Klotho ablation converts the biochemical and skeletal alterations in FGF23 (R176Q) transgenic mice to a Klotho-deficient phenotype

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Submitted 24 June 2008; accepted in final form 29 October 2008

Bai X, Dinghong Q, Miao D, Goltzman D, Karaplis AC. Klotho ablation converts the biochemical and skeletal alterations in FGF23 (R176Q) transgenic mice to a Klotho-deficient phenotype. Am J Physiol Endocrinol Metab 296: E79–E88, 2009. First published November 4, 2008; doi:10.1152/ajpendo.90539.2008.—Transgenic mice overexpressing fibroblast growth factor (FGF23) (R176Q) (FTr) exhibit biochemical [hypophosphatemia, phosphaturia, abnormal 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] metabolism] and skeletal (rickets and osteomalacia) abnormalities attributable to FGF23 action. In vitro studies now implicate the aging-related factor Klotho in the signaling mechanism of FGF23. In this study, we used a mouse genetic approach to validate in vivo the potential role of Klotho in the metabolic and skeletal derangements associated with FGF23 (R176Q) overexpression. This study, we crossed mice heterozygous for the hypomorphic Klotho allele (Kt+/−) to FTr mice and obtained FTr transgenic mice homozygous for the Kl-hypomorphic allele (FTr/Kt−/−). Mice were killed on postnatal day 50, and serum and tissues were procured for analysis and comparison with FTr, wild-type, and Kt−/− controls. From 4 wk onward, FTr/Kt−/− mice were clearly distinguishable from FTr mice and exhibited a striking phenotypic resemblance to the Kt−/− controls. Serum analysis for calcium, phosphorus, parathyroid hormone, 1,25(OH)2D3, and alkaline phosphatase activity, however, is not detected in recombinant Klotho protein. The Klotho gene encodes a single-pass transmembrane protein. The extracellular domain has two homologous domains with sequence similarity to β-glucosidase of bacteria and plants. β-Glucosidase-like activity, however, is not detected in recombinant Klotho protein. The Klotho gene is expressed in limited tissues, notably in kidney tubules and parathyroid (15). Klotho and FGFR1(IIIc) form a heterodimeric, high-affinity receptor for FGF23. In functioning as a cofactor of FGFRs, in vitro studies have shown that Klotho appears essential for FGF23 to activate FGF signaling. Klotho may also have additional functions, since the extracellular domain of Klotho protein is shed from the cell surface, and this soluble form may function as a humoral factor that regulates multiple growth factor signaling pathways (25).

Mice severely hypomorphic for Klotho (Kt−/−) have been reported to exhibit near-complete absence of Klotho expression (14) and display a variety of abnormalities, including prominent mineral and skeletal abnormalities. The biochemical alterations simulate those seen in mice with targeted deletion of Fg23 (22, 23). Furthermore, circulating immunoreactive levels of FGF23 are reported to be elevated in the Kt−/− mice, suggesting that resistance to FGF23 action occurs in this model. Consequently, both in vitro and in vivo studies suggest that functional crosstalk occurs between KL and FGF23 and underscores the observed direct interactions between KL, FGF23, and its cognate FGFRs. Nevertheless, distinct skeletal changes have been described in the Kl-deficient mice and the Fg23-null mouse, which appear to differ.

In this study, we have undertaken to examine in vivo the putative role of Klotho in FGF23 action on mineral and skeletal homeostasis using the mouse genetic approach. Crosses were made between transgenic mice expressing and secreting FGF23, and its cognate FGFRs (28). The Klotho gene encodes a single-pass transmembrane protein. The extracellular domain has two homologous domains with sequence similarity to β-glucosidase of bacteria and plants. β-Glucosidase-like activity, however, is not detected in recombinant Klotho protein. The Klotho gene is expressed in limited tissues, notably in kidney tubules and parathyroid (15). Klotho and FGFR1(IIIc) form a heterodimeric, high-affinity receptor for FGF23. In functioning as a cofactor of FGFRs, in vitro studies have shown that Klotho appears essential for FGF23 to activate FGF signaling. Klotho may also have additional functions, since the extracellular domain of Klotho protein is shed from the cell surface, and this soluble form may function as a humoral factor that regulates multiple growth factor signaling pathways (25).

