Published in final edited form as:

Bone. 2017 September ; 102: 31–39. doi:10.1016/j.bone.2017.01.034.

Heritable and acquired disorders of phosphate metabolism: etiologies involving FGF23 and current therapeutics

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Abstract

Phosphate is critical for many cellular processes and structural functions, including as a key molecule for nucleic acid synthesis and energy metabolism, as well as hydroxyapatite formation in bone. Therefore it is critical to maintain tight regulation of systemic phosphate levels. Based upon its broad biological importance, disruption of normal phosphate homeostasis has detrimental effects on skeletal integrity and overall health. Investigating heritable diseases of altered phosphate metabolism has led to key discoveries underlying the regulation and systemic actions of the phosphaturic hormone Fibroblast growth factor-23 (FGF23). Both molecular and clinical studies have revealed novel targets for the development and optimization of therapies for disorders of phosphate handling. This review will focus upon the bridge between genetic discoveries involving disorders of altered FGF23 bioactivity, as well as describe how these findings have translated into pharmacologic application.

Keywords

FGF-23; genetics; hypophosphatemia; hyperphosphatemia; Klotho; tumoral calcinosis

Introduction

The maintenance of serum phosphate concentrations is a multi-organ process involving phosphate absorption in the gut, reabsorption in the kidney and storage within the skeleton. Endocrine communication between these key sites provides stable phosphate and calcium balance. The kidney is the primary organ responsible for maintaining short-term phosphate concentrations. PTH action on the kidneys results in reduction of apical membrane expression of the proximal tubule Type II sodium phosphate co-transporters NPT2a and NPT2c (1), which decreases renal phosphate reabsorption (Figure 1). In parallel, PTH increases expression of the renal vitamin D 1-alpha-hydroxylase anabolic enzyme (Cyp27b1) which catalyzes the conversion of 25-hydroxy vitamin D to its active form,

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$1,25(OH)_2$ vitamin D (1,25D). This elevation in 1,25D increases phosphate and calcium absorption in the intestine (Figure 1). 1,25D and serum phosphate increases also elevate the production of FGF23 in osteoblasts/osteocytes (2), and like PTH, decreases the apical membrane expression of NPT2a. In contrast to PTH, FGF23 reduces the renal vitamin D 1-alpha-hydroxylase while increasing expression of the catabolic renal 24-hydroxylase (Cyp24a1) (3). Cyp24a1 initiates the degradation of 1,25D by hydroxylation of the side chain to 24,25D. 1,25D induces Cyp24a1, whereas hypocalcemia, through increased PTH, suppresses this enzyme, thus playing an important role in calcium-phosphate homeostasis and the vitamin D endocrine system. Importantly, loss of bioactive FGF23 through gene knockout experiments in mice or through human mutations involving inactivation of FGF23 protein, its signaling components, and FGF23 intracellular processing enzymes, has revealed that there are no other compensatory proteins for maintaining phosphate levels while suppressing 1,25D concentrations (4, 5).

A key finding in the phosphate field was the discovery that FGF23 required a co-receptor, αKlotho (KL), to signal within its target tissues (6). KL has highest expression in the kidney, a major site of FGF23 bioactivity, however KL also has robust expression in choroid plexus and in the parathyroid glands (7) (Figure 1). KL is known to be expressed as a full-length transmembrane protein (membrane form, or ‘mKL’) and as a circulating form (known as ‘cKL’) that arises from cleavage of mKL at the juxta-extracellular domain (8, 9). FGF23 initiates intracellular signaling through MAPK-related pathways via activation of a heteromeric complex, potentially involving pre-assembly of mKL and an FGF receptor (FGFR) (10). Although multiple FGFRs can interact with KL in vitro (6, 11), FGFR1 has become the leading candidate for in vivo binding to FGF23 and KL. Indeed, mice with kidney-specific KO of FGFR1 in the context of tandem deletion of FGFR4 appear to have renal resistance to FGF23 activity and manifest elevated serum phosphate and FGF23 (12).

Considering the above actions of FGF23, elevated FGF23 due to genetic or acquired diseases results in a hallmark biochemical phenotype of hypophosphatemia with inappropriately normal or low 1,25D. Over the long term, low 1,25D may result in secondary hyperparathyroidism. With loss of FGF23 bioactivity in patients and in *Fgf23*-KO mice, this results in the biochemical converse, although patients with familial hyperphosphatemic tumoral calcinosis (hfTC) due to loss of FGF23 bioactivity (discussed below) may not have elevated 1,25D. In addition to phosphate and 1,25D, and potentially PTH, other regulators of FGF23 production that lie outside the ‘typical’ feedback loops controlling phosphate and calcium balance have recently been discovered including anemia and hypoxia (13, 14). Indeed, the fundamental reasons for their links to FGF23 and phosphate metabolism remain to be completely understood, however these pathways may reveal novel regulation of phosphate as well as support new or optimized treatments for both rare and common syndromes involving FGF23.

Heritable disorders of hypophosphatemia involving FGF23

a. Autosomal dominant hypophosphatemic rickets (ADHR)

FGF23 was originally identified in a collaborative effort as the causative gene for the Mendelian disorder of renal phosphate wasting ADHR (OMIM 193100) (15). Consistent

with other diseases associated with a common denominator of elevated FGF23 described herein, patients with ADHR have biochemical pathologies of hypophosphatemia and normocalcemia, with inappropriately normal or low 1,25D. The sustained hypophosphatemia leads to rickets in children and osteomalacia in adults, also similar to other phosphate-wasting syndromes (16). Unique to ADHR however is incomplete penetrance with variable expressivity (16). Upon positional cloning of ADHR, it was determined gain of function mutations within the FGF23 subtilisin-like proprotein convertase (SPC) proteolytic cleavage site ($_{176}RH_{177}T_{178}R_{179}/S_{180}AE$) were responsible for the ADHR phenotype (Table 1). These missense mutations result in amino acid alterations of either of the arginine (R) residues at positions 176 or 179 to glutamine (Q) or tryptophan (W) (15). Transfection of FGF23 expression plasmids found that secreted FGF23 protein harboring the ADHR mutations was detected primarily as full-length (32kDa) whereas wild type (WT) FGF23 protein was detected as N-terminal (20 kDa) and *C*-terminal (12 kDa) fragments (17, 18). It was later confirmed that the full-length form of FGF23 contained the bioactive properties whereas the FGF23 *N*-terminal and *C*-terminal fragments were unable to elicit signaling with the FGFR/Klotho receptor complex (19).

