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Fibrous Dysplasia and Fibroblast Growth Factor-23 Regulation

Alison M Boyce^{1,2,4}, Nisan Bhattacharyya³, and Michael T Collins^{3,4}

¹Bone Health Program, Division of Orthopaedics and Sports Medicine, Children's National Medical Center, Washington, DC 20010

²Division of Endocrinology and Diabetes, Children's National Medical Center, Washington, D.C. 20010

³Skeletal Clinical Studies Unit, Craniofacial and Skeletal Diseases Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, 20910

Abstract

Fibrous dysplasia (FD) is a skeletal disorder caused by activating mutations in $G_s\alpha$ that result in elevations in cAMP. A feature of FD is elevated blood levels of the bone cell-derived phosphaturic hormone, fibroblast growth factor-23 (FGF23). FGF23 regulates serum phosphorus and active vitamin D levels by action on proximal renal tubule cells. An essential step in the production of biologically active FGF23 is glycosylation by the UDP-N-acetyl- α -D-galactosamine:polypeptide N-acetylgalactosaminyl transferase (ppGalNAc-T3). In the absence of glycosylation, FGF23 is processed into inactive N- and C-terminal proteins by a subtilisin proprotein convertase, probably furin. Normally, most if not all circulating FGF23 is intact. In FD, C-terminal levels are elevated, suggesting altered FGF23 processing. Altered processing in FD is the result of a cAMP-dependent, coordinated decrease in ppGalNAc-T3 and an increase in furin enzyme activity. These findings, and emerging data from other diseases, suggest regulation of FGF23 processing may be a physiologically important process.

Keywords

FGF23; fibrous dysplasia; phosphorus; vitamin D; mineral metabolism; bone

Fibrous Dysplasia Background

Fibrous dysplasia (FD; OMIM 174800) is an uncommon and debilitating skeletal disorder resulting in fracture, deformity, functional impairment, and pain^{1–3}. FD occurs along a broad clinical spectrum, and may affect one bone (monostotic FD) or many bones (polyostotic FD). FD may occur in association with extraskeletal features, most commonly café-au-lait macules and hyperfunctioning endocrinopathies, including precocious puberty, hyperthyroidism, growth hormone excess, hypercortisolism, and FGF23-mediated hypophosphatemia. FD in combination with one or more of these extraskeletal features is termed McCune-Albright syndrome^{4–7}.

⁴Corresponding authors: Michael T. Collins, MD, Chief, Skeletal Clinical Studies Unit, CSDB, NIDCR, NIH, 30 Convent Drive, Building 30, Room 228, MSC 4320, Bethesda, MD 20892-4320, 301-496-4913 direct; 301-728-0329 cell; 301-480-9962 fax, mc247k@nih.gov.

Disclosure

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FD arises from somatic activating mutations in the *GNAS* gene, which encodes the α -subunit of the G_s -stimulatory protein^{8,9}. Disease burden depends on the stage of embryogenesis at which the mutation occurs, and the specific clone in which the mutation occurs; this determines the locations to where mutated progenitor cells subsequently migrate^{10,11}. The vast majority of the mutations that occur in $G_s\alpha$ in FD arise at the R201 position, which includes the intrinsic GTPase domain of the molecule^{12,13}. This results in prolonged interaction of activated G_s with the effector molecule adenylyl cyclase, and increased production of the second messenger cyclic AMP (cAMP)¹⁴. In bone, constitutive $G_s\alpha$ activity results in proliferation of undifferentiated bone marrow stromal cells, which accumulate in marrow spaces leading to local loss of hematopoiesis and marrow fibrosis¹⁵. Osteogenic cells derived from these progenitors are functionally impaired, leading to abnormal matrix deposition and formation of bone that is morphologically and structurally unsound. FD demonstrates site-specific histologic features; in the craniofacial area it appears as dense with uninterrupted trabecular networks, while in the axial and appendicular skeleton the trabecular pattern is discontinuous (often referred to as having a 'Chinese writing' appearance)¹⁶. Typical features recognized at all skeletal sites include abnormal collagen formation as manifested by so-called Sharpey's fibers and loss of typical lamellation patterns, increased vascularization, and abnormal persistence of woven bone¹⁶. Undermineralized matrix and accumulation of osteoid is also a common and clinically important feature of FD¹⁷. It may be the lesional osteomalacia in FD tissue that lends plasticity to bones affected by FD and their tendency to bow and fracture^{18,19}.

