ENDOCRINE AND PARACRINE ROLE OF FGF23 AND KLOTHO IN HEALTH AND DISEASE

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ENDOCRINE AND PARACRINE ROLE OF FGF23 AND KLOTHO IN HEALTH AND DISEASE

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αKlotho and fibroblast growth factor-23 (FGF23) were discovered independently about 20 years ago. Since their initial description, a series of exciting discoveries have revealed the important role of endocrine and paracrine FGF23 and αKlotho signaling not only for the physiological regulation of mineral and bone homeostasis, but also for the pathophysiology of diseases such as chronic kidney disease, left ventricular hypertrophy, myocardial infarction, hypertension, and disorders characterized by impaired bone mineralization. The 11 articles compiled in this Research Topic consist of three Original Research articles and 8 Reviews or Mini Reviews, and are an excellent source of information about the state of the art in the FGF23/αKlotho field, covering almost all aspects of FGF23/αKlotho biology.

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Table of Contents

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EDITORIAL
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Editorial: Endocrine and Paracrine Role of FGF23 and Klotho in Health and Disease

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Keywords: Klotho, fibroblast growth factor (FCF-23), chronic kidney disease, X-linked hypophosphatemia, myocardial infarction, bone mineralization, left ventricular hypertrophy

Editorial on the Research Topic

Endocrine and Paracrine Role of FGF23 and Klotho in Health and Disease

αKlotho and fibroblast growth factor-23 (FGF23) were discovered independently about 20 years ago. The transmembrane protein αKlotho was originally described as an anti-aging factor (1), whereas FGF23 was discovered as a plasma phosphate-lowering hormone, suppressing phosphate reabsorption and vitamin D hormone production in the kidney (2–4). Typical for seminal discoveries, both findings have generated an avalanche of subsequent research. It took about 10 years after these initial, completely independent discoveries to find out that both molecules are part of the same signaling pathway (5). After 20 years of intensive research, there is still keen interest in the αKlotho/FGF23 biology field. This interest is currently mainly fueled by the so far unexplained finding that the levels of circulating FGF23 are a strong predictor of disease progression and mortality in patients with chronic kidney disease (CKD), and by the emerging role of FGF23 in cardiovascular diseases (6–8). Progress in science is often driven by controversies. The αKlotho/FGF23 field is actually a very good example of controversy-driven advancement of science.

The purpose of this Research Topic was to bring together Reviews/Mini-Reviews and Original Research articles, reflecting the state of the art and the current controversies in this rapidly expanding research area. The 11 accepted articles consist of three Original Research articles and 8 Reviews or Mini-Reviews.

The Original Research articles deal (i) with the bone-kidney-gut endocrine signaling network, (ii) with the role of FGF23 as pro-hypertrophic factor in the heart, and (iii) with the function of FGF23 as a local regulator of mineralization in bone. Fujii et al. investigated the role of osteocytes in the control of mineral homeostasis, using a diphtheria toxin-mediated osteocyte ablation model in mice. Intriguingly, they found an only transient reduction in circulating FGF23 after osteocyte ablation, which was followed by profoundly increased renal phosphate excretion and suppressed renal Klotho expression after serum FGF23 had normalized. Data from human studies have shown a strong association between left ventricular hypertrophy and circulating FGF23 (6). To elucidate further the pathophysiological role of FGF23 in the development of cardiac hypertrophy, Leifheit-Nestler et al. compared two mouse models characterized by increased circulating FGF23, Klotho deficient mice and *Hyp* mice. *Hyp* mice are a model of X-linked hypophosphatemia (XLH). Leifheit-Nestler et al. found that despite high circulating FGF23, increased cardiac abundance of *Fgf23* mRNA, and low renal Klotho expression, *Hyp* mice did not show pathological cardiac remodeling. These findings suggest that increased circulating FGF23 alone is not cardiotoxic in the absence of hyperphosphatemia/hypercalcemia. If extrapolated to humans, the data provided by this study suggest that chronically elevated FGF23 levels in XLH patients will not induce left ventricular

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hypertrophy *per se*. It is well-known that the mineral-bone-disorder in CKD patients (CKD-MBD) is associated with impaired bone mineralization (9). It was previously thought that the mineralization defect in CKD is mainly due to secondary hyperparathyroidism, metabolic acidosis, increased circulating Wnt inhibitors, and uremic toxins (9). The paper by Andrukhova et al. establishes a new paradigm, namely that excessive osteocytic FGF23 secretion locally contributes to the bone mineralization defect in CKD-MBD by suppression of alkaline phosphatase, which in turn leads to the accumulation of the mineralization inhibitor pyrophosphate.

The 8 Reviews/Mini-Reviews cover most of the current knowledge in the FGF23/αKlotho field. The physiological actions of FGF23 are reviewed in the paper by Erben. Current evidence suggests that under physiological conditions, kidney and bone are the main target organs of FGF23 activity. The endocrine actions of FGF23 in the kidney are mediated through the FGF receptor 1c/αKlotho receptor complex, whereas the paracrine actions of FGF23 in bone are αKlotho-independent. One of the burning questions in the field is the question whether soluble Klotho has FGF23-independent effects. It is firmly established that transmembrane αKlotho functions as a co-receptor for FGF23 signaling (5). In addition, the extracellular part of αKlotho can be shed by membrane-anchored sheddases, giving rise to soluble αKlotho circulating in the blood stream. The review by Dalton et al. summarizes the state of the art as of 2017, describing the FGF23-independent hormonal and enzymatic functions of soluble αKlotho reported previously. However, in January 2018 the paradigm-shifting paper by Chen et al. (10) demonstrated that soluble αKlotho functions as a co-receptor for FGF23 signaling, and lacks any FGF23-independent effects. The review by Richter and Faul was accepted after the landmark paper by Chen et al. (10) came out, and is an insightful and thought-provoking paper. Richter and Faul not only describe what is currently known about the actions of FGF23 in the heart, liver, leukocytes, skeletal muscle, endothelium, lung, and CNS. In addition, Richter and Faul hypothesize that the main function of soluble αKlotho may be to act as a decoy receptor for FGF23, preventing pathological FGF23 signaling, which appears to be mainly mediated through FGF receptors 3 and 4.

Emerging evidence suggests that FGF23 may have paracrine effects in the heart. Leifheit-Nestler and Haffner summarize what is known about the local secretion of FGF23 in the heart, and comprehensively discuss the putative paracrine functions of FGF23 in the development of left ventricular hypertrophy. From a different perspective, the review by Stöhr et al. describes our current knowledge about the mechanisms how cardiac disease induces FGF23 secretion and how FGF23 may impact cardiovascular disease via canonical and non-canonical effects. Furthermore, they discuss the potential for therapeutic interventions. An important aspect of cardiovascular disease is vascular calcification, which is often found in CKD patients. Although, it is generally accepted that hyperphosphatemia is the major driving force for vascular calcification in CKD patients, αKlotho deficiency may also play a role. The review by Lang et al. summarizes our knowledge about the pathophysiological mechanisms leading to vascular calcification in two hyperphosphatemia mouse models, αKlotho deficient mice and mice treated with a vitamin D overload. In addition, Lang et al. outline the possibilities for therapeutic interventions explored in these mouse models.

During recent years, it became clear that activated immune cells are able to produce FGF23, and that FGF23 also acts on immune cells. The review by Fitzpatrick et al. is dealing with the role of FGF23 in inflammation. Fitzpatrick et al. point out that it is important to distinguish between indirect effects of FGF23 on the immune system mediated through suppression of vitamin D hormone production, and direct αKlotho-independent and αKlotho-dependent effects on granulocytes and macrophages, respectively.

In February 2018, anti-FGF23 antibody therapy was approved by the European Medicines Agency for the treatment of XLH. The paper by Fukumoto is a comprehensive review of the therapeutic role of FGF23 inhibition by antibodies or synthetic inhibitors in phosphate-wasting disorders and CKD. Fukumoto concludes that while patients with phosphate-wasting diseases such as XLH may benefit from FGF23 blocking antibodies as long as the therapy is carefully monitored, current evidence is insufficient to justify the use of inhibition of FGF23 signaling in patients with CKD.

In summary, the articles in this research topic are an excellent source of information about the current knowledge in the FGF23/αKlotho field.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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ORIGINAL RESEARCH
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Effect of Osteocyte-Ablation on Inorganic Phosphate Metabolism: Analysis of Bone–Kidney–Gut Axis

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In response to kidney damage, osteocytes increase the production of several hormones critically involved in mineral metabolism. Recent studies suggest that osteocyte function is altered very early in the course of chronic kidney disease. In the present study, to clarify the role of osteocytes and the canalicular network in mineral homeostasis, we performed four experiments. In Experiment 1, we investigated renal and intestinal Pi handling in osteocyte-less (OCL) model mice [transgenic mice with the dentin matrix protein-1 promoter-driven diphtheria toxin (DT)-receptor that were injected with DT]. In Experiment 2, we administered granulocyte colony-stimulating factor to mice to disrupt the osteocyte canalicular network. In Experiment 3, we investigated the role of osteocytes in dietary Pi signaling. In Experiment 4, we analyzed gene expression level fluctuations in the intestine and liver by comparing mice fed a high Pi diet and OCL mice. Together, the findings of these experiments indicate that osteocyte ablation caused rapid renal Pi excretion ($P < 0.01$) before the plasma fibroblast growth factor 23 (FGF23) and parathyroid hormone (PTH) levels increased. At the same time, we observed a rapid suppression of renal Klotho ($P < 0.01$), type II sodium phosphate transporters Npt2a ($P < 0.01$) and Npt2c ($P < 0.05$), and an increase in intestinal Npt2b ($P < 0.01$) protein. In OCL mice, Pi excretion in feces was markedly reduced ($P < 0.01$). Together, these effects of osteocyte ablation are predicted to markedly increase intestinal Pi absorption ($P < 0.01$), thus suggesting that increased intestinal Pi absorption stimulates renal Pi excretion in OCL mice. In addition, the ablation of osteocytes and feeding of a high Pi diet affected FGF15/bile acid metabolism and controlled Npt2b expression. In conclusion, OCL mice exhibited increased renal Pi excretion due to enhanced intestinal Pi absorption. We discuss the role of FGF23–Klotho on renal and intestinal Pi metabolism in OCL mice.

Keywords: osteocyte, phosphate, fibroblast growth factor 23, Klotho, intestine, kidney, liver, bile acid

INTRODUCTION

Inorganic phosphate (Pi) homeostasis is maintained by complex interactions between vitamin D, parathyroid hormone (PTH), and fibroblast growth factor 23 (FGF23) (1–3). FGF23 promotes renal Pi excretion by decreasing its reabsorption in the proximal tubules while concurrently reducing plasma $1,25(OH)_2D$ by both decreasing its biosynthesis and increasing its metabolism (3–6). FGF23 requires an additional cofactor, α-Klotho (Klotho), to bind with high affinity

and signal efficiently through its cognate FGF receptor (7, 8). The FGF23–Klotho system directly participates in the bone–parathyroid axis as FGF23 inhibits PTH secretion (9–11). The sodium-dependent P_i cotransport system includes the Npt2a and Npt2c cotransporters, which locate in the apical membrane of the proximal tubular cells, and Npt2b cotransporter, which locates in the apical membrane of the intestinal epithelial cells (12–16). The FGF23–Klotho system suppresses renal Npt2a and Npt2c protein levels and decreases active vitamin D metabolism (17, 18). The reduction of plasma $1,25(OH)_2D$ levels leads to decreased intestinal Npt2b protein levels (19). The FGF23–Klotho system regulates the three transporters and controls systematic Pi homeostasis (12, 13).

Elevated circulating FGF23 levels are strongly related to adverse outcomes in patients with chronic kidney disease (CKD) of all stages (20–22). Circulating FGF23 levels increase early in the course of CKD and reach levels that are several hundred times the normal range in advanced CKD and end-stage renal disease (20–22). CKD is also associated with reduced Klotho expression (23). Klotho deficiency is not only an early biomarker of CKD but also a pathogenic intermediate for CKD development and progression, and extrarenal complications (23). The causes of the increased cardiovascular risk associated with kidney disease are partly related to the CKD-mineral bone disorder (CKD-MBD) syndrome, with the FGF23–Klotho system playing an important role in the pathogenesis of CKD-MBD (23).

Osteocytes are abundant in bone and comprise 95% of all bone cells (24–26). The specialized morphology of osteocytes allows them to function effectively to direct the balance between osteoblast and osteoclast activity, and to regulate systemic mineral metabolism (24–26). Osteocytes respond to kidney damage by increasing the production of secreted factors important for bone and mineral metabolism (27). Indeed, these cells are the primary production site of several factors important for bone and mineral metabolism, including FGF23 and sclerostin (SOST), a negative regulator of the Wnt/β catenin pathway in bone (27, 28). Several recent reports suggest that altered osteocyte function is manifested by changes in osteocytic FGF23, and dentin matrix acidic phosphoprotrein-1 (DMP1) and SOST expression are observed very early in the course of CKD (27). By contrast, the decline in renal Klotho is an early event that is followed by other changes (FGF23, $1,25(OH)_2D$, PTH) as CKD progresses (29). Thus, osteocytes may be the central organ regulating Pi metabolism and dietary Pi-sensing in the kidney–bone axis (1, 24, 30, 31). The roles of osteocytes in mineral metabolism, however, have not been fully elucidated.

Recently, Tatsumi et al. established osteocyte-ablated model mice based on the diphtheria toxin receptor-mediated cell knockout (TRECK) system (32, 33) and examined the role of osteocytes in bone metabolism (32). Osteocyte-less (OCL) mice exhibit osteoporosis. Studies of osteocyte ablation model mice have provided *in vivo* evidence that osteocytes sense loading to the skeleton and orchestrate bone remodeling by controlling both osteoblasts and osteoclasts (32). The effect of osteocyte ablation on mineral metabolism remains unknown. Especially, the effects on calcium and Pi absorption, such as by the kidney and intestine, are not known. In addition, the effects of osteocyte ablation on the fluctuation of hormones related to mineral metabolism (PTH, FGF23, and vitamin D) are unclear. In the present study, we investigated mineral (calcium and Pi) metabolism in OCL mice.

MATERIALS AND METHODS

Animal Experiments

In this study, we used the dentin matrix protein 1 promoter-diphtheria toxin receptor (DMP1-DTR) transgenic (Tg) mice established by Tatsumi et al. (32). Injecting these mice with diphtheria toxin (DT) achieves *in vivo* inducible and specific ablation of osteocytes. Ten-week-old DMP1-DTR Tg mice and wild-type (WT) mice as littermate controls (Cont) were maintained at 23°C on a 12-h light/dark cycle. The mice had unlimited access to water and a standard rodent diet. A metabolic cage was used to measure body weight, and to collect blood, urine, and feces. All animal studies were performed in accordance with the guidelines for the care and handling of laboratory animals and were approved by the animal care committee of Tokushima University.

For the dietary regulation experiments, 10-week-old male DT-injected mice (OCL and Cont mice) were placed on one of the following two isocaloric diets for 7 days: (1) control-Pi (CP) 0.6% Pi and (2) high-Pi (HP) 1.2% Pi (34, 35). For analysis of the osteocyte network-disrupted mice, we used granulocyte colony-stimulating factor (G-CSF)-injected mice. Injecting the mice with G-CSF induced hematopoietic stem/progenitor cell mobilization (36). Disruption of the osteocyte network was induced as described previously (37). Male C57 BL/6 mice were injected with recombinant human G-CSF (Filgrastim, Kyowa Kirin, 250 mg/kg body weight/day subcutaneously every 12 h in 10 divided doses unless otherwise indicated) in phosphate-buffered saline supplemented with 0.1% bovine serum albumin, and maintained for 1 day after administering the last dose of G-CSF for 24-h urine collection (37).

Establishment of Osteocyte-Ablated Mice (OCL Mice)

DMP1-DTR Tg mice were created based on the diphtheria TRECK system (32). The mouse DMP1 gene fused to human HB-EGF (DTR) cDNA with a polyadenylation signals were constructed. The DMP1-DTR Tg mice were mated with WT mice and the offspring were genotyped by polymerase chain reaction (PCR) targeting the inserted gene using genomic DNA extracted from the tail. The following primers were used: 5′-GGGTCCCTCTTCTTCCCTAGC-3′ (forward) and 5′-GTATCCACGGACCAGCTGCTAC-3′ (reverse) (200 bp product for the transgene). Injecting DMP1-DTR Tg mice with DT resulted in targeted and inducible ablation of osteocytes. DT (50 μg/kg body weight)-treated DMP1-DTR Tg mice were used as OCL mice (**Figure 1A**).

Calcium Absorption Analysis

Intestinal Ca^{2+} absorption was assessed in 8-week-old mice by measuring serum $^{45}Ca^{2+}$ at early time-points after oral gavage.

Mice were fasted 12 h before the test. Animals were hemodynamically stable under anesthesia (urethane 1.4 mg/g body weight) during the entire experiment. The test solution contained 0.1 mM $CaCl_2$, 125 mM NaCl, 17 mM Tris, and 1.8 g/l fructose, and was enriched with 20 μCi $^{45}CaCl_2$/ml for oral tests (Cont and OCL mice) (38). For oral tests, 15 μl/g body weight was administered orally by gavage. Blood samples were obtained at the indicated time intervals. Serum (10 μl) was analyzed by liquid scintillation

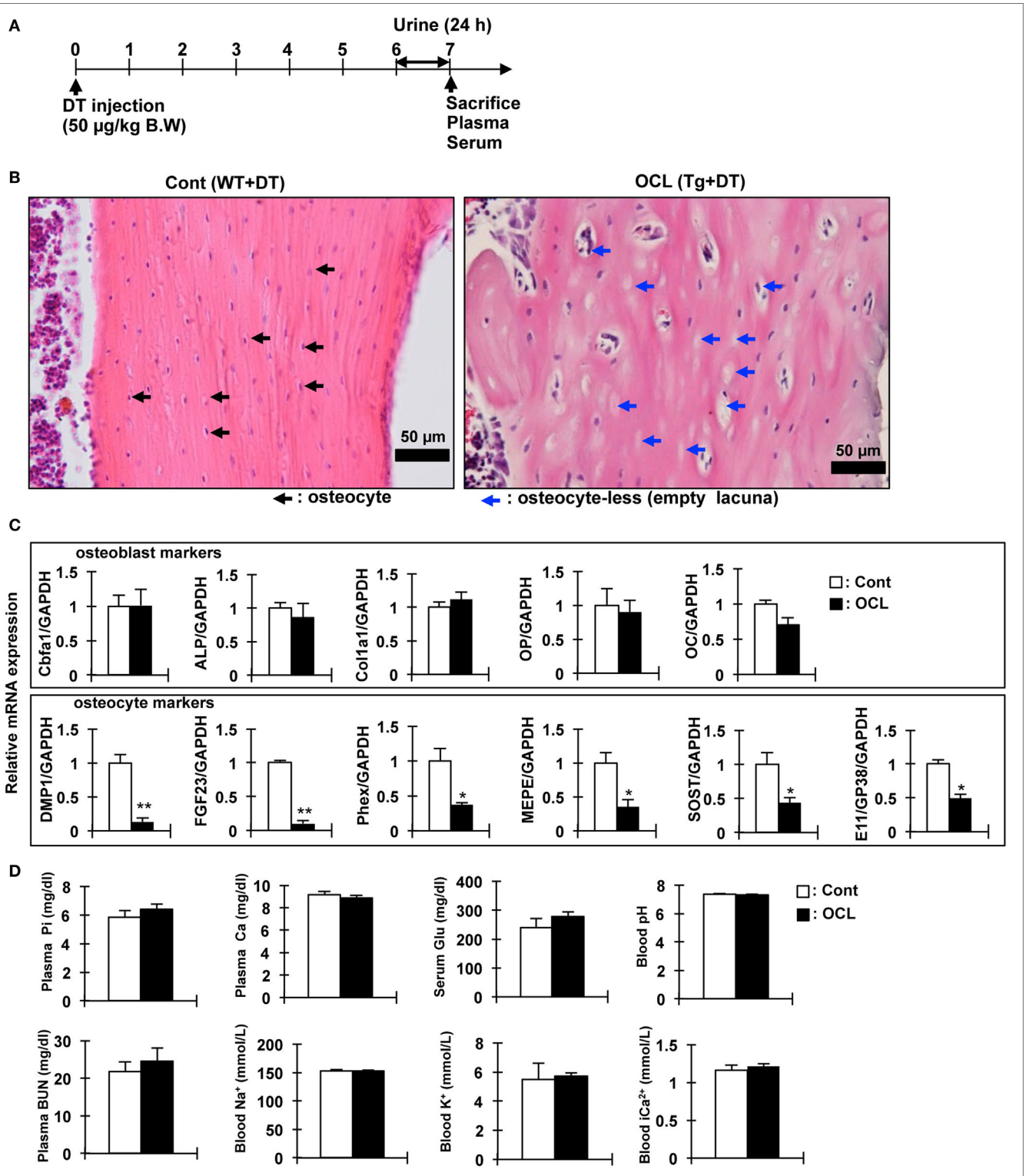

FIGURE 1 | Continued

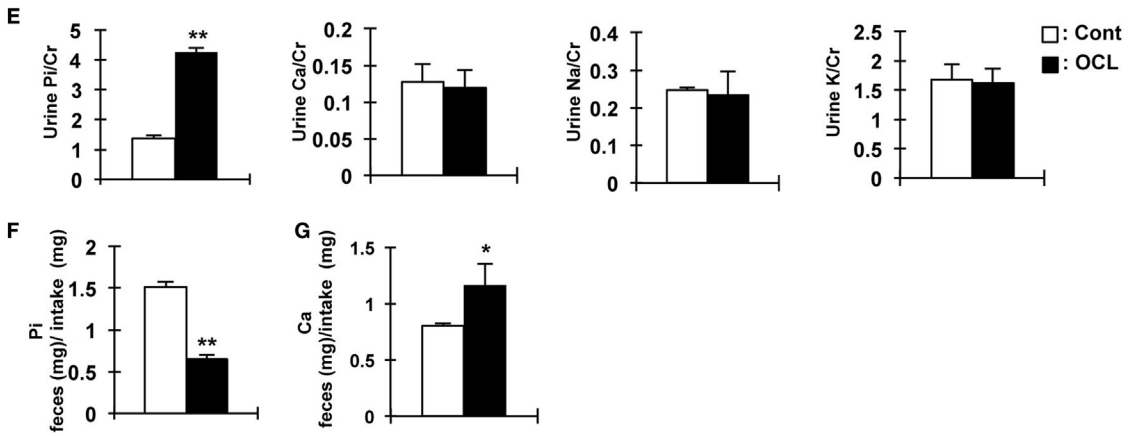

FIGURE 1 | Gene expression and biochemical analysis in osteocyte-less (OCL) mice. **(A)** Experimental design. Ten-week old male wild-type (WT) and transgenic (Tg) mice were intraperitoneally injected with 50 μg/kg body weight diphtheria toxin (DT) in 0.9% NaCl were assessed at 7 days after injection. Controls (Cont): WT + DT, OCL: Tg + DT. **(B)** Cortical bone of the femurs of Cont and OCL mice stained with hematoxylin and eosin at 7 days after DT injection. Scale bars indicate 50 μm. Magnification 200×. Black arrowheads indicate osteocytes and blue arrowheads indicate ablated osteocytes (n = 6 mice/group). **(C)** Gene expression of osteocyte markers in femurs of OCL mice or Cont mice. Gene expression of osteocyte markers [dentin matrix protein-1 (DMP-1), fibroblast growth factor 23 (FGF23), phosphate-regulating endopeptidase homolog X-linked (Phex), matrix extracellular phosphoglycoprotein (MEPE), Sclerostin (SOST), and E11/GP38] and osteoblast markers [Cbfa1, a Alkaline Phosphatase (ALP), Collagen type I alpha (Col1a1), osteopontin (OP), and osteocalcin (OC)] were assessed by real-time polymerase chain reaction (PCR) at 7 days after DT injection. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and pooled from two or three independent experiments (n = 6 mice/group). **(D,E)** Plasma, serum, whole blood, and urine biochemical markers in control and OCL mice at 7 days after DT injection (n = 6 mice/group). **(F,G)** From 6 to 7 days after DT injection, feces were collected for 24 h and food intake was measured (24 h) using a metabolic cage. **(F)** Inorganic phosphate (Pi) in feces (mg)/Pi intake (mg), **(G)** calcium in feces (mg)/Ca intake (mg) (n = 6 mice/group). Food consumption of the Cont mice was adjusted to that ingested by the OCL mice. Calcium and Pi levels in the feces were normalized to the amount of diet. The bar graphs are presented as arithmetic means ± SEM (n = 6/group). Two-tail unpaired t-test $^{**}P < 0.01$, $^{*}P < 0.05$ vs control.

counting (38). Changes in the plasma calcium concentration (Δμmol) were calculated from the ^{45}Ca content of the plasma samples and the specific activity of the administered calcium (38).

Gene Expression Studies

Total RNA was isolated from bone, liver, proximal intestine, and distal intestine with ISOGEN (Wako, Osaka, Japan). The proximal intestine refers to the duodenum and the proximal part of the jejunum. The distal intestine refers to the distal part of jejunum and ileum (39). For quantitative reverse transcription (RT)-PCR, total RNA (1 μg) was reverse-transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen), and samples were analyzed using the Applied Biosystems®StepOnePlus Real-Time PCR system (Thermo Fisher Scientific Inc., Japan). The primers and product size used are shown in Table S1 in Supplementary Material. The amount of target mRNA was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. RT-PCR was performed using SYBR® Premix Ex Taq II (Tli RNaseH Plus) (TAKARA, OSAKA, Japan). The PCRs contained 1 μl of cDNA (equivalent to 50 ng of total RNA), 2× SYBR® Premix Ex Taq II and 400 nM specific primers in a total of 25 μl. Relative expression values were evaluated with the $2^{-\Delta\Delta}Ct$ method. Data were normalized to GAPDH and pooled from three independent experiments.

