A homozygous missense mutation in human KLOTHO causes severe tumoral calcinosis

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Familial tumoral calcinosis is characterized by ectopic calcifications and hyperphosphatemia due to inactivating mutations in FGF23 or UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GALNT3). Herein we report a homozygous missense mutation (H193R) in the KLOTHO (KL) gene of a 13-year-old girl who presented with severe tumoral calcinosis with dural and carotid artery calcifications. This patient exhibited defects in mineral homeostasis with marked hyperphosphatemia and hypercalcemia as well as elevated serum levels of parathyroid hormone and FGF23. Mapping of H193R mutation onto the crystal structure of myrosinase, a plant homolog of KL, revealed that this histidine residue was at the base of the deep catalytic cleft and mutation of this histidine to arginine should destabilize the putative glycosidase domain (KL1) of KL, thereby attenuating production of membrane-bound and secreted KL. Indeed, compared with wild-type KL, expression and secretion of H193R KL were markedly reduced in vitro, resulting in diminished ability of FGF23 to signal via its cognate GFG receptors. Taken together, our findings provide what we believe to be the first evidence that loss-of-function mutations in human KL impair FGF23 bioactivity, underscoring the essential role of KL in FGF23-mediated phosphate and vitamin D homeostasis in humans.

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Introduction

Tumoral calcinosis (also referred to as hyperphosphatemic familial tumoral calcinosis, OMIM 211900) is a rare autosomal recessive metabolic disorder characterized by the development of often severe ectopic calcifications in soft tissues. Calcific masses are typically found around major joints such as the hip, elbow, shoulder, and knee. Biochemical abnormalities associated with tumoral calcinosis include hyperphosphatemia secondary to increased renal tubular phosphate reabsorption and elevated or inappropriately normal 1,25-dihydroxyvitamin D [1,25(OH)2D] levels. Furthermore, this disorder is occasionally associated with diaphysis, hyperostosis, arterial aneurysms, dental abnormalities, and angiod streaks of the retina.

Recent molecular genetic analyses demonstrated that tumoral calcinosis can result from biallelic inactivating mutations in genes encoding FGF23 (1–4) or the UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GALNT3) (5–8). FGF23 is a hormone that promotes renal phosphate excretion by decreasing phosphate reabsorption in the proximal tubule and also increasing metabolism of 1,25(OH)2D by both decreasing biosynthesis and increasing metabolism of 1,25(OH)2D (9). GALNT3 is a Golgi-associated enzyme that initiates O-glycosylation of mature polypeptides. This enzyme selectively O-glycosylates a furin-like convertase recognition sequence in FGF23, thereby preventing proteolytic processing of FGF23 and allowing secretion of intact FGF23 (10). Therefore, dysfunction of either FGF23 or GALNT3 decreases circulating intact, bioactive FGF23, which leads to hyperphosphatemia and ultimately to tumoral calcinosis.

Recent studies have shown that FGF23 requires an additional cofactor, klotho (KL), to bind and signal through its cognate FGF receptors (FGFRs) (11, 12). Diminished KL expression in mice results in a phenotype characterized by osteopenia, skin and muscle atrophy, pulmonary emphysema, infertility, hyperactivity, ectopic vascular and soft-tissue calcifications, and death by 60 days of age, which has been interpreted as a premature aging phenotype (13). Biochemical abnormalities of KL-deficient mice include severe hyperphosphatemia, hypercalcemia, hypoglycemia, and increased serum levels of 1,25(OH)2D (13–15). Of significance, the KL-deficient phenotype largely overlaps with the phenotype of Fgfg23-null mice (12, 16, 17), indicating functional crosstalk between KL and FGF23 and underscoring the observed direct interactions between KL, FGF23, and its cognate FGFRs (11, 12).

Herein we report a patient with severe vascular and soft-tissue calcifications as well as multiple abnormalities of mineral homeostasis due to a point mutation in the KL gene. Clinical and biochemical evaluation, together with functional studies, indicate that this mutation attenuates the ability of KL to support FGF23 signaling and causes deranged phosphate, vitamin D, and calcium homeostasis. To our knowledge, this is the first disease-causing mutation identified in the KL gene.

