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REVIEW

Fibroblast growth factor 23 and bone mineralisation

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Fibroblast growth factor 23 (FGF23) is a hormone that is mainly secreted by osteocytes and osteoblasts in bone. The critical role of FGF23 in mineral ion homeostasis was first identified in human genetic and acquired rachitic diseases and has been further characterised in animal models. Recent studies have revealed that the levels of FGF23 increase significantly at the very early stages of chronic kidney disease (CKD) and may play a critical role in mineral ion disorders and bone metabolism in these patients. Our recent publications have also shown that FGF23 and its cofactor, Klotho, may play an independent role in directly regulating bone mineralisation instead of producing a systematic effect. In this review, we will discuss the new role of FGF23 in bone mineralisation and the pathophysiology of CKD-related bone disorders.
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INTRODUCTION

Fibroblast growth factor (FGF) 23, a member of the FGF19 subfamily of FGFs, plays a key role in balancing mineral ion homeostasis and bone mineralisation.[1–4] FGF23 was discovered due to its characterisation as a cause of autosomal dominant hypophosphatemic rickets[4] and as the ectopically overproduced phosphaturic factor responsible for tumour-induced osteomalacia.[5] Patients with such diseases suffer from impaired bone mineralisation and hypophosphataemia associated with a phosphate-wasting syndrome caused by impaired renal phosphate reabsorption and unexpectedly low levels of calcitriol.[6] Observations in later studies allowed the identification of FGF23 as a key factor in the physiological regulation of phosphate and implied that it was involved in associated disorders of phosphate and bone metabolism.[1,4,7]

FGF23 is a 32-kDa protein that is mainly secreted by osteocytes and osteoblasts in bone. Human FGF23 consists of 251 amino acids and includes a signal peptide composed of 24 amino acids in the N-terminal portion of the protein. The half-life of intact FGF23 is 0.5–1 h in tumour-induced osteomalacia patients.[8–9] The major target of FGF23 is the kidney, where it downregulates the luminal expression of sodium-phosphate cotransporters in the proximal tubule to stimulate phosphaturia.[10] It also inhibits 25-hydroxyvitamin D-1α-hydroxylase and stimulates 24-hydroxylase to suppress the production of 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D).[11]

Circulating levels of FGF23 can be measured by an intact FGF23 assay, which captures intact FGF23 and C-terminal fragments. Because inactive C-terminal fragments may accumulate in patients with end-stage renal disease, we do not know whether the C-terminal FGF23 assay can provide comparable sensitivity to the intact FGF23 assay in such cases.[12]

FGF23–FGF RECEPTOR–KLOTHO COMPLEX

To date, five distinct membrane FGF receptors (FGFRs), including FGFR1, FGFR2, FGFR3, FGFR4, and FGFR5, which belong to the tyrosine kinase superfamily, have been identified in vertebrates.[13–16]

Klotho, a single-pass transmembrane protein, is the cofactor implicated in the binding and activation of FGFRs by FGF23. This discovery occurred during a serendipitous experiment that examined a mouse strain that displays a premature-ageing disorder.[17] Verifying that the phenotypes of Klotho-deficient mice were replicated in FGF23-deficient mice was the first step in identifying the presumptive cofactor required for FGFR binding by FGF23.[18]

The action of FGF23 is mediated by binding to FGF cell-surface receptors, including several types of FGFRs such as FGFR1, 3c and 4, and the FGF23 coreceptor, α-Klotho.[19] FGF23 binds more robustly to FGFRs in the presence of Klotho and triggers intracellular signalling pathways that mediate its biological function. This finding can explain the restricted, tissue-specific action of FGF23, which may be secondary to the relatively limited expression of α-Klotho in specific tissues, including the parathyroid, kidney and pituitary gland. Circulating α-Klotho transduces FGF23 activity in an *in vitro* system at a lower level than the membrane-bound form.[20] FGFs commonly signal through the extracellular signal-regulated kinases/early growth response protein 1 pathway.[19]