Biochemical Analyses

Plasma, feces, and urinary Pi and calcium and serum blood urea nitrogen (BUN) were determined by the Phospha-C test, Calcium-E test, or BUN-B test (Wako Pure Chemical Industries, Ltd., Osaka, Japan), respectively (40). To measure the fecal Pi and calcium levels, the collected feces (24 h) were dried at 110°C for 24 h, 250°C for 3 h, and at 350°C for 3 h. FGF23 and PTH concentrations were determined using an FGF23 ELISA kit (Kainos Laboratories, Inc., Tokyo, Japan) and mouse PTH 1-84 ELISA kit (Immutopics, CA, USA), respectively (40). $1,25(OH)_2D$ levels were measured using a radioreceptor assay (SRL, Inc., Tokyo, Japan). Heparinized mixed arterial–venous blood was collected and analyzed immediately for pH, blood gases, and electrolytes using an OPTI CCA TS blood gas analyzer (Sysmex Corporation, Kobe, Japan).

Bone Analysis

Eight- to ten-week-old Tg mice and their WT littermates were perfused with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4). Femurs and tibiae were immersed in the same fixative for 12 h prior to decalcification with 10% EDTA (pH 7.4) for 2 weeks. The specimens were embedded in paraffin and subjected to histochemistry with hematoxylin and eosin staining and immunohistochemical analysis using DMP1 antibody (TAKARA, Kyoto, Japan).

Preparation of Brush Border Membrane Vesicles (BBMVs) and Transport Assay

Brush border membrane vesicles were prepared from kidney and distal intestine using the Ca^{2+} precipitation method,

and used for immunoblot analysis as previously described (34, 41). Levels of leucine aminopeptidase, Na^+-K^+-ATPase, and cytochrome *c* oxidase were measured to assess membrane purity. Uptake of ^{32}P into the BBMVs was measured by the rapid filtration technique (34, 41).

Immunoblot Analyses

Protein samples were in denatured with 2-mercaptoethanol and subjected to 8 or 10% SDS-PAGE. The separated proteins were transferred by electrophoresis to Immobilon-P polyvinylidene difluoride (Millipore, Billerica, MA, USA) and then treated with the following diluted antibodies. Immunoblot analyses were performed using the following primary antibodies: affinity-purified anti-Npt2a (1:4,000) (34), anti-Npt2c (1:3,000) (41), and anti-Npt2b (1:2,000), as described previously (42). Anti-Klotho (for mouse total lysate; Trans Genic Inc., Fukuoka, Japan) was used following the manufacturer's instructions. Mouse anti-actin monoclonal antibody (Chemicon, Temecula, CA, USA) was used as an internal control. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG was utilized as the secondary antibody (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA), and signals were detected using Immobilon Western (Millipore). Membranes were exposed to standard X-ray film and densitometric quantification was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). All experiments were repeated at least five times.

Histochemical Analyses of Kidney Sections

Immunohistochemical analyses of rat kidney sections were performed as described previously with minor modifications (43). Specimens were embedded in paraffin and subjected to immunohistochemistry for affinity-purified Npt2a antibodies or affinity-purified Npt2c antibodies. Sections were then treated with Envision (+) rabbit peroxidase (Dako, Carpinteria, CA, USA) for 30 min at room temperature. Immunoreactivity was detected by incubating the sections with 0.8 mM diaminobenzidine (43). Masson trichrome staining was observed using a Trichrome Stain (Masson) Kit (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. Von kossa stain performed in OCL and klotho-deficient mice (43). Von Kossa staining for mineral deposition in the kidney was performed by applying 5% silver nitrate to the renal sections and exposing the sections to bright light for 30 min (44). Paraffin sections were counterstained with hematoxylin for evaluation of tissue and cell morphology.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using the Student's *t*-test. We evaluated differences between the two groups by an unpaired *t*-test and differences among multiple groups were analyzed by ANOVA followed by Tukey's post-test for multiple comparisons. All computations were performed using GraphPad Prism 5.0 Software. In all experiments, differences were considered statistically significant at $P < 0.05$.

RESULTS

Pi Regulatory Genes in Bone of the OCL Mice

We investigated the physiologic function of osteocytes in OCL mice. Expression of DT receptors on osteocytes was targeted specifically using the DMP1 promoter (32). A single intraperitoneal injection of DT into DMP1-DTR Tg mice killed osteocytes harboring the DT receptor and resulted in a number of empty lacunae containing no osteocytes, as reported previously (32). The OCL mice exhibited a non-significant tendency toward decreased food intake, consistent with a previous report (45). Histochemical analysis of the cortical bone revealed a low abundance of osteocytes compared with Cont mice (32). Consistent with previous findings (9), OCL mice exhibited a decreased osteocyte number at 7 days after DT injection (**Figures 1A,B**). After ablated osteocytes, bone resorption was increased, bone formation and mineralization are suppressed, resulting in osteoporosis (32).

We also measured the expression of Pi regulatory genes in the osteocyte-rich fractions as described previously (32). The mRNA levels of DMP1 ($P < 0.01$), FGF23 ($P < 0.01$), matrix extracellular phosphoglycoprotein (MEPE) ($P < 0.05$), and phosphate-regulating endopeptidase homolog X-linked (Phex) ($P < 0.05$) as Pi regulatory factors were significantly decreased in OCL mice. SOST ($P < 0.05$) and E11/GP38 ($P < 0.05$) as osteocyte markers were also significantly decreased in OCL mice (**Figure 1C**). On the other hand, osteoblast markers were not changed in OCL mice compared with Cont mice. Compared with DT-injected WT mice, DT (50 μg/kg, DT50)-injected Tg mice showed no changes in plasma Pi, calcium, glucose, pH, BUN, Na^+, K^+, and Ca^{2+} (**Figure 1D**). In addition, urinary Ca/Cr, Na/Cr, and K/Cr concentrations were not different between DT50 mice and Cont mice (**Figure 1E**). By contrast, OCL mice exhibited prominent hyperphosphaturia ($P < 0.01$) (**Figure 1E**). Food consumption by the OCL mice was slightly lower than that of Cont mice. Food consumption by the Cont mice was adjusted to the amount in OCL mice. Calcium and Pi levels in the feces were normalized to the amount of diet consumed. The Pi content in the feces was reduced by 56.9% that in the Cont mice ($P < 0.01$) (**Figure 1F**). The calcium content in the feces was 45.4% higher than that in Cont mice ($P < 0.05$) (**Figure 1G**). These findings suggest that renal Pi excretion and intestinal Pi and calcium absorption may be abnormal in OCL mice. In particular, it is expected that the increased Pi absorption in the small intestine influences systemic Pi metabolism.

Time-Course of the Changes in the Hormones Involved in Pi Homeostasis in OCL Mice

To investigate the initial triggers for the increase in renal Pi excretion, we analyzed the time-course of the changes in the Pi excretion levels, plasma PTH, FGF23, and renal Klotho protein after DT treatment. Renal Pi excretion was significantly increased at 5 days after DT injection (3.45-fold, $P < 0.01$) (**Figure 2A**). Plasma FGF23 levels first decreased at 1 day (32.4%, $P < 0.01$) and 3 days (32.4%, $P < 0.01$) after injection of DT and then recovered

(**Figure 2B**). By contrast, plasma PTH levels were significantly increased at 9 days (1.52-fold, $P < 0.01$) (**Figure 2C**). Renal Klotho levels were significantly (75.5%) decreased at 5 days after DT injection ($P < 0.01$) (**Figure 2D**). These data suggest that the beginning of the increase in Pi excretion is not consistent with that of the increase in plasma PTH and FGF23 levels. We analyzed renal fibrosis (**Figure 2E**). High dietary Pi loading increases renal fibrosis (46). In OCL mice, kidney samples were obtained at 7 days after DT injection. The kidney samples obtained from OCL mice were stained with Masson's trichrome to detect total collagen deposits (**Figure 2E**), which revealed interstitial fibrosis and increased renal Pi excretion independent of plasma PTH and FGF23 levels. The kidney calcification observed in Klotho-deficient mice was not detected in OCL mice (**Figure 2F**).

We then investigated if DT injection induced dose-dependent changes in the osteocyte number and renal Pi excretion (32). At 5 days after DT (0, 15, or 50 μg/kg BW) injection, we measured Pi excretion and renal Klotho and $1,25(OH)_2D$ levels (**Figure 2G**). At 5 days after DT injection, the number of osteocytes in the cortical bone decreased to 33.5 ± 1.7% (DT15) or 75.3 ± 4.2% (DT50) that in the DT0 mice ($P < 0.01$) (**Figure 2G**). The plasma $1,25(OH)_2D$ levels did not change after DT injection (**Figure 2H**).

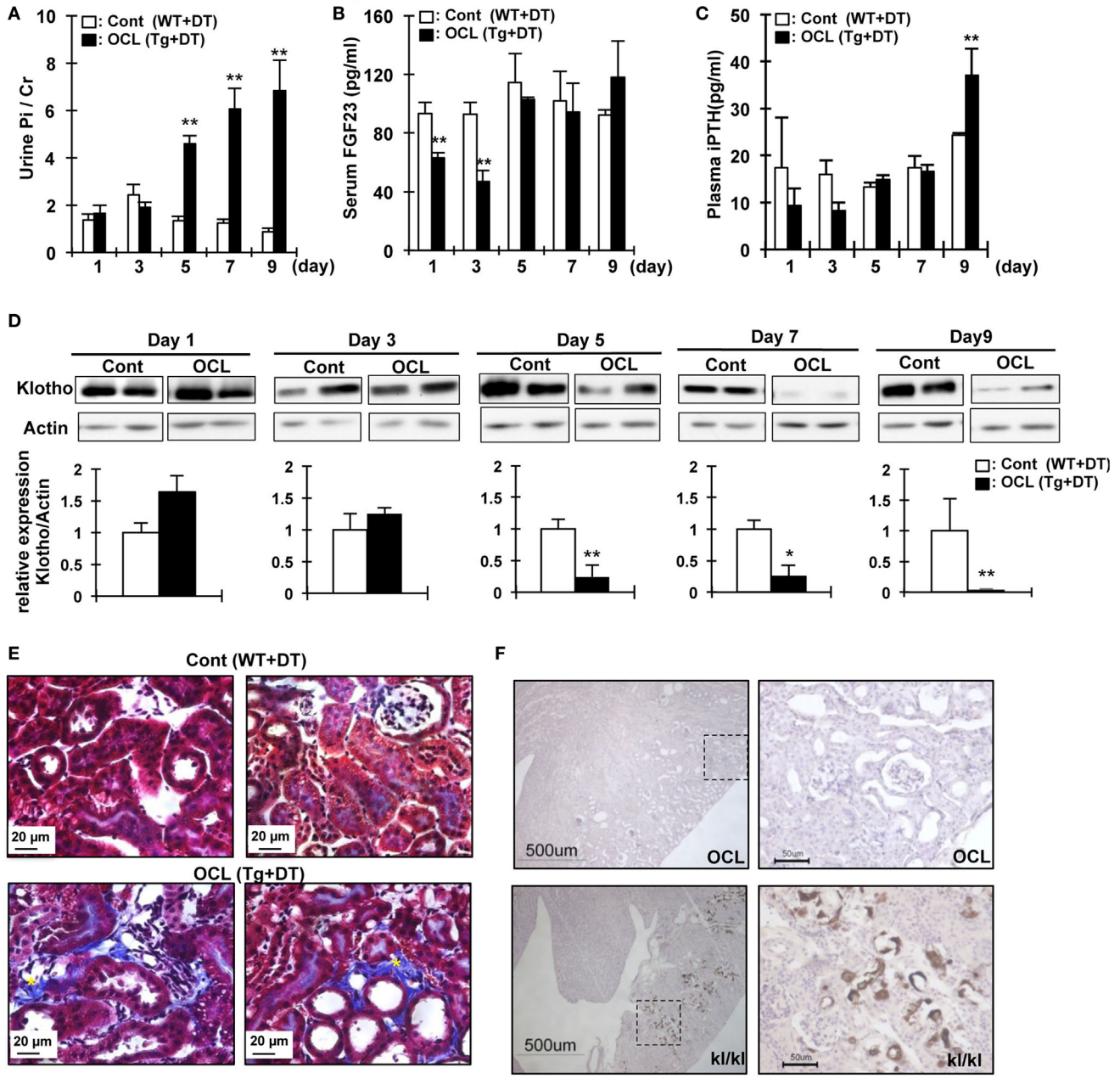

FIGURE 2 | Continued

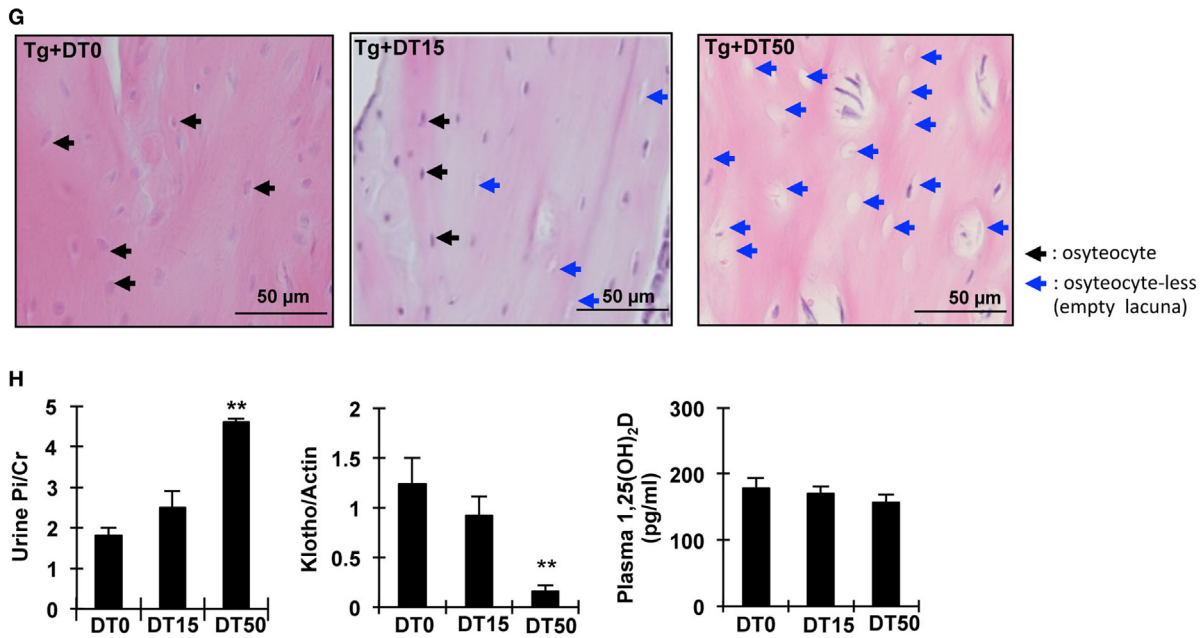

FIGURE 2 | Changes in urine phosphate and phosphate-regulating factor in osteocyte-less (OCL) mice. Ten-week old male transgenic (Tg) mice and wild-type (WT) mice were intraperitoneally injected with 50 μg/kg body weight diphtheria toxin (DT) in 0.9% NaCl. Controls (Cont): WT + DT, OCL: Tg + DT. Analysis of biochemical markers at the indicated times after DT administration. **(A)** Urine Pi/Cr, **(B)** serum fibroblast growth factor 23 (FGF23), and **(C)** plasma PTH were analyzed in Cont and OCL mice after DT injection. **(D)** Renal Klotho expression in OCL mice by immunoblotting analysis (n = 6 mice/group). **(E)** Masson trichrome staining of sequential kidney sections from Cont and OCL mice. Scale bars indicate 20 μm. Magnification 400×. In OCL mice, severe fibrosis was observed in the peritubular interstitium. Fibrotic areas are stained blue (yellow *). **(F)** Von Kossa staining for mineral deposition in the kidney. OCL: Tg + DT (50 μg/kg body weight DT injection), kl/kl: klotho-deficient mice. Scale bars indicate 500 μm, Magnification 100× (n = 6/group). **(G,H)** Ten-week-old male transgenic (Tg) mice were intraperitoneally injected with 15 or 50 μg/kg body weight DT in 0.9% NaCl. DT0: vehicle (0.9%NaCl), DT15: 15 μg/kg body weight DT injection, DT50; 50 μg/kg body weight DT injection (n = 6/group). **(G)** Cortical bone mouse femurs stained with hematoxylin and eosin at 5 days after DT injection. Scale bars indicate 50 μm. Magnification 200×. **(H)** Urine Pi excretion in OCL. Renal Klotho expression in OCL mice by immunoblotting analysis. Plasma 1,25$(OH)_2$D in OCL mice. The bar graphs are presented as arithmetic means ± SEM (n = 6/group). Two-tail unpaired t-test $^{**}P < 0.01$ vs control.

Urinary Pi excretion was significantly increased at 5 days after DT50 injection ($P < 0.01$) (**Figure 2C**). By contrast, DT50 injection markedly suppressed renal Klotho expression ($P < 0.01$) (**Figure 2D**). These data suggest that the increased renal Pi excretion was dependent on the number of osteocytes ablated.

Renal and Intestinal Pi Transport Activity in the OCL Mice

To examine whether the expression of renal Pi transporters was affected in OCL mice, Npt2a and Npt2c mRNA and protein levels were investigated (**Figure 3**). Npt2a and Npt2c mRNA levels were significantly decreased in OCL mice ($P < 0.01$) (**Figure 3A**). PiT2 mRNA levels were not changed in OCL mice compared with Cont mice (**Figure 3A**). 1α(OH)ase mRNA levels were markedly increased in OCL mice (4.9-fold, $P < 0.01$), whereas 24(OH)ase mRNA expression levels were not changed (54.4% decreases, $P < 0.01$) (**Figure 3A**). Klotho mRNA expression levels were significantly (44.4%) decreased in OCL mice ($P < 0.01$) (**Figure 3A**).

The mRNA levels of the transcription factor early growth response 1 (Egr-1) were significantly increased in OCL mice (2.4-fold, $P < 0.01$), whereas FGF receptor 1 (FGFR1) mRNA levels were not changed. The mRNA expression levels of FGF receptors 3 (FGFR3) were significantly increased (2.1-fold, $P < 0.01$) and FGF receptors 4 (FGFR4) were significantly decreased (34%, $P < 0.01$) (**Figure 3A**). Npt2a and Npt2c protein levels were markedly decreased in OCL mice ($P < 0.01$, $P < 0.05$) (**Figure 3C**). In addition, sodium-dependent Pi cotransport activity in the BBMVs was also significantly reduced in OCL mice compared with Cont mice ($P < 0.01$) (**Figure 3D**). Immunohistochemical analysis revealed reduced Npt2a and Npt2c immunoreactivity in the proximal tubular cells (**Figure 3B**). Intestinal Npt2b protein levels were significantly increased in OCL mice compared with Cont mice (fourfold, $P < 0.01$) (**Figure 3E**). Intestinal Pi transport activity in OCL mice was 4.2-fold higher than that in the Cont mice ($P < 0.01$) (**Figure 3F**).

Intestinal Calcium Absorption

We then investigated calcium absorption in the small intestine. In contrast to intestinal Pi absorption, the calcium content in the feces in OCL mice was 45.4% higher than that in Cont mice ($P < 0.05$) (**Figure 1G**).

Oral calcium absorption analysis using $^{45}Ca^{2+}$ solution indicated that calcium absorption (plasma $^{45}Ca^{2+}$) levels were significantly (62–69%) decreased in OCL mice ($P < 0.01$) (**Figure 4A**). Intestinal transient receptor potential vanilloid 6 (TRPV6), calbindin-D9k (CaBP D9k), and plasma membrane calcium

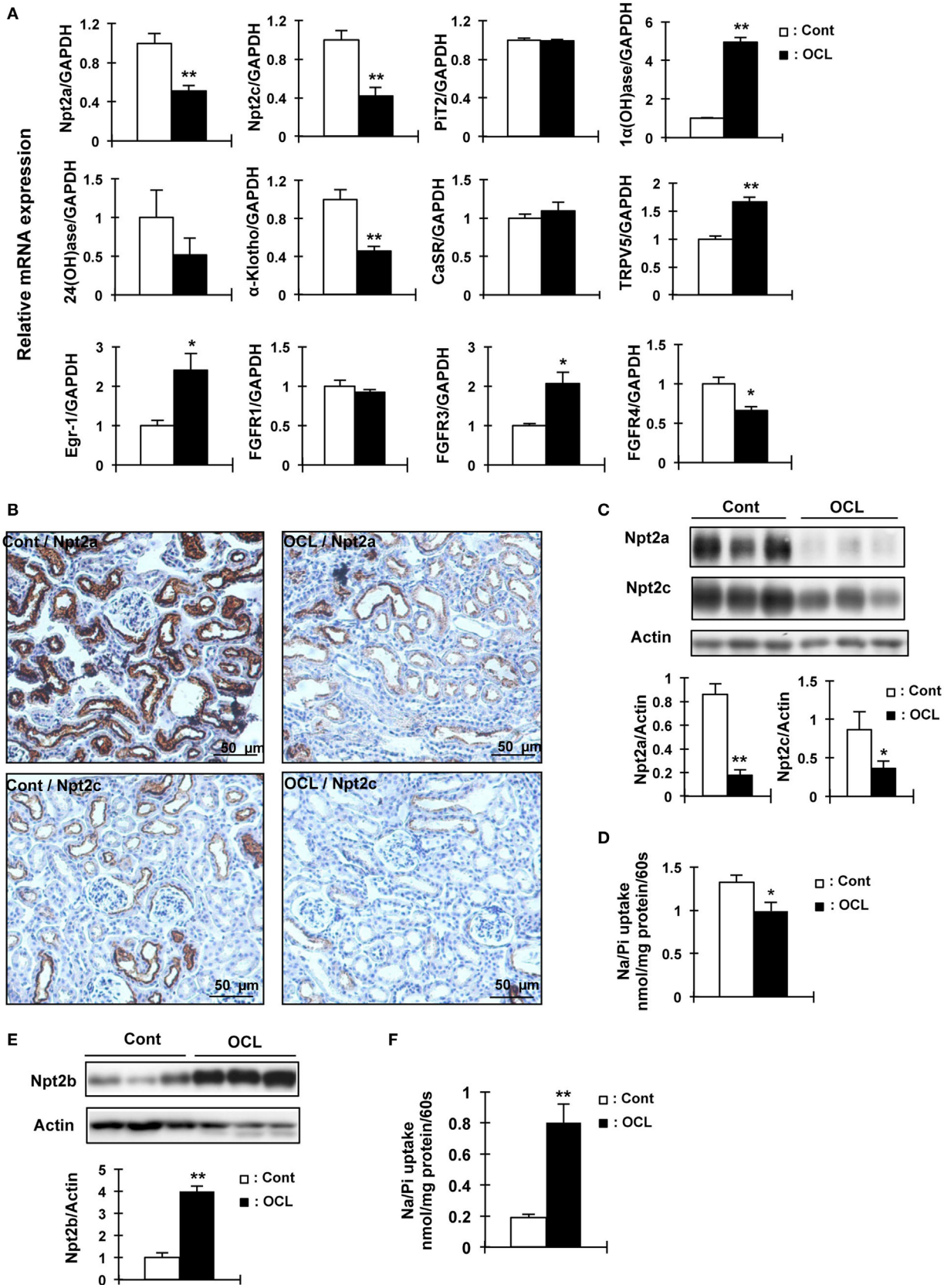

FIGURE 3 | Continued

FIGURE 3 | Expression of renal Npt2a, Npt2c, and intestinal Npt2b in osteocyte-less (OCL) mice. Ten-week-old male wild-type (WT) and transgenic (Tg) mice were intraperitoneally injected with 50 μg/kg body weight DT in 0.9% NaCl and assessed at 5 days after injection. Controls (Cont): WT + DT, OCL: Tg + DT. **(A)** Quantitative polymerase chain reaction (PCR) of Npt2a, Npt2c, PiT2, 1α(OH)ase, 24(OH)ase, Klotho, CasR, TRPV5, early growth response 1 (Egr-1), FGF receptor 1 (FGFR1), FGFR3, and FGF receptors 4 (FGFR4) mRNA in mouse kidney at 5 days after DT injection. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and pooled from two or three independent experiments ($n = 7$ mice/group). **(B)** Immunohistochemical analysis of renal Npt2a and Npt2c proteins. Scale bars indicate 50 μm. Magnification 200×. **(C)** Immunoblotting analysis of Npt2a and Npt2c proteins in renal brush-border membrane vesicles (BBMVs). All membranes were reprobed for actin. Actin was used as an internal control. The bar graphs are presented as arithmetic means ± SEM ($n = 5$/group). **(D)** Renal Na/Pi transport activity in mice. Na/Pi transport activity was determined by ^{32}P uptake in kidney BBMVs ($n = 7$/group). **(E)** Immunoblotting analysis of Npt2b proteins in intestinal (jejunum and ileum) brush-border membrane vesicles. All membranes were reprobed for actin. Actin was used as an internal control ($n = 7$ each/group). **(F)** Intestinal (distal jejunum and ileum) Na/Pi transport activity in mice. Na/Pi transport activity was determined by ^{32}P uptake in intestinal BBMVs ($n = 7$/group). The bar graphs are presented as arithmetic means ± SEM, Two-tail unpaired *t*-test $^{**}P < 0.01$, $^{*}P < 0.05$ vs control.