Results

Case report. A 13-year-old girl initially presented with complaints of fatigue and nightly frontal headaches without other neurological symptoms. She denied polyuria or polydipsia, and her past medical

Nonstandard abbreviations used: 1,25(OH)2D, 1,25-dihydroxyvitamin D; EGR1, early growth response 1; FGFR, FGF receptor; GALNT3, UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3; KL, klotho; PTH, parathyroid hormone.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Due to the biochemical and radiographic presentation PTX low-PO diet as well as sevelamer (2,400 mg TID) improved her serum phosphorous, although it remained elevated (6.9 mg/dl).

Serum FGF23 levels. Due to the biochemical and radiographic diagnosis of tumoral calcinosis, serum FGF23 levels in the patient were measured using 2 different ELISAs. FGF23 levels in our patient were 150- to 550-fold higher than the normal mean in both ELISAs. The intact FGF23 level was 16,140 pg/ml (normal mean, 29.7 ± 20.7 pg/ml; ref. 18), and the C-terminal FGF23 level (which includes both intact and C-terminal fragments) was 10,900 RU/ml (normal mean, 72.9 ± 38.2 RU/ml; ref. 18). In contrast, tumoral calcinosis patients with FGF23 and GALNT3 loss-of-function mutations have high C-terminal but low intact serum FGF23 levels (1, 4, 19, 20).

Mutational and structural analyses of KL. To identify the molecular defect associated with the observed clinical manifestations, mutational analyses were performed on candidate genes. The patient showed no disease-causing changes in the gene responsible for familial hypocalciuric hypercalcemia, the calcium sensing receptor (CASR), or the 2 genes previously associated with tumoral calcinosis, FGF23 and GALNT3. Due to similarities in the biochemical phenotype between Kl-deficient mice and our patient, direct DNA sequencing of Kl was performed and revealed that the patient had a homozygous A-to-G transition (c.578A>G) in exon 1 (Figure 2A). This nucleotide change was not found in 230 control white individuals by PCR-RFLP analysis with BsrI (data not shown), confirming the change to be a mutation.

This mutation replaced histidine 193 with arginine (H193R) in the set of hyperphosphatemia.

Skeletal radiographs revealed diffuse osteopenia; patchy sclerosis in the hands, feet, long bones and calvariae; intracranial calcifications (Figure 1A); as well as metacarpal periosteal reaction (Figure 1B). CT of the head demonstrated calcification of the dura and carotid arteries, without parenchymal calcifications (Figure 1C). Upon radiography, multiple punctuate calcifications were also visible in the Achilles tendon (Figure 1D). Renal ultrasound and echocardiogram were normal. Subsequent treatment with phosphate restriction (600–800 mg/day) and sevelamer (2,400 mg, 3 times a day) improved her serum phosphorous, although it remained elevated (6.9 mg/dl).

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal ranges</th>
<th>At presentation</th>
<th>After PTX</th>
<th>Prior to starting low PO diet</th>
<th>While on low-PO diet</th>
<th>After sevelamer treatment (2,400 mg TID)</th>
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<tr>
<td>Phosphorous (mg/dl)</td>
<td>3.0–4.5</td>
<td>6.4</td>
<td>7.9</td>
<td>7.8</td>
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<td>0.5</td>
<td>0.5</td>
<td>0.6</td>
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<td>Alkaline phosphatase (IU/l)</td>
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<td>117</td>
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<td>Intact PTH (pg/ml)</td>
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<td>121</td>
<td>11</td>
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<td>Urine creatinine (mg/dl)</td>
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<tr>
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<td>98%</td>
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<td>Urine phosphorous (mg/dl)</td>
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<td>24.8</td>
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<td>C-terminal FGF23 (RU/ml)</td>
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<tr>
<td>Intact FGF23 (pg/ml)</td>
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Additional laboratory assessments included the following: normal complete blood count; IGF-1, 238.2 ng/ml (normal, 192–640 ng/ml); IGF binding protein-3, 2.7 mg/l (normal, 1.7–5.0 mg/l); proctolin, 13.7 ng/ml (normal, 2–17 ng/ml); fasting glucose, 70 mg/dl; fasting insulin, 8.1 μU/ml (normal, 2–17 μU/ml); total cholesterol, 150 mg/dl (normal, 112–208 mg/dl); HDL, 60.0 mg/dl (normal, 30–70 mg/dl); LDL, 74 mg/dl (normal, <100 mg/dl); and triglycerides 82 mg/dl (normal, 41–138 mg/dl). For FGF23, the normal mean ± SD is shown. PTX, parathyroidectomy; TRP, tubular reabsorption of phosphorous.
Residue was situated at the base of the deep catalytic pocket (Figure 2C). Replacement of the histidine with an arginine was predicted to introduce steric clashes with neighboring residues, and these spatial constraints should have destabilized the tertiary fold of the KL1 domain, leading to reduced expression and secretion of KL.