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In this study, we have undertaken to examine in vivo the putative role of Klotho in FGF23 action on mineral and skeletal homeostasis using the mouse genetic approach. Crosses were made between transgenic mice expressing and secreting from the liver human FGF23 (R176Q), a mutant form that fails to be degraded by furin proteases (ETr) (1), and Kt−/− mice. Using this approach, we introduced the FGF23 (R176Q) transgene on the Kl−/− genetic background. The compound mutant mice (ETr/Kt−/−) were then evaluated for the characteristic FGF23-induced biochemical and skeletal abnormalities. We show that the absence of KL completely reverses the biochemical alterations that characterize the FGF23 (R176Q) transgenic mice, but distinct skeletal abnormalities are seen.

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MATERIALS AND METHODS

Animals. Male mice heterozygous for the hypomorphic Klotho allele (Kl+/−) were purchased fromCLEA Japan. Female FGF23 (R176Q) transgenic mice (FTR) were generated and bred in our laboratory (2). The two strains of mice were crossed to obtain FGF23 (R176Q) transgenic mice homozygous for the Klotho-hypomorphic allele (FTR/Kl−/−). Regular rodent diet (501 Rodent Laboratory Chow) was purchased from Harlan Teklad (Indianapolis, IN). Dinking water for all experimental mice was tap water. All animal experiments were reviewed and approved by the institutional animal care committee. Mice were killed around 50 days postpartum. Serum and tissues were procured for analysis and compared with wild-type (WT), Kl−/−, and FTR controls.

Genotyping of mice. Mice were screened for the FGF23 (R176Q) transgene by Southern blot of tail-tip DNA prepared from a 1-cm portion of their tails. In brief, 10 μg of genomic DNA were digested with BamHI, separated by 1% agarose gel electrophoresis, transferred to nitrocellulose membranes by upward capillary transfer in 20× saline–sodium citrate (SSC) overnight, and hybridized to the radiolabeled whole cDNA of human FGF23 (48% formamide, 10% dextran sulfate, 5× SSC, 1× Denhardt’s solution, and 100 μg/ml salmon sperm DNA) at 42°C overnight. The membranes were washed in 0.1% SDS plus 2× SSC for 15 min at room temperature with rotation and then in 0.1% SDS plus 0.1% SSC for another 15 min at 60°C. The autoradiograms were prepared using Kodak BioMax film at −80°C with intensifying screens.

DNA PCR was used for Kl genotyping and was performed as follows: 94°C for 5 min, then 30 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 45 s, then 72°C for 10 min with sense primer GKL01 (5′-TTGGAGATTGAATGGGGACGAAAGAG) and antisense primer GKL02 (wild type allele; 5′-CTCAAGAAGCTGGATAGT). Amplified fragments from wild-type and mutant alleles are 815 and 491 bp, respectively.

Serum biochemistry. Serum concentrations of calcium, phosphorus, creatinine, and serum alkaline phosphatase (ALP) were determined by routine methods following the manufacturer’s instructions (Point Scientific, Brussels, Belgium). Serum intact parathyroid hormone (PTH; Immutopics) and FGF23 (Kainos Laboratories, Tokyo, Japan) were measured using enzyme-linked immunosorbent assays. 1α,25(OH)2D3 determinations were performed using a commercially available radioimmunoassay kit (Immunodiagnostic Systems).

Northern blot analysis. cDNA fragments corresponding to nucleotides 535–1586 of mouse 25-hydroxyvitamin D3 1α-hydroxylase (Cyp24; accession no. D49438) and to nucleotides 421–1471 of mouse 25-hydroxyvitamin D3 1α-hydroxylase (Cyp27b1; accession number AB006034) were prepared by RT-PCR of mouse kidney total RNA, subcloned, and sequenced. DNA probes for hydroxylases and glyceraldehyde-3-phosphate dehydrogenase were prepared using a random primed DNA labeling kit (Roche Molecular Biosciences) and [α-32P]dCTP (800 Ci/mmol; PerkinElmer Life Sciences). Total RNA was isolated from kidney with Tripure Isolation Reagent (Roche Molecular Biosciences) and 20-μg aliquots were fractionated by electrophoresis on a 1% formaldehyde agarose gel, transferred to nitrocellulose membranes by upward capillary transfer in 20× SSC overnight and hybridized to the radiolabeled DNA fragments (48% formamide, 10% dextran sulfate, 5× SSC, 1× Denhardt’s solution, and 100 μg/ml salmon sperm DNA) at 42°C overnight. The membranes were washed in 0.1% SDS plus 2× SSC for 15 min at room temperature with rotation and then in 0.1% SDS plus 0.1% SSC for another 15 min at 60°C. The autoradiograms were prepared using Kodak BioMax film at −80°C with intensifying screens. Quantification of signal intensity on autoradiograms was performed using ImageJ software (http://rsb.info.nih.gov/ij/).

