGF23* or *Klotho* gene express severe ectopic calcification. For instance, familial tumoral calcinosis (FTC) is an autosomal recessive disorder characterized by hyperphosphatemia and ectopic calcifications; and is associated with diaphysitis, hyperostosis, arterial aneurysms, dental abnormalities, and angioid streaks of the retina. Genetic studies have shown evidence that missense mutations in the human *FGF23* gene or *GALNT3* gene (UDP – N – acetyl – alpha – D - galactosamine: polypeptide N-acetylgalactosaminyltransferase 3) cause the disease³⁵. Mutations in *GLANT3* prevent its ability to selectively O-glycosylate a furin-like convertase recognition sequence in FGF23, thus preventing the proteolytic processing of FGF23 and the secretion of intact FGF23 protein³⁶. Hence, mutations in either *FGF23* or *GALNT3* genes reduce FGF23 activities, which lead to hyperphosphatemia and eventually to tumoral calcinosis in patients with FTC.

Klotho is a recently identified co-factor in FGF23 signaling. Experimental studies have demonstrated convincingly that FGF23, in the absence of *klotho*, cannot exert its bioactivities. For instance, despite extremely high serum levels of *Fgf23* (about 2000-fold higher) in *klotho* ablated mice, *Fgf23* is unable to exert its phosphaturic effects in these mice¹². The lack of phosphaturic activity despite extremely high levels of *Fgf23* in *klotho* ablated mice signifies that *Fgf23* is incapable to perform its physiological functions in the absence of *klotho*³². Recently, a point mutation in the human *Klotho* gene was reported in a 13 year old patient with severe vascular and soft-tissue calcification despite significantly high serum level of FGF23. Lack of function of the *Klotho* gene in this patient can attenuate the ability of FGF23 to exert

its phosphate lowering effects, which can eventually lead to the severe vascular and soft tissue calcifications³⁷.

Can FGF23 suppress calcification?

Since reduced FGF23 activity is associated with vascular and soft tissue calcification in both experimental and human studies, the clinically relevant question would be: does FGF23 have any calcification inhibitory effects. Current observations suggest that the manipulation of FGF23 activity can delay calcification through the lowering of serum calcium and phosphate levels. Also, Shimada and coworkers demonstrated that FGF23 can suppress the renal expression of 1 α -hydroxylase, the rate limiting enzyme that converts the inactive vitamin-D metabolite to its active form³⁸. It is possible that FGF23 can reduce calcification by inhibiting vitamin-D activity. Inaba et al. recently reported that FGF23 is an independent factor that is negatively associated with hand-artery, but not aortic calcification in haemodialysis patients, and proposed plasma FGF23 levels as a reliable marker for medial peripheral artery calcification in these patients³⁹. Nevertheless, extensive cardiovascular calcification is the leading cause of death in chronic kidney disease patients undergoing dialysis, despite their significantly high serum FGF23 levels^{40–42}. Variability in the degree of failing kidneys can account for this apparent contradiction: diminishing renal function interferes to various extents with the ability of FGF23 to exert its inhibitory effects due to potential defects in *klotho* and/or *FGFR* expression, leading to both elevated serum FGF23 levels and vascular calcification. Finally, a recent study on subjects with normal kidney function found no correlation between serum intact FGF23 and/or fetuin-A levels, and coronary artery score⁴³. These findings suggest that under normal renal function, where the kidneys are effectively maintaining a normal phosphate balance, FGF23 is not a suitable marker for coronary artery calcification. Further studies are needed to better understand the role of FGF23 in vascular and soft tissue calcification under various pathological conditions.