Analysis of KL protein expression. To test this hypothesis, we transiently expressed membrane-bound and secreted isoforms of wild-type and mutant KL in HEK293 cells. Compared with wild-type KL, total expression of the membrane-bound isoform was markedly reduced in mutant KL (Figure 3A). As previously demonstrated by others (11, 21), cells expressing wild-type KL produced 2 different species of membrane-bound KL. However, cells expressing the mutant KL had reduced or undetectable levels of fully glycosylated (mature) KL (Figure 3, A and B). Because of its location in the KL gene, the H193R mutation could also alter secreted KL protein. In this regard, there was prominent KL secretion from cells expressing wild-type KL, whereas almost no KL protein was detected in the media of cells expressing mutant KL (Figure 3A). However, when secreted KL was stably expressed for an extended period, minute amounts of mutant KL protein were detectable in the culture media of the cell line (data not shown). These data suggest that mutant KL is less stable than wild-type KL.

Since protein stability can often be improved by culturing cells at a lower temperature (22), we cultured cells stably expressing KL at 28.5°C and 37°C. Indeed, we found an increased expression of the mature mutant KL in cells cultured at lower temperature compared with cells maintained at 37°C, whereas the expression of wild-type KL was insensitive to changes in cell culture temperature (Figure 3B). These data are consistent with our structural prediction that the KL mutation reduces KL protein stability, manifesting in reduced protein expression and maturation.

FGF23 biological activity. KL is required for high affinity FGFR binding and bioactivity of FGF23 (11, 12). The effect of the H193R KL mutation on FGF23 biological activity was assessed by quantifying transcripts of the human early growth response 1 (EGR1) gene, a known downstream mediator of FGF23 signaling, in HEK293 cells (12). Stimulation of cells with FGF23 resulted in a 9-fold increase in EGR1 mRNA in cells expressing wild-type KL, compared with control cells expressing vector alone (Figure 4). In contrast, consistent with reduced membrane-bound KL expression, FGF23-mediated EGR1 expression was 6-fold lower in cells transfected with H193R KL (P < 0.01).

Coimmunoprecipitation analyses. To further investigate how the H193R mutation impairs the ability of KL to support FGF23 signaling, we studied the impact of the KL mutation on formation of the ternary FGF23-KL-FGFR1c complex. As shown in Figure 5 (top panel), FGF23 coprecipitated with wild-type KL but not with H193R KL. The KL mutation also affected the interaction between

Figure 1
Radiographic imaging of the patient carrying the KL H193R mutation. (A) Intracranial calcification and short bulbous tooth roots. (B) Sclerosis in the left hand. (C) Head CT demonstrating midline areas of dural calcification and posterior fossa calcifications (arrows). (D) Plain radiograph of ankle with Achilles tendon calcifications (arrow).
KL and FGFR1c, although to a much lower extent than the effects on FGF23-KL binding. In complimentary experiments, KL was coprecipitated with FGF23, and the H193R mutation was found to markedly reduce KL coprecipitation with FGF23 (Figure 5, bottom panel). This reduced KL coprecipitation was consistent with low EGR1 activation by FGF23 in the presence of mutant KL (Figure 4). The difference in binding between immunoprecipitations targeting epitopes in FGF23 and KL is likely due to the fact that precipitation with KL would pull down all mutant KL regardless of maturity and cellular location of the protein, whereas the precipitation with FGF23 would likely pull down only the mature KL on the cell surface, which appears to be the major species capable of interacting with FGFRs and FGF23 (11). In contrast, although FGF23 bound FGFR1c less efficiently with the H193R KL, FGFR1c still coprecipitated with FGF23 under these conditions (Figure 5, bottom panel). It should be noted, however, that FGFR1c was...