Radiographs. Femurs and aortas were removed and dissected free of soft tissue, and radiographs were taken using a Faxitron X-ray, Wheeling, IL) under constant conditions (22 kV, 4 min exposure) using Kodak X-Omat TL film (Eastman Kodak).

Micro-CT analysis. Tibias were dissected free of soft tissue, fixed overnight in 70% ethanol, and analyzed by micro-CT using a SkyScan 1072 scanner and associated analysis software (SkyScan, Antwerp, Belgium) as described (19). Briefly, image acquisition was performed at 100 kV and 98 mA with a 0.9° rotation between frames. During scanning, the samples were enclosed in tightly fitting plastic wrap to prevent movement and dehydration. Thresholding was applied to the images to segment the bone from the background. Two-dimensional images were used to generate three-dimensional renderings using the 3D Creator software supplied with the instrument. The resolution of the micro-CT images was 18.2 μm.

Histology. Tissues were removed and fixed in PLP fixative (2% paraformaldehyde containing 0.075 M lysine and 0.01 M sodium periodate solution) overnight at 4°C and processed histologically as described (18). Proximal ends of tibias were decalcified in EDTA glycerol solution for 5–7 days at 4°C. Decalcified tibias were dehydrated and embedded in paraffin for which 5-μm sections were cut on a rotary microtome and stained with hematoxylin and eosin (H&E), or histochemically for ALP and for tartrate-resistant acid phosphatase (TRAP). Undecalcified bones were embedded in LR White acrylic resin (London Resin, Theale, UK). Sections 1 μm thick were cut on an ultramicrotome and stained for mineral with the von Kossa staining procedure using toluidine blue as counterstain.

Histochemical staining for ALP and TRAP. Enzyme histochemistry for ALP activity was performed as described (20). Briefly, following preincubation overnight in 1% magnesium chloride in 100 mm Tris-maleate buffer (pH 9.2), dewaxed sections were incubated for 2 h at room temperature in a 100 mM Tris-maleate buffer containing naphthol AS-MX phosphate (0.2 mg/ml; Sigma) dissolved in ethylene glycol monomethyl ether (Sigma) as substrate, and fast red TR (0.4 mg/ml; Sigma) as a stain for the reaction product. After being washed with distilled water, the sections were counterstained with Vector methyl green nuclear counterstain (Vector Laboratories) and mounted with Kaiser’s glycerol jelly.

Enzyme histochemistry for TRAP was performed as previously described (21). Dewaxed sections were preincubated for 20 min in buffer containing 50 mM sodium acetate and 40 mM sodium tartrate at pH 5.0. Sections were then incubated for 15 min at room temperature in the same buffer containing 2.5 mg/ml naphthol AS-MX phosphate (Sigma) in dimethylformamide as substrate, and 0.5 mg/ml fast garnet GBC (Sigma) as a color indicator for the reaction product. After being washed with distilled water, the sections were counterstained with methyl green and mounted in Kaiser’s glycerol jelly.

Immunohistochemical staining. The type I collagen were determined by immunohistochemistry as described previously (18). Affinity-purified goat antihuman type I collagen antibody (Southern Bio-technology Associates, Birmingham, AL) was applied to dewaxed paraffin sections overnight at room temperature. After being washed with high-salt buffer (50 mM Tris·HCl, 2.5% NaCl, 0.05% Tween 20, pH 7.6) for 10 min at room temperature followed by two 10-min washes with TBS, the sections were incubated with secondary antibody (biotinylated rabbit anti-goat IgG, Sigma), washed as before, and incubated with the Vectastain ABC-AP kit (Vector Laboratories) for 45 min. After washing as before, red pigmentation to demarcate the regions of immunostaining was produced by a 10- to 15-min treatment with Fast Red TR/Naphthol AS-MX phosphate (containing 1 mM levamisole as endogenous ALP inhibitor; Sigma). After being washed with distilled water, the sections were counterstained with methyl green and mounted with Kaiser’s glycerol jelly.