Concluding remarks

The recent understanding of the systemic regulation of mineral ion homeostasis and vitamin D metabolism by FGF23-*klotho* signaling leads us to revisit the mechanistic aspect of calcification^{44–47}. Whether the FGF23-*klotho* duo can directly inhibit calcification or the effect is indirect due to reduced availability of calcification promoting mineral ions, needs additional study. Hypothetically, enhanced FGF23-*klotho* activity can delay the process of calcification by negatively impacting the serum phosphate balance. Further studies will determine if the pharmacological manipulation of FGF23 activity can be beneficial in fine-tuning existing treatments of vascular and/or soft tissue calcification⁴⁸. Such studies will also expand our understanding of the fundamental aspects of mineral ion metabolism under physiological and pathological conditions, including calcification.

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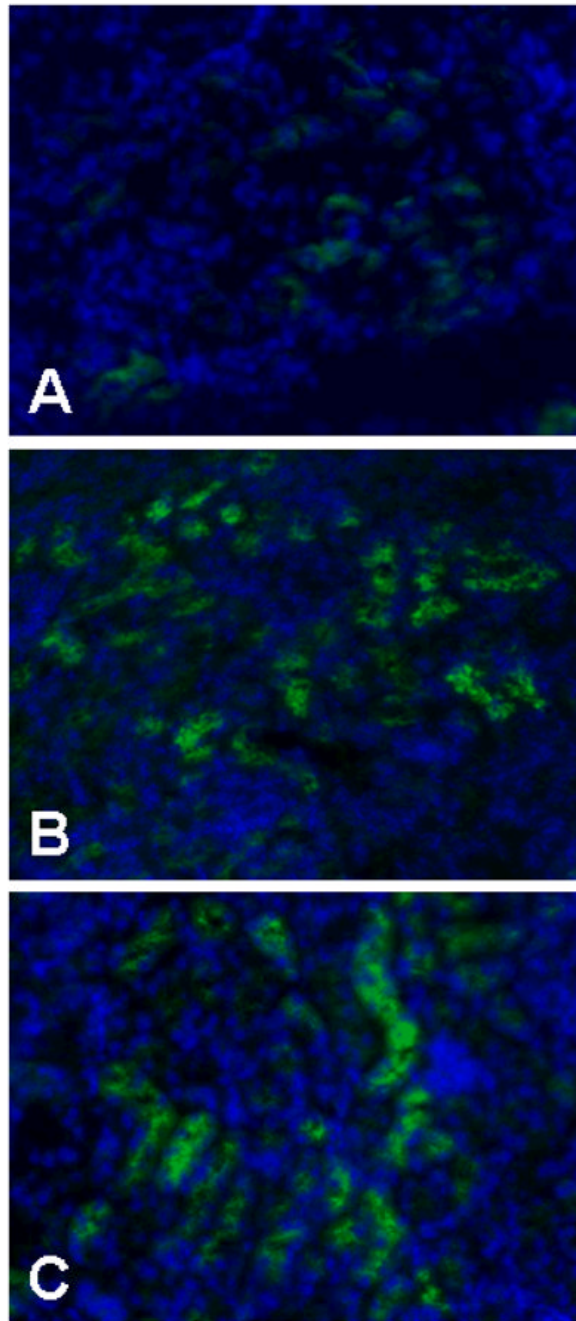


Figure 1. Immunostaining of NaPi2a protein in kidneys obtained from control (A), *Fgf23*^{-/-} (B) and *klotho*^{-/-} (C) mice. An increased expression of NaPi2a protein is detected in *Fgf23*^{-/-} and *klotho*^{-/-} mice, compared to wild-type mice. Please note that NaPi2a protein is exclusively present in the luminal side of the proximal tubular epithelial cells.