Figure 2
Mutational and structural analyses of the KL H193R mutation. (A) Mutational analysis of the KL gene. DNA sequencing of the KL exons revealed an A-to-G transition in exon 1 (boxes). Left, normal control; right, patient. c.578A>G, homozgyous A-to-G transition. (B) Sequence alignment of the region encompassing the mutated histidine residue of KL and related family-1 glycosidases. LPH, lactase phlorizinhydrolyase; MYR, myrosinase. Residue numbers are in parentheses to the left of the alignment. For myrosinase, secondary structure elements are denoted below the sequence, and the locations and lengths of the secondary structure elements are indicated by boxes in the sequence. H, α helix; β, β strand; g, g helix. A dash in the sequence represents a gap introduced to optimize the alignment. The histidine residue, which is mutated in KL of the patent presented in this study, is colored red. Note that this residue is conserved among family-1 glycosidases and their orthologs. (C) Ribbon representation of the crystal structure of myrosinase (Protein Data Bank [PDB] ID, 1E6S) and surface representation of catalytic site residues. White mustard myrosinase is a plant homolog of human KL. A close-up view of the catalytic cavity with bound substrate (gluco-hydroximolactam) is shown on the right, and to orient the reader, a view of the whole structure is shown on the left. Note that histidine 141 of the catalytic site is homologous to H193 of human KL.
In this study, we identified what we believe to be the first disease-causing mutation in KL, H193R, in a patient presenting with severe tumoral calcinosis manifested by marked hyperphosphatemia with ectopic and vascular calcifications. The mutation drastically decreased protein expression of the membrane-bound and secreted forms of KL, which led to reduced FGF23-KL-FGFR1c complex formation on the cell surface and, ultimately, to impaired FGF23 signaling. Although substitution of H193 with arginine affects the tertiary fold of KL1 domain in KL, we cannot rule out the possibility that defective enzymatic activity of KL is implicated in impaired FGF23 signaling. Regardless, our data clearly show that KL dysfunction due to H193R mutation leads to a clinical phenotype reflecting impaired biological activity of FGF23.

KL was originally described as a gene involved in aging, as KL-deficient mice exhibited shortened lifespan and multiple aging-related phenotypes (13), whereas overexpression of the KL gene in mice extended lifespan (24). Importantly, the phenotype of KL-deficient mice closely parallels that of Fgf23-null mice (16, 17), indicating that KL and FGF23 have functions in the same metabolic pathway. Similar to mice deficient in KL or Fgf23, our patient developed hypercalcemia, hyperphosphatemia, and increased 1,25(OH)2D levels, resulting in ectopic calcifications and osteopenia. In contrast to KL-deficient mice, however, our patient did not have a phenotype suggestive of abnormal or early aging. In addition, there was also no clinical evidence of emphysema, skin atrophy, delayed puberty (hypogonadotropic hypogonadism), or neural degeneration as was observed in the KL-deficient mice. Likewise, tumoral calcinosis patients with FGF23 or GALNT3 mutations do not show signs of premature aging, although early dementia, most likely from cerebral calcifications, may occur (25). These observations, together with our present findings, suggest that KL likely serves as a factor controlling mineral ion homeostasis rather than aging. However, there are potential explanations for a lack of aging features in our patient. The KL mutation found in our patient did not result in complete loss of KL expression or secretion. Therefore, the patient retained partial KL function, as indicated in Figure 4. Possibly due to this partial activity of KL, the patient had not developed features of premature aging, although we cannot rule out that she may develop such features over time. It is also possible that humans are able to partially compensate for deficient KL function in a manner that lessens the effect on phosphate metabolism or other metabolic pathways, such that the phenotype may vary in severity and in details from the mouse model. Finally, this study demonstrated that the KL mutation impaired FGF23 signaling but it may not have affected other less well-characterized functions of KL, which could be more relevant to premature aging.

Under normal physiological circumstances, hyperphosphatemia and FGF23 inhibit renal 1,25(OH)2D synthesis, thereby leading to low 1,25(OH)2D levels. However, compromised FGF23 signaling due to mutations in any of the 3 genes involved in tumoral calcinosi (FGF23, GALNT3, and now KL) disrupts this negative feedback mechanism, resulting in increased serum 1,25(OH)2D concentrations and hyperphosphatemia (1, 4, 19, 20). In response to severe