These various forms of FGF23 can be detected in the circulation using FGF23 specific enzyme-linked immunosorbent assays (ELISAs) for humans and rodents (Kainos, Inc (20) and Qidel, Inc (21)). Intact FGF23 measurements utilize two antibodies that bind at both the *N*- and *C*-terminal portions of the full-length, bioactive form of the hormone (or 'iFGF23'). '*C*-terminal' FGF23 ('cFGF23' or 'Total FGF23') human- (22) or rodent-specific (13) ELISA measurements represent levels of iFGF23 plus its proteolytic fragments in plasma. In this instance, both ELISA antibodies recognize peptides that lie within the *C*-terminal side of the SPC-cleavage site.

ADHR differs from the other hereditary disorders of hypophosphatemia, due to the fact that those with ADHR FGF23 mutations can exhibit either an early or delayed onset of disease presentation (16, 23). In some instances, patients clearly documented with high FGF23 and hypophosphatemia had a reversal of the phosphate wasting phenotype with minor therapeutic intervention (24). Based upon these key observations, novel aspects of the underlying molecular mechanism were identified in human and pre-clinical mouse models linking FGF23 with iron-handling. Indeed, patients with late onset ADHR were more often women and began exhibiting symptoms during puberty, a state often associated with anemia (16, 24). From this observation, Imel *et al* tested the association between serum FGF23 and serum iron levels in ADHR patients and normal individuals. A significant negative correlation was found in both normal and ADHR patients between iron and cFGF23 (25). Interestingly, a negative correlation between serum iron and iFGF23 was only found in ADHR patients. These findings suggested that while low iron concentrations dictated increased production of FGF23, patients with normal FGF23 alleles cleaved the protein to maintain proper serum phosphate levels. On the other hand, ADHR patients appeared to be resistant to FGF23 cleavage and inactivation, thus promoting the accumulation of bioactive hormone. These results were recapitulated in mice containing ADHR R176Q-*Fgf23* knock-in alleles. Adult mice homozygous for the R176Q mutant (ADHR mice) and WT mice were placed on a low iron diet for 8 or 12 weeks. The low-iron diet elevated cFGF23 serum levels in both genotypes, however iFGF23 only increased in a significant percentage of the ADHR

mice, which had decreased serum phosphate leading to osteomalacia (13). These studies revealed that the control of circulating iFGF23 during anemia occurs by increasing bone Fgf23 mRNA. Further, the processing of FGF23 was discovered to occur post-translationally, within a dynamic system that could potentially be important for sensing and modifying short term serum phosphate concentrations (see ARHR type 3).

A model of early onset ADHR was also tested in which pregnant females were placed on a low-iron diet at 14 days of gestation, mimicking anemia in the last trimester of pregnancy. In this model, both the WT offspring in addition to the ADHR pups showed a significant increase in iFGF23 when the dams received the low iron diet (14). These data demonstrated a robust link between iron and FGF23 levels in that the normal inhibitory feedback of low phosphate on FGF23 production is negated by iron deficiency anemia. Thus both genetic and environmental elements contribute to the development of ADHR, with stimuli outside of the normal feedback loops for phosphate handling acting as powerful inducers of FGF23 expression.

b. X-linked hypophosphatemia (XLH)

The commonest heritable form of phosphate wasting is XLH (OMIM 307800) which occurs in 1:20,000 births, with over 325 loss of function mutations characterized from patients within the *PHEX* gene (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) (26) (Table1). The XLH clinical features are characterized by a biochemical profile of elevated FGF23 with hypophosphatemia and normocalcemia, with inappropriately normal or low 1,25D. Similar to ADHR, XLH patients have osteomalacia/rickets due to the prevailing hypophosphatemia, but in contrast to ADHR, XLH is fully penetrant and onset is from birth. PHEX is an ectoenzyme exhibiting high expression levels in bone and teeth with lower levels also observed in skin, muscle and brain (27). Whereas the mechanism of PHEX actions in vivo are incompletely understood, patients with XLH have elevated iFGF23 (20, 22). It has been postulated that the PHEX mutations cause a cell differentiation defect, as osteoblasts isolated from *Hyp* mice (animal model of XLH; (27)) are unable to mineralize properly (28, 29). The interactions between FGF23 and PHEX are indirect as FGF23 is not a PHEX substrate (30, 31). Further, the increased Fgf23 mRNA levels in *Hyp* bone support that the increase in serum FGF23 in XLH is due to both over production by skeletal cells, as well as potentially a decrease in FGF23 proteolysis (32). The knowledge that iFGF23 is elevated in XLH from birth has led to clinical trials using an antibody-based therapy to nullify circulating FGF23 (see below).