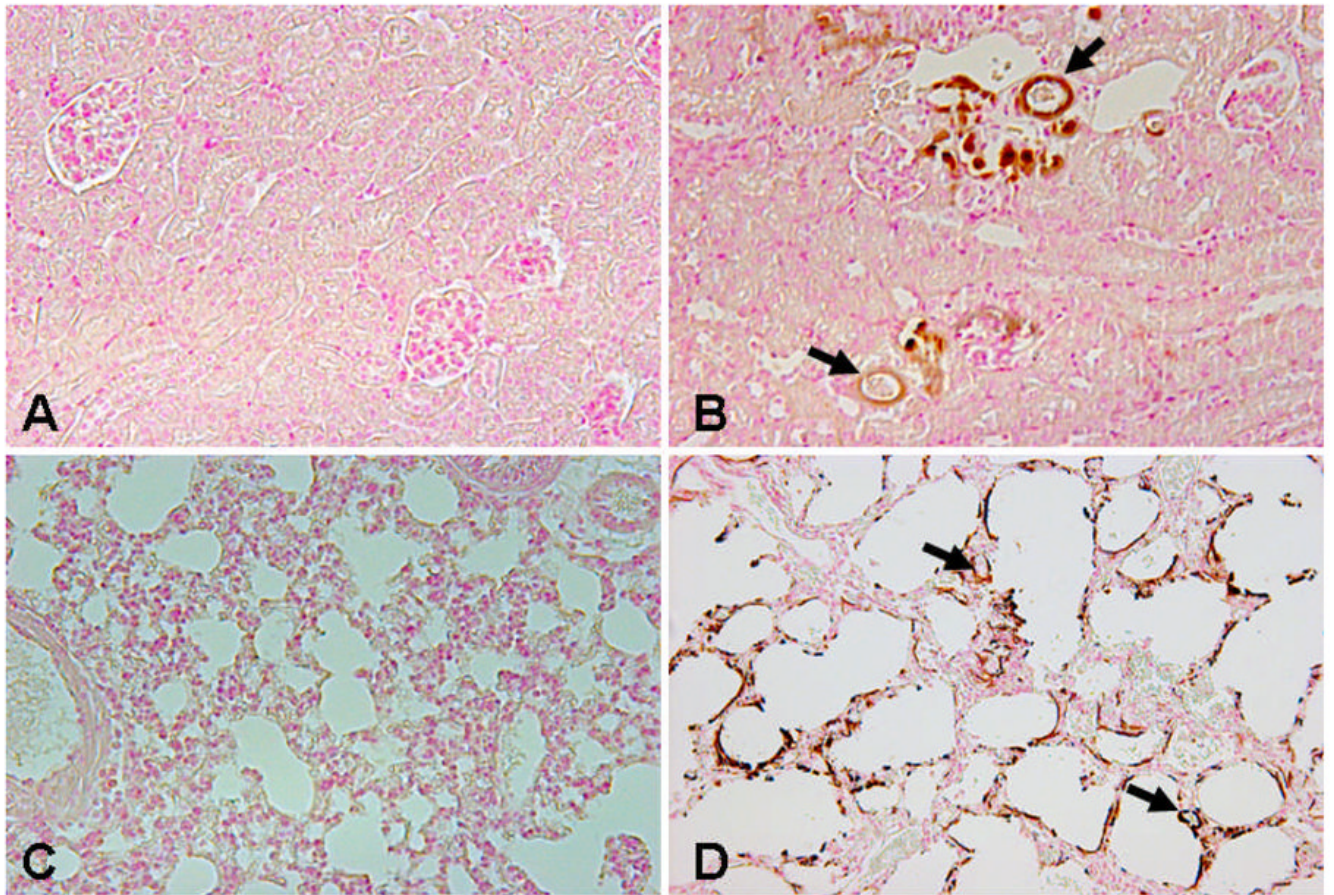


Figure 2. Soft tissue and vascular calcification in *Fgf-23*^{-/-} mice
von Kossa staining on paraffin sections of the kidney (A, B) and lung (C, D), showing widespread renal (B) and pulmonary (D) calcifications in *Fgf-23*^{-/-} mice. No such calcification is noted in the wild-type littermates (A, C). Arrows depict the calcified vessels in the kidney and lung of the *Fgf-23*^{-/-} mice. (Magnification: kidney $\times 20$; lung $\times 10$).