Figure 4

The KL mutation impairs KL-dependent FGF23 signaling. HEK293 cells were transiently transfected with vector alone (BLK), wild-type, or mutant (MUT) membrane-bound KL. The cells were treated for 30 minutes with 100 ng/ml FGF23 or vehicle. Relative EGR1 mRNA expression was determined by real-time quantitative RT-PCR using β-actin as the internal standard (n = 6–8). Results are presented as fold change (mean ± SEM) compared to unstimulated cells. *P < 0.01, compared with BLK; †P < 0.01, compared with the same plasmid transfection without FGF23 treatment; ††P < 0.01, compared with WT+FGF23.
Multiple sequence alignments were performed. Routine clinical biochemistries were
measured. In contrast, our patient had normal fasting glucose and insulin
excretion (29), our patient did not have hypercalciuria. Second, although
FGF23 and hyperphosphatemia (12), there exist several biochemical
different functional roles of the mutated genes. These differing clinical phenotypes most likely result from the
mutations described herein have only been reported in 2 other patients
of this disorder, our patient appeared to be more severely affected
calcifications (8, 19). Even considering the phenotypic variability
of this disorder, our patient appeared to be more severely affected
than previously reported cases. In this regard, the brain calcifications
described herein have only been reported in 2 other patients
with GALNT3 mutations (in the choroid plexus [ref. 5] and dura
[refs. 6, 26]). Furthermore, our patient had clinical features such as
osteopenia and hypercalciemia that are seen in Kl-deficient mice
(13, 27, 28) but have not been reported in previous tumoral calcinosis cases. Interestingly, both our patient and the Kl-deficient mouse have a combination of osteopoenic and hyperostotic bones
(13, 27, 28), similar to the hyperostotic lesions seen in a variant of
tumoral calcinosis, hyperostosis-hyperphosphatemia syndrome.
These differing clinical phenotypes most likely result from the
different functional roles of the mutated genes.

Despite biochemical similarities such as high circulating levels of
FGF23 and hyperphosphatemia (12), there exist several biochemical
differences between the Kl-deficient mice and our patient. First,
although Kl-deficient mice have increased urinary calcium excretion
(29), our patient did not have hypercalciuria. Second, Kl-deficient mice have abnormal regulation of insulin/IGF-1 sig-
naling, resulting in hypoglycemia and increased insulin sensitivity.
In contrast, our patient had normal fasting glucose and insulin
concentrations. Third, PTH levels are low in the Kl-deficient mice when compared with wild-type mice (15) and similarly are
predominantly low or normal in tumoral calcinosis cases that result from FGF23 or GALNT3 mutations. However, our patient had
hyperparathyroidism at presentation and subsequently underwent
subtotal parathyroidectomy of hyperplastic glands. The reason for the hyperparathyroidism was unclear, as elevated serum phos-
phate is a stimulus for PTH but high calcium and 1,25(OH)₂D inhibit PTH production. Since Kl is expressed in the parathyroid glands (30), KL may have a direct role in the regulation of PTH in humans. Alternatively, this may be the result of tertiary hyperpara-
thyroidism caused by persistent hyperphosphatemia. The lack of
hyperparathyroidism in other tumoral calcinosis patients implies
that this may be a feature limited to this particular patient. Further
study of additional human cases will be required to clarify
whether hyperparathyroidism is a unique response of our patient or a feature inherent to the disorder in humans.

In conclusion, we demonstrated that a homozygous loss-of-
function Kl mutation in a human resulted in severe tumoral
calcinosis, not early aging as previously proposed. Despite the
elevated circulating FGF23 concentrations, the H193R mutation
diminished the ability of KL to function as a cofactor necessary for
FGF23 signaling through FGFRs, leading to hyperphosphatemia,
increased 1,25(OH)₂D concentrations, and subsequent ectopic calcifi-
cations. The fact that a mutation in KL was found in a patient
with tumoral calcinosis demonstrates that, in addition to FGF23
and GALNT3, KL should now be considered in the molecular diag-
nosis of tumoral calcinosis.

Methods

Patient and control individuals. The study was approved by the Institutional
Review Board of Indiana University — Purdue University Indianapolis.
Written informed consent was obtained from participants prior to partic-
ipation in the study. Blood samples were collected from the patient with
tumoral calcinosis and healthy race-matched individuals.

Measurement of serum biochemistries. Routine clinical biochemistries were
analyzed by standard methods. FGF23 concentrations were determined
using 2 different assays: FGF23 ELISA Kit (Kainos Laboratories Inc.),
which recognizes only intact FGF23 with monoclonal antibodies, and
Human FGF-23 (C-Term) ELISA Kit (Immutopics International), which
detects both intact and C-terminal fragments of FGF23.

Mutational analyses. Genomic DNA was extracted from blood samples
using the QiAamp DNA Blood Mini Kit (Qiagen). All exons and their
adjacent intronic sequences for the FGF23, GALNT3, and KL genes were
amplified using Taq DNA polymerase (New England Biolabs) or a Multi-
plex PCR Kit (Qiagen). Each PCR amplicon was directly sequenced from
forward and/or reverse PCR primers using Big-Dye Terminator Cycle
Sequencing Kit and the ABI PRISM 3100 Genetic Analyzer (Applied Bio-
systems). Mutational analysis for the calcium sensing receptor (CASR) gene
was performed by GeneDx.