PRODUCTION OF FGF23

FGF23 is mainly secreted by osteocytes,[21–23] which are cells embedded in the mineralised bone matrix that are connected to the cells outside the bone through a bone fluid-filled lacunocanalicular system.[24] FGF23 shows the highest expression levels in bone, especially in osteocytes.[25–26] The expression of FGF23 is increased in the osteocytes of

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patients with hypophosphataemic rickets and chronic kidney disease (CKD) relative to control subjects.[27–28]

Young and mature osteocytes regulate mineralisation and phosphate homeostasis mainly through the release of FGF23. They also regulate biomineralisation and FGF23 signalling via molecules that include phosphate-regulating genes with homologies to endopeptidases on the X chromosome (PHEX), dentin matrix protein-1 (DMP1) and matrix extracellular phosphor-glycoprotein (MEPE). PHEX, DMP1 and MEPE are all highly expressed in osteocytes.[29–31] Mutations in PHEX can cause X-linked hypophosphataemic rickets, while mutations in DMP1 can cause autosomal recessive hypophosphataemic rickets.[25,29] If the function of DMP1 or PHEX becomes altered, FGF23 can become elevated in osteocytes and the circulation, leading to the excretion of phosphate by the kidney, resulting in osteomalacia and rickets. Loss-of-function mutations and knock-out murine models have been constructed for PHEX, DMP1 and MEPE.[25,29,32–34] In recent studies, Dmp1-null and Hyp mouse models show enhanced FGFR signalling compared with wild-type (WT) control mice, and the inhibition of FGFR signalling results in a reduction of FGF23 in the bone marrow stromal cells from both mouse models.[35] These data suggest that the inhibitory effects of DMP1 and PHEX are mediated by FGFR signalling in the osteocytes and that FGFRs play a role in endocrine signalling through the osteocytes.

$1,25(OH)_2D$ can induce the expression of FGF23 in osteocytes,[36–37] and parathyroid hormone (PTH) may also regulate FGF23 levels. FGF23 mRNA expression in the calvaria and serum FGF23 levels are increased in mice after the infusion of PTH.[38]

The extent of glycosylation and proteolytic processing can also regulate FGF23 activity at the protein level. Intact FGF23 is protected against furin-mediated cleavage to generate the C-terminal form of the peptide by glycosylation.

In different disease states, the location of the osteocytes that release FGF23 may vary. FGF23 is mainly released by the osteocytes of both cortical and trabecular bone in mouse models of hereditary hypophosphataemia.[26,36] However, in a study conducted on the Col4a3-null mouse, researchers found different results. FGF23 was primarily expressed in trabecular osteocytes in this study, and significant increases in FGF23 gene expression in the bone did not occur until the later stages of CKD. The post-transcriptional processing, excretion, or breakdown of FGF23 may be altered during the early stages of CKD because FGF23 becomes elevated during this period, and osteocytes are the main site of FGF23 production.[39]

FGF23-ASSOCIATED BONE DISEASES

FGF23-associated bone diseases refer to a group of conditions in which the function and/or amount of FGF23 is abnormal.[40] This abnormality may consist of primary or secondary changes in FGF23 function (Table 1).

In cases of high FGF23 levels, patients with autosomal dominant hypophosphatemic rickets,[41] X-linked hypophosphataemia,[29] oncogenic osteomalacia,[42] autosomal recessive hypophosphataemia, autosomal recessive hypophosphataemic rickets and fibrous dysplasia[23] have the common characteristics of elevated FGF23 in the serum and hypophosphataemia. Most changes in FGF23 are non-PTH/vitamin D-dependent. Congenital or acquired dysfunction of the reabsorption of phosphorus in the kidney tubules and vitamin D deficiency syndrome are the most common causes of non-genetic hypophosphataemia. All types of hypophosphatemic rickets share the characteristic of excess or overactive FGF23,[43–44] and such changes in FGF23 are most likely caused by a mutation in PHEX or MEPE.[45–48] Because PTH stimulates the secretion of FGF23, high FGF23 syndrome, chronic nephropathy and cardiovascular diseases are usually aggravated by secondary parathyroidism.