Areas of skeletal involvement are established early, with 90% of craniofacial lesions evident by age 5 years, and 75% of all FD lesions established by age 15 years²⁰. Although any area of the skeleton may be affected, the most commonly involved sites include the proximal femora and skull base^{21,22}. Femoral disease may present with a classic coxa vara, or shepherd's crook deformity. Skull disease may result in facial asymmetry, and rarely damage to the cranial nerves leading to loss of vision and/or hearing. Complications of craniofacial FD are more common in patients with uncontrolled growth hormone excess^{23,24}. The radiographic appearance of FD varies depending on the location and activity of the affected bone. During childhood, FD in the appendicular and craniofacial skeleton typically demonstrates a homogeneous 'ground glass' appearance. Disease activity may wane during adulthood, at which point FD often adopts a more sclerotic and less homogeneous appearance. X-ray findings in the axial skeleton (including the ribs, pelvis, and spine) may be subtle, and FD in these areas is often more easily detected using bone scintigraphy²².

The Role of FGF23 in Mineral Metabolism in Fibrous Dysplasia

Andrea Prader was the first to propose the existence of a phosphaturic factor. In 1959 he reported the case of an 11-year old girl who developed severe rickets over the course of a year that resolved after resection of a benign connective tissue tumor. Prader speculated that the tumor produced a circulating factor responsible for her phosphate wasting²⁵, a condition later described as tumor-induced osteomalacia (reviewed in^{26,27}). Since then, FGF23 overproduction has been identified as the underlying etiology of tumor-induced osteomalacia and other hypophosphatemic disorders, including X-linked, autosomal dominant, and autosomal recessive hypophosphatemic rickets, and FD²⁸⁻³¹.

Although frank hypophosphatemia in patients with FD is infrequent, a renal tubulopathy including some degree of phosphate wasting is one of the most commonly associated extraskeletal manifestations^{32,33}. In 2003 it was recognized that FD-associated phosphate wasting arises from overproduction of FGF23 by abnormal osteogenic precursors, and that in normal bone FGF23 is produced by both osteoblasts and osteocytes³¹. This provided the

first evidence that bone is the physiologic source of FGF23 production, a finding that was subsequently supported by other investigators^{34–36}. The degree of FGF23 overproduction in FD is correlated with disease severity and tissue burden; thus significant hypophosphatemia is seen only in patients with a substantial FD burden^{31,32}. In contrast to most other features of McCune-Albright syndrome, hypophosphatemia may resolve as patients become older^{7,37}. This likely reflects intrinsic changes in FD that occur with age, including decline in the number of mutated progenitor cells and the tendency of histologic features to improve over time³⁷.

FGF23 reduces serum phosphate levels by three mechanisms: 1) directly by down-regulation of the NaPi 2a and 2c transporters in the proximal tubule, leading to reduced renal phosphate reabsorption at the level of the kidney, 2) directly by down-regulation of renal 1 α -hydroxylase, which decreases production of active 1,25-dihydroxyvitamin D and as a result decreases intestinal phosphate absorption, and 3) indirectly by the compensatory increase in PTH that develops in order to maintain eucalcemia in response to relatively or frankly low 1,25-dihydroxyvitamin D^{38–40}. Elevated PTH exacerbates the phosphate wasting effect of FGF23 and worsens the disease.

Clinical sequelae of FGF23-mediated hypophosphatemia in FD include earlier onset and increased risk of fractures relative to patients with hypophosphatemia, and FD-related bone pain¹⁹. Treatment is similar to other forms of FGF23-mediated hypophosphatemia, and includes supplementation with phosphorus and vitamin D analogs^{6,7}.