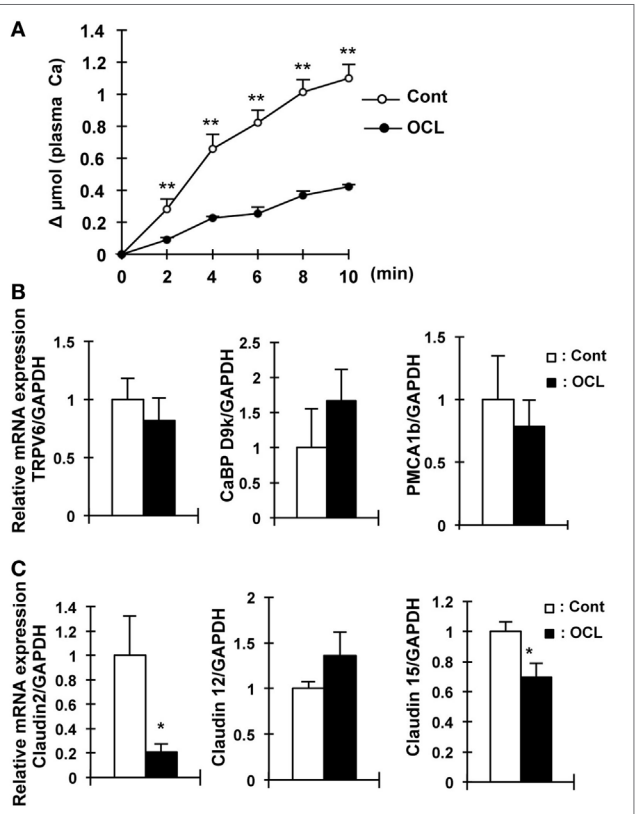

FIGURE 4 | Calcium absorption in osteocyte-less (OCL) mice. Ten-week-old male wild-type (WT) and transgenic (Tg) mice were intraperitoneally injected with 50 μg/kg body weight DT in 0.9% NaCl at 5 days after DT injection. Control (Cont): WT + DT, OCL: Tg + DT. **(A)** Intestinal calcium absorption, assessed by whole blood levels of ^{45}Ca (Δμmol) after oral gavage ($n = 5$/group). **(B)** Gene expression of calcium transport and its regulating factor in intestinal of OCL mice or Cont mice. Gene expression of transient receptor potential vanilloid channel 6 (TRPV6) as apical calcium transport, calbindin-D_{9k} (CaBP-9k) as cytoplasmic calcium-binding proteins, and plasma membrane calcium ATPase 1b (PMCA1b) as basolateral calcium transporter were assessed by real-time polymerase chain reaction (PCR) at 5 days after DT injection. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and pooled from two or three independent experiments ($n = 7$ mice/group). **(C)** Gene expression of intestinal claudin 2, 12, and 15 in OCL mice or Cont mice. Gene expression of intestinal (proximal part of intestine) claudin 2, 12, and 15 were assessed by real-time PCR at 5 days after DT injection. Data were normalized to GAPDH and pooled from two or three independent experiments ($n = 7$ mice/group). The bar graphs are presented as arithmetic means ± SEM, two-tail unpaired *t*-test $^{**}P < 0.01$, $^{*}P < 0.05$ vs control.

ATPase 1b (PMCA1) mRNA levels, however, were not altered in OCL mice (**Figure 4B**). On the other hand, mRNA expression levels of claudin 2, which regulates intestinal Ca^{2+} absorption *via* paracellular pathways, were dramatically (79.1%) decreased in OCL mice ($P < 0.01$) (**Figure 4C**). Although claudin 12 mRNA expression levels were not changed in OCL mice, claudin 15 mRNA expression levels were decreased in OCL mice (30.4% decreases, $P < 0.05$). Thus, intestinal calcium absorption was significantly decreased in OCL mice. On the other hand, renal excretion of calcium was not affected in OCL mice. In the kidney, calcium-sensing receptor mRNA levels were not changed and TRPV5 mRNA expression was significantly (1.7-fold) increased in OCL mice ($P < 0.01$) (**Figure 3A**).

Analysis of Osteocyte Canalicular Network-Disrupted Mice

We investigated other mouse models of osteocyte network disruption. Eight-week-old C57BL/6 mice were administered recombinant human G-CSF (250 μg/kg body weight/day) and analyzed 1 day after the last dose of G-CSF (**Figure 5A**). In the G-CSF injected mice, hematopoietic stem cells were removed from the medullary cavity (data not shown). RNA was extracted from the tibias and femurs of vehicle- and G-CSF-treated mice after flushing out the bone marrow. Gene expression was assessed by quantitative RT-PCR and the results were normalized to GAPDH mRNA. In G-CSF injected mice, Col1a1 (64.6%, $P < 0.01$), osteocalcin (53.8%, $P < 0.01$), and osteopontin mRNA (58.8%, $P < 0.05$) as osteoblastic markers were decreased; and FGF23 (83.8%), DMP1 (54.8%), and Phex (62.4%) as osteocytic markers were also decreased ($P < 0.01$), suggesting that differentiation of osteoblasts into osteocytes was suppressed by G-CSF injection (**Figure 5C**). Immunohistochemical analysis using DMP1 antibody showed that the length and number of canaliculi were markedly decreased in the femoral bone of G-CSF treated mice (**Figure 5B**). Remarkably, canaliculi did not reach the surface of the bone (**Figure 5C**). Therefore, these results indicate that the osteocytic canalicular network was disrupted. The serum Pi concentration did not significantly differ between the G-CSF-injected mice and vehicle-injected mice (**Figure 5D**) and was similar to that in the OCL mice (**Figure 1D**). Urinary Pi levels, however, were significantly higher (2.2-fold, $P < 0.05$) in the G-CSF-injected mice than in the vehicle-injected mice (**Figure 5D**), same as in OCL mice (**Figure 1E**). In the renal BBM of G-CSF mice, Npt2a and Npt2c levels were significantly

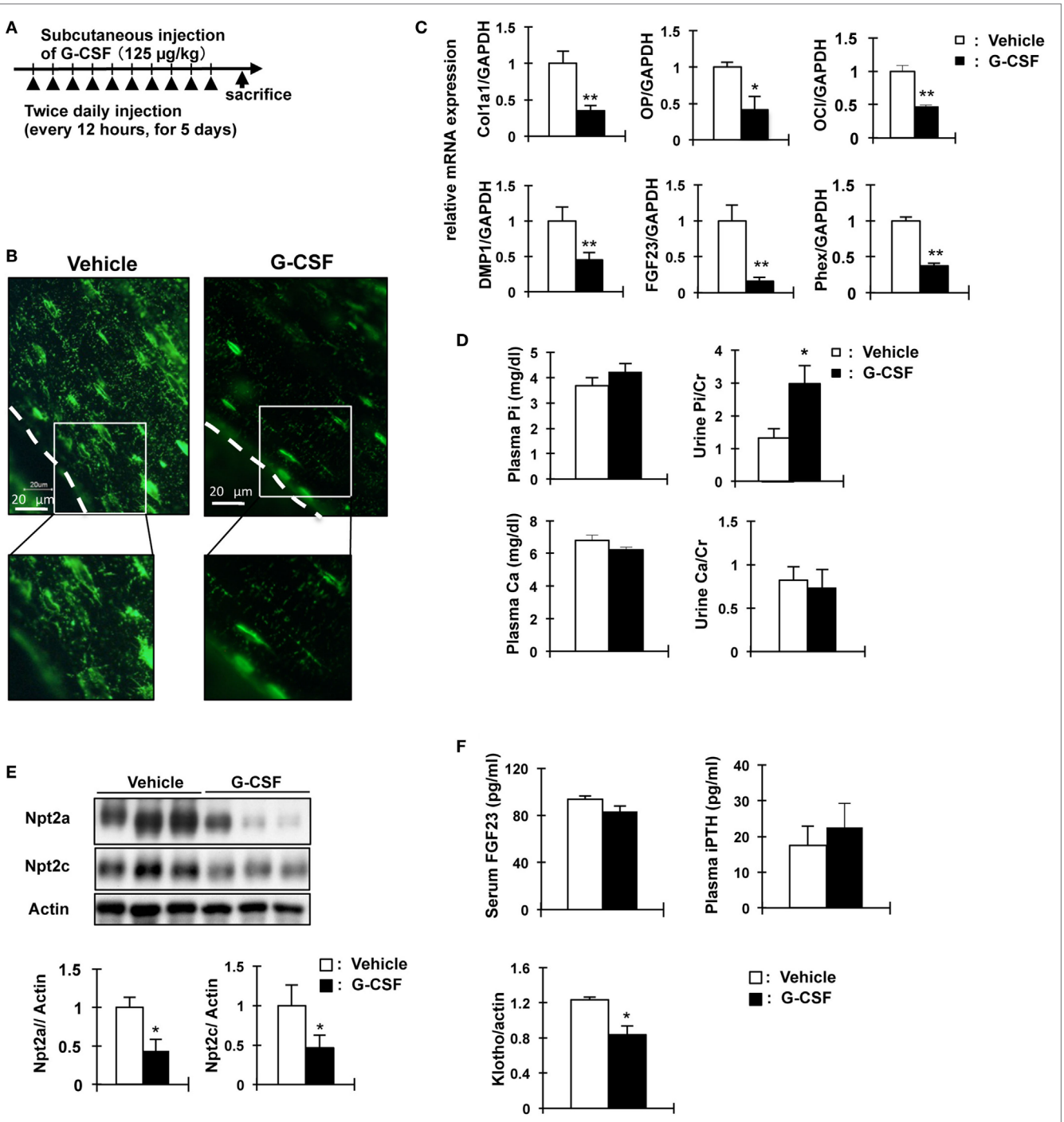

FIGURE 5 | Analysis of granulocyte colony stimulating factor (G-CSF)- injected mice in phosphate metabolism. **(A)** Experimental design for osteocytic canaliculi network disruption by G-CSF (filgrastim 125 mg/kg/dose, s.c.). **(B)** Immunofluorescence staining of dentin matrix acidic phosphoprotrein-1 (DMP1) in osteocytes and lacuna-canaliculi network of the femur of vehicle and G-CSF injected mice. Green: DMP1. The scale bars indicate 20 μm. Magnification 400×. **(C)** Real-time polymerase chain reaction (PCR) analysis of markers of osteoblast and osteocytes. RNA was extracted from the femurs of 8-week-old WT mice injected with phosphate-buffered saline (white bars) or G-CSF (black bars) for 6days. Gene expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression levels. Collagen type I alpha (Col1a1), osteopontin (OP) osteocalcin (OC), FGF23, DMP1, and Phex. Data are presented as mean ± SEM. $^{**}P < 0.01$ $^{**}P < 0.05$ ($n = 6$/group). **(D)** Biochemical analysis of plasma Inorganic phosphate (Pi), plasma calcium, urinary Pi/Cr, and urinary Ca/Cr levels in G-CSF or vehicle-injected mice. Urine was collected for 24 h from days 5 to 6. **(E)** Immunoblotting analysis of Npt2a and Npt2c proteins in renal brush-border membrane vesicles of G-CSF-treated mice and vehicle-injected mice. All membranes were reprobed for actin. Actin was used as an internal control. The bar graphs are presented as arithmetic means ± SEM ($n = 6$ each group). **(F)** Serum FGF23 and plasma iPTH and renal Klotho levels in G-CSF treated mice and vehicle-injected mice. $^{**}P < 0.01$; $^{*}P < 0.05$ ($n = 6$ mice/group). Renal Klotho protein levels were observed by immunoblotting analysis in renal total lysates. All membranes were reprobed for actin. Actin was used as an internal control. The bar graphs are presented as arithmetic means ± SEM ($n = 6$/group). Two-tail unpaired t-test $^{**}P < 0.01$, $^{*}P < 0.05$ vs control (vehicle injection).

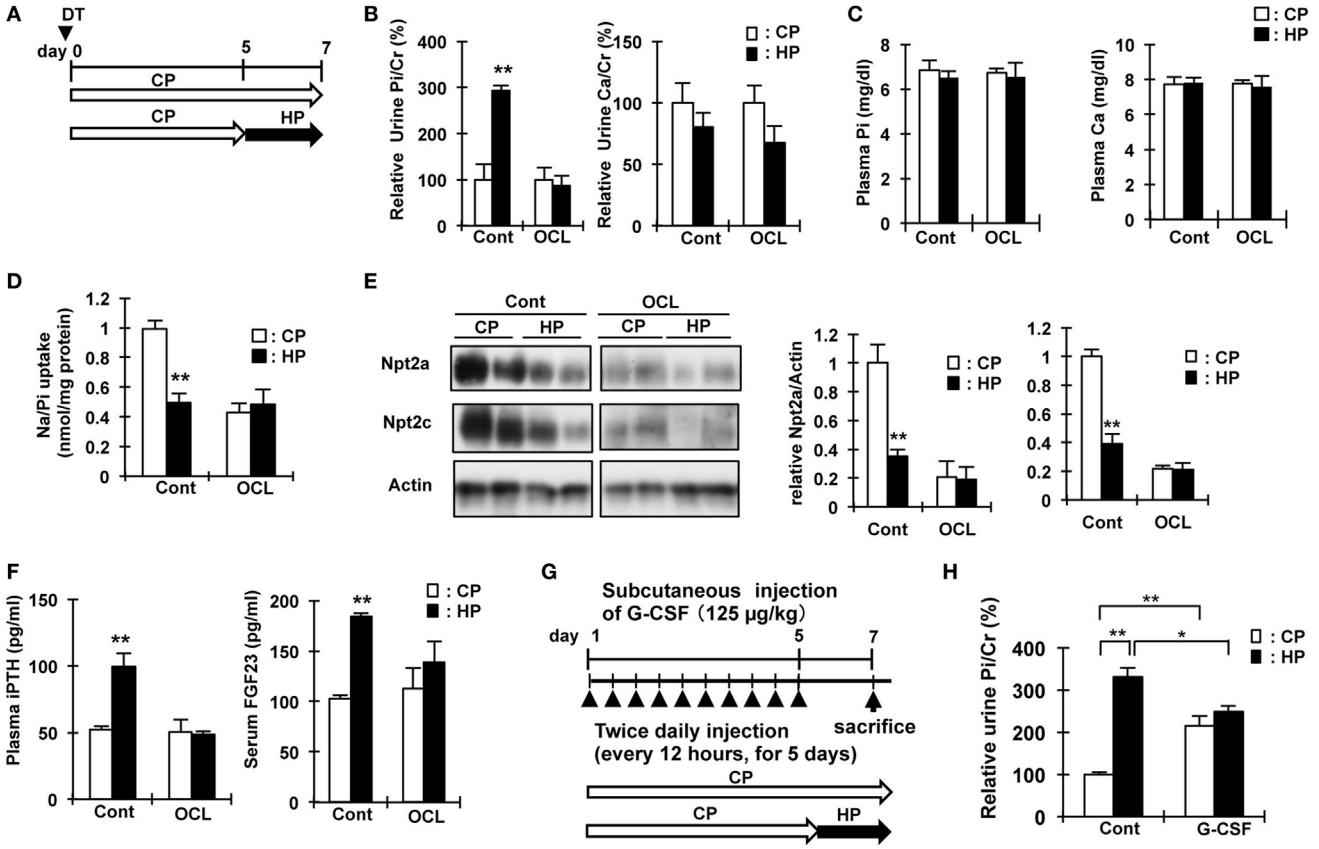

FIGURE 6 | High phosphate diet response in osteocyte-less (OCL) and granulocyte colony stimulating factor (G-CSF) mice. **(A)** Experimental design of high-Pi (HP) dietary loading in OCL mice. Ten-week-old male transgenic (Tg) mice and wild-type (WT) mice were intraperitoneally injected with 50 μg/kg body weight DT in 0.9% NaCl. Control (Cont): WT + DT, OCL: Tg + DT. Control and OCL mice were fed the control inorganic phosphate (Pi) diet until day 5 and from days 5 to 7 were fed either the high Pi diet (HP) or control diet (CP). **(B)** Relative urine Pi/Cr (%) and Ca/Cr (24 h urine collection) at 6–7 days after diphtheria toxin (DT) injection. **(C)** Plasma Pi and calcium at 7 d after DT injection. **(D)** Renal Na/Pi transport activity in mice. Na/Pi transport activity was determined by ^{32}P uptake in kidney brush border membrane vesicles (BBMVs) (n = 6/group). **(E)** Immunoblotting analysis of Npt2a and Npt2c proteins in renal BBMVs. All membranes were reprobed for actin. Actin was used as an internal control. **(F)** Plasma intact PTH and serum FGF23 at 7 days after DT injection. The bar graphs are presented as arithmetic means ± SEM (n = 6/group). Two-tail unpaired t-test $^{**}P < 0.01$, $^{*}P < 0.05$. (CP VS HP). **(G)** Experimental design of HP dietary loading in G-CSF mice. Seven-week-old male mice were injected with PBS or G-CSF (filgrastim 125 mg/kg/dose, s.c.). Control (Cont): WT + PBS, G-CSF: WT + G-CSF. Control and OCL mice were fed the control Pi diet until day 5 and fed either the high Pi diet (HP) or control diet (CP) from days 5 to 7. **(H)** Relative urine Pi/Cr (%) (24 h urine collection) at 6–7 days. The bar graphs are presented as arithmetic means ± SEM (n = 6/group). ANOVA followed by Tukey's post-test for multiple comparisons. $^{**}P < 0.01$, $^{*}P < 0.05$ (CP vs HP, Cont vs G-CSF).

decreased ($P < 0.05$) (**Figure 5E**), similar to the renal Npt2a and Npt2c protein levels in OCL mice (**Figure 3C**). In G-CSF-injected mice, plasma iPTH and FGF23 levels did not differ significantly from those in the vehicle-injected mice (**Figure 5F**). On the other hand, renal Klotho protein levels were decreased in G-CSF-injected mice ($P < 0.05$) (**Figure 5F**). Thus, mice with G-CSF-induced osteocyte network disruption showed hyperphosphaturia independent of the plasma PTH and FGF23 levels.

Feeding on a High Pi Diet Does Not Stimulate Renal Pi Excretion in OCL and G-CSF Mice

The increased renal Pi excretion in OCL and G-CSF mice may be due to the increase in Pi released from the bone and intestinal Pi absorption. Therefore, we investigated whether feeding mice a high Pi diet increases urinary Pi excretion. **Figures 6A,G** show the schedule for dietary phosphate loading in OCL and G-CSF mice. After feeding on a high Pi diet, Pi excretion was significantly increased in the Cont mice ($P < 0.01$), but not changed in the OCL and G-CSF mice (**Figures 6B,H**). After feeding on a high Pi diet, plasma Pi and calcium levels were not changed in the OCL mice (**Figure 6C**).

In Cont mice, feeding on a high Pi diet significantly reduced renal Npt2a and Npt2c protein levels ($P < 0.01$) and Na/Pi uptake in BBMs ($P < 0.01$), whereas no changes were observed in OCL mice (**Figures 6D,E**). In the mice fed a high Pi diet, the levels of plasma intact PTH were not changed in OCL mice, but significantly increased in Cont mice (1.9-fold, $P < 0.01$) (**Figure 6F**). The increase in plasma PTH levels and serum FGF23 levels

induced by a high Pi diet in Cont mice was not observed in OCL mice. Therefore, for dietary Pi adaptation, signaling between the intestine and parathyroid gland, and between the intestine and kidney may be disturbed in OCL mice.

Analysis of Gene Expression Profiles in OCL Mice

We performed DNA microarray analysis of the intestine, liver, and kidney in OCL mice (data not shown). In these analyses, we focused on bile acid metabolism, a commonly changing metabolic system, in OCL mice and G-CSF-administered mice, because we did not detect prominent differences in the expression of genes related to glucose, amino acid, other nutrient metabolic pathways. Bile acid metabolism was markedly disturbed in the intestine and liver in OCL mice compared with Cont mice (Figure S1 in Supplementary Material). In a previous study, Sato et al. reported that osteocytes regulate fat metabolism, that OCL mice lack visible white adipose tissue, and plasma leptin levels decrease in association with fat loss (45). Bile acids serve as ligands for the nuclear receptor farnesoid X receptor (FXR) (46, 47). Bile acids act on FXR in ileal enterocytes to induce the expression of FGF15 (47, 48). FGF15 also stimulates gallbladder filling (47, 48). FGF15 inhibits bile acid synthesis by repressing the transcription of Cyp7al, which encodes the first and rate-limiting enzyme in the classic bile acid synthesis pathway (43). FGF15 acts through a cell surface receptor complex composed of the FGF receptor 4 and β-Klotho (49, 50). In liver, transcription of the small heterodimer partner (SHP) gene is induced by bile acids *via* FXR, and SHP, in turn, binds to the CYP7A1 promoter to repress gene transcription (47). SHP is required for FGF15 to efficiently repress bile acid synthesis (48). In OCL mice compared with Cont mice, transcripts of FXR (liver: $P < 0.05$, distal intestine: $P < 0.01$), Cyp7A1 ($P < 0.05$), FGF receptor 4 (FGFR4) ($P < 0.05$), β-Klotho ($P < 0.01$), SHP (distal intestine; $P < 0.01$), and FGF15 ($P < 0.01$), the concentrations of serum bile acid ($P < 0.05$), and feces bile acids ($P < 0.05$) intestinal bile acids ($P < 0005$) were significantly increased in the liver and/or distal intestine compared with Cont mice (Figures S1B–D in Supplementary Material). Intestinal vitamin D receptor, calcium-sensing receptor, and parathyroid hormone 1 receptor mRNA levels were not changed in OCL mice (data not shown).

Analysis of Gene Expression Profiles in High Pi Diet-Fed Mice

Next we investigated whether OCL mice are in a high Pi state. Lipid accumulation in white adipocytes was decreased in OCL and high Pi diet-fed mice (Figures S1A and S2A in Supplementary Material). We observed alterations in the genes involved in bile acid metabolism ($P < 0.01$) (Figure S1 in Supplementary Material). Quantitative PCR analysis revealed that, compared with Control Pi-fed mice (CP mice), high Pi-fed mice (HP mice) had significantly lower transcript levels of Cyp7A1 ($P < 0.01$), Cyp8b1 ($P < 0.01$), FGFR4 ($P < 0.05$), and β-Klotho ($P < 0.05$) in the liver. FXR ($P < 0.01$), OST-α ($P < 0.05$), and FGF15 ($P < 0.01$) levels in the distal intestine were also significantly decreased in HP mice (Figures S2B,C in Supplementary Material). On the other hand, the concentrations of serum bile acid and feces bile acids were not changed in the liver or distal intestine in HP mice compared with CP mice (Figure S2D in Supplementary Material). The serum triglyceride and cholesterol concentrations were lower in HP mice than in CP mice (Figure S2D in Supplementary Material).

DISCUSSION

In the present study, we investigated the role of osteocytes in Pi metabolism using OCL mice. In this experiment, osteocyte ablation revealed that renal Pi excretion was enhanced before the plasma PTH and FGF23 levels increased. In particular, renal Klotho protein levels declined already in the early stage. In OCL mice, Pi excretion in the feces was markedly reduced and intestinal Pi absorption (Npt2b levels and Pi uptake in BBMV) was significantly enhanced. The present study indicates that the increased renal Pi excretion is due to increased intestinal Pi absorption in OCL mice (**Figure 7**). Similar findings regarding Pi metabolism were obtained in canalicular network-disrupted mice. Furthermore, the response to dietary Pi loading was suppressed in OCL mice.

With regard to the mechanisms underlying the suppression of renal Pi transport, we observed that renal Npt2a and Npt2c protein levels were significantly decreased in the OCL mice. The transcription factor Egr-1 is downstream of ERK1/2 and is a biomarker of ERK1/2 signaling activation by FGF23 (7). Renal Egr-1 mRNA levels were significantly increased, while Klotho protein levels were markedly decreased (7). Renal 1alpha-hydroxylase (1α(OH)ase) mRNA was upregulated in the OCL mice, suggesting that klotho downregulation is involved in the stimulation of $1,25(OH)_2D$ synthesis.

Recent studies demonstrated that FGFR1c/Klotho is the primary receptor complex responsible for mediating Pi metabolism (51), whereas there is a redundant requirement for FGFR3/Klotho and FGFR4/Klotho in the control of $1,25\ (OH)_2D$ levels (52, 53). Thus, the two pathways are expected to be involved in vitamin D and Pi transport by the FGF23–Klotho system (6, 54). Portale et al. reported that suppression of renal $1,25(OH)_2D$ synthesis was intact in egr-$1^{-/-}$ mice treated with FGF23 (55). They found that downstream of FGFR binding and ERK1/2 signaling, the pathways diverge such that egr-1 is required for FGF23-dependent inhibition of Pi transport but not for inhibition of $1,25(OH)_2D$ synthesis (55, 56). The mechanisms underlying the promotion of renal Pi excretion in OCL mice remain unknown, but the increase in egr-1 mRNA suggests the involvement of FGF23-dependent Pi inhibition. We are currently studying the molecular mechanism of the enhancement of Pi excretion in OCL mice.

Osteocyte-less (OCL) mice are thought to have abnormally high Pi absorption from the small intestine. If this hypothesis is correct, OCL mice would be in a state of high Pi and, thus, it is expected that the response of renal Pi excretion to a high Pi diet would be attenuated (43). Osteocytes extend dendrites to each other in the osteocytic canaliculi, and osteoblasts and osteoclasts connect with each other on the bone surface by gap junctions, referred to as the osteocytic canalicular network (24, 57–61). The network is complicated and extends through

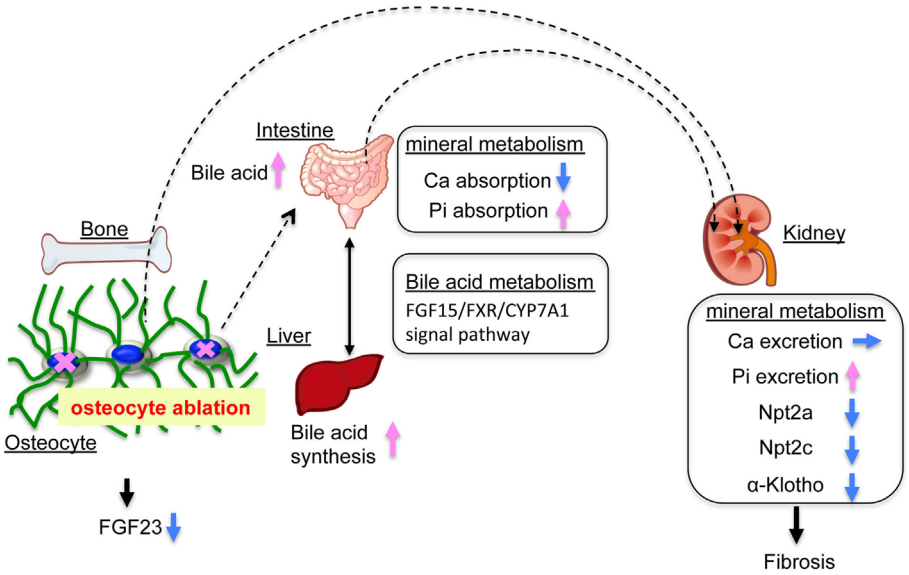

FIGURE 7 | Mineral balance in osteocyte-less (OCL) mice. Osteocyte ablation affects mineral metabolism in the kidney and intestinal tract. The OCL mice showed increased renal inorganic phosphate (Pi) excretion (suppression of Npt2a and Npt2c) by enhanced intestinal Pi absorption (elevation of Npt2b). The OCL mice exhibited markedly suppressed renal Klotho expression, possibly reflecting a disrupted FGF23–Klotho system. OCL causes abnormalities in the bile acid (FGF15/FXR/CYP7A1) signaling pathway in the intestine–liver axis and stimulates intestinal Pi absorption. Thus, osteocytes may be an important regulator of mineral balance in addition to bile acid metabolism.

the entire bone, possibly forming gap junctional intercellular communication pathways (24, 57–61). Osteocyte canalicular networks are thought to be important for bone formation as the structure seems to be ideal for sensing mechanical stress and mechanotransduction (24, 57–61). A recent study demonstrated that osteocytic canalicular network-disrupted mice are created by injection of G-CSF (37). Disruption of the osteocyte canalicular network by G-CSF increased Pi excretion and decreased renal klotho levels independent of PTH and FGF23. In addition, in G-CSF-treated mice or OCL mice, renal Pi excretion did not increase in response to a high Pi diet. These observations suggest that OCL mice already had a high Pi state, and excessive Pi from intestinal absorption may promote renal Pi excretion.