Low FGF23 conditions can be divided into primary and secondary conditions. Primary low FGF23 syndrome and FGF23 deficiency are commonly found in tumoral calcinosis[49] and hyperostosis hyperphosphataemia syndrome. Mutations of FGF23, Klotho, GALNT3 or SAMD9 cause an inactivating mutation of FGF23. Secondary low FGF23 syndrome is mainly accompanied by normal or decreased blood phosphorus and elevated $1,25(OH)_2D$ and found in patients with a low phosphorus diet, mutated vitamin D receptors, mutated 1α-hydroxylase, mutated or deficient NaPi-2a or mutated NaPi-2c.

FGF23 INHIBITS BONE MINERALISATION

FGF23 itself is an inhibitor of mineralisation, but whether it acts directly or indirectly is not yet known.[50] Wang *et al.*[50] showed that adenoviral overexpression of FGF23 in rat calvarial cells *in vitro* inhibits bone mineralisation independent of its systemic effects on phosphate homeostasis. Our group and others have also demonstrated that FGF23 treatment of primary calvarial osteoblast cultures from WT mice or from the osteoblastic MC3T3-E1 cell line leads to an inhibition of mineralisation,[51–52] thus showing an effect on mineralisation independent of circulating factors.

Previously, our group and others have shown that mice lacking FGF23 function ($Fgf23^{-/-}$) exhibit a phenotype of biochemical disorders including hyperphosphataemia, hypercalcaemia, high serum $1,25(OH)_2D$ levels and decreased serum PTH levels.[49,53–54] Despite the presence of a high serum mineral ion content,[55–56] $Fgf23^{-/-}$ mice present with severe defects in skeletal mineralisation (osteomalacia/osteoidosis). The reason that this reduced skeletal mineralisation occurs in the presence of high serum calcium and phosphate is largely unknown.

Table 1 List of FGF23-related bone diseases

FGF23 levels	Primary changes	Secondary changes
High	Autosomal dominant hypophosphataemic rickets (ADHR)	Chronic nephrosis
	X-linked hypophosphataemia (XLH)	High phosphate diet
	Oncogenic osteomalacia (OOM)	Klotho deficiency disease
	Autosomal recessive hypercholesterolaemia rickets (ARHR)	
	Autosomal recessive hypophosphataemia (ARHP)	
	Fibrous dysplasia (FD)	
Low	Tumoural calcinosis (TC)	Low phosphate diet
	Hyperglycaemic hyperosmolar state (HHS)	Vitamin D receptor mutation
	Inactive FGF23	1α-hydroxylase mutation
		NaPi-2a deficiency/mutation
		NaPi-2c mutation

Recently, we discovered that the expression of osteopontin (OPN), a well-known inhibitor of bone mineralisation,[57] is substantially elevated in bone, suggesting a possible explanation for the defective mineralisation in the bones of *Fgf23*$^{-/-}$ mice.[58] Both *in situ* hybridisation and immunohistochemistry were performed on femur sections of 6-week-old animals and showed enhanced OPN signal in the bones of *Fgf23*$^{-/-}$ mice. Moreover, to examine the accumulation of OPN at a higher resolution, we performed transmission electron microscopy after immunogold labelling for OPN in undecalcified calvarial bone. Compared with the moderate extent of immunolabelling in *WT* bone, seen as patches of gold particles dispersed throughout the matrix and surrounding osteocyte lacunae, the bones of *Fgf23*$^{-/-}$ mice were intensely labelled at several locations. Abundant gold particle labelling was observed over the aborted mineralisation foci in the osteoids, at the sharply demarcated mineralisation front, and immediately lining the lacunar wall surrounding the osteocytes. Osteocytes found in these heavily OPN-labelled regions of osteoid bone matrix showed prominent secretory granules that were intensely labelled for OPN, indicating the local secretion of this protein by bone cells. Furthermore, we demonstrated that ablation of *Opn* (*Spp1*) from *Fgf23*$^{-/-}$ mice significantly ameliorated the osteomalacia. Collectively, these findings indicate that increased OPN levels are responsible, in part, for the skeletal mineralisation defect observed in *Fgf23*$^{-/-}$ mice.