PCR-RFLP analysis of the A-to-G transition in KL was performed by
amplifying genomic DNA harboring the transition, followed by restric-
digest with BsrI (New England Biolabs). Forward and reverse PCR
primers were 5′-AGACTCCCCGAACGCGT-3′ and 5′-GTGGTCCAAGT-
GAAAGCTCGT-3′, respectively. The wild-type A allele produced 4 frag-
ments (258, 230, 120, and 19 bp), whereas the G allele found in the patient
produced 3 fragments (378, 230, and 19 bp) as the change disrupts 1 of the
3 BsrI restriction sites.

Computational analysis. Multiple sequence alignments were performed
using Accelrys GCG software package (version 11.1.3). Structural analysis

Figure 5
The Kl mutation impairs the ability of membrane-bound KL to form
a ternary complex with FGF23 and FGFR1c. HEK293 cells overex-
pressing wild-type or mutant mKL were transfected with FGFR1c
and treated with FGF23. KL or FGF23 were immunoprecipitated, and
the immunoprecipitates were analyzed for coprecipitated proteins by
immunoblotting with the antibodies indicated.
Research Article

of myosinase was performed using an o software program (version 11.0.4; http://xray.bmc.uu.se/~alwyn/).

Expression vectors. The membrane-bound and secreted forms of human KL cDNAs cloned into pCDNA3.1/V5-His expression vector (Invitrogen) were a generous gift from Harold Dietz (Howard Hughes Medical Institute, Johns Hopkins University, Baltimore, Maryland, USA). Mutant KL-H193R cDNA was generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Human FGFR1c was PCR amplified from hFGFR-1 (ATCC) and cloned into pcDNA3.1+ expression vector (Invitrogen). Human FGF23 mutant (R176Q) cloned into pFLAG-CMV-3 expression vector (Sigma-Aldrich) was previously described (31). All constructs were sequenced to verify introduction of the correct mutation and the absence of cloning artifacts.

HEK293 cell culture and transfection. HEK293 cells (ATCC) were cultured in DMEM/F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone; Thermo Fisher Scientific), 1% L-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin at 37°C and 5% CO2. Subconfluent cells were transiently transfected with wild-type KL or H193R KL plasmid DNAs, using Lipofectamine 2000 (Invitrogen). After transfection, the cells were maintained in culture for additional 48-96 hours before cell harvest and collection of conditioned media. For the generation of stable cell lines, cells were transfected with wild-type and H193R KL plasmid DNAs, reseeded into 20-cm dishes, and selected in medium containing 450 µg/ml Geneticin (Invitrogen) for 2 weeks. Neomycin-resistant monoclonals were picked for expansion. For temperature rescue experiments, cells of the stable cell lines were equally divided into two 60-mm plates and grown for 48 hours at either 37°C or 28.5°C.

Immunoblotting. Cell lysates and conditioned media were electrophoresed on 10% Tris-HCl Ready Gel precast gels (Bio-Rad) and blotted onto nitrocellulose membranes. Membranes were incubated with HRP-conjugated V5 antibody (Invitrogen). Immunoblots were developed with ECL Plus Western Blotting Detection Reagents (GE Healthcare).

Analysis of EGR1 gene expression. HEK293 cells (2 × 105 cells/well) were transiently transfected with 0.5 µg of plasmid DNA containing wild-type KL or mutant membrane-bound KL, using FuGENE 6 reagent (Roche Applied Science). Cells transfected with empty vector DNA served as a negative control. Transfected cells were starved with serum-free media and then incubated with conditioned media from cells secreting FGF23. Following a 2-hour incubation, the cells were washed and lysed with RIPA buffer (Sigma-Aldrich) containing protease inhibitors (Complete Mini Tablet; Roche Applied Science). Cell lysates were incubated overnight at 4°C with agarose beads carrying anti-FLAG or anti-V5 antibodies (Sigma-Aldrich). Agarose beads were washed, and immunoprecipitated proteins were analyzed by immunoblotting with antibodies against V5 (KL; Invitrogen), FLAG (FGF23; Sigma-Aldrich), FGFR1 (Cell Signaling Technology), or FGF23 (33).

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