Computer-assisted image analysis. After H&E staining or histochemical or immunohistochemical staining of sections from five mice of each genotype, images of fields of images were photographed with a Sony digital camera. Images of micrographs from single sections were digitally recorded using a rectangular template, and recordings were radiographic inspection system (Faxitron X-ray, Wheeling, IL) under constant conditions (22 kV, 4 min exposure) using Kodak X-Omat TL film (Eastman Kodak).
processed and analyzed using Northern Eclipse image analysis software as previously described (18).

Statistical analysis. Differences between groups were analyzed by one-way ANOVA, followed by the Bonferroni posttest using the software program Prism, version 4 (GraphPad Software, San Diego, CA) and were expressed as means ± SE. The value of $P < 0.05$ was considered significant.

**RESULTS**

Introduction of the FGF23 (R176Q) transgene on the Kl−/− background. To evaluate in vivo the pivotal role of Klotho in FGF23 action, we devised a strategy to introduce the FGF23 (R176Q) transgene on the Kl−/− genetic background. To this end, we bred FTr mice with Kl+/− ice to generate a strain of mice that are null for the Kl allele but have also incorporated the FGF23 (R176Q) transgene in their genome (Fig. 1, A and B). The FTr/Kl−/− mice had similar phenotypic features (Fig. 1C) and body weight (Fig. 2A) as the Kl−/− controls and were very distinct from the FTr controls with complete absence of the rachitic features and short tail that are distinct characteristics of the latter group.

Loss of Kl completely reverses the FTr serum biochemical profile. In parallel to the reversal of the dysmorphic alterations, reversible changes were also observed in the serum biochemical profile of the FTr/Kl−/− mice compared with FTr controls (Fig. 2, B–F). Thus serum phosphorus and 1,25(OH)2D3 were elevated rather than reduced in the FTr/Kl−/− animals while the increased PTH and ALP levels seen in the FTr mice were reduced in the FTr/Kl−/− animals. Additionally, serum calcium levels were increased. The FTr/Kl−/− mice therefore exhibited a profile similar to that of the Kl−/− controls. These changes occurred despite a profound 146-fold increase in serum intact FGF23 levels (Fig. 2G) and similar serum creatinine (Fig. 2H) concentration in the FTr/Kl−/− mutants.

Expression of renal hydroxylases. We observed that the expression of renal Cyp27b1 and Cyp24 was profoundly altered in the presence of high FGF23 levels. Hence, in the FTr mice, the mRNA level of Cyp27b1 was decreased by 50%, whereas the gene expression of Cyp27b1 was increased nearly fourfold compared with wild-type animals (Fig. 3, A and B). Thus the concomitant decrease in Cyp27b1 and increase in Cyp24 activity resulted in a decrease in serum levels of 1,25(OH)2D3 (Fig. 2F). In contrast, both in Kl−/− and FTr/Kl−/− mice, the increase in Cyp27b1 expression was associated with raised circulating levels of the hormone (Fig. 2F), implying increased protein expression and/or enzymatic activity of the elevated Cyp27b1. Here, the lack of Klotho protein in the FTr/Kl−/− mice appears to abrogate FGF23 inhibition of the Cyp27b1 renal hydroxylase enzymatic activity. Moreover, the increase in Cyp24 may have been in response to the elevated 1,25(OH)2D3 and likely prevented the serum concentrations of 1,25(OH)2D3 from being even more highly elevated.

Effect of Klotho ablation on the skeletal mineralization in FTr mice. Radiographic examination of femurs procured from all four strains of mice disclosed that the bone length was shorter in Kl−/−, FTr, and FTr/Kl−/− mice compared with wild-type littersmates (Fig. 4A). Trabecular radiolucency of Kl−/− and FTr/Kl−/− mice was reduced relative to that of FTr mice. From three-dimensional reconstructed anterior views (Fig. 4B), longitudinal sections (Fig. 4C), and cross sections (Fig. 4D) of the proximal ends of tibias, it was observed that the epiphyses were smaller, unmineralized growth plate spaces were wider, and mineralized cortical bone volume was reduced in FTr mice, as previously reported (1), whereas Kl ablation in FTr mice resulted in marked improvement of all these rachitic changes, paralleling the phenotype of the Kl−/− mice. Bones from both Kl−/− and FTr/Kl−/− displayed narrower unmineralized growth plate spaces and greater trabecular bone volume and cortical bone volumes compared with bones from FTr mice (Fig. 4, B–D); however, the cortical bone volumes remained less and cortical thinning was greater than in wild-type mice.