c. ARHR Type 1, loss of function *DMP1* mutations

Autosomal recessive hypophosphatemic rickets type 1 (ARHR-1) is a disorder with elevated serum FGF23 and osteomalacia (OMIM 241520), and was found to be due to inactivating mutations in Dentin Matrix Protein-1 (*DMP1*) (Table 1). DMP1 is highly expressed in osteocytes and as a member of the Small Integrin-Binding LIgand, N-linked Glycoprotein (SIBLING) family may aid in hydroxyapatite nucleation (33). In consanguineous ARHR families, genetic analyses identified mutations in the *DMP1* start codon, far *C*-terminus, as well as in exon splicing sites (34, 35). Examination of the *Dmp1*-null mouse model demonstrated that homozygous loss of Dmp1 causes defective osteocyte maturation, leading

to elevated iFGF23 expression and pathological changes in bone mineralization through an undefined mechanism (34). This differentiation defect appears to phenocopy the *Hyp* mouse (32). Collectively, the work supports the hypothesis that PHEX and DMP1 influence overlapping pathways during osteocyte maturation and to downstream inappropriate expression of FGF23. It has been postulated that SIBLING proteins may interact with PHEX and suppresses FGF23 expression, however the molecular mechanisms underlying this finding have yet to be fully determined (36). DMP1 is found in the bone extracellular matrix as *N*-terminal (37 kDa) and *C*-terminal (57 kDa) fragments and its processing appears to be important for maintaining function. The inability of Dmp1 to be cleaved due to introduction of a D213A mutation resulted in a bone phenotype similar to the *Dmp1*-null mouse (37). Conversely, over-expression of the DMP1 57 kDa *C*-terminal fragment on the *Dmp1*-null background rescued the bone phenotype (38).

d. ARHR Type 2, loss of function ENPP1 mutations

Loss of function mutations in ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1) lead to the development of ARHR type 2 (OMIM 613312) (Table 1) (39–41). ENPP1 is the 'reciprocal' enzyme to alkaline phosphatase and converts inorganic phosphate to extracellular inorganic pyrophosphate (PPi), a potent inhibitor of skeletal mineralization. Indeed, hypophosphatemia is observed downstream of elevated serum iFGF23 in patients with recessive *ENPP1* mutations which is paralleled in the *Enpp1*-null mouse (42). This leads to weakened long bones with reduced trabecular number, trabecular volume and cortical thickness in both patients and *Enpp1*-null mice. ENPP1 is responsive to extracellular inorganic phosphate (Pi) and helps maintain proper Pi/PPi ratios for appropriate skeletal mineralization, thus this enzyme may provide signals for the control of FGF23 production. Previous studies demonstrated osteoblasts lacking Enpp1 display an aberration in their ability to differentiate. Maturation markers including osteocalcin (OCN), bone sialoprotein (BSP) and tissue non-specific alkaline phosphatase (TNAP) fail to increase in osteogenic media with either a knockdown or deletion of Enpp1 (43). While these studies did not directly measure PHEX or DMP1 levels, the presence of the differentiation defect may contribute to the over production of FGF23 in ARHR-2 in a similar manner to that observed in XLH or ARHR-1. Whereas hypomineralization occurs within bone with the loss of ENPP1, ectopic calcification is evident within tissues such as aorta and kidney and hyperostosis is radiologically detected within the joints. The role of ENPP1 as an inhibitor of tissue calcification is underscored by the fact that additional, distinct mutations are pathogenic in idiopathic infantile arterial calcification (IIAC), as well as ossification of the posterior longitudinal ligament of the spine (OPLL)(41).

e. ARHR Type 3, loss of function FAM20C mutations

Family with sequence similarity 20, member C (FAM20c) loss of function mutations give rise to ARHR-3, also known as Raine syndrome. Originally thought to be lethal, especially when the mutations arise in the conserved *C*-terminal domain of FAM20C (Table 1), some mutations have been described in surviving Raine syndrome patients (44–46). Clinical manifestations include craniofacial malformation and osteosclerosis of the skull and long bones. In one case a non-lethal mutation in FAM20C (R408W) also displayed a rachitic phenotype. Indeed, the patient was hypophosphatemic due to high serum iFGF23 driving

renal phosphate wasting. Iliac crest bone biopsies showed pronounced osteomalacia, likely contributing to the patient's shortened stature (47). Mutational analysis of FAM20C in a single case also revealed co-manifestion of sclerosing and hypophosphatemic rickets phenotypes (48). Functional studies demonstrated FAM20C is a casein kinase and phosphorylates secreted proteins containing an S-X-E recognition sequence (49). Deletion of *Fam20c* in mice resulted in a significant rise in serum iFGF23 along with a phosphate wasting phenotype and severe tooth defects (50). Tagliabracci *et al* found the SIBLING family member of proteins, including DMP1, are substrates for FAM20c phosphorylation activity. Despite the reduced levels of DMP1 in FAM20C knockdown cells (68), crossing a Dmp1-transgene onto the *Fam20c* conditional knockout mouse failed to rescue the bone phenotype and elevated FGF23 (51). Thus, loss of FAM20C, in part, may block or reduce functional DMP1 which phenocopies the *Dmp1*-null mice.

The *FAM20C* loss of function mutations in Raine syndrome patients with elevated iFGF23 also gave rise to the idea that there may be more direct interactions between these genes. Of note, the Fam20C S-X-E recognition sequence is found within the FGF23 protein at the SPC cleavage site R_{179}/S_{180}-A_{181}-E_{182}. In vitro data demonstrated that the FAM20C-mediated phosphorylation of FGF23 S180 blocked GALNT3 mediated *O*-glycosylation at FGF23 residue T178, thereby promoting furin cleavage of FGF23 (52). Conversely, mutation of the S180 residue inhibited FGF23 phosphorylation, permitting GALNT3 glycosylation and stabilization of FGF23. Introduction of a Raine-mutant FAM20C cDNA demonstrated reduced efficiency of FGF23 phosphorylation and thus partial stabilization of FGF23, explaining the increased iFGF23 and hypophosphatemia in some ARHR-3 patients (52). It was noted that an S-x-E/D site at S212 within FGF23 is a target of FAM20C phosphorylation yet it is unclear whether this modification affects the processing or function of the intact hormone (53). Further studies are needed to examine the various FAM20C mutations as disparities remain between the severe osteosclerosis documented in patients with ARHR-3 compared to the rachitic bone phenotype observed *Fam20c*-null mice.