In the present study, OCL mice exhibited abnormal intestinal Pi and calcium absorption. Previous studies demonstrated that the kidney rapidly increases Pi excretion when intestinal Pi absorption is markedly elevated (62, 63). In these conditions, the increase in the plasma PTH or FGF23 levels leads to increased renal Pi excretion in response to an increase in intestinal Pi absorption (62, 63). In OCL mice, however, we observed no increase in PTH or FGF 23. Therefore, the phosphaturic factors acting on the kidney are unknown. In OCL mice, $1,25(OH)_2D$ may be a factor that enhances intestinal Pi absorption. With respect to the FGF23–Klotho system, plasma $1,25(OH)_2D$ levels are an important factor for intestinal mineral absorption (3). In the OCL mice, plasma $1,25(OH)_2D$ levels were not changed while renal 1α(OH)ase mRNA was significantly increased. We investigated the mechanism of the reduction of calcium absorption and the elevation of Pi absorption. To clarify intestinal Pi absorption, we measured intestinal Pi transport activity in BBMV and found the elevation of Npt2b protein and Pi uptake in the OCL mice. In addition, the transfer of ^{45}Ca into the blood was markedly reduced. The calcium concentration in the feces was significantly increased and the gene expression levels of claudin 2 and claudin 15, which are involved in calcium transport, were reduced. No change in the plasma $1,25(OH)_2D$ concentration, however, was observed. In the OCL mice, we observed the suppression of a vitamin D-responsive gene (claudin 2) involved in calcium absorption, but no decrease in TRPV6 or calbindin D9K mRNA (64). Based on these findings, we suspect that a system other than vitamin D metabolism is involved in the abnormal intestinal mineral absorption in OCL mice. Although the reasons for the abnormal intestinal mineral absorption remain unknown, osteocytes are considered to be extremely important for intestinal Pi and calcium absorption.

To elucidate the mechanism for abnormal mineral absorption in the OCL mice, we performed DNA microarray analyses of the intestine and liver. We observed alterations in the genes involved in fat and bile acid metabolism (Figures S1 and S2 in Supplementary Material). Metabolic analysis of the OCL mice and G-CSF-treated mice suggested a functional abnormality of the FGF15–FXR–CYP7A1 system in bile acid metabolism (43). Indeed, we observed increased serum bile acid and feces bile acid levels in the OCL mice. A recent study demonstrated that feeding of a lithocholic acid-rich diet increased intestinal Pi absorption and suppressed renal Pi absorption in WT mice (American Society of Nephrology, Kidney Week 2017 Abstract FR-PO258, Hashimoto N, Sakaguchi Y, Hamano T, Isaka Y, Matsui I, Mori D, Matsumoto A, Shimada K, Yamaguchi S, Kubota K, Oka T, Yonemoto S). Lithocholic acid is a known VDR ligand (65). The details of these mechanisms are unknown. However, bile

acid metabolism is considered to be a regulator of important for intestinal and renal Pi absorption (66). Bile acids are natural detergents that may solubilize calcium-phosphate particles in the small intestine (67). Bile acids are also important signaling molecules and have a role in energy metabolism, metabolic syndrome, obesity, and diabetes (68). Because Pi is deeply involved in energy metabolism, it is expected to be involved in the regulation of energy metabolism by bile acids.

We investigated whether the enhanced intestinal Pi absorption is linked to changes in FGF15/CYP7A1/bile acid in the OCL mice. Dietary Pi load is a powerful factor to suppress intestinal Npt2b protein levels (42). We investigated the possibility that the FGF15/CYP7A1 system responds to the dietary Pi load. Based on quantitative PCR analysis, the increased gene expression levels in the FGF15–FXR–CYP7A1 system observed in OCL mice were largely suppressed in control mice fed a HP diet. These findings suggest that dietary Pi levels affect the FGF15–FXR–CYP7A1 system in the intestines and liver, in addition to vitamin D metabolism.

Previous studies showed that vitamin D exerts a negative feedback on bile acid synthesis by decreasing Cyp7a1 expression (69, 70). FGF15 and SHP play central roles in the feedback regulation of bile acid synthesis by bile acids and FXR (46–48). FGF15 is integral to the mechanism of CYP7A1 regulation of vitamin D (69, 71). VDR regulates FGF15 in the intestine and this pathway is essential for the vitamin D-induced suppression of bile acid synthesis (72). In the liver, the FGF15/FGFR4/β-klotho complex suppresses SHP expression and increases Cyp7A1 (47, 49, 50). The molecular mechanisms of the vitamin D and bile acid abnormalities in OCL mice are not clear. We speculate that OCL mice have disturbed vitamin D and bile acid (FGF15/FXR/CYP7A1) metabolism due to disruptions of the FGF23–Klotho system (**Figure 7**).

In conclusion, the findings of the present study suggest that osteocyte ablation stimulates intestinal Pi absorption and enhances renal Pi excretion. The phosphaturic factors active in OCL mice are unknown, but increased Pi absorption is prominent in OCL mice. The marked increase in intestinal Pi absorption may be due to disruption of the FGF23–Klotho system by osteocyte ablation affecting bile acids and vitamin D metabolism. Further studies are needed to clarify the factors involved in the increased renal Pi excretion in OCL mice.

AUTHOR CONTRIBUTIONS

ST and K-iM contributed to the study design. OF, MO, TA, HS, KN, AM, KI, AH, IK, HS, ST, and K-iM collected and analyzed data. ST and K-iM wrote and revised the final draft of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fendo.2017.00359/full#supplementary-material.

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Impact of Altered Mineral Metabolism on Pathological Cardiac Remodeling in Elevated Fibroblast Growth Factor 23

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Clinical and experimental studies indicate a possible link between high serum levels of fibroblast growth factor 23 (FGF23), phosphate, and parathyroid hormone (PTH), deficiency of active vitamin D (1,25D) and klotho with the development of pathological cardiac remodeling, i.e., left ventricular hypertrophy and myocardial fibrosis, but a causal link has not been established so far. Here, we investigated the cardiac phenotype in klotho hypomorphic (*kl/kl*) mice and *Hyp* mice, two mouse models of elevated FGF23 levels and klotho deficiency, but differing in parameters of mineral metabolism, by using histology, quantitative real-time PCR, immunoblot analysis, and serum and urine biochemistry. Additionally, the specific impact of calcium, phosphate, PTH, and 1,25D on hypertrophic growth of isolated neonatal rat cardiac myocytes was investigated *in vitro*. *Kl/kl* mice displayed high serum Fgf23 levels, increased relative heart weight, enhanced cross-sectional area of individual cardiac myocytes, activated cardiac Fgf23/Fgf receptor (Fgfr) 4/calcineurin/nuclear factor of activated T cell (NFAT) signaling, and induction of pro-hypertrophic NFAT target genes including *Rcan1*, *bMHC*, brain natriuretic peptide (*BNP*), and atrial natriuretic peptide (*ANP*) as compared to corresponding wild-type (WT) mice. Investigation of fibrosis-related molecules characteristic for pathological cardiac remodeling processes demonstrated ERK1/2 activation and enhanced expression of Tgf-β1, *collagen I*, and Mmp2 in *kl/kl* mice than in WT mice. In contrast, despite significantly elevation of serum and cardiac Fgf23, and reduced renal *klotho* expression, *Hyp* mice showed no signs of pathological cardiac remodeling. *Kl/kl* mice showed enhanced serum calcium and phosphate levels, while *Hyp* mice showed unchanged serum calcium levels, lower serum phosphate, and elevated serum iPTH concentrations compared to corresponding WT mice. In cultured cardiac myocytes, treatment with both calcium or phosphate significantly upregulated endogenous *Fgf23* mRNA expression and stimulated hypertrophic cell growth and expression of pro-hypertrophic genes. The treatment with PTH induced hypertrophic cell growth only, and stimulation with 1,25D had no significant effects.

In conclusion, our data indicate that *Hyp* mice, in contrast to *kl/kl* mice appear to be protected from pathological cardiac remodeling during conditions of high FGF23 levels and klotho deficiency, which may be due, at least in part, to differences in mineral metabolism alterations, i.e., hypophosphatemia and lack of hypercalcemia.

Keywords: fibroblast growth factor 23, klotho deficiency, mineral metabolism, left ventricular hypertrophy, cardiac fibrosis, klotho hypomorphic mice, *Hyp* mice

INTRODUCTION

Patients with chronic kidney disease (CKD) (1–3) and patients with heart failure (4–8) display abnormalities in mineral metabolism, which are associated with cardiovascular diseases (CVD). The phosphaturic hormone fibroblast growth factor (FGF) 23 is synthesized and secreted by osteocytes in response to various stimuli (9) to increase renal phosphate excretion by inhibiting the expression of sodium–phosphate co-transporters NaPi-2a and NaPi-2c (10, 11). Moreover, FGF23 suppresses parathyroid hormone (PTH) expression and secretion (12, 13) and inhibits renal formation of $1,25(OH)_2D_3$ (1,25D, calcitriol) by downregulating 1α-hydroxylase (Cyp27b1) and upregulating 24-hydroxylase (Cyp24a1) (11, 14, 15). Klotho is an endocrine hormone primarily produced in the kidney (16) and functions as co-receptor for FGF23, whereby it increases the binding affinity of FGF23 to its FGF receptors (FGFRs) and, therefore, mediates different signaling pathways including renal phosphate and 1,25D metabolism (17–19).

With declining kidney function, the endocrine network of mineral metabolism becomes altered, which leads to elevated serum levels of FGF23, phosphate, and PTH in addition to a deficiency of 1,25D and klotho (20). All these parameter represent key risk factors for the development of endothelial dysfunction, left ventricular hypertrophy (LVH), myocardial fibrosis, and contribute to the overall cardiovascular mortality in CKD and non-CKD patients (1, 6, 21–26). We previously showed that FGF23 directly targets the heart and promotes LVH by binding to FGFR4 on cardiac myocytes activating phospholipase C gamma (PLCγ)/calcineurin/nuclear factor of activated T cells (NFAT) to induce pro-hypertrophic gene expression independently of its co-receptor klotho that is not expressed in the heart (16, 27–29). Moreover, we demonstrated that FGF23 contributes to pathologic cardiac remodeling and promotes the pro-fibrotic crosstalk between cardiac myocytes and fibroblasts resulting in enhanced cardiac hypertrophy and fibrosis in the absence of klotho (30).

Besides high FGF23 levels, klotho deficiency also seems to be associated with cardiac dysfunction in humans and rodents (25, 29, 31), and rescuing the availability of klotho by genetic overexpression or intravenous delivery of soluble klotho shows beneficial outcomes including amelioration of cardiac hypertrophy in klotho-deficient uremic mice and suppression of cardiac fibroblast activation and collagen synthesis (31–33). In addition, klotho ameliorates FGF23-mediated oxidative stress by inducing nitric oxide synthesis and degrading reactive oxygen species in endothelial cells (34). Furthermore, klotho protects against myocardial hypertrophy by reducing indoxyl sulfate-mediated oxidative stress *in vitro* and *in vivo* (35).

To date, the impact of high phosphate levels for the development of cardiomyopathy in the setting of high FGF23 levels is controversially discussed. Increased serum phosphate is associated with cardiovascular mortality in CKD (25, 36) and FGF23 mediates renal phosphate excretion only in the presence of klotho (19). Normalization of serum phosphate and FGF23 levels by dietary phosphate restriction in 5/6 nephrectomized klotho-deficient mice did not abrogate the development of cardiac hypertrophy suggesting that reduced klotho contributes to uremic cardiomyopathy independent of phosphate and FGF23 (32). In contrast, cardiac hypertrophy and fibrosis correlated with high phosphate levels in uremic and non-uremic klotho-deficient mice, and genetically induced elevation of soluble klotho ameliorated phosphate-induced hypertrophic growth of cardiac myocytes *in vivo* (31). The association between FGF23 and cardiac remodeling in klotho-deficient animals suggests that FGF23 affects cardiac hypertrophy and fibrosis only in states of high phosphate and klotho deficiency. Thus, the interplay between increased FGF23, phosphate, and reduced klotho participates in the development of CVD. However, the direct impact of each single factor on pathologic cardiac remodeling is still not clear. Here, we test whether elevated FGF23 levels in klotho deficiency invariably leads to cardiac hypertrophy and fibrosis and whether different alterations in mineral metabolism play a role in these effects. Therefore, we compared two mouse models with genetically elevated serum FGF23 levels and reduced renal klotho availability, *Hyp* mice, and klotho hypomorphic (*kl/kl*) mice, with respect to the development of a pathologic cardiac phenotype. The *Hyp* mouse is a murine homolog to the human disease of X-linked dominant hypophosphatemia resembling elevated circulating FGF23 concentrations resulting in renal phosphate wasting, hypophosphatemia, decreased renal 1,25D synthesis, and defects in bone mineralization (37, 38). Klotho hypomorphic mice present with high plasma concentrations of FGF23, 1,25D, and phosphate, resulting in severe soft tissue calcification and premature aging (16). In addition, we used neonatal rat ventricular myocytes (NRVM) to study the role of parameters of altered mineral metabolism including calcium, phosphate, PTH, and 1,25D for the induction of cardiac hypertrophy.

MATERIALS AND METHODS

Animal Experiments

All experimental procedures were approved by the State Office committee on animal welfare Lower Saxony for *Hyp* mice and Baden-Württemberg for *kl/kl* mice and performed in accordance with national animal protection guidelines from Directive 2010/63/

EU of the European Parliament on the protection of animals used for scientific purposes.

Hemizygous male B6.Cg-Phex^{Hyp}/J (*Hyp*) mice (strain no. 000528; Jackson Laboratory, Bar Harbor, ME, USA) with X-linked semidominant mutation in the *Phex* gene causing defects in phosphate metabolism and male wild-type (WT) littermates produced from breeding of heterozygous females (*Hyp*/+) with C57BL/6J WT males were used in this study. Mice were fed normal rodent chow containing 600 IU/kg cholecalciferol, 0.7% calcium, and 0.5% phosphate (#1324, Altromin, Lage, Germany) *ad lib*. The origin of homozygous klotho hypomorphic mice (*kl/kl*), breeding, and genotyping were described previously (16). Five to 13 mice per group were used in this study and sacrificed at 6–8 weeks of age. Blood was collected *via* cardiac puncture, and hearts were isolated and prepared for histological and biochemical analyses. For serology, blood was centrifuged at 4°C and 13,000 rcf for 20 min. Serum supernatant was collected, stored at −80°C, and subsequently analyzed *via* ELISA techniques for C-term FGF23, intact FGF23, and 1-84 PTH (each from Immutopics, San Clemente, CA, USA), and spectrophotometrically for calcium and phosphate (each from DiaSys Diagnostic Systems GmbH, Holzheim, Germany).

Isolation of NRVM

Neonatal rat ventricular myocytes (NRVM) were isolated using a standard isolation system (Worthington Biochemical Corporation) (39). In brief, hearts from 1- to 2-days-old Sprague Dawley rats were harvested, minced in calcium- and magnesium-free Hank's Balanced Salt Solution (HBSS) followed by tissue digestion with 50 µg/mL trypsin at 4°C for 20–24 h. Soybean trypsin inhibitor in HBSS was added and the tissue was further digested with collagenase (in Leibovitz L-15 medium) under slow rotation (15 rpm) at 37°C for 45 min. Cells were homogenized and resuspended 20 times with a standard 10 mL serological pipette and filtered twice through a 70-µm cell strainer (BD Falcon). After incubation at room temperature for 20 min, cells were centrifuged at 100 × *g* for 5 min and cell pellet was re-suspended in plating medium Dulbecco's Modified Eagle Medium (DMEM) with 20% M199 (Invitrogen), 15% fetal bovine serum (FBS; Invitrogen), and 1% penicillin/streptomycin solution (P/S; Invitrogen).

Cells were plated on glass and plastic surfaces pre-coated with laminin (Invitrogen; 10 µg/mL in PBS) at room temperature for 1 h. For immunofluorescence analysis, 3×10^5 cells were seeded per well on pre-coated glass coverslips in 24-well plates and for RNA isolations, 8×10^5 cells were seeded in 6 cm-culture dishes. Cells were left in plating medium at 37°C over night. After starvation in maintenance medium DMEM with 20% M199, 1% insulin-transferrin-sodium selenite solution (ITS; Sigma-Aldrich), and 1% P/S, isolated NRVM were stimulated in duplicates in maintenance medium in the presence of 3 mM calcium, 1 mM phosphate, 10 nM PTH, 10 nM calcitriol, or vehicle, respectively, for 48 h. At least six independent cell isolations were used for all experiments.

Histological Analysis

Formalin-fixed paraffin-embedded heart tissue samples were deparaffinized in xylene, hydrated through a series of graded alcohols. For the quantification of cardiac myocyte size, fixed cardiac mid-chamber (MC) (33) sections were incubated with wheat germ agglutinin (WGA) Alexa Fluor555 (Invitrogen) at 5 µg/mL in PBS for 1 h to visualize cellular borders of individual cardiac myocytes. 4′,6-diamidino-2-phenylindole (DAPI; 0.2 µg/mL) was used for nuclear staining in the dark for 15 min. Representative immunofluorescence images of cardiac tissue were taken on a Zeiss AxioObserver Z1 microscope (Carl Zeiss) with a Plan-Apo 63×/N.A. 1.4 oil objective. ZEN Software (Carl Zeiss) was used to measure myocardial cross-sectional area in square micrometer of 100 cardiac myocytes in average.

For the detection of myocardial fibrosis and visualization of fibrillar collagen fibers, MC sections were stained with picrosirius red (Sigma-Aldrich) as described previously (28). In brief, deparaffinized MC sections were incubated with picrosirius red for 60 min followed by mounting in non-aqueous mounting medium and analyzed by bright field and polarized microcopy using a Keyence BZ-9000® microscope with a 20× objective. In addition, for polarized light microscopy, two polarization filters were used in a rectangular orientation, positioned above and below the sample.

Immunocytochemistry and Morphometry of NRVM

To analyze hypertrophic growth of isolated NRVM on laminin-coated glass coverslips after 48 h of treatment with calcium, phosphate, PTH, and 1,25D, NRVM were fixed in 2% PFA in 5 mg/mL sucrose for 5 min and permeabilized in 1% Triton X-100 in PBS for 10 min followed by incubation with mouse monoclonal antibody against sarcomeric α-actinin (1:1,000 dilution; EA-53; Sigma-Aldrich). Cy3-conjugated goat-anti mouse (Jackson Immuno Research) was used as secondary antibody at 1:300 dilution. To visualize nuclei, fixed cells were incubated with DAPI (400 ng/mL in PBS) for 10 min. Immunofluorescence images were taken on a Zeiss AxioObserver Z1 microscope (Carl Zeiss) with a 40× objective. Myocyte cross-sectional area was measured based on α-actinin-positive staining using Carl Zeiss ZEN software. At least 100 cells per stimulation were quantified for the determination of cardiac myocyte cross-sectional area.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR) Analysis

For RNA isolation of snap-frozen mouse myocardial tissue or NRVM, RNeasy Mini Kit (Qiagen) was used according to the manufacturer protocol. Total RNA (500 ng) was transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen) and qRT-PCR was performed in triplicates (20 ng cDNA per reaction) with appropriate primers in 5′–3′orientation (**Table 1**) using QuantiFAST SYBR Green PCR Kit including ROX dye (Qiagen). Forty-five cycles (95°C, 10 s; 60°C, 30 s) were performed on an ABI prism 7900HT Fast system (Applied Biosystems). Relative gene expression values, adjusted for the same CT-threshold and baseline settings, were calculated according to the $2^{-\Delta\Delta CT}$ method (40) using *Gapdh* as housekeeping gene (SDS Software v2.3, Applied Biosystems).

TABLE 1 | The following oligonucleotides shown in 5′–3′orientation were used as primers for quantitative RT-PCR analyses.

Gene	Orientation	Primer sequence (5′–3′)
Mouse Fgf23		Mm_Fgf23_1_SG QuantiTect Primer Assay QT01066772 (Qiagen, Hilden, Germany)
Mouse Fgfr1	Forward	TGC CAG CTG CCA AGA CGG TG
	Reverse	AAG GAT GGG CCG GTG AGG GG
Mouse Fgfr4	Forward	GGC TAT GCT GTG GCC GCA CT
	Reverse	GGT CTG AGG GCA CCA CGC TC
Mouse Rcan1	Forward	CCC GTG AAA AAG CAG AAT GC
	Reverse	TCC TTG TCA TAT GTT CTG AAG AGG G
Mouse aMHC	Forward	ACT GTG GTG CCT CGT TCC
	Reverse	GCC TCT AGG CGT TCC TTC TC
Mouse bMHC	Forward	AGG CAA GGC AAA GAA AGG CTC ATC
	Reverse	GCG TGG AGC GCA AGT TTG TCA TAA
Mouse ANP	Forward	ATT GAC AGG ATT GGA GCC CAG AGT
	Reverse	GA CAC ACC ACA AGG GCT TAG GAT
Mouse BNP	Forward	CCA GAT GAT TCT GCT CCT GC
	Reverse	TGA ACT ATG TGC CAT CTT GG
Mouse Col1	Forward	CCG CTG GTC AAG ATG GTC
	Reverse	CCT CGC TCT CCA GCC TTT
Mouse Mmp2	Forward	AAC TAC GAT GAT GAC CGG AAG TG
	Reverse	TGG CAT GGC CGA ACT CA
Mouse Tgfb1	Forward	TTG CTT CAG CTC CAC AGA GA
	Reverse	TGG TTG TAG AGG GCA AGG AC
Mouse Klotho	Forward	TGG GAA GGT TTT GTC CAG AAG A
	Reverse	AGA AAC GAG ATG AAG ACC AGC A
Mouse Gapdh	Forward	TAT GTC GTG GAG TCT ACT GG
	Reverse	AGT GAT GGC ATG GAC TGT GG
Rat Fgf23	Forward	GCA ACA TTT TTG GAT CGT ATC A
	Reverse	GAT GCT TCG GTG ACA GGT AGA
Rat Rcan1	Forward	CTC ACA CAC GTG GAC CAC CA
	Reverse	CGC CCA ATC CAG ACA AAC AG
Rat bMHC	Forward	CTC CAG AAG AGA AGA ACT CC
	Reverse	CCA CCT GCT GGA CAT TCT GC
Rat ANP	Forward	AAA TCC CGT ATA CAG TGC GG
	Reverse	GGA GGC ATG ACC TCA TCT TC
Rat BNP	Forward	CCA GAA CAA TCC ACG ATG C
	Reverse	TCG AAG TCT CTC CTG GAT CC
Rat Gapdh	Forward	ACT CCA CGA CAT ACT CAG CAC
	Reverse	CAT CAA CGA CCC CTT CAT T

Protein Isolation and Immunoblotting

For protein extraction from snap-frozen myocardial specimens of the left ventricle of *Hyp* and *kl/kl* mice, 30 mg tissue was homogenized in 250 µL RIPA extraction buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-desoxycholate, 1 mM EDTA) with protease (P8340, Sigma-Aldrich) and phosphatase (S65208, Sigma-Aldrich) inhibitors, sonicated two times and incubated on ice for 30 min. Cell lysates were centrifuged at 13,000 rpm and 4°C for 10 min, protein concentration was quantified using BCA test, 100 µg total protein was boiled in sample buffer and analyzed by SDS-PAGE and immunoblotting. Antibodies to FGF23 (1:1,000; ab98000, Abcam), Calcineurin A (1:10,000; ab52761, Abcam), NFATc4 (1:200; sc-13036, Santa Crus Biotechnology Inc.), Rcan1 (1:1,000; SAB2101967, Sigma), pERK1/2 and ERK1/2 (each 1:1,000; #4370 and #9107, Cell Signaling Technology), connective tissue growth factor (Ctgf) (1:1,000; ab6992, Abcam), and GAPDH (1:1,000; #2118, Cell Signaling Technology) were used as primary antibodies in 5% BSA in LI-COR blocking buffer/TBS (1:2) over night, and IRDye® secondary goat-anti-mouse and goat-anti-rabbit (LI-COR Biosciences) were used as secondary antibodies. Primary antibodies to collagen 1 (1:1,000; ab34710, Abcam), MMP2 (1:500; ab92536, Abcam), and TGF-β1 (1:500; ab179695, Abcam) were used in 5% milk in TBS-T over night followed by using HRP-labeled secondary antibodies and enhanced chemiluminescent substrate. The Odyssey Imager (LI-COR Biosciences) was used for protein detection and quantification.

Statistical Analysis

Data are presented as mean ± SEM if not indicated otherwise. Comparison between groups of *Hyp* or *kl/kl* mice and its respective WT littermates were done by unpaired *t*-test in case of normally distributed data or Mann–Whitney *U* test in case of non-Gaussian distributions, respectively (GraphPad Prism Software version 6.0). Different groups of NRVM were compared by one-way ANOVA and Bonferroni's multiple comparison *post hoc* tests. Two-tailed *P* values of <0.05 were considered statistically significant.