To further evaluate the effect of Kl ablation in FTr mice on skeletal mineralization, undecalcified sections of the distal ends of femurs were stained with the von Kossa procedure, and trabecular bone volume, mineralized area of the growth plates, and osteoid volume in trabecular and cortical bone were measured by computer-assisted image analysis. The epiphyseal ends were enlarged (Fig. 5A) and the trabecular bone volume (Fig. 5, A, B, and D) and mineralized area of the growth plates were markedly decreased (Fig. 5, B and E), whereas osteoid volume in both trabeculas (Fig. 5, B and F) and cortex (Fig. 4, C and G) were

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**Fig. 1.** Generation of Kl−/−/FTr mice. A: PCR amplification of wild-type (WT) and mutant Klotho (Kl) alleles. B: Southern blot analysis used to identify the neonatal murine Fgf23 and the human FGF23 (R176Q) transgene. C: illustrative representation of the various mouse strains, as indicated.
increased significantly in $F^T_r$ mice compared with $Kl^{-/-}$ and $F^T_r/Kl^{-/-}$. Following ablation of $KL$ in $F^T_r$ mice, increased skeletal mineralization was observed in $F^T_r/Kl^{-/-}$ mice, similar to that seen in the $Kl^{-/-}$ controls (Fig. 5). In both $Kl^{-/-}$ and $F^T_r/Kl^{-/-}$ strains, the trabecular bone volume was greater (Fig. 5, A, B, and D), the mineralized area of the growth plate was higher (Fig. 5, B and E), and osteoid volume as a percent of trabecular (Fig. 5, B and F) and cortical (Fig. 5, C and G) bone volume was similar compared with wild-type littermates.

**Effect of KL ablation in $F^T_r$ mice on bone turnover.** To determine the effect of KL ablation in $F^T_r$ mice on osteoblastic bone formation, we analyzed sections from the proximal ends of tibias stained with H&E (Fig. 6A), stained histochemically for ALP (Fig. 6B), and processed immunohistochemically for type I collagen (Fig. 6C). The number of osteoblasts was determined (Fig. 6E), and ALP and type I collagen positive area were measured (Fig. 6, F and G) by computer-assisted image analysis. As shown, the number of osteoblasts was increased, and both ALP activity and type I collagen deposition were increased in bone matrix from $F^T_r$ mice. In contrast, the number of osteoblasts was significantly decreased, and both ALP activity and type I collagen deposition in bone matrix were markedly decreased in the skeletons from $Kl^{-/-}$ and $F^T_r/Kl^{-/-}$ animals.

To assess the effect of KL ablation in $F^T_r$ mice on osteoclastic bone resorption, sections from proximal ends of tibias were stained histochemically for TRAP (Fig. 6D), and the number of TRAP positive osteoclasts was determined (Fig. 6H). The number of osteoclasts was markedly decreased in $F^T_r/Kl^{-/-}$ and $Kl^{-/-}$ specimens compared with $F^T_r$ bones, but all were decreased compared with wild-type mice.

**Effect of KL ablation on ectopic calcification in $F^T_r$ mice.** Ectopic calcification is also a hallmark of the $Kl^{-/-}$ phenotype. To determine the effect of KL ablation in $F^T_r$ mice on ectopic...
An analysis of aortas and paraffin sections from kidneys, and lung were stained with the von Kossa procedure (Fig. 7). Ectopic calcifications were not detected in tissues from FTr and wild-type controls but were extensively present in FTr/KI-/- tissues, similar to the KI-/- controls. Despite the renal calcification, serum creatinine was not significantly reduced at this stage (Fig. 2).

**DISCUSSION**

Using a mouse genetic approach, we have demonstrated in vivo the pivotal role that Klotho plays in the action of FGF23. In the absence of Klotho, nearly all of the biochemical features associated with FGF23 overexpression are completely reversed. Hence, the resultant dominant features are those associated with the KI-null genotype such as hyperphosphatemia, increased 1,25(OH)2D3 concentrations, and ectopic calcifications, despite the elevated circulating FGF23 concentrations. These studies thereby further substantiate in vitro studies showing that Klotho functions as a cofactor necessary for FGF23 signaling through FGFRs (28).