Genomic and acquired influences on FGF23 expression

a. Tumor induced osteomalacia (TIO)

Soon after the positional cloning of FGF23 as the gene mutated in ADHR, it was reported that a rare, mesenchymal tumor type that caused an XLH- and ADHR-mimetic biochemical syndrome, tumor induced osteomalacia (TIO; OMIM 605380), produced high levels of FGF23 (3, 54). These tumors were histologically classified under the collective term of 'phosphaturic mesenchymal tumor, mixed connective tissue variant (PMTMCT)' or 'phosphaturic mesenchymal tumor' (55). In studies performed in parallel to the ADHR genetic work, SAGE analysis identified FGF23 as a highly expressed transcript in PMTMCT and functional studies identified the FGF23 actions of reducing kidney NPT2a expression and the renal 1-alpha-hydroxylase mRNA (3). With the development of the first FGF23 ELISA, it was discovered that most patients with TIO had elevated serum FGF23, which was reduced following tumor resection (22). Using this tool, FGF23-producing tumors have also been localized in patients with venous sampling (56–58). Others have had modest success using tracers such as radiolabeled octreotide and octreotide therapy (59) as well as

MRI (57), computed tomography(CT) (60), whole body sestamibi scanning (61), and Ga68-DOTA-octreotide PET/CT imaging (62). A key clinical issue with PMTMCTs is that many are small in size and can arise in difficult places to image such as the nasal cavities and the base of the skull. Considering the difficulty with localization, a plausible approach for treating TIO patients until the tumor is found and removed could be using the anti-FGF23 antibody therapy KRN-23 (see below). Indeed, this strategy is currently being evaluated in an ongoing clinical trial (*clinicaltrials.gov*).

It has recently come to light that in a group of 15 TIO tumors analyzed through next-generation RNA sequencing, that a fibronectin (*FN1*)-*FGFR1* fusion gene was detected in 9 of the tested samples (63) (Table 1). Interestingly, the PMT-associated FN1-FGFR1 fusion protein is predicted to retain at least some of the three extracellular FGF-binding (Ig-like) domains (63), therefore overexpression of most of FGFR1 as well as ligand-activated receptor signaling could potentially occur. Whether the fusion gene is responsible for tumorigenesis or is a consequence of the cancer phenotype is not established. Since activating mutations in *FGFR1* are associated with elevated FGF23 in some patients with osteoglophonic dysplasia (OGD), a disease of dwarfism as well as craniosynostosis (64), and in multiple cancers (65–67), another potential therapy could be the use of FGFR inhibitors. Although in terms of patient treatment there could be concern that FGFR1 is ubiquitously expressed, it is possible that reducing FGFR activity could provide some benefit. This regimen, potentially in combination with anti-FGF23, may reduce possible autocrine signaling through FGFR1, as well as inhibit bioactivity of circulating FGF23.

b. *αKLOTHO* balanced translocation

The molecular interactions between FGF23, KL, and FGFRs are critical to systemic phosphate handling, as highlighted by the fact that disruptions in any of these genes results in loss of the phosphaturic actions of the kidney. A case report described a patient with a 9:13 translocation who was negative for *FGF23*, *FGFR1* and *DMP1* mutations (68), but had the hallmark clinical symptoms of markedly increased serum FGF23 during prevailing hypophosphatemia, with inappropriately normal 1,25D and secondary hyperparathyroidism. Diagnostic genomic probes and DNA sequencing reveled that this patient had a fusion of the APRIN (a paralog of the cohesin-associated Pds5 gene lineage that may regulate chromatin) and *αKLOTHO* genes, leading to elevated plasma cKL levels versus normal controls (68). Although KL has been associated with direct reduction of proximal tubule NPT2a expression (69), due to its known renal actions the sustained highly elevated FGF23 likely contributed to the hypophosphatemic phenotype observed in this patient.

To test the mechanisms whereby over expression of cKL would affect phosphate handling, an approach using sustained delivery cKL adeno-associated virus 2/8 and a liver-specific promoter was undertaken in normal mice. Upon delivery of cKL for 8 weeks, there was a dose-dependent increase in plasma FGF23, as well as hypophosphatemia and hypocalcemia, similar to the *KLOTHO* translocation patient (70). Bone Fgf23 mRNA was stably, and highly expressed in the AAV-cKL treated mice versus control animals. This increase in FGF23 was associated with enhanced renal signaling including up-regulated Egr1 mRNA, and down regulation of NPT2a (70). In vitro, co-expression of cKL and FGFR1 with FGF23

elicited positive p-FGFR1 and p-ERK1/2 activation supporting stimulation of the heteromeric signaling complex, potentially eliciting FGF23 actions in kidney and FGFR1-mediated FGF23 production in bone (70).

c. Linear sebaceous nevus syndrome, Schimmelpenning-Feuerstein-Mims (SFM); also Epidermal nevus syndrome (ENS)

Linear sebaceous nevus syndrome or Schimmelpenning-Feuerstein-Mims (SFM) disease are more specific terms for Epidermal nevus syndrome (ENS; OMIM 163200), and are defined by skin lesions that have been previously associated with hypophosphatemia in some cases. Elevated FGF23 was linked with this syndrome as the likely cause of the low serum phosphate in later studies (71, 72). Mutation analyses of skin biopsies showed that these patients can have somatic mosaicism for mutations in *FGFR3*, *PIK3CA*, and in Schimmelpenning-Feuerstein-Mims (SFM) syndrome the *RAS* genes, including *KRAS*, *HRAS*, and *NRAS* (Table 1), which were not found in biopsies of normal adjacent tissue. The RAS mutations occur in 'hot spot' mutational domains, and have been previously associated with increased cell proliferation (72). Conceptually, SFM appears to be similar to TIO, where ectopic expression of FGF23 leads to elevated plasma levels and hypophosphatemia. Interestingly, a case report suggested that the skin lesions affects the underlying bone, which was the source of elevated circulating FGF23 (71).