RESULTS

Circulating Fgf23 Levels and Endogenous Cardiac Fgf23 Synthesis Are Elevated in *Hyp* and *kl/kl* Mice

First, we investigated circulating and cardiac levels of Fgf23 in *Hyp* and *kl/kl* mice. *Hyp* mice presented with 10-fold higher levels of both serum C-term and intact Fgf23 when compared to WT littermates. In *kl/kl* mice, circulating C-term and intact Fgf23 concentrations were 2,000- and 3,300-fold higher compared to respective WT controls (**Figures 1A,B**). In the heart tissue of *Hyp* and *kl/kl* mice, endogenous cardiac *Fgf23* mRNA expression and full-length biological active Fgf23 protein were significantly upregulated compared to respective WT controls (**Figures 1C,D**). Thus, both mouse models showed elevated circulating and cardiac Fgf23 levels, although serum Fgf23 concentrations appeared to be higher in *kl/kl* compared to *Hyp* mice.

Homozygous *kl/kl*, but Not *Hyp* Mice, Develop Cardiac Hypertrophy

Next, we investigated the development of cardiac hypertrophy in both mouse models. Due to growth retardation caused by abnormal mineral metabolism in *Hyp* mice, we calculated the relative heart weight on the basis of heart weight to body weight ratio. When compared to WT controls, the relative heart weight of *kl/kl* mice was 0.84 ± 0.15 mg/g higher (**Figure 2A**). In addition, *kl/kl* mice presented with 282 ± 13 µm^2 cross-sectional area of individual cardiac myocytes compared to 189 ± 14 µm^2 in WT

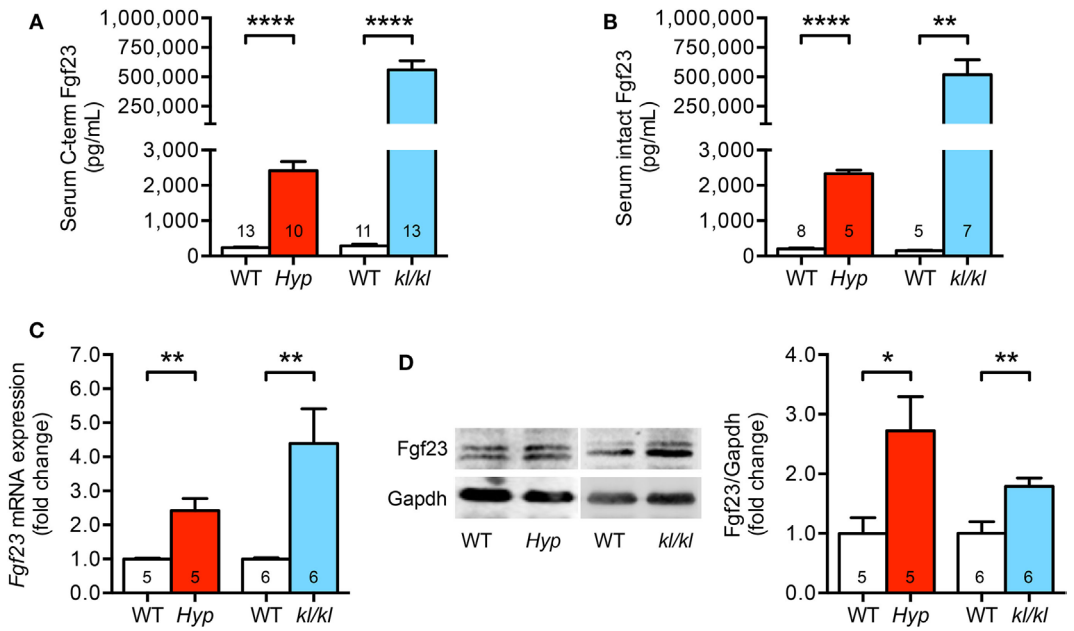

FIGURE 1 | Serum Fgf23 levels and cardiac Fgf23 expression are elevated in *Hyp* and *kl/kl* mice. **(A)** Increased serum C-term Fgf23 levels in both *Hyp* and *kl/kl* mice as compared to respective wild-type mice. **(B)** Elevation of intact Fgf23 concentrations in the serum of *Hyp* and *kl/kl* mice than in respective control mice. **(C)** *Fgf23* mRNA levels are increased in cardiac tissue of both mouse lines demonstrated by quantitative real-time PCR after normalization to *Gapdh*. **(D)** Representative immunoblot and respective quantification show the elevation of Fgf23 protein levels in total heart lysates of *Hyp* and *kl/kl* animals (Gapdh served as loading control). Values are presented as mean ± SEM; numbers in each bar graph represent the *n*-values used for the respective measurement; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

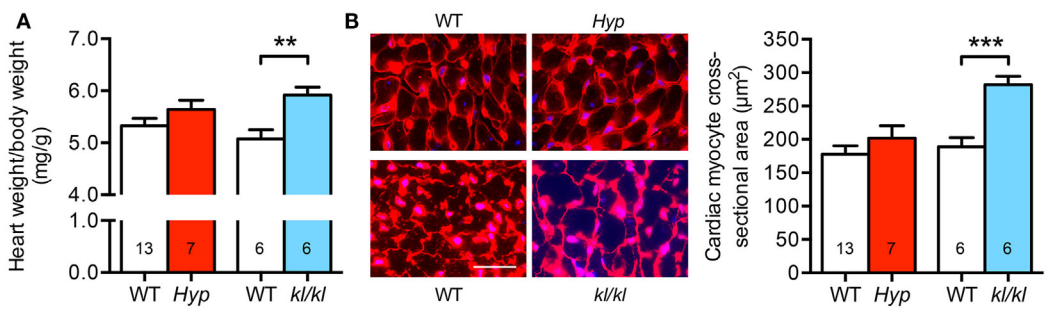

FIGURE 2 | Development of cardiac hypertrophy in *kl/kl* mice. **(A)** The relative heart weight to body weight ratio is increased only in *kl/kl* mice. **(B)** Immunofluorescence co-staining for wheat germ agglutinin (WGA) Alexa Fluor555 (red) and cell nuclei (blue) demonstrates enlarged size of cardiac myocytes in *kl/kl* mice but not in *Hyp* mice (magnification, 63×; scale bar, 25 μm). Values are presented as mean ± SEM; numbers in each bar graph represent the *n*-values used for the respective measurement; $^{**}P < 0.01$, $^{***}P < 0.001$.

littermates demonstrated by WGA staining of the myocyte cell borders in heart tissue sections. In contrast, *Hyp* mice showed unaltered cardiac myocytes cell size compared to WT littermates (202 ± 19 versus 178 ± 13 μm²; $P = 0.287$) (**Figure 2B**). Thus, it appears that only *kl/kl* mice display the development of cardiac hypertrophy.

Cardiac-Specific Fgfr4/Calcineurin/Nfat Signaling Is Only Activated in *kl/kl* Mice

Since Fgfr1 and Fgfr4 are the main Fgfrs expressed in the myocardial tissue of humans and rodents (27, 29), and each induces pathological cardiac remodeling through a different pathway, we next wanted to know whether both receptors were altered in the heart of *Hyp* and *kl/kl* mice. Cardiac *Fgfr1* mRNA levels were unchanged in *kl/kl* mice, but tended to be lower in *Hyp* mice; however, the difference did not reach statistical significance ($P = 0.184$) (**Figure 3A**). In contrast, cardiac *Fgfr4* mRNA expression was 3.8 ± 0.3-fold upregulated in *kl/kl* mice compared to controls. In *Hyp* mice, a trend of *Fgfr4* elevation was recognized when compared to their respective WT controls (2.4 ± 0.7-fold; $P = 0.097$) (**Figure 3B**). Calcineurin protein levels, activated by Fgfr4 *via* PLCγ, were 1.6 ± 0.1-fold enhanced in heart tissue lysates from *kl/kl* mice ($P = 0.017$), but not from *Hyp* mice

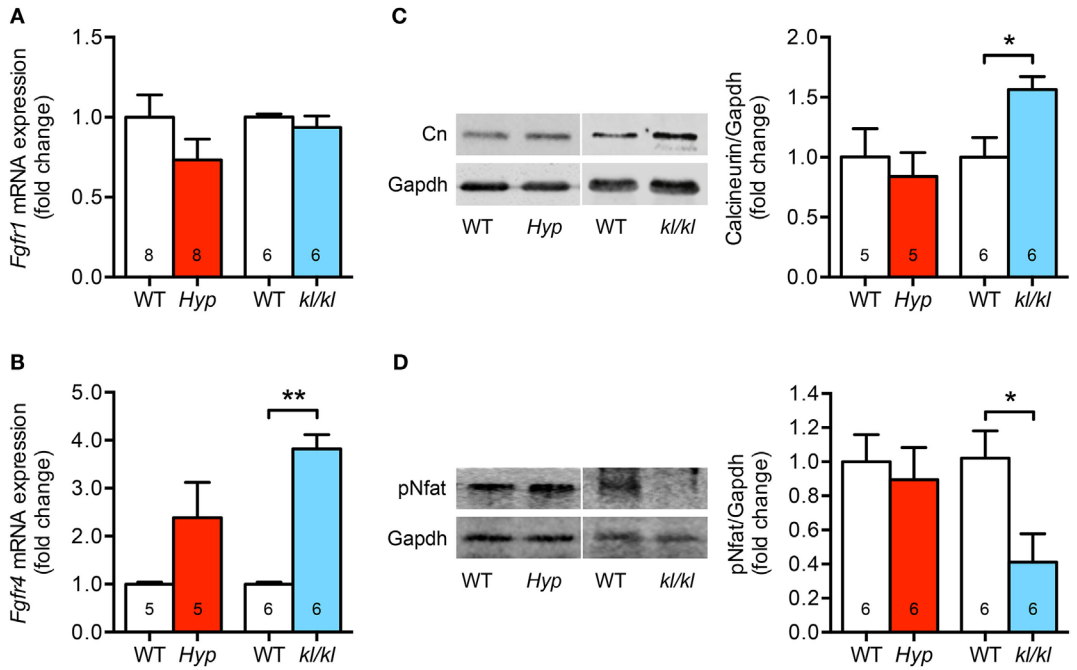

FIGURE 3 | The cardiac Fgfr4/calcineurin/nuclear factor of activated T cell signaling is activated in *kl/kl* mice. **(A)** Quantitative real-time PCR analysis reveals no significant differences in *Fgfr1* mRNA expression in cardiac tissue from both mouse lines compared to their respective wild-type controls. **(B)** *Fgfr4* mRNA expression is elevated in hearts from *kl/kl* mice as well as *Hyp* mice; however, the differences are statistically significant only for the *kl/kl* animals. **(C)** Representative immunoblot and quantification indicate an upregulation of cardiac calcineurin protein expression only for *kl/kl* mice (Gapdh served as loading control). **(D)** Representative immunoblot and quantification of Nfat activation show reduced Nfat phosphorylation in cardiac tissue from *kl/kl* mice. Values are presented as mean ± SEM; numbers in each bar graph represent the *n*-values used for the respective measurement; $^{*}P < 0.05$, $^{**}P < 0.01$.

(**Figure 3C**). Moreover, the transcription factor Nfat was clearly de-phosphorylated (0.41 ± 0.16-fold; $P = 0.027$) and thereby activated in heart tissue only from *kl/kl* mice compared to WT littermates (**Figure 3D**). Taken together, the pro-hypertrophic Fgfr4/calcineurin/NFAT signaling pathway is induced in *kl/kl*, but not in *Hyp* mice.

Pro-Hypertrophic NFAT Target Markers Are Upregulated in *kl/kl* but Not in *Hyp* Mice

In order to evaluate pro-hypertrophic genes targeted by activated calcineurin/NFAT pathway, we analyzed the expression of regulator of calcineurin 1 (Rcan1), brain natriuretic peptide (BNP), atrial natriuretic peptide (ANP), and both alpha- and beta-myosin heavy chain (aMHC, bMHC) in myocardial tissue of these two mouse models. In *kl/kl* mice, cardiac Rcan1 expression was 2.1-fold upregulated on both mRNA and protein level when compared to WT controls but not in *Hyp* mice (**Figures 4A,B**). The switch to a fetal cardiac gene expression pattern was only detected in *kl/kl* mice demonstrated by an enhanced *bMHC* to *aMHC* ratio when compared to respective WT littermates (**Figure 4C**). Moreover, the mRNA expression of the pro-hypertrophic factors *ANP* and *BNP* was clearly induced in *kl/kl* mice (5.3 ± 1.1-fold, $P = 0.0035$; 2.0 ± 0.3-fold, $P = 0.0094$) (**Figures 4D,E**). Interestingly, *BNP* levels were even 0.6-fold lower in *Hyp* mice in comparison to their WT controls ($P = 0.010$).

Only klotho Hypomorphic Mice Display Enhanced Cardiac Fibrosis

One major characteristic of pathologic cardiac hypertrophy is the concomitant development of myocardial fibrosis (3, 41). Therefore, we next investigated collagen synthesis and remodeling as well as the expression levels of fibrosis-related molecules involved in the transforming growth factor-beta (Tgf-β) signaling cascade. Demonstrated by picrosirius red staining of myocardial tissue sections, the accumulation of fibrillar collagens was clearly increased in *kl/kl* mice but not in *Hyp* mice (**Figure 5A**). This was confirmed by a 2.0-fold enhanced mRNA expression of both collagen 1 (*Col1*) and matrix metallopeptidase 2 (*Mmp2*) only in *kl/kl* mice compared to WT littermates (**Figures 5B,C**). One major pro-fibrotic pathway in cardiomyopathy among others is the induction of connective tissue growth factor (Ctgf) *via* Tgf-β1-mediated activation of extracellular signal-regulated kinases (ERK)1/2 (41–43). Only in *kl/kl* mice, *Tgfb1* mRNA levels were 3.0-fold upregulated compared to respective WT controls (**Figure 5D**). The enhanced pro-fibrotic gene expressions in *kl/kl* mice were confirmed by higher Col1, Mmp2, and Tgf-β1 protein expressions in myocardial tissue of *kl/kl* mice but not in *Hyp* mice (**Figures 5E–G**). Moreover, ERK1/2 was activated in *kl/kl* mice demonstrated by a 2.6-fold enhanced phosphorylation in total heart tissue lysates (**Figures 5H,I**). Finally, Ctgf protein expression was 1.5-fold induced in *kl/kl* mice but not in *Hyp* mice (**Figures 5H,J**). In summary, interstitial cardiac fibrosis was only present in *kl/kl* mice.

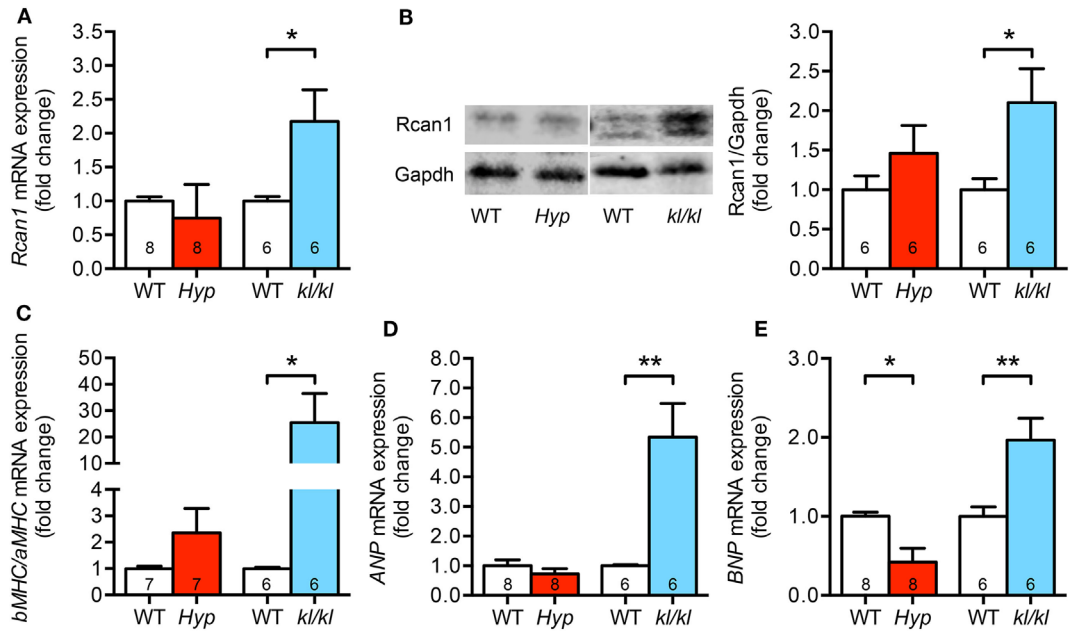

FIGURE 4 | *Kl/kl* mice show an upregulation of cardiac pro-hypertrophic nuclear factor of activated T cell target genes. **(A,B)** Rcan1 expression is increased only in cardiac tissue from *kl/kl* mice demonstrated by quantification of mRNA levels by real-time PCR and protein levels by western blotting. **(C)** Quantification of *bMHC* and *aMHC* mRNA expression shows the induction of fetal gene program in heart tissue from *kl/kl* mice but not from *Hyp* mice. **(D)** *ANP* mRNA expression is induced in the myocardium of *kl/kl* mice in relation to respective wild-type animals. **(E)** Cardiac *BNP* mRNA expression is reduced in *Hyp* mice, but upregulated in *kl/kl* mice. Values are presented as mean ± SEM; numbers in each bar graph represent the *n*-values used for the respective measurement; $^{*}P < 0.05$, $^{**}P < 0.01$.

Mineral Metabolism Differs Between *Hyp* and *kl/kl* Mice

As we presented in this study, circulating Fgf23 levels and cardiac Fgf23 synthesis were significantly enhanced in both *Hyp* and *kl/kl* mice. However, only *kl/kl* mice developed cardiac hypertrophy and fibrosis. To identify additional causes mediating pathologic cardiac remodeling in *kl/kl* mice and to evaluate why *Hyp* mice might be protected from cardiovascular disease, we next investigated parameters of mineral metabolism. Serum calcium levels were normal to slightly reduced in *Hyp* mice (8.7 ± 0.8 versus 9.6 ± 0.5 mg/dL; $P = 0.327$) but significantly enhanced in *kl/kl* mice compared to WT littermates (11.3 ± 0.5 versus 9.5 ± 0.3 mg/dL; $P = 0.007$) (**Figure 6A**). In addition, *Hyp* mice displayed hypophosphatemia (5.6 ± 0.5 versus 8.7 ± 0.3 mg/dL; $P < 0.0001$) while *kl/kl* mice were hyperphosphatemic (9.9 ± 0.6 versus 7.4 ± 0.2 mg/dL; $P = 0.0012$) (**Figure 6B**). Furthermore, *Hyp* mice showed 6.5-fold enhanced serum PTH concentrations compared to WT animals (**Figure 6C**), whereas previous reports describe suppressed PTH levels in *kl/kl* mice (44, 45). Others and we showed previously that *Hyp* mice are deficient for 1,25D, whereas *kl/kl* mice have significantly higher serum 1,25D levels (37, 44–46). Finally, *Hyp* mice showed markedly reduced renal *Klotho* mRNA expression levels (0.6 ± 0.07-fold; $P = 0.0012$), which were barely detectable in renal tissue of *kl/kl* mice (**Figure 6D**). Taken together, despite enhanced circulating Fgf23 levels in both mouse models, serum calcium, phosphate, and 1,25D levels were increased in *kl/kl* mice. In *Hyp* mice, serum calcium was unchanged and serum phosphate and 1,25D levels were decreased compared to their WT littermates. In contrast, serum PTH levels were increased in *Hyp* mice, but suppressed in *kl/kl* mice.

Calcium and Phosphate Stimulate Hypertrophic Cell Growth and Induce Pro-Hypertrophic Genes in Isolated Cardiac Myocytes

Since both animal models significantly differ with respect to serum calcium, phosphate, PTH, and 1,25D levels but only *kl/kl* mice developed cardiac remodeling processes (**Table 2**), we next investigated whether each single parameter was able to affect the endogenous *Fgf23* expression and to promote cardiac hypertrophy *in vitro*. Therefore, we stimulated NRVM, which do not express klotho, with calcium, phosphate, PTH, and 1,25D and evaluated hypertrophic growth of individual cardiac myocytes, changes of endogenous *Fgf23* expression, and finally induction of pro-hypertrophic markers. Treatment of NRVM with calcium, phosphate, and PTH but not with 1,25D significantly enhanced cardiac myocytes cross-sectional area demonstrated by immunocytochemical staining with anti-α-actinin antibody followed by quantification of the cell size (**Figures 7A,B**). Interestingly, *Fgf23* mRNA levels of NRVM were significantly induced by calcium and phosphate treatment but neither by PTH nor by 1,25D (**Figure 7C**), which is in contrast to their

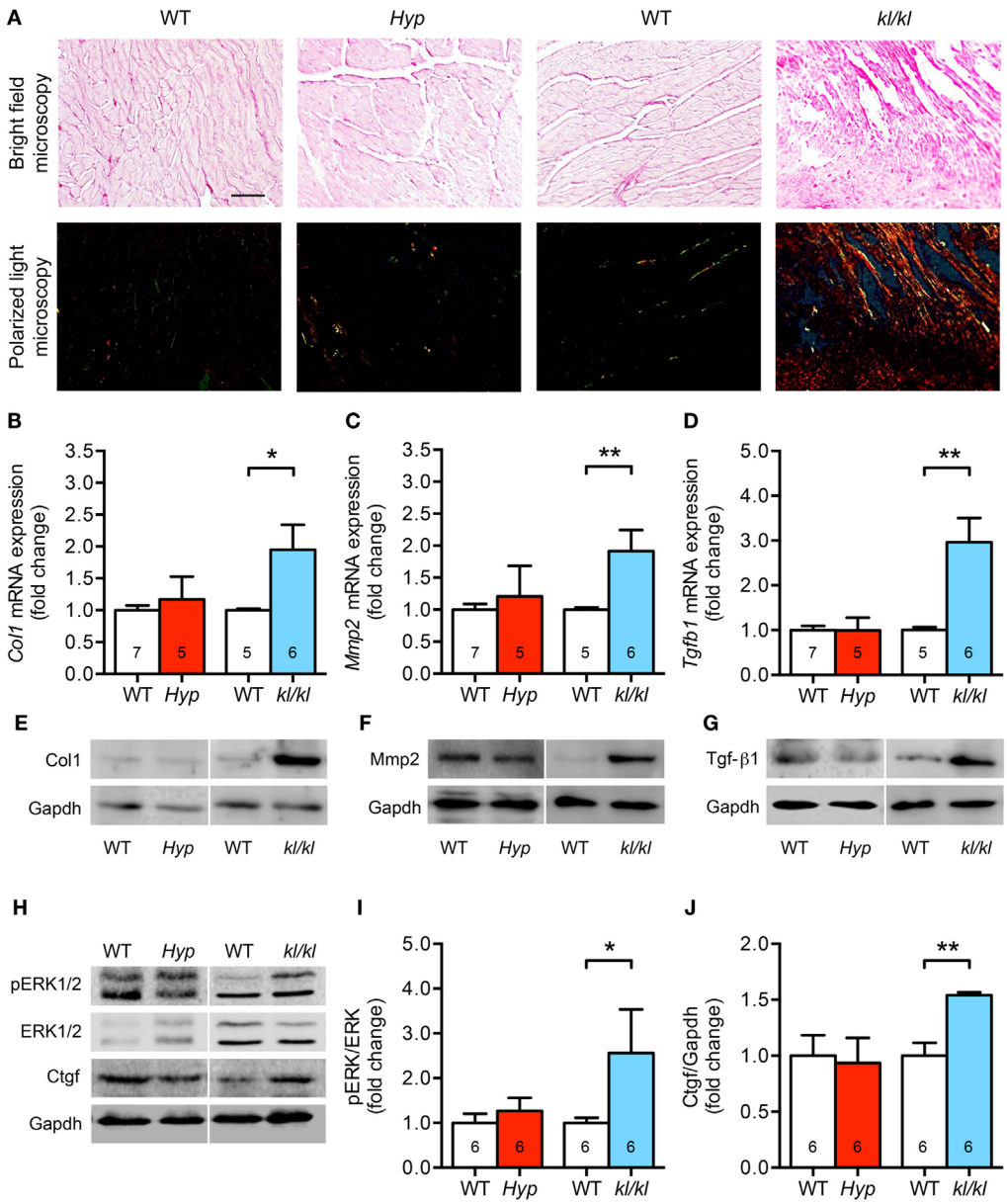

FIGURE 5 | Development of cardiac fibrosis in *kl/kl* mice but not in *Hyp* mice. **(A)** Representative images of murine myocardial sections stained for fibrillar collagens by picrosirius red demonstrate increased accumulation of fibrillar collagens in *kl/kl* mice but not in *Hyp* mice (magnification, 20×; scale bar, 50 μm). **(B)** The mRNA levels of *Col1* are higher in heart tissue of *kl/kl* mice and unchanged in *Hyp* animals. **(C)** Likewise, *kl/kl* mice show an enhanced *Mmp2* mRNA expression. **(D)** The mRNA expression of *Tgfb1* is significantly different between both mouse lines whereby it is upregulated in *kl/kl* animals. The protein expression of **(E)** Col1, **(F)** Mmp2, and **(G)** Tgf-β1 in heart tissue lysates is markedly enhanced in *kl/kl* mice but not in *Hyp* mice. **(H)** ERK1/2 is only activated in *kl/kl* mice demonstrated by increased phosphorylated protein levels in immunoblot analysis followed by **(I)** densitometric quantification. **(J)** The levels of Ctgf protein are increased in *kl/kl* mice but not in *Hyp* mice. Values are presented as mean ± SEM; numbers in each bar graph represent the *n*-values used for the respective measurement; $^{*}P < 0.05$, $^{**}P < 0.01$.

Fgf23-stimulating properties reported for bone (9, 47). *Rcan1* mRNA expression was significantly induced by treatment of NRVM with phosphate and tended to be increased by calcium treatment, a difference, however, not reaching statistical significance (**Figure 7D**). Moreover, the mRNA levels of *bMHC*, *BNP*, and *ANP* were significantly induced by both calcium and phosphate but not significantly modified after stimulation with PTH or 1,25D (**Figures 7E–G**). Taken together, among the altered parameters of mineral metabolism differing between *Hyp* and *kl/kl* mice, only calcium and phosphate clearly induced cardiac *Fgf23* expression and promoted myocytes hypertrophy *in vitro*, suggesting that elevated phosphate and calcium levels may promote Fgf23-mediated cardiac hypertrophy in states of klotho deficiency.