Elevated 1,25(OH)2D3, serum calcium, and serum phosphorus along with ectopic calcifications were previously described in KI-/- (14) and Fgf23-/- (22, 23) mice and were also observed in our study in both the KI-/- and FTr/KI-/- mutants. Ablation of the gene encoding the enzyme synthesizing 1,25(OH)2D3 (Cyp27b1) (24) or of vitamin D signaling (9) in Fgf23-/- mice have been reported to result in normocalcemia, normophosphatemia, and absence of ectopic calcifications. Recent studies have suggested that normalization of the hyperphosphatemia per se in Fgf23-/- mice reduces vascular calcification, even in the presence of elevated 1,25(OH)2D3, implicating the elevated blood phosphorus per se as an independent factor, in addition to the elevated 1,25(OH)2D3, in the pathophysiology of the ectopic calcification (26).

A severe autosomal recessive metabolic disorder in humans, familial tumoral calcinosis (OMIM 211900), also manifests with hyperphosphatemia and massive calcium deposits in the skin and subcutaneous tissues. Previous molecular genetic analyses demonstrated that tumoral calcinosis can result from biallelic inactivating mutations in genes encoding FGF23 (6, 16) or the UDP-N-acetyl-α-D-galactosamine-polypeptide N-acetylgalactosaminyltransferase 3 (GALNT3), a Golgi-associated glycosyltransferase responsible for initiating mucin-type...
O-glycosylation (10, 27). The first disease-causing mutation in Klotho, H193R, was recently described in a patient presenting with severe tumoral calcinosis manifested by marked hyperphosphatemia with ectopic and vascular calcifications (10). The homozygous loss-of-function KL mutation drastically decreased protein expression of the membrane-bound and secreted forms of Klotho, which led to reduced FGF23-KL-FGFR1c complex formation on the cell surface and, ultimately, to impaired FGF23 signaling. 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 diagnosis of tumoral calcinosis. Moreover, these findings add credence to our observation of the in vivo importance of Klotho in the mechanism of action of FGF23.

Recent studies have reported both in vitro and in vivo that FGF23 is a direct negative regulator of PTH synthesis and secretion, provided FGFR signaling is intact (5, 12). Furthermore, the proband carrying the human KL mutation leading to tumoral calcinosis and FGF23 resistance was reported to have hyperparathyroidism due to parathyroid hyperplasia (10). In contrast, in our studies, serum PTH concentrations were elevated in FTr mice, which have high circulating FGF23 concentrations and in which signaling is intact, and serum PTH concentrations were reduced rather than elevated in both KL−/− and FTr/KL−/− mice, which have reduced FGF23 functionality. Furthermore, we recently reported that deletion of the gene encoding PTH in mice with X-linked hypophosphatemic rickets (Hyp), which have increased circulating FGF23, resulted in
hyperphosphatemia and premature demise of the animals due to hypocalcemia (1). Consequently, elevated PTH appears not only to be an accompaniment of high circulating FGF23 but also an essential compensatory mechanism for a high FGF23 state. The recent description of a patient with a 9;13 translocation causing a primary increase in Klotho expression with an accompanying rise in circulating FGF23 levels, hypophosphatemic rickets, and refractory hyperparathyroidism adds further credence to this contention (7). Finally, increased PTH is an invariable accompaniment of late-stage chronic kidney disease in humans and animals in association with increased circulating FGF23. Consequently, in both animals and humans, any direct inhibitory actions of FGF23 on the parathyroid cells appear, in the vast majority of cases, to be amply compensated for by the stimulatory action of modulators such as hypocalcemia and reduced serum 1,25(OH)2D (8). Consequently, FGF23 may have a direct stimulatory role on the parathyroids in vivo, at least under some circumstances.

The bone phenotype of the Kl−/− mice was previously reported as displaying abnormal elongation of trabecular bone in the epiphyses of long bones with an increase in three-dimensional bone volume fraction and an increase in the number and thickness of the trabecular bones, resulting in high radio-opacity in the metaphyseal region (30, 31). A similar phenotype was also observed in the metaphysis of both the Kl−/− and FTr−/Kl−/− mutants in our study but has not been reported in Fgf23−/− mice. In addition, a defect in Klotho gene expression has been reported to lead to the independent impairment of osteoblast and osteoclast differentiation, which can result in low bone turnover (11), and, indeed, we observed a decrease in both osteoblastic and osteoclastic activity in both the Kl−/− and FTr−/Kl−/− mice. This decrease in bone turnover could be due to the associated reduction in circulating PTH levels (19). In contrast, in Fgf23−/− mutants, which also have