Hyperphosphatemic Familial Tumoral Calcinosis (hfTC)

a. hfTC due to loss of function *FGF23* mutations

Familial hyperphosphatemic tumoral calcinosis (hfTC; OMIM 211900) is characterized by elevated serum phosphate and ectopic and vascular calcifications, often occurring as periarticular. This disorder is the 'biochemical reciprocal' to XLH, ADHR, and TIO, and has a wide range of severity. hfTC is genetically heterogeneous. In this regard, recessive missense mutations in *FGF23* are associated with altered intracellular processing of the mature, bioactive iFGF23 (73–77) (Table 1). The locations of the FGF23 mutations occurred within the *N*-terminus, which is largely conserved among the FGF family members. The patients with these mutations had 'signature' FGF23 ELISA assay profiles; analysis of patients using the cFGF23 ELISA demonstrated markedly elevated serum cFGF23, but the iFGF23 ELISAs showed normal, or low, serum concentrations (74, 75). Co-modifying the RXXR/SAE furin cleavage site with an ADHR R176Q mutation produced hfTC-mutant FGF23 proteins that were stabilized, supporting that the missense mutations either slightly altered glycosylation (see GALNT3-hfTC below) or potentially exposed the mature FGF23 to intracellular SPC degradation (73, 77). The unique serum FGF23 ELISA pattern supports the concept of a positive feedback loop where the hyperphosphatemia increases FGF23 transcription, but the hfTC mutations inhibit iFGF23 production and thus activity, further driving FGF23 expression.

b. *GALNT3-hfTC* and Hyperostosis-hyperphosphatemia syndrome (HHS)

The gene encoding the GalNac-transferase 3 (*GALNT3*) is an ER and trans-Golgi network protein that *O*-glycosylates target substrates. FGF23 is predicted to have multiple GALNT3 recognition motifs, with a key site on T178 within the $R_{176}H_{177}T_{178}R_{179}/S_{180}AE$ motif.

Loss of function mutations in GALNT3 are responsible for hfTC that is essentially indistinguishable from FGF23-hfTC (78, 79) (Table 1). Interestingly, the signature ELISA profile that predicts FGF23-hfTC is the same in GALNT3-hfTC, with high cFGF23 and low or normal iFGF23, supporting a direct relationship between these genes. Work using the *Galnt3*-null mouse further demonstrated that bone Fgf23 mRNA was elevated whereas serum iFGF23 was normal (80). The similarities between FGF23- and GALNT3-hfTC suggested that at the protein level, T178 protects FGF23 from SPC degradation, which was confirmed through in vitro analyses (81). Interestingly, a form of hfTC, Hyperostosis-hyperphosphatemia syndrome (HHS), was found to be caused by mutations in *GALNT3* (81–83). Prior to genetic identification of the *GALNT3* mutations in HHS, patients were diagnosed with this form of hfTC if they presented with radiographic findings of periosteal reaction and cortical hyperostosis in the long bones. Whether genetic background is the determinant of the long bone involvement remains to be determined.

c. *hfTC due to an αKLOTHO* loss of function mutation

A case of hfTC in a child with numerous ectopic calcifications, including in brain, was reported in which the serum values for hfTC were ‘atypical’. In this regard, ELISA results showed elevated iFGF23 and cFGF23, suggesting a molecular pathogenesis distinct from FGF23- and GALNT3-hfTC, and end organ resistance to iFGF23 (84). Upon DNA sequencing of this patient, a missense mutation (H193R) in the predicted extracellular domain of the *αKLOTHO* gene was discovered (Table 1). When expressed *in vitro*, this alteration led to a mature mKL form that was poorly expressed at the cell membrane, and unable to fully transmit intracellular MAPK signaling when exposed to FGF23 (84). These findings supported that the patient was producing excess FGF23 due to the inability to fully activate the mutant KL and maintain proper renal phosphate balance.

Novel therapies based upon clinical and genetic discoveries

a. KRN-23 neutralizing antibody

The standard of care for diseases associated with elevated FGF23 are suboptimal and is comprised of phosphate repletion and low dose calcitriol. This current therapy has a high burden of treatment, including being medicated 3–5 times per day, diarrhea, risk of nephrocalcinosis, as well as secondary/tertiary hyperparathyroidism. Additionally, multiple painful surgeries are often required to correct bowed or fractured limbs. Whereas the genetic basis of the hypophosphatemic diseases described above varies, the ultimate result is elevated serum bioactive iFGF23 causing renal phosphate wasting and suppressed 1,25D concentrations. Since FGF23 has no compensatory molecules and considering the complications associated with current treatments, it was hypothesized that an antibody based therapy would be advantageous in FGF23-related hypophosphatemic disorders. Two monoclonal antibodies were developed by Kirin-Hakko-Kyowa Pharma and found *in vitro*, using Egr-1 gene activity as downstream readout for FGF23-Klotho signaling, that a combination of antibodies blocked the induction of Egr-1 in a dose dependent manner (85). Injections of these antibodies *in vivo* in WT mice demonstrated an increase in serum phosphate due to elevated proximal tubule Npt2a. Serum 1,25D also increased in treated animals from a reversal of the FGF23 directed effects on *Cyp24a1* and *Cyp27b1* (85). Thus,

these data supported that specific antibodies had the ability to neutralize FGF23 activity by interrupting FGF23/FGFR/Klotho interactions. The antibodies were next tested in the *Hyp* XLH mouse model. Single subcutaneous injections of either low (4 mg/kg) or high doses (16 mg/kg) of anti-FGF23 significantly improved serum phosphate levels out to 3 days post injection due to a reduction in urine phosphate excretion (86). 1,25D levels were also dramatically increased and maintained for up to 7 days post injection for the high dose treatment. Performing weekly injections beginning at 4 weeks of age demonstrated an increase in femur and tibia length about one month later compared to the control injected mice, with BV/TV% returning to near WT levels in the anti-FGF23 treated *Hyp* mice (86). Osteomalacia is a well characterized feature of *Hyp* long bones due to the lack of mineral content for hydroxyapatite deposition. The amount of non-mineralized bone was significantly reduced in the treated mice and approached WT levels in the *Hyp* mice receiving high dose anti-FGF23. These data, in conjunction with bone specific flox-*Fgf23* knockout studies in *Hyp* mice (21), demonstrated the key driver of the hypophosphatemia phenotype is FGF23 and therefore validates the anti-FGF23 antibody treatment regimen.