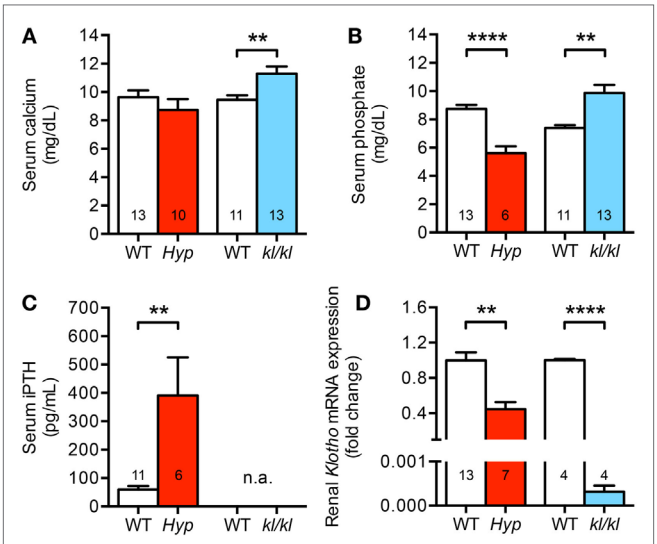

FIGURE 6 | Different mineral metabolism between *Hyp* and *kl/kl* mice. **(A)** Serum calcium levels are normal to slightly reduced in *Hyp* mice and elevated in *kl/kl* animals. **(B)** *Hyp* mice are hypophosphatemic and *kl/kl* mice display hyperphosphatemia. **(C)** Serum parathyroid hormone (PTH) levels in *Hyp* mice are extensively elevated. For *kl/kl* mice, the PTH levels in the serum were not available (n.a.). **(D)** Renal *Klotho* mRNA expression is reduced in *Hyp* mice than in respective wild-type mice and barely detectable in *kl/kl* mice. Values are presented as mean ± SEM; numbers in each bar graph represent the *n*-values used for the respective measurement; $^{**}P < 0.01$, $^{****}P < 0.0001$.

TABLE 2 | Differences between *Hyp* and *kl/kl* mice regarding characteristics of cardiovascular and kidney disease, cardiac FGF23/FGFR4 system, and parameters of mineral metabolism.

Characteristics	*Hyp* mouse	*kl/kl* mouse
Cardiac hypertrophy	–	+
Cardiac fibrosis	–	+
Kidney injury (48, 49)	–	–
Blood pressure (44, 50)	↑	↓
Calcification (44, 51)	–	+
Serum Fgf23	↑	↑↑
Cardiac Fgf23	↑	↑
Cardiac Fgfr4	↔	↑
Renal klotho	↓	↓↓
Serum calcium	↔ ↓	↑
Serum phosphate	↓	↑
Serum parathyroid hormone (44, 51)	↑	↓
Serum 1,25D (37, 44, 51)	↓	↑↑

DISCUSSION

High FGF23 levels and klotho deficiency are postulated as key risk factors for the development of uremic cardiomyopathy (2, 23, 25, 31, 52) and increase of FGF23 is further associated with poor outcome in patients with heart failure, cardiogenic shock, or arrhythmia at normal kidney function (4–8). Even though FGF23 directly targets the heart and contributes to hypertrophy and fibrosis independent of klotho (27, 28), it is not known whether the increase of FGF23 in parallel with reduction of klotho alone is sufficient to lead to CVD. Here, we show that enhanced FGF23 synthesis in *kl/kl* mice led to the development of cardiac hypertrophy and fibrosis while *Hyp* mice, despite of high circulating and cardiac FGF23 levels and reduced renal klotho expression, did not show any cardiac phenotype. This suggests that elevated FGF23 levels in the presence of klotho deficiency do not necessarily promote cardiac toxicity of FGF23 *in vivo per se*. Among additional key factors of altered mineral metabolism in *Hyp* and *kl/kl* mice, only calcium and phosphate stimulated endogenous FGF23 expression in cultured cardiac myocytes and induced pro-hypertrophic gene expression resulting in hypertrophic cell growth *in vitro*. The present results suggest that the differences in serum calcium and phosphate levels between the two mouse models may contribute to the effects on cardiac hypertrophy and fibrosis at high FGF23 levels. Concomitant normal serum calcium levels and hypophosphatemia may prevent *Hyp* mice from the development of LVH, whereas concomitant hypercalcemia and hyperphosphatemia may have further promoted pathological cardiac remodeling in *kl/kl* mice.

In the present study, both *Hyp* and *kl/kl* mice presented with enhanced circulating levels of C-term and intact FGF23 as well as upregulated cardiac FGF23 synthesis in addition to reduced renal *Klotho* expression, although both alterations were more pronounced in *kl/kl* mice. However, *kl/kl* mice, but not *Hyp* mice, developed cardiac hypertrophy, demonstrated by an increase in relative heart weight and cardiac myocyte cross-sectional area. In addition, we demonstrated for the first time that FGFR4 is upregulated in heart tissue of *kl/kl* mice and this resulted in activation of calcineurin/NFAT signaling and finally induction of pro-hypertrophic NFAT target genes *Rcan1*, *bMHC*, *ANP*, and *BNP*. Furthermore, *kl/kl* mice further displayed enhanced myocardial fibrosis with concomitant induction of collagen 1, Mmp2 and Tgf-β1 expression, activated ERK1/2 and Ctgf protein. Thereby, we support the findings of Hu et al. showing that pERK1/2 is elevated and myocardial fibrosis is present in heart tissue of homozygous *kl/kl* mice as well (31). Moreover, this group investigated the Tgf-β1-mediated activation of ERK1/2 in neonatal rat cardiac myocytes and fibroblasts *in vitro*, which was only present in the absence of klotho suggesting that there is a strong connection between klotho deficiency and the induction of pro-fibrotic pathways. In addition, treatment of neonatal mouse cardiac fibroblasts with the 65 kDa soluble klotho isoform was shown to suppress myofibroblast proliferation and collagen synthesis (33). Taken together, our data suggest that the well-established cardiac FGF23/FGFR4 signaling involved in the development of pathologic cardiac hypertrophy with the parallel appearance of cardiac fibrosis is induced in *kl/kl* mice with high FGF23 levels and klotho deficiency.

Our results in homozygous *kl/kl* mice are well in line with Yang and colleagues who showed that heterozygous *kl/+* mice had elevated relative heart weight and enhanced left ventricular posterior wall thickness with reduced left ventricular internal diastolic diameter (35). In another study by Hu et al., heterozygous *kl/+* mice showed reduced ejection fraction, stroke volume, and cardiac output in addition to enlarged septum and posterior wall thickness within the diastole (31). In contrast, neither *kl/+* nor *kl/kl* mice with comparable age had baseline cardiac

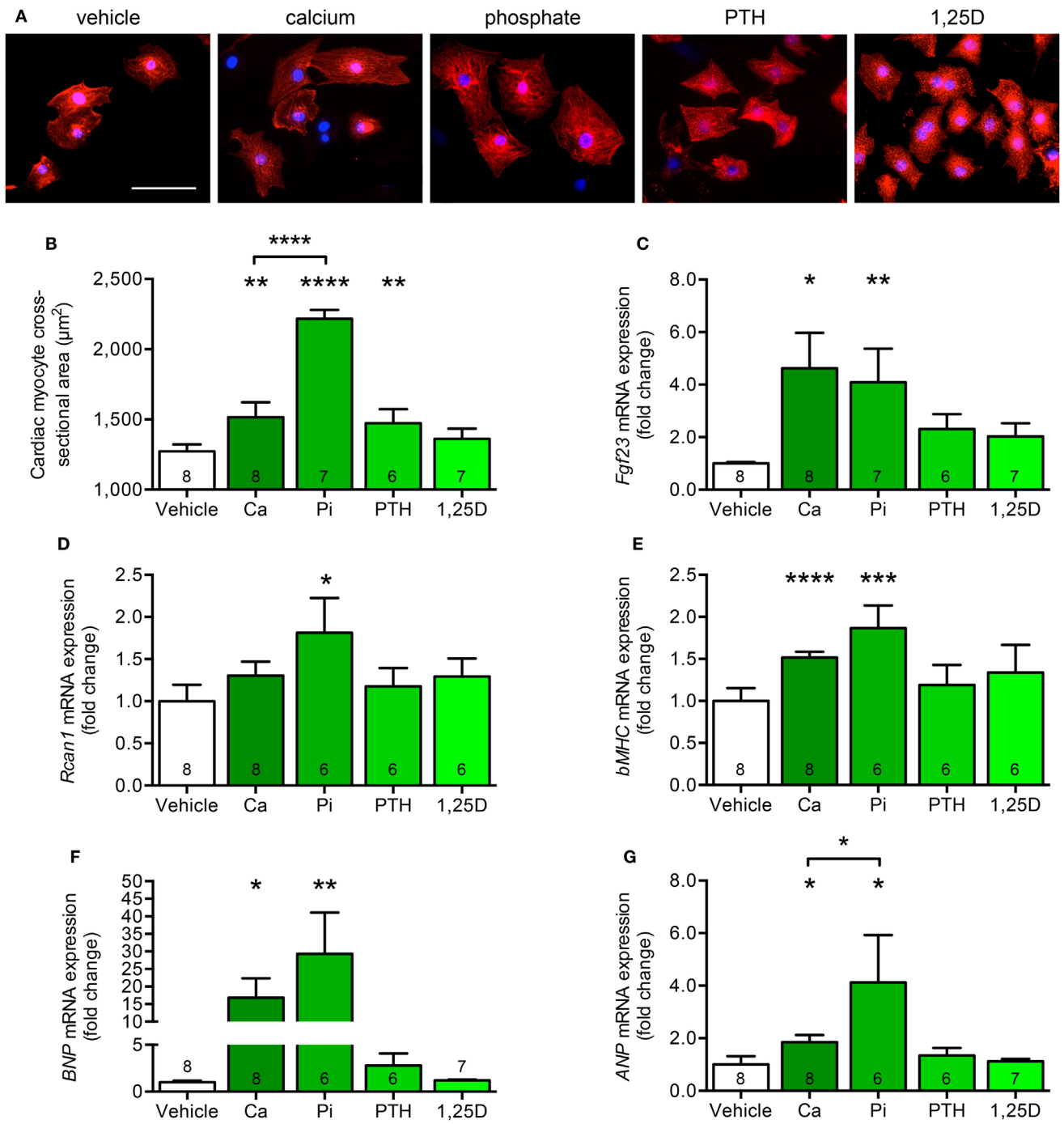

FIGURE 7 | Calcium and phosphate treatment induces cell growth and pro-hypertrophic gene expression in cultured neonatal rat ventricular myocytes (NRVM). **(A,B)** Immunofluorescence staining for sarcomeric α-actinin followed by quantification demonstrates enhanced cardiac myocyte cross-sectional area after treatment of NRVM with calcium, phosphate, and PTH, but not 1,25D (magnification, 40×; scale bar, 50 µm). **(C)** Calcium and phosphate stimulation significantly increases *Fgf23* mRNA expression in NRVM. **(D)** *Rcan1* mRNA levels are significantly enhanced by treatment of NRVM with phosphate. The mRNA levels of **(E)** *bMHC*, **(F)** *BNP*, and **(G)** *ANP* are significantly upregulated after stimulation of NRVM with calcium and phosphate. Values are presented as mean ± SEM; numbers in each bar graph represent the *n*-values used for the respective measurement; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Abbreviations: Ca, calcium; Pi, phosphate; PTH, parathyroid hormone; 1,25D, $1,25(OH)_2D_3$.

abnormalities published in other studies by Xie and colleagues (32, 53). Interestingly, according to this study, serum FGF23 and phosphate levels were similar in WT and *kl*/+ mice. The different cardiac outcome in klotho-deficient mice within these studies might be, at least partially, due to differences in dietary phosphate content ranging from 0.35 to 0.6% (31, 32, 35, 53).

The development of pathologic cardiac remodeling including hypertrophy and fibrosis is less clear in *Hyp* mice. Recently, it was described that 8-week-old *Hyp* mice showed increased relative heart weight, and administration of an FGFR1 activating antibody resulted in normalization of relative heart weight, left ventricular wall thickness, and blood pressure. Treatment of *Hyp* mice with soluble klotho ameliorated systolic, diastolic, and mean arterial blood pressure (MAP) (54). Andrukhova and colleagues showed enhanced FGF23 serum levels in 3-month-old *Hyp* mice with enhanced MAP in addition to significantly higher relative heart weight compared to WT littermates (50). Thus, both studies suggest that *Hyp* mice on a regular phosphate diet present with enhanced blood pressure and cardiac hypertrophy. However, a detailed evaluation of the cardiac phenotype in *Hyp* mice, i.e., investigation of cardiac myocyte cross-sectional area, pro-hypertrophic signaling pathways, and concomitant fibrosis, in order to evalute pathological cardiac hypertrophy in-depth, was not yet been performed in *Hyp* mice. In the present study, we investigated histological and molecular biological analyses of heart tissue in 6- to 8-week-old *Hyp* mice on a regular phosphate diet and compared our findings to their WT littermates. In contrast to the abovementioned studies (50, 54), relative heart weight was not significantly altered in *Hyp* mice in the present study, and additionally, cardiac myocyte cross-sectional area was similar to controls. Although, *Hyp* mice showed comparably increased circulating FGF23 levels and the presence of enhanced cardiac FGF23 synthesis in the present study, FGFR4 and its respective downstream signaling pathway, i.e., calcineurin/NFAT, *Rcan1*, *bMHC*, *ANP*, and *BNP*, were not induced at all. Neither accumulation of fibrillar collagens nor fibrosis-related pathways, including collagen 1 expression, Tgf-β1-mediated activation of ERK1/2, or Ctgf were induced in *Hyp* mice compared to controls. Thus, our data point out that high FGF23 levels in addition to klotho deficiency does not necessarily result in pathological cardiac remodeling *in vivo*. The fact that blood pressure is elevated in *Hyp* mice (50) while low in *kl/kl* mice (44) further supported the hypothesis that FGF23-mediated cardiac hypertrophy might be blood pressure independent.

Therefore, the question arises if alterations in the mineral metabolism may modulate the cardiac phenotype in these two mouse models. In line with previous published studies by others (37, 55) and us (46), *Hyp* mice showed normal to reduced serum calcium levels, presented with hypophosphatemia, secondary hyperparathyroidism, and vitamin D deficiency. In contrast, *kl/kl* mice were hypercalcemic and hyperphosphatemic, had elevated 1,25D levels, and suppressed serum PTH levels (44, 45). Thus, the observed differences in calcium and phosphate serum concentrations may at least partly explain the different cardiac phenotypes in the two mouse models. The importance of phosphate as a risk factor for cardiomyopathy was previously demonstrated in a variety of experimental studies showing that high-phosphate diet induced cardiac hypertrophy and fibrosis in rodents (25, 28, 56). Moreover, stimulation with phosphate induced pERK1/2, Ctgf, and collagen 1 in isolated neonatal rat cardiac fibroblasts and in addition pERK1/2, Ctgf, and pSmad2/3 in cardiac myocytes *in vitro* (31). Interestingly, co-treatment with soluble klotho only ameliorated Ctgf and collagen 1 levels in cardiac fibroblasts and Smad2/3 phosphorylation in myocytes suggesting that klotho primarily targets phosphate-induced pro-fibrotic signaling pathways. The impact of phosphate on the development of cardiac hypertrophy on cellular level was not investigated so far. Here, we present that both calcium and phosphate stimulated hypertrophic growth of cultured cardiac myocytes with upregulation of *Rcan1*, *bMHC*, *ANP*, and *BNP*, respectively. Interestingly, among the known parameters of mineral metabolism that induce FGF23 synthesis in bone (9), only calcium and phosphate upregulated endogenous *Fgf23* expression in NRVM.

Circulating FGF23 levels were considerably higher and the degree of klotho deficiency was lower in *kl/kl* mice compared to *Hyp* mice in the present study. Therefore, we cannot exclude that further stimulation of circulating FGF23, e.g., by increased phosphate load may result in pathological cardiac remodeling in *Hyp* mice despite the presence of hypophosphatemia. In addition, *kl/kl* mice presented with hypercalcemia, which was shown to stimulate cardiac myocyte hypertrophy *in vitro*, although the effects of calcium were lower compared to phosphate. However, concomitant hypercalcemia may have further promoted the cardiac phenotype in *kl/kl* mice. Furthermore, in *kl/kl* mice, the regulation of cardiac hypertrophy and fibrosis by other pathological factors cannot be ruled out (57–59). Clearly, further studies are needed to confirm an association of altered mineral metabolism at high FGF23 levels and pathological cardiac remodeling.

In extension to previously published studies, our results support the hypothesis that both altered parameters of mineral metabolism and elevated FGF23 levels contribute to cardiac hypertrophy and fibrosis in the setting of klotho deficiency, and consequently, *Hyp* mice might be protected from pathologic cardiac remodeling by the presence of concomitant hypophosphatemia and lack of hypercalcemia. In addition, high FGF23 levels are only cardiotoxic in the presence of high calcium and/or high phosphate and independent of klotho deficiency. The latter supports the concept of early initiation of phosphate lowering treatment in states of elevated FGF23 and klotho deficiency, e.g., uremia, in order to prevent pathological cardiac remodeling.

ETHICS STATEMENT

All experimental procedures were approved by the State Office committee on animal welfare Lower Saxony for *Hyp* mice and Baden-Württemberg for *kl/kl* mice, and performed in accordance with national animal protection guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

AUTHOR CONTRIBUTIONS

Design: ML-N, BR, JV, and DH; experiments: BR, MB, JN, and IV; experimental support and critical revision of the manuscript: IA, JV, FL, JH, and SK; data evaluation: ML-N, BR, MB, JN, and IV; preparation of manuscript: ML-N, BR, and DH.

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Augmented Fibroblast Growth Factor-23 Secretion in Bone Locally Contributes to Impaired Bone Mineralization in Chronic Kidney Disease in Mice

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Chronic kidney disease-mineral and bone disorder (CKD-MBD) is a systemic disorder of mineral and bone metabolism caused by CKD. Impaired bone mineralization together with increased bony secretion of fibroblast growth factor-23 (FGF23) are hallmarks of CKD-MBD. We recently showed that FGF23 suppresses the expression of tissue nonspecific alkaline phosphatase (TNAP) in bone cells by a Klotho-independent, FGF receptor-3-mediated signaling axis, leading to the accumulation of the mineralization inhibitor pyrophosphate. Therefore, we hypothesized that excessive FGF23 secretion may locally impair bone mineralization in CKD-MBD. To test this hypothesis, we induced CKD by 5/6 nephrectomy in 3-month-old wild-type (WT) mice and *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ (*Fgf23*/VDR) compound mutant mice maintained on a diet enriched with calcium, phosphate, and lactose. Eight weeks postsurgery, WT CKD mice were characterized by reduced bone mineral density at the axial and appendicular skeleton, hyperphosphatemia, secondary hyperparathyroidism, increased serum intact Fgf23, and impaired bone mineralization as evidenced by bone histomorphometry. Laser capture microdissection in bone cryosections showed that both osteoblasts and osteocytes contributed to the CKD-induced increase in *Fgf23* mRNA abundance. In line with our hypothesis, osteoblastic and osteocytic activity of alkaline phosphatase was reduced, and bone pyrophosphate concentration was ~2.5-fold higher in CKD mice, relative to Sham controls. In *Fgf23*/VDR compound mice lacking Fgf23, 5/6-Nx induced secondary hyperparathyroidism and bone loss. However, 5/6-Nx failed to suppress TNAP activity, and bone pyrophosphate concentrations remained unchanged in *Fgf23*/VDR CKD mice. Collectively, our data suggest that elevated Fgf23 production in bone contributes to the mineralization defect in CKD-MBD by auto-/paracrine suppression of TNAP and subsequent accumulation of pyrophosphate in bone. Hence, our study has identified a novel mechanism involved in the pathogenesis of CKD-MBD.

Keywords: fibroblast growth factor-23, chronic kidney disease, bone mineralization, osteocytes, pyrophosphate, alkaline phosphatase

INTRODUCTION

The progressive decline in kidney function associated with chronic kidney disease (CKD) leads to complex changes in mineral and bone metabolism. These changes include alterations in circulating biomarkers, metabolic bone disease, and ectopic, especially vascular calcifications. The term chronic kidney disease-mineral and bone disorder (CKD-MBD) has been coined to better illustrate the association between altered mineral and bone metabolism and cardiovascular morbidity in CKD patients (1). CKD-MBD encompasses metabolic bone disease, vascular calcifications, as well as changes in blood biochemistry such as secondary hyperparathyroidism, hyperphosphatemia, lowered levels of the vitamin D hormone 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], chronic metabolic acidosis, elevated circulating Wnt (Wingless/integrated-1) inhibitors, and increased concentrations of intact fibroblast growth factor-23 (FGF23) (1, 2).

Fibroblast growth factor-23 is a bone-derived hormone, suppressing urinary phosphate reabsorption by a downregulation of apical membrane expression of sodium phosphate co-transporters in proximal renal tubular epithelium (3, 4). Moreover, FGF23 is a strong transcriptional suppressor of proximal tubular 1α-hydroxylase, the key enzyme for $1,25(OH)_2D_3$ production (3). In distal renal tubules, FGF23 augments renal calcium and sodium reabsorption (5, 6). Only the intact form of FGF23 is biologically active (7). High-affinity binding of FGF23 requires the concomitant presence of FGF receptors (FGFRs) and of the co-receptor αKlotho (Klotho) in target tissues (8, 9).

Increased blood concentrations of intact FGF23 are one of the earliest biomarkers of CKD. The decline in kidney function in CKD patients causes the circulating intact FGF23 levels to rise. In advanced renal failure, FGF23 serum concentrations can reach levels 1,000-fold above the normal range (10, 11). The reason underlying the upregulation of circulating intact FGF23 in CKD patients is still not entirely clear. One component may be reduced renal elimination through impaired glomerular filtration rate (GFR) (12), but lower renal elimination alone is insufficient to explain the pronounced rise in intact FGF23 in CKD patients. Parathyroid hormone (PTH) and extracellular phosphate are known stimulators of FGF23 secretion (13). However, several studies in human CKD patients have shown that the early increase in circulating intact FGF23 occurs independent of hyperphosphatemia and increased PTH (14). It is conceivable that the CKD-induced increase in circulating pro-inflammatory cytokines may play an important role in causing the rise in FGF23 in patients with early CKD (15, 16).

In bone, the combination of secondary hyperparathyroidism, low $1,25(OH)_2D_3$, hyperphosphatemia, and metabolic acidosis leads to a syndrome named renal osteodystrophy. The hallmarks of renal osteodystrophy are altered bone turnover (high or low) and impaired bone mineralization (1). Bone turnover in CKD patients is thought to be mainly driven by secondary hyperparathyroidism (1, 17). The mechanisms underlying the impaired bone mineralization are less clear, and may involve secondary hyperparathyroidism, metabolic acidosis, increased circulating Wnt inhibitors, or uremic toxins (1). We recently showed that FGF23 not only targets the kidney in an endocrine manner but also acts as an auto-/paracrine regulator of bone mineralization by suppressing tissue nonspecific alkaline phosphatase (TNAP) expression in osteoblasts and osteocytes (18). One of the substrates of TNAP in bone is pyrophosphate, which is produced by osteoblasts and osteocytes for the regulation of bone mineralization. Pyrophosphate is a potent inhibitor of mineralization by binding to hydroxyapatite crystals (19). In bones of *Hyp* mice, which are characterized by profoundly increased endogenous bony production of Fgf23, we found suppressed osteocytic TNAP activity and accumulation of pyrophosphate (20). Both effects could be rescued by bone-specific ablation of *Fgf23* (20). This finding led us to hypothesize that the CKD-driven upregulation of bony Fgf23 secretion may also locally contribute to impaired bone mineralization in CKD-MBD by suppressing TNAP, leading to accumulation of pyrophosphate. To test this hypothesis, we induced CKD by 5/6-nephrectomy (5/6-Nx) in wild-type (WT) and *Fgf23* deficient mice, and examined mineral and bone metabolism, 8 weeks after 5/6-Nx.

MATERIALS AND METHODS

Animals

All animal studies were approved by the Ethical Committee of the University of Veterinary Medicine, Vienna and by the Austrian Federal Ministry of Science and Research and were undertaken in strict accordance with prevailing guidelines for animal care (permit No. BMWF-68.205/0054-II/3b/2013). All efforts were made to minimize animal suffering. All experiments were performed on 3-month-old male WT and $Fgf23^{-/-}$/$VDR^{\Delta/\Delta}$ mice on C57BL/6N genetic background. To generate $Fgf23^{-/-}$/$VDR^{\Delta/\Delta}$ mice, $VDR^{+/\Delta}$/$Fgf23^{+/-}$ double heterozygous mice were mated, and the offspring was genotyped by multiplex PCR using genomic DNA extracted from the tail as described (21, 22). All mice were kept at 24°C with a 12/12 h light/dark cycle, and were allowed free access to tap water and a diet containing 2.0% calcium, 1.25% phosphorus, 20% lactose, and 600 IU vitamin D/kg (Ssniff, Soest, Germany). This diet was shown to normalize mineral homeostasis in vitamin D deficient mice and rats, and in mice lacking a functional VDR (23). Urine was collected in metabolic cages for a 12-h period overnight before necropsy. All mice received subcutaneous calcein double labeling (20 mg/kg, s.c.), 4 and 2 days before necropsy. At necropsy, mice were exsanguinated from the abdominal V. cava under general anesthesia (ketamine/xylazine, 100/6 mg/kg i.p.) for serum collection.

5/6-Nephrectomy (5/6-Nx) Model

The 5/6 nephrectomy (5/6 Nx) was performed by a two-stage procedure (24) under isoflurane anesthesia. Pain was managed by s.c. injections of buprenorphine, metamizol, and meloxicam. In addition, the animals received the analgesic piritramid *via* the drinking water for 3 days postsurgery, starting 6 h postsurgery. At the first stage (week 1), the left kidney was exposed *via* a left flank incision, and decapsulated to avoid ureter and adrenal damage. Thereafter, the upper and lower poles were resected. Bleeding was controlled by microfibrillar collagen hemostasis (Gelaspon,

Chauvin Ankerpharm, Berlin). The resected upper and lower poles were weighed to ascertain 2/3-resection. One week later, the entire right kidney was removed *via* a right flank incision. In the Sham control mice, the left kidney was exposed *via* a left flank incision at step 1, and the right renal artery was identified after a right flank incision at step 2. After each flank incision, muscles and skin were appropriately repositioned and sewed.