The regulation of FGF23

The physiologic mechanisms by which FGF23 transcription and translation, and ultimately blood levels are regulated have not yet been clearly defined. Clinically, changes in FGF23 levels likely involve feedback from serum phosphorus and 1,25-dihydroxyvitamin D^{41–45}. Molecularly, the fact that mutations in phosphate regulating endopeptidase homolog, X-linked (*PHEX*)²⁸ and dentin matrix protein-1 (*DMP-1*)³⁶, (the genes mutated in X-linked hypophosphatemic rickets (XLH) and autosomal recessive hypophosphatemic rickets, respectively), cause dysregulated elevations in blood FGF23 and hypophosphatemia/rickets, implicates *PHEX* and *DMP-1* in FGF23 regulation. There is also evidence that pathologically elevated levels of α -Klotho (α KL) are able to directly elevate FGF23 levels^{46,47}. In addition, mutations in the *FAM20C* gene that encodes the phosphorylation enzyme Golgi-enriched fraction casein kinase (GEF-CK) have recently been reported in several families with FGF23-mediated hypophosphatemic rickets⁴⁸.

Human FGF23 is a 251 amino acid protein with a high degree of homology across many species that includes a 24 amino acid signal peptide, indicating it is a secretory hormone^{28,38,49}. The FGF23 protein has two major functional domains: the N-terminal portion, which includes homology to other FGFs, and a unique C-terminal domain. FGF23 exerts its action via interaction with various FGF cell-surface receptors including FGFR1, 3c, and 4. FGF23 signaling is dependent upon its interaction not only with an FGFR, but also with its co-receptor, α KL. Evidence indicates that the most potent signal transduction is exhibited when FGF23 binds to FGFR1^{50,51}. However, recent evidence also suggests that in certain pathophysiologic states, such as renal failure where FGF23 levels may be elevated by two to three orders of magnitude, FGF23 may also interact with other FGFRs such as FGFR4 to mediate pathophysiologic effects⁵². While FGFRs including FGFR1 are widely expressed, it may be the relatively narrow tissue expression profile of α KL that lends specificity to FGF23 action under normal physiologic conditions. FGF23 receptor binding induces various cell signaling events, including activation of extracellular signal-regulatory

kinases (ERK) and mitogen activated kinases (MAPK), that lead to activation of the early growth response protein 1 (EGR1) ⁵⁰.

Recent evidence suggests the mineral metabolism effects of FGF23 may be PTH-dependent ⁵³, with PTH acting synergistically as part of a coordinated physiological process to induce phosphate excretion via the action of sodium-hydrogen exchanger regulatory factor-1 (NHERF-1), a PDZ domain containing scaffolding protein ⁵⁴. Upon addition of PTH and/or FGF23, specific receptor molecules induce signal transduction pathways by activating either PKC or MAPK pathways, causing the dissociation of NHERF-1 from the Npt2a complexes, which in turn induces Npt2a endocytosis and reduces renal phosphate reabsorption⁵⁵.

FGF23 processing

FGF23 circulates in several forms: biologically active intact FGF23 (iFGF23), and inactive C-terminal (cFGF23) and N-terminal FGF23 (nFGF23) fragments (⁵⁶, reviewed in ⁵⁷). There are two assays commonly used for the measurement of FGF23. One assay detects only iFGF23 (Kainos, Tokyo, Japan). The other assay uses capture and detection antibodies that are both directed to the C-terminus, and as a result detects both iFGF23 and cFGF23. Currently there are no nFGF23-specific assays.

FGF23 processing appears to be primarily regulated by two major steps: 1) post-translational O-linked glycosylation by UDP-N-acetyl- α -D-galactosamine:polypeptide N-acetylgalactosaminyl transferase (ppGalNAc-T3) at Ser or Thr residues, and 2) protein cleavage around amino acids 176–179 (RXXR, ¹⁷⁶RHTR¹⁷⁹) (⁵⁸, reviewed in ⁵⁹). Studies from cell culture models suggest that production and secretion of active iFGF23 requires glycosylation, and that under-glycosylated FGF23 is prone to cleavage to inactive fragments ^{56,58,60}. In the absence of glycosylation, the FGF23 cleavage site is recognized by subtilisin-like proprotein convertase enzymes such as furin. In support of this, FGF23 has been co-localized with ppGalNAc-T3 and furin by confocal microscopy⁶¹. In addition, when LoVo cells, a cell line that lacks furin, are transfected with FGF23 there is no FGF23 processing, in spite of the fact that the other subtilisin-like proprotein convertases are expressed (unpublished data, NB and MTC). However recent evidence suggests other proprotein convertases, such as PC2, may also play a role ⁶². Mass spectrometric analyses of purified FGF23 indicates that several Ser and Thr residues may be O-glycosylated and therefore important for protection against cleavage of FGF23 by furin ^{56,60}. However, demonstration of this in clinical specimens remains to be seen.