A humanized anti-FGF23 antibody (KRN23) was taken into phase I clinical trials and tested in adult XLH patients. The trial design tested several concentrations of KRN23 through either subcutaneous (s.c.) or intravenous (i.v.) injection versus placebo in a total of 38 patients (87). This study recapitulated the observations in the preclinical *Hyp* model where a single administration of KRN23 potently improved serum phosphate levels in a dose dependent manner. Compared to i.v. administration, activity of KRN23 was sustained within the s.c. group and phosphate remained above baseline up to 50 days post treatment. 1,25D levels also increased after treatment, but returned to baseline in 8 and 29 days after administration in the i.v. and s.c. groups, respectively (87). Importantly, calcium levels were not found to be dramatically altered in this study regardless of administration route and the therapy was well tolerated. In a follow-up study, 28 patients were given KRN23 subcutaneously every 28 days for a total of 16 doses. The first four doses contained increasing KRN23 concentrations (considered the escalation phase) whereas the extension phase doses were only adjusted on a patient basis to prevent hyperphosphatemia (88). Serum phosphate peaked 7 days after KRN23 dosing but did not return to the patient baseline in between doses during the extension phase. Serum 1,25D levels displayed a similar pattern with KRN23 dosing, however the peak levels declined further into the extension phase. Of note, nephrocalcinosis did not arise in the patients treated with KRN23, a common adverse event when this patient population is given the current regimen of combined calcitriol and phosphate therapy. These initial studies show pharmacological inhibition of FGF23 in hypophosphatemic disorders can function to maintain serum phosphate levels in a normal and therapeutic range that is likely beneficial to XLH patients (Figure 2). One aspect of the bone phenotype observed in *Hyp* mice and XLH patients includes a grossly disrupted growth plate. 1,25D is important for hypertrophic chondrocyte apoptosis and thus growth plate maturation. Lui, *et al* found that daily injections of 1,25D in *Hyp* mice beginning at 2 days of age rescued *Hyp* phenotypes similarly, if not better than anti-FGF23 (89). Thus, additional studies could potentially address combinations of anti-FGF23 and 1,25D.

b. Iron repletion for ADHR

As described above, iron deficiency anemia and hypoxia are known to increase FGF23 in mice and humans, and in the context of a stabilizing ADHR FGF23 mutation, results in elevated iFGF23 and hypophosphatemic bone disease (25). Considering this effect of anemia on FGF23 and the fact that iron supplementation to ADHR mice rescued the low serum phosphate (14), a case was reported using iron replacement therapy to treat ADHR. The family under study was positive for an R176Q FGF23 mutation, and had an affected father and daughter with a history of disease onset and remission (90). The child was removed from standard therapy and placed on iron II sulfate, which corresponded with improvement of the renal phosphate leak as well as elevated plasma 1,25D (90). Over a six month period, her serum iron stabilized and after all treatments ceased, serum FGF23 normalized. Although the patient was followed for almost a decade, the potential use of iron therapy in anemic ADHR patient could provide additional benefit considering the side effects of the current treatment regimen involving phosphate and 1,25D provision. In a clinical study, although mean plasma FGF23 was elevated in patients with XLH compared to normal controls, it was reported that FGF23 correlated negatively with serum iron in both patients and normal controls (91). Thus, elevated iFGF23 is the common denominator in ADHR, XLH, ARHR, TIO, KL-translocation, and ENS, however iron therapy likely applies solely to ADHR as it is the only FGF23-related disorder with clear late-onset associated with physiological situations of anemia.

c. Innovative therapies for hfTC

The treatment option for the heritable forms of hyperphosphatemia were previously very limited. Surgical removal or drainage of surface calcifications often results in temporary relief, with the lesions recurring with time. Groups have reported success in some patients with the carbonic anhydrase inhibitor acetazolamide in reducing calcifications with limited reductions in serum phosphate (79), suggesting that pH may be critical for development of the calcinosis phenotype. Similarly, the use of restricted phosphate diet and phosphate binders for management may also produce variable relief. Theoretically, the most relevant therapy for FGF23- and GALNT3-hfTC would likely be recombinant iFGF23 to restore kidney phosphate excretion and reduce serum levels. Additionally, it is possible that the ADHR-mutant form of recombinant FGF23 could be favorable due to the more stable nature of this species versus wild type protein (17, 92). Of significance, a topical treatment containing sodium thiosulfate was recently developed and virtually ablated the surface ectopic calcifications in confirmed FGF23- and GALNT3-hfTC patients (93). This analog is not favorable for clinical use through oral administration as it produces side effects of digestive symptoms, and metabolic acidosis. Although the exact mechanisms of action are unknown, it is hypothesized that the calcifications may have been eliminated due to the compound’s ability to chelate calcium salts. Radiological images of three patients showed a dramatic improvement in joint calcifications between 5 to 8 months after starting the regimen, as well as improvement in overall limb mobility and flexibility (93). In this small study it was also noted that the patients did not suffer any adverse effects typically observed with oral or intravenous administration of sodium thiosulfate, thus potentially deriving a very promising step forward for care of these patients (Figure 3).

Summary

In summary, the study of Mendelian and acquired disorders of renal phosphate handling involving FGF23 continues to reveal important mechanisms dictating the systemic control of phosphate. The dovetailed combinations of molecular genetic, clinical and translational science have been important for the advancement of these findings from the laboratory to practical application. There is still much to be learned in both rare and common diseases of mineral metabolism, however the studies to date have fostered development of innovative therapies as well as potential optimization of current treatment paradigms which are providing high impact to patient care.

Acknowledgments

The authors would like to acknowledge NIH grants DK063934, DK95784, and AR070329 (KEW); F32-AR065389 (ELC); the Indiana Genomics Initiative (INGEN), supported in part by the Lilly Endowment, Inc.; The David Weaver Professorship, and a Showalter Scholar award through the Ralph W. and Grace M. Showalter Research Trust (KEW).