As mentioned above, FGF23 requires Klotho for its actions. In the absence of Klotho, FGF23 has a very low affinity for FGFR1 and cannot induce signal transduction (phosphorylation).[59–61] The direct effect of FGF23 on bone mineralisation was unclear before Klotho was detected in the osteoblastic cell linage.[62] This expression pattern was confirmed by our recent study, which showed that Klotho is expressed in both cultured osteoblasts and isolated cortical bone, although the level is much lower than that of the kidney.[63]

In addition to being a cofactor of FGF23, Klotho may play a specific function in osteoblasts. *Kl*$^{-/-}$ and *Fgf23*$^{-/-}$ mice share very similar phenotypes.[64] Klotho knockout (*Kl*$^{-/-}$) mice have elevated bone volume compared with the normal bone volume of *Fgf23*$^{-/-}$ mice.[63,65–66] Cultured osteoblasts isolated from *Kl*$^{-/-}$ pups at the age of 2 days showed markedly impaired mineralisation, suggesting that Klotho may play a specific role in osteoblastic mineralisation. Interestingly, we recently showed that deletion of *PTH* might be responsible for the normalisation of the increased Opn levels in *Kl*$^{-/-}$ mice and subsequently rescue the mineralisation defect, but this same deletion did not improve the defect in *Fgf23*$^{-/-}$ animals.[56] An osteoblast-specific *Klotho* knockout mouse model may be needed to determine the independent role of Klotho in skeletal mineralisation.

FGF 23 AND CKD BONE DISEASES

FGF23 levels increase progressively during the decline of renal function.[67–68] Several studies of patients with CKD have demonstrated that the levels of circulating FGF23 begin to increase early in the course of kidney dysfunction.[67,69–71] The expression levels of FGF23 in osteocytes increase,[28] which provided an excellent explanation for the observations that the levels of 1,25$(OH)_2$D begin to decline and the levels of PTH progressively increase early in the course of CKD.[72] The precise mechanism by which FGF23 secretion is stimulated in response to the phosphate load is not yet clear. Studies of healthy individuals have shown that several days of a high-phosphate or low-phosphate diet results in an increase or decrease in FGF23 levels, respectively;[73–74] however, FGF23 does not respond rapidly to dietary phosphate loading or intravenous administration of phosphate.[75–76] Despite a significant reduction in serum phosphate levels, a session of dialysis did not decrease serum FGF23 levels in patients receiving haemodialysis,[77] and elevated FGF23 levels persist for weeks to months after kidney transplantation.[78–79] Together, these data suggest that FGF23 secretion is regulated by chronic phosphate load.

Normophosphataemia is maintained by FGF23 and PTH before patients reach end stage renal disease (ESRD). However, renal Klotho expression is decreased during the progression of CKD,[80] leading to a reduction in the kidney's ability to excrete urinary phosphate, which will finally overcome the compensatory effects of these phosphaturia hormones. Such processes lead to an increase in serum phosphate levels, a progressive reduction in 1,25$(OH)_2$D levels and stimulated PTH secretion. Therefore, patients with ESRD commonly suffer from hyperphosphataemia, decreased levels of 1,25$(OH)_2$D and secondary hyperparathyroidism.[72] FGF23 levels will increase markedly and often reach 100- to 1 000-fold above the normal range by the time patients start dialysis.[81–95] Hyperphosphataemia, chronic phosphate retention, PTH levels and vitamin D will increase serum FGF23 levels, resulting in extremely high FGF23 levels.[38,92,94,96–97]

Elevated FGF23 levels have been linked by numerous reports to the main adverse clinical outcomes in CKD, such as progression to ESRD, cardiovascular disease and death. In a 1 : 1 case–control study of 400 participants in a prospective cohort of patients with incident ESRD, elevated levels of FGF23 were independently associated with a greater risk of mortality.[81] This observations helped support the concept that elevated FGF23 is not just a biomarker of phosphate-mediated cardiovascular toxicity, but is directly toxic itself.[98] Another study of 219 prevalent haemodialysis patients confirmed that elevated FGF23 levels were independently associated with a greater risk of mortality during ESRD.[82] Elevated FGF23 levels were independently associated with greater subsequent risks of mortality, allograft loss and composite reactions in a study of 984 prevalent kidney transplant recipients with a median transplant age at enrolment of 6 years.[99]