Fig. 6. Effect of Kl ablation on osteoblastic bone formation and osteoclastic bone resorption in FTr mice. Representative micrographs of decalcified paraffin-embedded sections of tibial metaphysis stained with hematoxylin and eosin (magnification ×100; bar = 80 μm; A), histochemically for ALP (magnification ×100; bar = 80 μm; B), immunohistochemically for type I collagen (magnification ×400; bar = 20 μm; C), and histochemically for tartrate-resistant acid phosphatase activity (magnification ×400; bar = 20 μm; D) in 50-day-old mice of the respective genotype. E: no. of osteoblasts/mm² was counted in the metaphyseal regions of H&E-stained tibias of the mice. ALP positive (F) and type I collagen (Col I; G) positive areas as percent of tissue area, and the number of TRAP positive osteoclasts [N.Oc/T.Ar (no./mm²); H] were determined in the metaphyseal regions for each group. Each value is the mean ± SE of determinations of 5 mice in each group. **P < 0.01 and ***P < 0.001 compared with WT mice. ##P < 0.01 and ###P < 0.001 compared with Kl−/− and FTr−/Kl−/− mice.
reduced PTH levels, although reduced bone formation has also been reported (22), mineralized bone volume/tissue volume ratio was significantly decreased in these mice (22, 23). Consequently, it is possible that the decreased bone turnovers in the Fgf23−/−, Kl−/−, and FTr/Kt−/− mutants are all related to reduced PTH but that the increased mineralized trabecular bone volume observed in our studies in Kl−/− and FTr/Kt−/− mutants reflects a direct effect of Klotho. Thus signaling via an intact Klotho protein, which appears to be present in both FTr+ mice and Fgf23−/− mice, may contribute in a significant way to the development of impaired mineralization irrespective of whether serum FGF23, phosphorus, or 1,25(OH)2D3 is elevated or reduced. Inasmuch as Klotho has been reported to interfere with insulin-like growth factor (IGF)-I signaling (4, 29) and deletion of IGF-I from osteoblasts has been associated with impaired bone matrix mineralization (32), this may rep-

Fig. 7. Effect of Kl ablation on ectopic calcification in FTr mice. Contact radiographs of aortas (A) and representative micrographs of histological sections from kidney (B) and lung (C) stained with the von Kossa procedure (magnification ×400) from 50-day-old mice of the indicated genotype. Ectopic calcium deposition (red arrows) was evident in tissues from Kl−/− and FTr/Kt−/− mice but not from WT or FTr+ mice, likely a consequence of the elevated calcium-phosphorus product evident in the former two groups of animals.
resent a mechanism for Klotho to impede mineralization independent of FGF23, phosphorus, and 1,25(OH)2D.

In contrast to the findings in the metaphysis, in the diaphysis of long bones, relative osteopenia has been reported as the major lesion in Kl−/− mice (30, 31). The decreased cortical bone volume observed in our studies, in both Kl−/− and FGF23/Kl−/− mice relative to the wild-type mice, is compatible with this observation. Interestingly, cortical radiolucency has also been reported in Fg23−/− mice, which have an intact Klotho, but has been ascribed to impaired mineralization of cortical bone (22). Recent studies have indicated that Klotho acts as a Wnt antagonist (17), and studies in mice and humans have now established that augmented Wnt signaling leads to increased bone mass (13). However, in the absence of Klotho, continuous Wnt exposure whether in vitro or in vivo triggers accelerated cellular senescence (17). Consequently, chronic unopposed Wnt stimulation may contribute to stem cell deple- tion and the diaphyseal osteopenia observed in the Klotho-deficient phenotypes.

In summary, our findings here substantiate the essential role of Klotho in the mechanism of action of FGF23 in vivo, since its concurrent ablation reverses the near complete spectrum of biochemical alterations attributed to FGF23 actions. Neverthe- less, the presence in FGF23-overexpressing animals of discrete skeletal phenotypic changes in both the metaphysis and the diaphysis that have not been reported in the Fgf23−/− null mutant is compatible with the hypothesis that Klotho deficiency and suggests that Klotho signaling may modulate bone function independent of FGF23.

ACKNOWLEDGMENTS

We thank M. Papageorgiou for assistance with preparation of the manuscript.

GRANTS

This work was supported by operating grants from the Canadian Institutes for Health Research to A. C. Karaplis and D. Goltzman.

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