The most dramatic example of altered FGF23 processing is the disease of hyperphosphatemic familial tumoral calcinosis (HFTC). These patients present with reduced urinary phosphate excretion, hyperphosphatemia, and inappropriately normal or elevated 1,25-dihydroxyvitamin D. HFTC can result from mutations in three different genes. The most common form is due to loss of function mutations in ppGalNAc-T3, and is sometimes referred to as HFTC type 1 ⁶³. The second most common form is HFTC type 2, which is due to mutations in FGF23 ⁶⁴. The least common (to date only one case has been reported) is HFTC type 3 due to mutations in α KL, the FGF23 co-receptor, which results in a form of FGF23 resistance ⁶⁵. In types 1 and 2 FGF23 processing is altered, resulting in elevation of nFGF23 and cFGF23 and low levels of iFGF23. In Type 1 loss of function mutations in ppGalNAc-T3 presumably leads to decreased glycosylation and increased FGF23 cleavage. FGF23 mutations in type 2 HFTC typically arise at Ser or Thr residues, which are potential sites for O-glycosylation by ppGalNAc-T3. However, mutations at sites other than Ser or Thr have also been reported (H41Q, Q54K and M96T). It has been speculated that disrupted protein folding may also play important roles in FGF23 stability and processing ⁶⁶.

FGF23 Processing in Fibrous Dysplasia

Studies in FD have provided novel insights into both production and processing of FGF23. The first insight into differential regulation of FGF23 in FD was the recognition that in spite of high levels of circulating FGF23, frank hypophosphatemia and/or rickets was relatively uncommon^{31,32}. Analysis of FD patient serum confirmed that while total FGF23 levels (a combination of iFGF23 and cFGF23) were elevated, there was a proportionally greater elevation in cFGF23 levels relative to iFGF23, consistent with increased FGF23 processing⁶¹.

The mechanism of FGF23 processing in FD was studied in detail in both human bone marrow stromal cells from FD patients, as well as an FGF23 producing cell line that was transiently transfected with either mutant or wild type $G_s\alpha$ ⁶¹. Patient bone marrow stromal cells, which had elevated cAMP levels due to the activating mutation in $G_s\alpha$, showed a cAMP-dependent coordinated increase in furin enzyme activity and a decrease in ppGalNAC-T3 enzyme activity. These data are consistent with increased cleavage of active iFGF23 into its inactive fragments. Also consistent with this was the demonstration of perinuclear co-localization by confocal microscopy of FGF23, furin, and ppGalNAC-T3 in patient bone marrow stromal cells. These studies were validated and extended by studies in FGF23 processing in HEK293 cells, a cell line that overexpresses FGF23. When HEK293 cells were treated with either cAMP-inducing doses of forskolin or co-transfected with mutant $G_s\alpha$, there was an increase in cFGF23 relative to iFGF23, as well as a coordinated increase in furin activity and decrease ppGalNAC-T3 enzyme activity. These data explain the biochemical phenotype observed in patients with FD.

There is precedent for the sort of coordinated O-glycosylation and furin protein processing that is seen in FD. Semenov and colleagues demonstrated that the mechanism of inhibition of pro-brain natriuretic peptide processing is a coordinated inhibition of O-glycosylation and increase in furin activity⁶⁷.

Other examples of altered FGF23 processing

A pattern similar to what has been observed in FD can be seen in Jansen metaphyseal chondrodysplasia. Jansen metaphyseal chondrodysplasia is a skeletal dysplasia caused by activating mutations in the PTH/PTHrP receptor. Constitutive receptor activation in Jansen metaphyseal chondrodysplasia leads to elevations in cAMP, as is seen in FD. In a report of one patient, levels of total FGF23 (cFGF23 + iFGF23) were elevated out of proportion to iFGF23, supporting a model of cAMP-mediated FGF23 overproduction with a compensatory increase in FGF23 processing⁶⁸.