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Highlights

- Phosphate is critical for nucleic acids, cellular energy, and bone hydroxyapatite.
- Heritable disorders revealed critical insight into FGF23 and Klotho actions.
- Genetic/clinical findings have led to novel therapies targeting phosphate diseases.

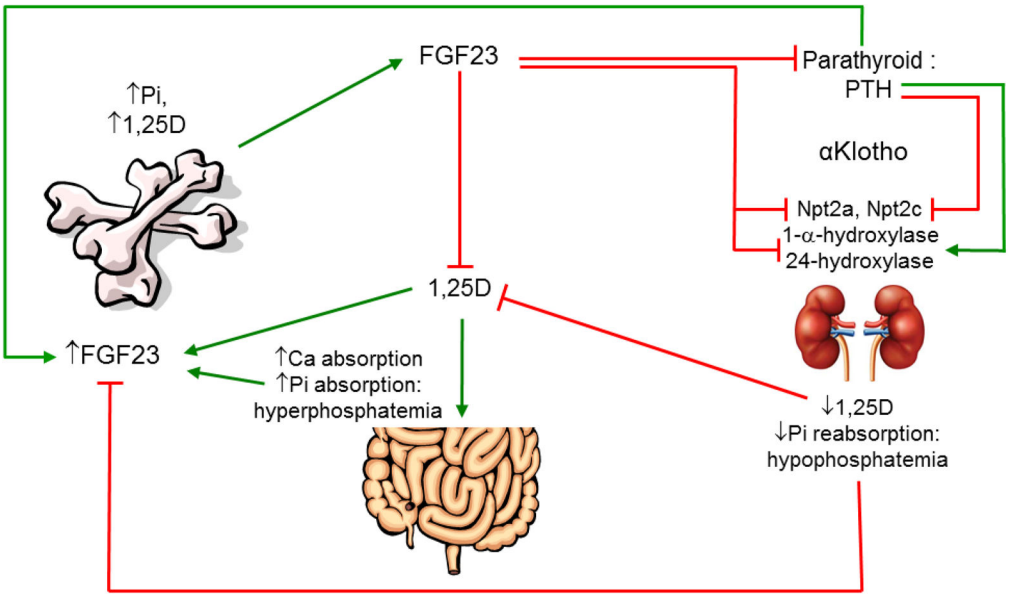

Figure 1.

FGF23 regulatory systems. FGF23 is produced in bone and secreted into the circulation, potentially in response to increased phosphate (Pi), 1,25D, and PTH. FGF23 acts in the kidney through αKlotho to decrease Npt2a and Npt2c expression and decrease 1,25D production, resulting in hypophosphatemia. 1,25D acts in the intestine to increase calcium (Ca) and Pi absorption. FGF23 acts in the parathyroid glands to reduce PTH. Hypophosphatemia and reduced 1,25D complete the feedback loop and inhibit FGF23 production.

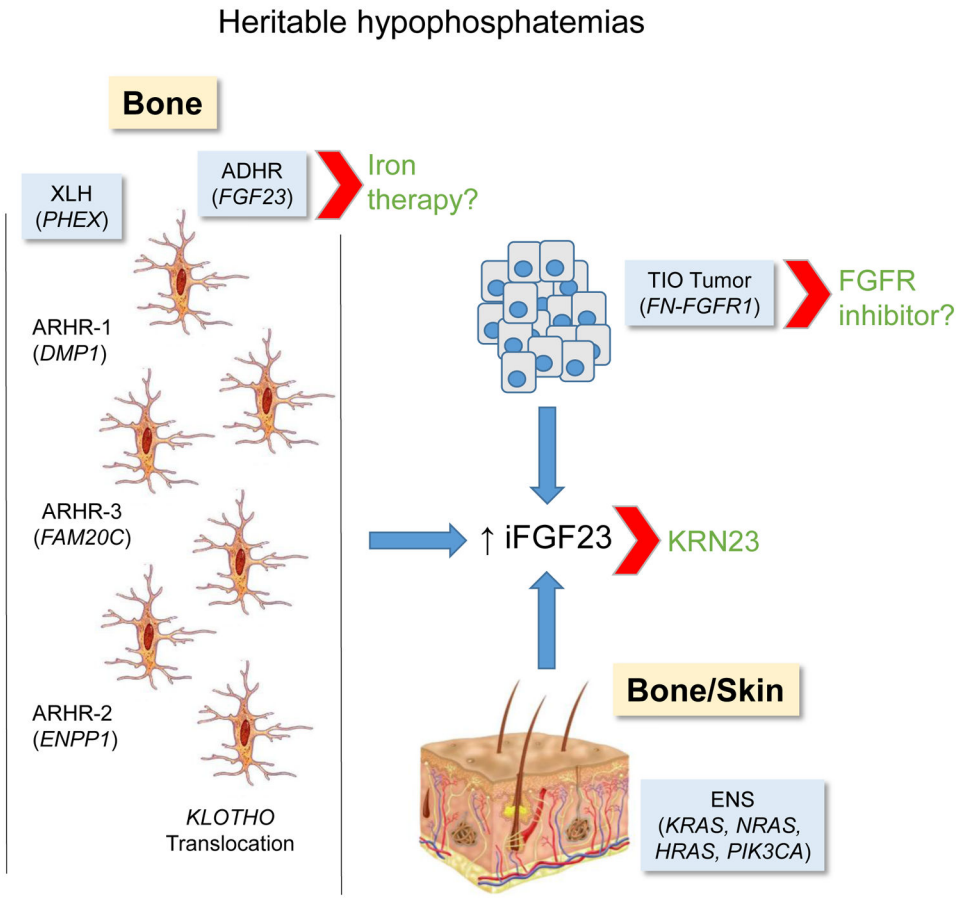

Figure 2.

Emerging therapeutics for heritable hypophosphatemias. The genetic discovery of the underlying pathogenesis of diseases associated with gain of FGF23 bioactivity has resulted in new therapeutic approaches. In theory, all of the heritable and acquired disorders of elevated iFGF23, including ADHR, XLH, TIO, ENS, KLOTHO translocation, and ARHR1–3 may benefit from anti-FGF23 therapy (KRN23). In anemic ADHR patients, iron supplementation may reduce serum FGF23. The blue boxes indicate diseases under evaluation or recruiting clinical trials (www.clinicaltrials.gov), with KRN23 for XLH, ENS, and TIO; and iron supplementation for ADHR.