The mechanisms underlying the association of FGF23 with poor outcomes have not been established, but several possible explanations for these mechanisms have been proposed. One possible explanation is the link between inflammation and FGF23. Inflammation is common in CKD/ESRD and is associated with significantly poorer outcomes.[100–101] FGF23 increases the production of inflammatory markers such as lipocalin-2, transforming growth factor-beta and tumour necrosis factor-alpha.[102] In the future, the clinical relevance of the effects of FGF23 on inflammation should be tested. In a series of experiments from a single group of investigators, FGF23 induced left ventricular hypertrophy (LVH) *in vitro* and in experimental animals by inducing the molecular mechanisms that are typical of pathological LVH.[103] These experiments challenge the prevailing paradigm that the effects of FGF23 on FGFR are weak without Klotho, even at high concentrations of FGF23, and demonstrate that FGF23 may exert direct effects on organs that do not express α-Klotho; however, more testing is needed to confirm such unorthodox hypotheses. Another mechanism could be that FGF23 acts to suppress vitamin D metabolism. By lowering vitamin D levels, FGF23 could be instrumental in producing adverse consequences, which would, under this paradigm, be related to the multiple and complex end-organ effects of low vitamin D, such as the activation of the RAAS, higher blood pressure, vascular calcification, inflammation, infections and malignancies.[104] Additionally, low vitamin D levels have shown a strong association with general adverse outcomes in patients with CKD and ESRD.[105–107]

The association between FGF23 and bone in the case of CKD differs greatly from that in general cases. High circulating levels of FGF23 in paediatric dialysis patients are associated with improved indices of

skeletal mineralisation in a cross-sectional analysis of 49 paediatric dialysis patients with secondary hyperparathyroidism.[108] A study of FGF23, DMP1 and MEPE expression in the bone tissue of 32 paediatric and young adult patients with CKD was performed to confirm this association and demonstrated that both FGF23 and DMP1 expression were upregulated in trabecular bone in early CKD, while MEPE expression remained unchanged from normal control levels. During all stages of CKD, the expression level of bone FGF23 was directly correlated with bone DMP1 expression, and the expression of each was inversely related to osteoid accumulation. However, MEPE expression was not related to skeletal mineralisation, but was inversely related to bone volume. The simultaneous increase in both DMP1 and FGF23 expression appears to contradict previous data suggesting that DMP1 suppresses FGF23 expression, but other data have suggested that the over-expression of DMP1 does not suppress FGF23 expression.[109] Additionally, DMP1 promoter activity increases in response to increasing phosphate concentrations.[110] Therefore, the simultaneous increase in bone DMP1 and FGF23 expression may reflect the increasing phosphate burden associated with progressive renal failure.

To date, the pathological role of elevated FGF23 in CKD remains unclear. Researchers have developed a monoclonal FGF23 antibody to evaluate the impact of chronic FGF23 neutralisation on chronic kidney disease-mineral and bone disorder (CKD–MBD).[111] CKD–MBD rats fed a high-phosphate diet were treated with low or high doses of FGF23-Ab or an isotype control antibody. Neutralisation of FGF23 led to sustained reductions in secondary HPT, including decreased parathyroid hormone; increased vitamin D; increased serum calcium; and normalisation of bone markers such as cancellous bone volume, trabecular number, osteoblast surface area, osteoid surface area and bone formation rate. However, dose-dependent increases in serum phosphate and aortic calcification associated with an increased risk of mortality in CKD–MBD rats treated with FGF23-Ab were observed. Thus, the mineral disturbances caused by neutralisation of FGF23 limit the efficacy of the FGF23-Ab and likely contribute to the increased mortality observed in this CKD–MBD rat model.[111] Further studies are needed to determine whether neutralising FGF23 in CKD could ameliorate the proposed deleterious extrarenal effects of supraphysiological FGF23 on the cardiovascular system while obviating the impairments of mineral homeostasis.

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