An important new finding is a potential role for serum iron levels in regulation of FGF23 synthesis and processing. Durham et al demonstrated elevated cFGF23 but normal iFGF23 levels in a group of anemic patients, suggesting low iron levels caused an increase in both FGF23 synthesis and processing⁶⁹. Imel et al studied the relationship between serum iron concentrations and plasma cFGF23 and iFGF23 levels in ADHR subjects and healthy controls⁷⁰. In individuals with ADHR, serum iron levels were negatively correlated with both iFGF23 and cFGF23, while in controls low serum iron was associated with elevated cFGF23, but not iFGF23. These findings suggest that in spite of increased transcription and translation of FGF23 in normal subjects, serum iFGF23 levels are maintained in the normal range, possibly by increased FGF23 processing. It appears that FGF23 mutations in subjects with ADHR (most of which are at the ¹⁷⁶RHTR¹⁷⁹ site) do not allow for the increased processing seen in normal subjects. These findings were supported in studies by Farrow et al⁷¹. In a mouse model of ADHR they showed that on a low iron diet, both ADHR and wild type mice had increased FGF23 transcription and translation, but only the ADHR mice had

elevated iFGF23 and were hypophosphatemic. The wild type mice were able to compensate by processing iFGF23 to cFGF23, which was elevated in the wild type mice. They further showed that, as might be expected in an iron-regulated pathway, the effects on FGF23 transcription, translation and processing may be mediated by the HIF-1 α pathway.

It has been speculated that excess levels of C-terminal FGF23 may exert an inhibitory effect on the action of iFGF23⁷². This would suggest increased FGF23 processing may serve a physiologic role in diseases such as FD by inhibiting the effects of excess FGF23. However, evidence from the *phex* and *galnt3* knockout mouse suggests otherwise⁷³. This double knockout mouse is frankly hypophosphatemic in the setting of extremely elevated cFGF23 and only moderately elevated iFGF23 levels, suggesting the excess cFGF23 was unable to inhibit the effects of iFGF23. The answer to the question as to whether or not excess cFGF23 can inhibit iFGF23 action is important, as it will determine whether or not FGF23 replacement would be an effective treatment for patients with iFGF23-deficient HFTC.

Taken together, these findings support a model in which FGF23 processing is a regulated process mediated by the relative activities of ppGalNAcT3 glycosylation and furin degradation; a process in which FGF23 that is fully glycosylated by ppGalNAcT3 is protected from degradation by furin. In FD and Jansen metaphyseal dysplasia cAMP pathways are operative, and in ADHR the HIF-1 α pathway may play an important role. Further elucidation of this exciting area of investigation is awaited.

Conclusions

The overall levels of intact biologically active FGF23 reflect the balance between the activity of a subtilisin-like proprotein convertase (most likely furin) that degrades iFGF23 and the galactosyl transferase enzyme, ppGalNAcT3, which by glycosylating FGF23, protects it from degradation (Figure). The processing of iFGF23 to its inactive degradation products is a regulated process that likely has physiological significance. In FD, there is a coordinated, cAMP-mediated regulation of both furin and ppGalNAcT3 enzyme activity that results in lower ppGalNAcT3 activity and elevated furin activity, with the net result of increased FGF23 processing and a proportionally elevated increase in cFGF23 relative to iFGF23. Evidence of altered FGF23 processing has also been observed in other disease states, such as ADHR, and suggest that FGF23 processing may represent a possible target for therapeutic intervention in FGF23-mediated diseases.

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Figure.

Bone cells (osteocytes and osteoblasts) are responsible for production of FGF23 (blue boxes). The galactosyl transferase UDP-N-acetyl- α -D-galactosamine:polypeptide N-acetylgalactosaminyl transferase (GALNT3, green box) glycosylates FGF23 and by doing so protects it from enzymatic degradation by a subtilisin proprotein convertase, probably furin (scissors). Unglycosylated, unprotected FGF23 (lower blue box) is degraded and C- and N-terminal degradation products (probably biologically inactive) are secreted into the circulation. In fibrous dysplasia, under the regulation of cAMP, the process of degradation is enhanced (blue arrow). This degradation process may also be increased in low iron states, possibly mediated by the HIF-1 pathway.