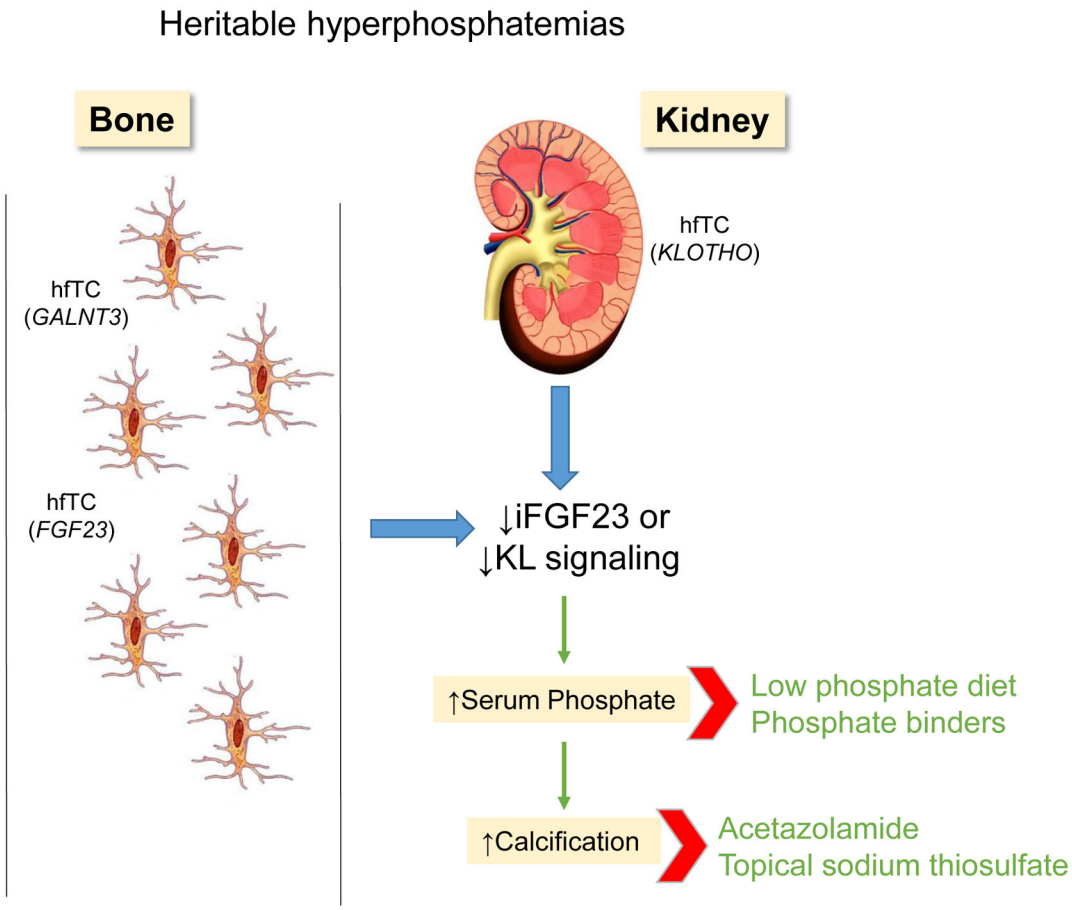

Figure 3.

Emerging therapeutics for heritable hyperphosphatemias. Treatments for hfTC currently lag behind those for the genetic hypophosphatemias and are often combination therapies of phosphate binders and low phosphate diet to attempt to reduce serum phosphate that occurs from lack of iFGF23 production or loss of KL signaling. In FGF23- and GALNT3-hfTC, patients have benefited from topical sodium thiosulfate as well as the carbonic anhydrase inhibitor acetazolamide to reduce calcification burden.

Table 1

Heritable and acquired disorders involving FGF23.

Disorder	Genes Affected	Gain or loss of function mutation	Phenotypic effects	Effect on serum Pi	Effect on serum 1,25D	iFGF23 ELISA conc.	cFGF23 ELISA conc.	Gross bone involvement
ADHR	*FGF23*	Gain of function	Stabilize active iFGF23	↓	↔	↔ or ↑	↔ or ↑	Rickets/ osteomalacia
XLH	*PHEX*	Loss of function	Increased iFGF23 production in osteocytes	↓	↔	↔ or ↑	↔ or ↑	Rickets/ osteomalacia
ARHR-1	*DMP1*	Loss of function	Increased iFGF23 production in osteocytes	↓	↔	↔ or ↑	↔ or ↑	Rickets/ osteomalacia
ARHR-2	*ENPP1*	Loss of function	Increased iFGF23	↓	↔	↔ or ↑	↔	Rickets/ osteomalacia
ARHR-3	*FAM20C*	Loss of function	Increased iFGF23	↓	↔	↑	↑	Rickets/ osteomalacia; sclerosis
TIO	*FN1-FGFR1* fusion	Gain of function?	iFGF23 over-produced by PMTMCT	↓	↔ or ↓	↔or↑	↔ or ↑	Osteomalacia
SFM/ ENS	*KRAS, NRAS, HRAS/ FGFR3, PIK3CA*	Gain of function	iFGF23 over-produced in bone/skin	↓	↔	↔ or ↑	↔ or ↑	Osteomalacia; focal bone lesions ipsilateral to affected skin
hfTC/ (HHS)	*FGF23,* or *GALNT3 (HHS)*	Loss of function	Destabilize active iFGF23	↑	↔ or ↑	↔ or ↓	↑↑	Potential cortical hyperostosis
hfTC	*αKLOTHO*	Loss of function	Reduced FGF23-dependent signaling	↑	↔ or ↑	↑↑	↑↑	Diffuse osteopenia; patchy sclerosis