Serum and Urine Biochemistry

Serum phosphorus and creatinine (Crea) as well as urinary creatinine were analyzed on a Cobas c111 analyzer (Roche). GFR was calculated based on the endogenous creatinine clearance (GFR = Urinary Crea/Serum Crea × Urine volume per min). Serum intact PTH (Immutopics) and serum intact Fgf23 (Kainos) were determined by ELISA.

Bone Mineral Density (BMD) Measurements

Bone mineral density of the left tibia and L2 lumbar vertebra was measured by peripheral quantitative computed tomography (pQCT) using an XCT Research M+ pQCT machine (Stratec Medizintechnik). The voxel size was 70 µm. One 0.2-mm-thick slice in the tibial shaft at 2 mm proximal to the tibiofibular junction, and three slices in the proximal tibial metaphysis located 1.5, 2, and 2.5 mm distal to the proximal tibial growth plate were measured. In the L2 vertebra, three slices were measured, one in a mid-transversal plane, and two located 0.5 mm rostral and caudal of the mid-transversal plane. BMD values of the tibial metaphysis and L2 lumbar vertebral body were calculated as the mean over three slices. A threshold of 600 mg/cm^3 were used for calculation of cortical BMD at the tibial shaft, and a threshold of 450 mg/cm^3 was used for the discrimination between trabecular and cortical BMD in both the tibial metaphysis and the L2 vertebra.

Bone Histology and Histomorphometry

Proximal tibiae were fixed in 4% paraformaldehyde for 24 h, processed for methylmethacrylate embedding, sectioned at 3 µm thickness using a HM 355S microtome (Microm, Walldorf, Germany), and stained with von Kossa/McNeal and for tartrate resistant acid phosphatase enzyme activity as described (25). Undeplasticized and unstained sections mounted with Fluoromount (Serva) were used for calcein-based measurements. All histomorphometric measurements were made with the help of a semiautomatic system (OsteoMeasure, OsteoMetrics) as described (26).

TNAP Histochemistry

Histochemical TNAP staining was performed as described previously (20). In brief, deplastified sections were incubated with Vector Red alkaline phosphatase staining kit (Vector Laboratories) and counterstained with DAPI. The sections were analyzed using a Zeiss Axioskop 2 microscope. The fluorescence signal was quantified using Image J. Relative fluorescence of osteoblasts was quantified along the bone surface. For the quantification of relative fluorescence in osteocytes, at least 90 osteocytes per animal were used, and fluorescence was normalized to cell number.

Pyrophosphate Measurement

Femurs were cut in half at the diaphysis, and the bone marrow was flushed out. Minerals and pyrophosphate were extracted using 300 µL 1.2 M HCl at 4°C overnight under light protection. Subsequently, HCl was evaporated at 99°C within 1–2 h, and samples were re-suspended in 0.5–1 mL of assay buffer. The amount of pyrophosphate was quantified using the PhosphoWorks Fluorimetric Pyrophosphate Assay (AAT Bioquest #21611) according to the manufacturer's protocol and was normalized to wet weight of the bone.

Laser Capture Microdissection (LCM)

Distal femurs were snap-frozen in liquid nitrogen with OCT compound (Sakura Finetek, Zoeterwoude, Netherlands). 4-µm-thick cryosections were cut on a cryotome (Leica Kryostat 1720), using the cryotape method as described (27). Cryosections were quickly stained with HistoStain (Arcturus), and cancellous and cortical bone osteoblasts and osteocytes (~100–200 cells per sample each) were harvested using a Veritas (Arcturus) LCM system as described (28).

RNA Isolation and Quantitative RT-PCR

RNA was extracted from LCM-harvested samples using the PicoPure RNA isolation kit (Thermo Fisher Scientific), and RNA quality was determined using the 2100 Bioanalyzer (Agilent Technologies). After first-strand cDNA synthesis (iScript cDNA Synthesis Kit, Bio-Rad), quantitative RT-PCR was performed on a Rotor-Gene 6000 (Corbett Life Science) using SsoFastTM EvaGreen PCR kit (Bio-Rad). A melting curve analysis was done for all assays. Primer sequences are available on request. Efficiencies were examined by standard curve. Gene expression data were corrected for efficiency and normalized to ornithine decarboxylase antizyme-1 (*Oaz1*) as housekeeping gene.

Statistical Analyses

The aim of the study was to compare Sham and CKD C57BL/6 mice and Sham and CKD *Fgf23*/VDR mice. The investigators were not blinded as to the type of intervention. We used 10–12 mice per group. For some parameters, data from two identical experiments were pooled. The power analysis of the study was based on our experience with similarly designed mouse experiments, which have shown that the SD of total proximal tibial BMD is about 35 mg/cm^3 in 4- to 5-month-old male C57BL/6 mice. Based on this SD, a group size of 10–12 animals is sufficient to detect a difference of about 45 mg/cm^3 total BMD (corresponding to an about 10% change in total BMD) with a power $\beta = 80\%$ and an error $\alpha = 5\%$. Statistics were computed using Prism 7.03 (GraphPad Software Inc.). The data from two groups (Sham and CKD) were analyzed by unpaired *t*-test. Equality of variances was tested by *F*-test. In case the variances were not equal, the data were analyzed using the non-parametric Mann–Whitney *U*-test. When more than two groups were compared, the data were analyzed by one-way analysis of variance followed by Holm–Sidak's multiple comparisons test. *P* values of less than 0.05 were considered significant. The data are presented as the mean ± SEM.

RESULTS

5/6-Nephrectomy-Induced CKD Is Associated With Increased Osteoblastic and Osteocytic Fgf23 Secretion

It is known that C57BL/6 mice are relatively resistant against the development of CKD after 5/6 nephrectomy (5/6-Nx), probably due to low renin activity in this strain of mice (24, 29, 30). Therefore, all mice were maintained on a diet enriched with calcium, phosphate, and lactose. This diet was named rescue diet because it normalizes mineral homeostasis in mice with deficient vitamin D signaling (23). It is well known that phosphate-rich diets accelerate disease progression in 5/6-Nx mice (31). In the rescue diet, lactose stimulates intestinal calcium and phosphate uptake by a vitamin D-independent, paracellular mechanism (32). Eight weeks after 5/6-Nx, GFR as measured by endogenous creatinine clearance was reduced by about 60% relative to Sham controls (**Figure 1A**). Serum creatinine was distinctly increased in 5/6-Nx mice (**Figure 1A**). Furthermore, 5/6-Nx mice showed hyperphosphatemia, increased total serum alkaline phosphatase

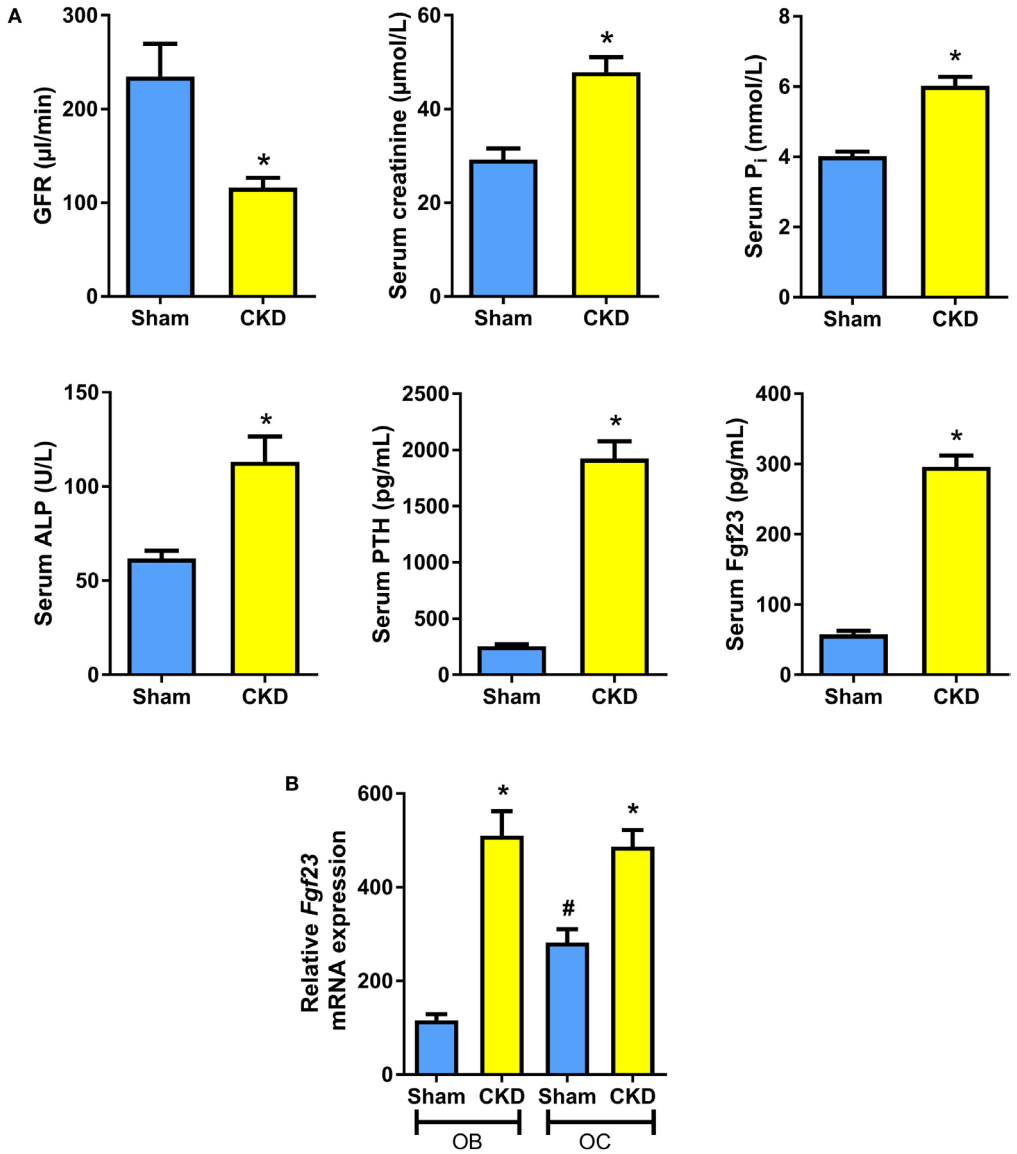

FIGURE 1 | 5/6-nephrectomy induces chronic kidney disease (CKD) and increased osteoblastic and osteocytic *Fgf23* mRNA expression in mice on high phosphate diet. **(A)** Glomerular filtration rate (GFR) (n = 12–13 each), serum creatinine (n = 12 each), serum phosphate (n = 12 each), serum alkaline phosphatase (ALP) activity (14 Sham, 20 CKD), serum parathyroid hormone (PTH) (n = 12 each), and serum intact Fgf23 (n = 5 each) in 5-month-old male C57BL/6 Sham and CKD mice on the phosphate-rich rescue diet, 8 weeks postsurgery. *P < 0.05 vs. Sham by t-test or Mann–Whitney U-test as appropriate. **(B)** *In situ* mRNA expression profiling of osteoblasts (OB) and osteocytes (OC) harvested by laser capture microdissection in 4-µm-thick distal femoral cryosections from 5-month-old male C57BL/6 Sham (n = 7–8) and CKD (n = 2) mice on rescue diet, 8 weeks postsurgery. *P < 0.05 vs. Sham, #P < 0.05 vs. Sham OB by one-way analysis of variance followed by Holm–Sidak's multiple comparisons test. Data in **(A,B)** are mean ± SEM.

(ALP) activity, severe secondary hyperparathyroidism, and elevated serum intact Fgf23 levels, relative to Sham controls (**Figure 1A**). Collectively, these data demonstrate that 5/6-Nx mice on a phosphate-rich diet develop CKD within 8 weeks postsurgery, which corresponds in severity approximately to stage 3 of human CKD (2).

It is still controversial whether osteoblasts or osteocytes are the major Fgf23-producing cells in CKD. To shed more light on this issue, we harvested osteoblasts and osteocytes in bone cryosections of Sham and CKD mice, employing LCM, a recently established technology (28). In Sham mice, *Fgf23* mRNA expression in osteocytes was about threefold higher compared with osteoblasts (**Figure 1B**). CKD induced an upregulation of *Fgf23* mRNA abundance in both osteoblasts and osteocytes (**Figure 1B**). Although it is clear that Fgf23 protein secretion may not be directly related to changes in *Fgf23* mRNA expression, our findings suggest that both cell types are involved in the CKD-driven increase in bony Fgf23 production.

CKD Mice Are Characterized by Osteopenia and Impaired Bone Mineralization

In agreement with the well-known fact that the CKD is associated with metabolic bone disease, CKD mice in our study were characterized by small, but significant reductions in total, cortical/subcortical, and trabecular BMD at the spine and at the proximal tibial metaphysis compared with Sham mice (**Figures 2A,B**).

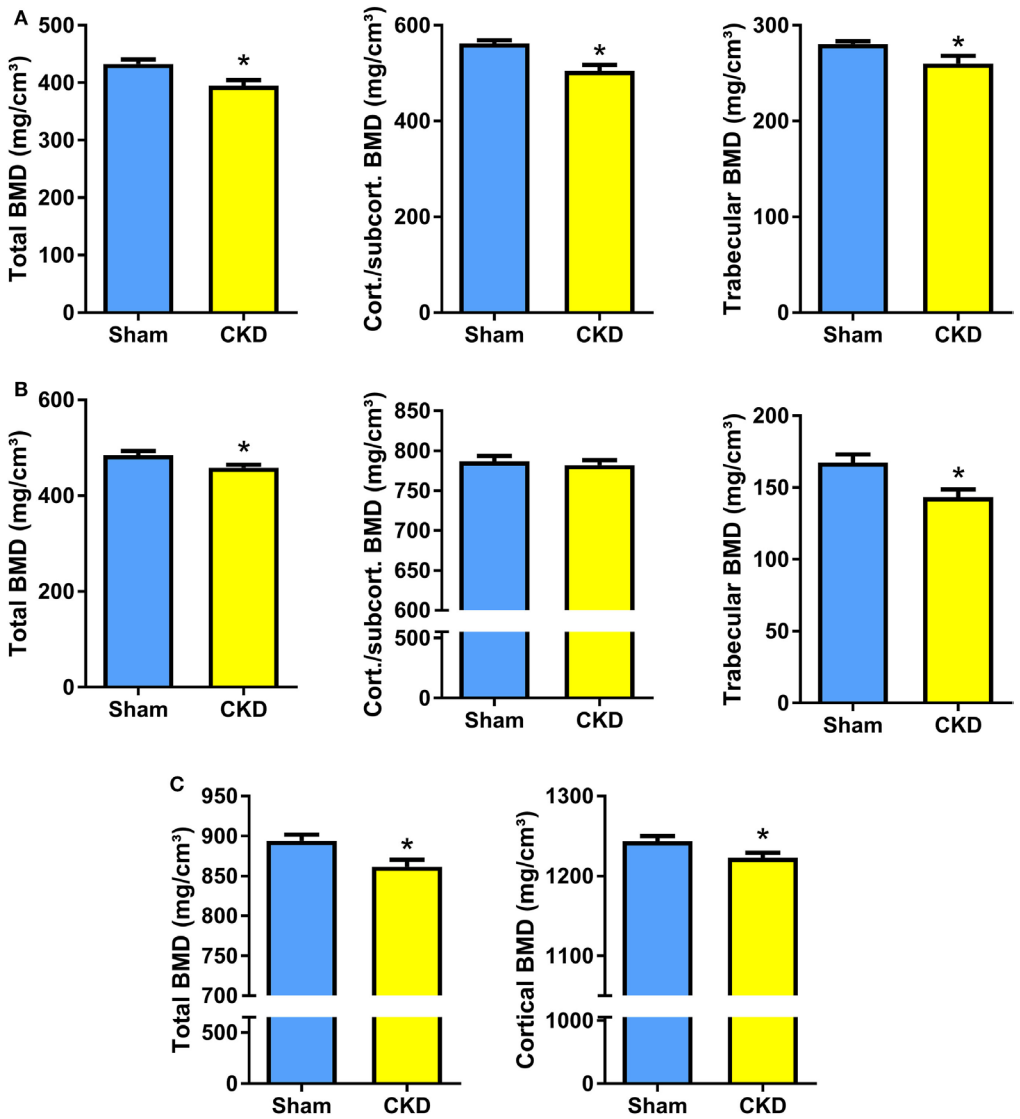

FIGURE 2 | Chronic kidney disease (CKD) induces bone loss in the axial and appendicular skeleton. **(A–C)** Total, cortical/subcortical, and trabecular bone mineral density (BMD) of the L2 lumbar vertebra (17 Sham, 7–9 CKD for each parameter) **(A)** and of the proximal tibial metaphysis (n = 16–17 each) **(B)**, as well as total and cortical BMD of the tibial shaft (n = 17 each) **(C)** in 5-month-old male C57BL/6 Sham and CKD mice on rescue diet, 8 weeks postsurgery. Data in **(A–C)** are mean ± SEM. *P < 0.05 vs. Sham by t-test or Mann–Whitney U-test as appropriate.

In addition, total and cortical BMD at the tibial shaft were lower in CKD mice, relative to Sham controls (**Figure 2C**). Cancellous bone histomorphometry showed that the CKD-induced osteopenia was associated with distinctly increased osteoid volume, osteoid surface, osteoblast surface, and osteoclast numbers (**Figures 3A,C**). Overt signs of impaired bone mineralization were absent in CKD

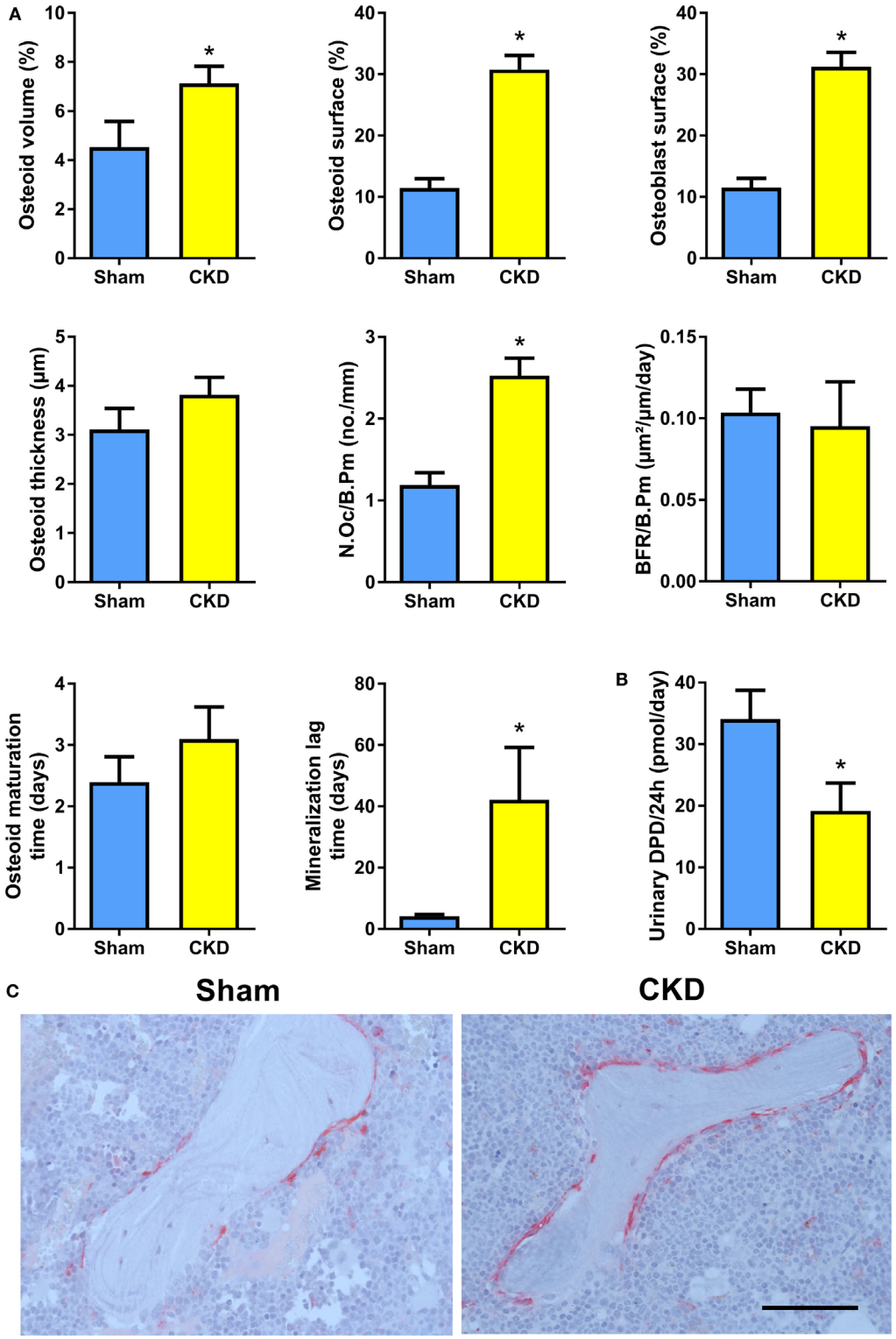

FIGURE 3 | Continued

FIGURE 3 | Chronic kidney disease (CKD) mice are characterized by impaired bone mineralization. **(A)** Osteoid volume (14 Sham, 24 CKD), osteoid surface (14 Sham, 24 CKD), osteoblast surface (14 Sham, 24 CKD), osteoid thickness (14 Sham, 24 CKD), osteoclast number per bone perimeter (N.Oc/B.Pm) (14 Sham, 24 CKD), bone formation rate per bone perimeter (BFR/B.Pm) (13 Sham, 8 CKD), osteoid maturation time (13 Sham, 8 CKD), and mineralization lag time (13 Sham, 8 CKD) measured by histomorphometry in cancellous bone of the proximal tibial metaphysis in 5-month-old male C57BL/6 Sham and CKD mice on rescue diet, 8 weeks postsurgery. *$P < 0.05$ vs. Sham by *t*-test or Mann–Whitney *U*-test as appropriate. **(B)** 24-h urinary excretion of deoxypyridinoline (DPD) ($n = 16–17$ each) in 5-month-old male C57BL/6 Sham and CKD mice on rescue diet, 8 weeks postsurgery. *$P < 0.05$ vs. Sham by *t*-test. Data in **(A,B)** are mean ± SEM. **(C)** Representative images of tartrate resistant acid phosphatase (TRACP)-stained proximal tibial sections of Sham and CKD mice, showing increased numbers of TRACP-positive osteoclasts in CKD mice. Bar = 100 µm in **(C)**.

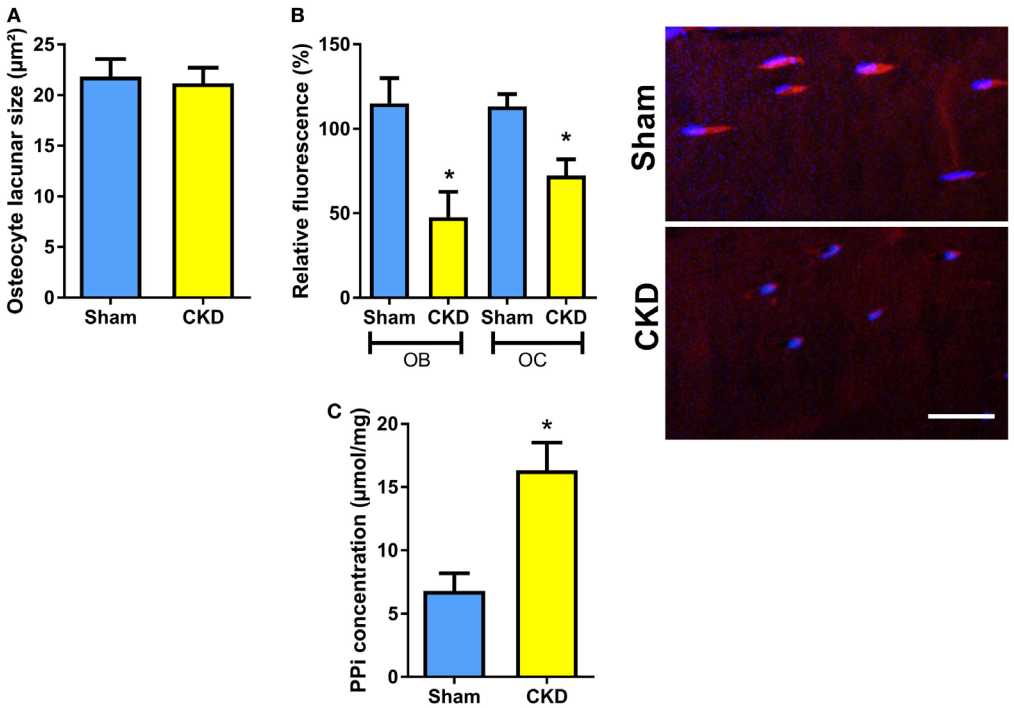

FIGURE 4 | Decreased tissue nonspecific alkaline phosphatase (TNAP) enzyme activity and increased pyrophosphate concentrations in bones of chronic kidney disease (CKD) mice. **(A)** Mean area of osteocyte lacunae measured by histomorphometry in tibial cortical bone ($n = 13–14$ each), **(B)** quantification of relative fluorescence after histochemical TNAP staining of osteoblasts (OB) and osteocytes (OC) in undecalcified sections of proximal tibiae ($n = 3$ each) and representative images of cortical bone osteocytes in Sham and CKD mice, and **(C)** pyrophosphate (PPi) concentration in extracts of femurs ($n = 3–5$ each) from 5-month-old male C57BL/6 Sham and CKD mice on rescue diet, 8 weeks postsurgery. Data in **(A–C)** are mean ± SEM. In **(B)**, * denotes $P < 0.05$ vs. Sham by one-way analysis of variance followed by Holm–Sidak's multiple comparisons test. In **(C)**, * denotes $P < 0.05$ vs. Sham by *t*-test. Bar = 20 µm in **(B)**.

mice, as evidenced by unchanged osteoid thickness, osteoid maturation time, and bone formation rate (**Figure 3A**). However, CKD mice showed a ~10-fold increase in mineralization lag time (**Figure 3A**), relative to Sham controls. The calculation of mineralization lag time is based on the adjusted apposition rate, which includes OFF periods in which osteoblasts are not actively mineralizing (33, 34). The pronounced CKD-induced increase in mineralization lag time is indicative of impaired bone mineralization, because a much smaller percentage of osteoblasts was actively mineralizing in CKD vs. Sham mice. In contrast to the elevated osteoclast numbers in CKD mice (**Figures 3A,C**), 24-h urinary excretion of collagen crosslinks was found to be reduced in CKD mice (**Figure 3B**). Hence, bone resorption at the functional, whole body level appeared to be decreased in CKD relative to Sham mice. Taken together, these results indicate that 5/6-Nx-induced bone loss was associated with impaired bone mineralization. However, despite the profound elevation in circulating intact PTH in CKD mice (**Figure 1A**), clear functional evidence of increased bone turnover, i.e., increased bone formation rate or collagen crosslink excretion, was absent in CKD mice, 8 weeks postsurgery.

Osteoblastic and Osteocytic TNAP Activity Is Suppressed, and Pyrophosphate Concentrations Are Increased in Bones of CKD Mice

To test whether the CKD-induced impairment in bone mineralization in cancellous bone would also have implications for mineralization in osteocyte lacunae, we measured osteocyte lacunar size in tibial cortical bone of Sham and CKD mice in von Kossa-stained bone sections. However, osteocyte lacunar size remained unchanged in CKD animals, relative to Sham controls (**Figure 4A**). We reported earlier that increased local endogenous

production of Fgf23 suppresses TNAP activity in osteocytes of *Hyp* mice (20). To examine whether a similar mechanism is also operative in CKD mice, we measured TNAP activity by histochemical analysis in plastic-embedded bone sections. Relative TNAP activity was indeed lower in osteocytes and osteoblasts of CKD mice compared with Sham controls (**Figure 4B**). The decrease in TNAP activity was associated with increased pyrophosphate concentrations in bones of CKD mice (**Figure 4C**). These findings support that notion that locally secreted Fgf23 suppresses TNAP in osteocytes, which leads to secondary accumulation of the mineralization inhibitor pyrophosphate.

Genetic Ablation of Fgf23 Rescues CKD-Induced TNAP Suppression and Pyrophosphate Accumulation

To test whether genetic ablation of *Fgf23* would rescue the suppression of TNAP activity and pyrophosphate accumulation in CKD mice, we performed 5/6-Nx in *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ (*Fgf23*/VDR) compound mutants, which are characterized by concomitant *Fgf23* deficiency and lack of a functioning vitamin D receptor (VDR$^{\Delta/\Delta}$). Single *Fgf23* knockout mice have a severe phenotype (35, 36), making it impossible studying 5/6-Nx-induced bone loss in these mice. However, the phenotype and early lethality of *Fgf23*$^{-/-}$ mice can be rescued by ablation of the vitamin D signaling pathway (21). *Fgf23*/VDR compound mutants kept on rescue diet are healthy and can be studied until old ages (37). The rescue diet enriched with calcium, phosphate, and lactose is an elegant dietary tool to normalize blood calcium and PTH levels in VDR-ablated mice (23, 38). Employing this genetic loss-of-function model, we found that 5/6-Nx induced secondary hyperparathyroidism and bone loss in *Fgf23*/VDR mice, similar to WT mice (**Figures 5A,B**). In line with our previously reported finding that *Fgf23*/VDR mice are characterized by partial renal and skeletal PTH resistance, intact PTH levels were several-fold higher in Sham *Fgf23*/VDR mice than

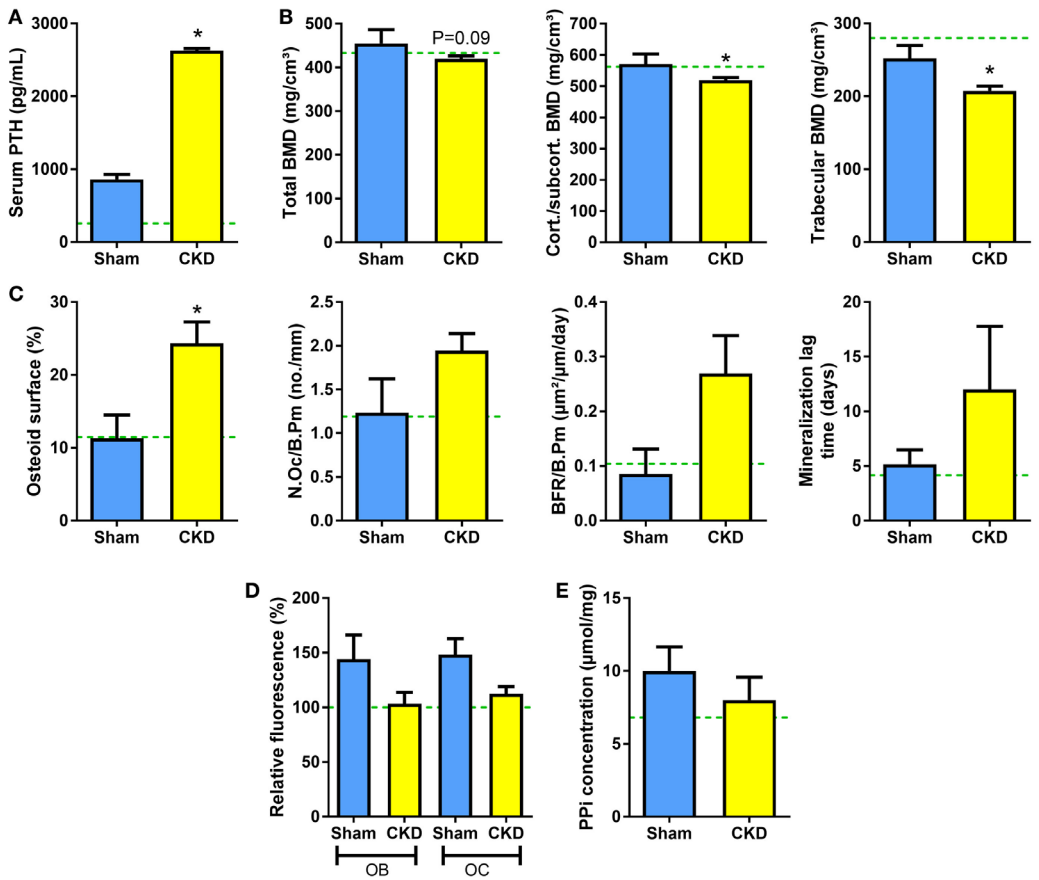

FIGURE 5 | 5/6-Nx induces bone loss in *Fgf23*/VDR compound mutant mice, but does not alter tissue nonspecific alkaline phosphatase (TNAP) activity or pyrophosphate concentration in bone. **(A)** Serum parathyroid hormone (PTH) (n = 13 each), **(B)** total, cortical/subcortical, and trabecular bone mineral density (BMD) of the L2 lumbar vertebra [4 Sham, 17 chronic kidney disease (CKD)], **(C)** osteoid surface (4 Sham, 14 CKD), number of osteoclasts (4 Sham, 14 CKD), bone formation rate (3 Sham, 10 CKD), and mineralization lag time (3 Sham, 10 CKD) in proximal tibial cancellous bone, **(D)** histochemical TNAP staining of undecalcified sections of proximal tibiae and quantification of relative fluorescence in osteoblasts (OB) and osteocytes (OC) (n = 3–5 each), and **(E)** pyrophosphate (PPi) concentration in extracts of femurs (n = 4–6 each) from 5-month-old male Sham and CKD *Fgf23*/VDR compound mutant mice on rescue diet, 8 weeks postsurgery. Data are mean ± SEM. Green dotted lines represent mean values found in wild-type (WT) Sham mice. The data in **(D)** were normalized to the WT Sham group. In **(A–C)**, * denotes $P < 0.05$ vs. Sham by t-test or Mann–Whitney U-test, as appropriate.

in Sham WT mice (**Figure 5A**). Nevertheless, 5/6-Nx still led to a ~3-fold upregulation of serum intact PTH in *Fgf23*/VDR mice (**Figure 5A**). Similar to WT mice, the CKD-induced bone loss in *Fgf23*/VDR mice was associated with increased osteoid surface (**Figure 5C**). However, in contrast to WT mice, osteoclast numbers and mineralization lag time in CKD *Fgf23*/VDR mice were not significantly different from Sham *Fgf23*/VDR mice, and showed, together with bone formation rate, only non-significant trends toward higher levels in CKD vs. Sham mice (**Figure 5C**). In agreement with the notion that the CKD-induced upregulation in osteoblastic and osteocytic Fgf23 secretion contributes to the mineralization defect in CKD mice, 5/6-Nx failed to significantly suppress TNAP activity in *Fgf23*/VDR mice (**Figure 5D**), and pyrophosphate concentrations remained unchanged in bones of CKD *Fgf23*/VDR mice (**Figure 5E**).

DISCUSSION

The current study has shown that 5/6-Nx C57BL/6 mice on the phosphate-rich rescue diet develop bone loss and impaired bone mineralization within 8 weeks postsurgery. Furthermore, we found that the CKD-driven increase in osteoblastic and osteocytic Fgf23 secretion contributes to the mineralization defect in murine CKD-MDB by auto-/paracrine suppression of TNAP and subsequent accumulation of pyrophosphate in bone. Hence, our study has uncovered a novel mechanism involved in the pathogenesis of CKD-MBD, i.e., Fgf23-driven accumulation of the mineralization inhibitor pyrophosphate. This new paradigm is shown in **Figure 6**.

We previously showed that the Fgf23-induced auto-/paracrine suppression of TNAP in osteoblasts and osteocytes is caused by a Klotho-independent, mainly FGFR3-mediated signaling mechanism (18). The presence of transmembrane Klotho increases the affinity of FGFR1c to FGF23 by a factor of about 20 (39). Therefore, one of the key questions in this context is whether the concentrations of Fgf23 in the extracellular fluid surrounding osteoblasts/osteocytes is high enough for Klotho-independent FGFR signaling. The true concentration of Fgf23 within the osteocyte canalicular network is currently unknown. However, due to the fact that Fgf23 is secreted locally from bone cells, it is likely that the concentration is much higher within the canalicular network compared with the blood. In addition, differentiation of osteoblasts into osteocytes is associated with a profound upregulation of FGFR1 and 3 mRNA expression *in vivo* (20). Therefore, it is well conceivable that the Fgf23 concentration within the canalicular network is indeed sufficient to suppress TNAP transcription *via* a Klotho-independent, auto-/paracrine feedback mechanism. The situation is more complicated in osteoblasts. Osteoblasts are polar cells, and it is currently unknown whether FGF23 is secreted in a polar manner. In addition, it is unknown whether the membrane distribution of FGFRs is uniform or whether it is also organized in a polar fashion in osteoblasts. Therefore, it is difficult to estimate the concentrations of locally produced Fgf23 in the extracellular fluid surrounding osteoblasts, and to judge the possibilities for local feedback mechanisms. Nevertheless, our finding that the CKD-induced suppression of osteoblastic and osteocytic TNAP activity and accumulation of pyrophosphate in bone did not occur in *Fgf23*/VDR mutant mice lacking *Fgf23* lends additional support to the notion that the CKD-driven increase in Fgf23 secretion suppresses TNAP transcription in an auto-/paracrine manner.

Despite the increase in pyrophosphate concentrations in bone, 5/6-Nx did not increase the size of osteocyte lacunae in cortical bone in the current study. This finding is not unexpected for two reasons. First, we did not observe major increases in osteoid thickness in cancellous bone, making it unlikely to see major changes in the amount of osteoid in osteocyte lacunae, and, therefore, in lacunar size. Second, murine cortical bone

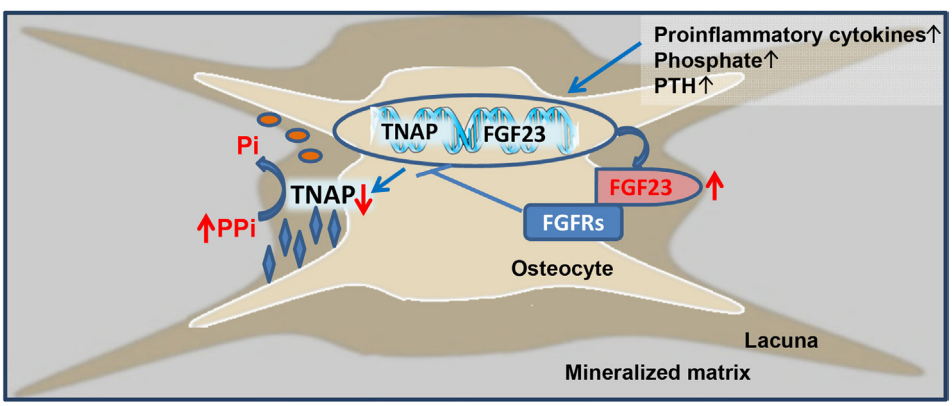

FIGURE 6 | Proposed model of fibroblast growth factor-23 (FGF23)-induced suppression of tissue nonspecific alkaline phosphatase (TNAP) and accumulation of pyrophosphate in chronic kidney disease (CKD). Driven by increased circulating concentrations of pro-inflammatory cytokines, phosphate, parathyroid hormone (PTH), and other unknown factors, osteoblastic and osteocytic FGF23 secretion is increased in CKD. Excessive concentrations of FGF23 in the extracellular fluid surrounding osteocytes lead to FGF receptor (FGFR)-mediated, Klotho-independent suppression of *TNAP* transcription in a para-/autocrine manner. One of the main functions of TNAP in the mineralization process is the hydrolysis of the mineralization inhibitor pyrophosphate (PPi), thus providing inorganic phosphate (Pi) for mineralization. Hence, excessive FGF23 concentrations in the osteocyte canalicular network lead to accumulation of PPi and inhibition of bone mineralization in CKD.

does not show Haversian remodeling (33). Since 5/6-Nx was performed in adult mice, analysis of osteocyte lacunar size necessarily included mostly osteocytes formed prior to the onset of renal disease.

Total serum ALP activity was increased in CKD mice in our study. Similarly, bone-specific ALP is typically increased in patients with high turnover CKD-MBD (40). ALP activity in serum originates in about equal parts from bone and liver, with TNAP being the major isoenzyme found in blood (41). How can increased circulating ALP activity be reconciled with our finding that excessive Fgf23 secretion locally suppresses TNAP in bone cells? We do not have a conclusive answer to this question. However, the most likely explanation is that the number of osteoblasts and of osteoblast precursor cells is increased due to secondary hyperparathyroidism in CKD. Therefore, serum ALP activity may be mainly reflecting the number of osteoblasts and of osteoblast precursors rather than the ALP activity of individual bone cells. In line with this idea, we found a ~3-fold increase in osteoblast surface in WT CKD mice, relative to Sham controls.

Despite an approximately twofold increase in osteoclast numbers in proximal tibial cancellous bone, 24-h excretion of collagen crosslinks was reduced in CKD mice in our study. The plasma clearance of collagen crosslinks depends on kidney function (42). Therefore, serum collagen crosslink concentrations are not considered suitable markers of bone resorption in CKD patients (40). Under normal circumstances, urinary collagen crosslink excretion is the best estimate of whole body bone resorption activity in mice, because osteoclast numbers are often not indicative of osteoclast resorptive activity (33). Similarly, osteoblast surface and calcein-based bone formation rate were dissociated in the current study, underscoring the potential discrepancies between cell morphology and functional readouts in disease models. Whether, despite potential accumulation in serum, urinary collagen crosslink excretion accurately reflects bone resorption in CKD mice is not known. However, in a steady state, urinary excretion of collagen crosslinks should equal collagen breakdown in bone also in animals with compromized kidney function. Nevertheless, it remains unclear whether bone resorption in our CKD mouse model was increased as suggested by histomorphometry or decreased as suggested by urinary collagen crosslink excretion.

It is well known that circulating intact PTH is one of the main drivers of bone turnover in CKD patients. Hence, serum intact PTH is a useful biomarker to discriminate between low and high bone turnover in CKD patients (17), albeit its sensitivity to dissect high and low bone turnover disease is only about 65% (17, 40). What is actually causing the often observed impairment in bone mineralization in bone biopsies of CKD patients is not well known (1). One possibility is that secondary hyperparathyroidism *per se* leads to disturbed mineralization due to excessive stimulation of matrix synthesis and woven bone formation (1). Moreover, kidney-derived circulating Wnt inhibitors such as dickkopf-1 or sclerostin may inhibit bone formation and mineralization (43). In addition, FGF23 has been shown to directly suppress Wnt signaling in osteoblasts, and circulating uremic toxins and metabolic acidosis may interfere with normal osteoblast function and maturation (44–46). Our study has added an additional mechanism, namely the Fgf23-induced suppression of TNAP in osteoblasts and osteocytes. TNAP is essential for the initiation of bone mineralization by cleavage of pyrophosphate (19, 47). It is currently technically challenging to assess differences in local pyrophosphate concentration within the bone matrix. However, we hypothesize that increased pyrophosphate concentrations are at least a partial explanation for the profound increase in non-mineralizing osteoid seams in CKD mice in our study. This model may also help to explain the hungry bone syndrome in CKD patients after parathyroidectomy or kidney transplantation, because the decline in bony FGF23 secretion postsurgery will lift the FGF23-induced suppression of TNAP, leading to increased mineralization of previously unmineralized osteoid, and, hence, increased flux of calcium and phosphate into bone.

In conclusion, here we report a novel mechanism leading to impaired bone mineralization in CKD-MBD. We found that excessive CKD-driven Fgf23 secretion in osteoblasts and osteocytes is leading to auto-/paracrine suppression of TNAP and subsequent accumulation of the mineralization inhibitor pyrophosphate.

ETHICS STATEMENT

All animal studies were approved by the Ethical Committee of the University of Veterinary Medicine, Vienna and by the Austrian Federal Ministry of Science and Research and were undertaken in strict accordance with prevailing guidelines for animal care (permit No. BMWF-68.205/0054-II/3b/2013). All efforts were made to minimize animal suffering.

AUTHOR CONTRIBUTIONS

OA and RE conceived and designed the experiments, analyzed the data, and wrote the manuscript; OA, CS, CB, and AP performed experiments.

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We thank U. Zeitz and S. K. Murali for help with the animal experiments, S. Sasgary for help with the genotyping, and G. Hofer for help with the pQCT measurements. OA passed away before the submission of the final version of this manuscript. RE accepts responsibility for the integrity and validity of the data collected and analyzed. This work was supported by a grant from the Austrian Science Fund (FWF P24186-B21) to RE.

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frontiers in Endocrinology

MINI REVIEW
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Physiological Actions of Fibroblast Growth Factor-23

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Fibroblast growth factor-23 (FGF23) is a bone-derived hormone suppressing phosphate reabsorption and vitamin D hormone synthesis in the kidney. At physiological concentrations of the hormone, the endocrine actions of FGF23 in the kidney are αKlotho-dependent, because high-affinity binding of FGF23 to FGF receptors requires the presence of the co-receptor αKlotho on target cells. It is well established that excessive concentrations of intact FGF23 in the blood lead to phosphate wasting in patients with normal kidney function. Based on the importance of diseases associated with gain of FGF23 function such as phosphate-wasting diseases and chronic kidney disease, a large body of literature has focused on the pathophysiological consequences of FGF23 excess. Less emphasis has been put on the role of FGF23 in normal physiology. Nevertheless, during recent years, lessons we have learned from loss-of-function models have shown that besides the paramount physiological roles of FGF23 in the control of 1α-hydroxylase expression and of apical membrane expression of sodium-phosphate co-transporters in proximal renal tubules, FGF23 also is an important stimulator of calcium and sodium reabsorption in distal renal tubules. In addition, there is an emerging role of FGF23 as an auto-/paracrine regulator of alkaline phosphatase expression and mineralization in bone. In contrast to the renal actions of FGF23, the FGF23-mediated suppression of alkaline phosphatase in bone is αKlotho-independent. Moreover, FGF23 may be a physiological suppressor of differentiation of hematopoietic stem cells into the erythroid lineage in the bone microenvironment. At present, there is little evidence for a physiological role of FGF23 in organs other than kidney and bone. The purpose of this mini-review is to highlight the current knowledge about the complex physiological functions of FGF23.

Keywords: fibroblast growth factor-23, Klotho, vitamin D, 1α-hydroxylase, bone mineralization, phosphate metabolism, alkaline phosphatase

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INTRODUCTION

In the year 2000, gain-of-function mutations in fibroblast growth factor-23 (FGF23) were identified as the genetic cause of autosomal dominant hypophosphatemic rickets (ADHR), an inherited renal phosphate-wasting disease (1). In the following years, FGF23 turned out to be the long-sought "phosphatonin" that had already been postulated in the 1980s, when parabiosis experiments in hypophosphatemic *Hyp* mice had shown that the renal phosphate wasting and the hypophosphatemia in these mice were caused by a factor circulating in the blood (2).

Fibroblast growth factor-23 is a 32 kDa glycoprotein mainly produced in bone by osteoblasts and osteocytes under physiological circumstances. FGF23 is inactivated by cleavage at the ^{176}RXXR^{179} site, a site that is mutated in ADHR patients. Together with FGF19 and FGF21, FGF23 belongs to

the group of endocrine FGFs (3). All endocrine FGFs require the co-receptors α- and β-Klotho for high-affinity binding to the ubiquitously expressed FGF receptors (FGFR1-4) in target cells (4–7). The co-receptor needed for binding of FGF23 to FGF receptors is transmembrane or soluble αKlotho (4, 8). Among the four different FGFRs, FGF receptor-1c (FGFR1c) is probably the most important FGFR for FGF23 signaling, at least under physiological conditions (4, 9). αKlotho enhances the binding affinity of FGFR1c to FGF23 by a factor of approximately 20 (6). The "c" in FGFR1 stands for a splice variant which occurs in FGFR1, 2, and 3.

The principal action of FGF23 on mineral metabolism that led to its discovery as a hormone is the suppressive effect on phosphate reabsorption from the urine (10, 11). In addition, FGF23 suppresses the synthesis of the vitamin D hormone, 1α, 25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], in the kidney (10, 11). It is now well known that diseases characterized by excessive blood concentrations of intact FGF23 lead to renal phosphate wasting and inappropriately low-circulating 1,25$(OH)_2D_3$ levels in patients with a normal kidney function (12). Examples of human disorders associated with elevated intact FGF23 are ADHR with a defective cleavage site of FGF23, X-linked hypophosphatemic rickets (XLH), and autosomal recessive hypophosphatemic rickets 1 (ARHR1) caused by overproduction of FGF23 in bone, and tumor-induced osteomalacia caused by FGF23-producing tumors (12). The molecular mechanism underlying the increased bony FGF23 secretion in XLH and ARHR1 patients is still unclear. In terms of factors that may drive FGF23 secretion, the common denominator in both diseases is impaired bone mineralization. XLH is caused by loss-of-function mutations in *PHEX* (13). It has been shown in *Hyp* mice, the murine model of XLH, that lack of the endopeptidase PHEX leads to accumulation of osteopontin and ASARM (acidic serine- and aspartate-rich MEPE-associated motif) peptides in the matrix, which both inhibit mineralization (14–17). ARHR1 is caused by loss-of-function mutations in dentin matrix protein-1, which is required for normal mineralization of bone (18). It is currently believed that the excessive osteocytic and osteoblastic FGF23 secretion in both diseases is either driven by the impaired mineralization of the extracellular matrix, which may be detected by matrix-embedded bone cells through a putative sensing mechanism that may involve FGF receptors (19, 20), or by an altered set point for phosphate sensing in bone cells (21, 22). Circulating intact FGF23 is also elevated in patients with chronic kidney disease (CKD), and can reach blood levels as high as 1,000-fold above the normal range (23, 24). Although elevated intact FGF23 may help to maintain normophosphatemia in early stages of CKD, serum phosphate levels typically increase at later stages of the disease despite very high-serum intact FGF23. Therefore, in the setting of impaired kidney function, the phosphaturic action of FGF23 is not able to correct the hyperphosphatemia in more advanced CKD.

Collectively, there is very good evidence that gain of FGF23 function results in renal phosphate wasting in patients with normal kidney function. However, what is the role of FGF23 in normal physiology? The purpose of this mini-review is to answer this question, and to highlight the current knowledge about the complex physiological functions of FGF23 in mice and men.

PHYSIOLOGICAL FUNCTIONS OF FGF23 IN THE KIDNEY

Knockout experiments in mice have revealed that the paramount physiological function of FGF23 is not its phosphaturic function, but its suppressive role in the control of renal 1α-hydroxylase (CYP27B1) transcription, the key enzyme for 1,25$(OH)_2D_3$ synthesis. Notably, in the absence of the ligand FGF23 or of its co-receptor αKlotho, the stringent endocrine control of 1α-hydroxylase transcription fails, leading to inappropriately high expression and activity of this enzyme. The sequel of 1α-hydroxylase overexpression are elevated 1,25$(OH)_2D_3$ levels, causing hypercalcemia, hyperphosphatemia, ectopic calcifications, impaired bone mineralization, and early lethality in α*Klotho* and *Fgf23* deficient mice (25–27). The major function of 1,25$(OH)_2D_3$ in mineral metabolism is the stimulation of intestinal calcium and phosphorus absorption. The crucial role of 1,25$(OH)_2D_3$ overproduction in mediating the phenotype of α$Klotho^{-/-}$ and $Fgf23^{-/-}$ mice is underscored by the well documented finding that ablation of vitamin D signaling almost completely rescues the phenotype of $Fgf23^{-/-}$ and α$Klotho^{-/-}$ mice (28–30). In analogy to the phenotype of α*Klotho* and *Fgf23* deficient mice, humans with loss-of-function mutations in *FGF23* or α*Klotho* are characterized by elevated circulating vitamin D hormone levels and soft tissue calcifications (31–34), corroborating the mouse data.

Despite the pivotal physiological importance of the FGF23-mediated suppression of 1α-hydroxylase transcription, the knowledge of the intracellular signaling pathway involved in this regulation is still fragmentary. Expression of 1α-hydroxylase is mainly localized in proximal renal tubules. Proximal and distal renal tubules express the co-receptor αKlotho as well as FGFR1, 3, and 4, but only little FGFR2 (35, 36). All FGFRs are receptor tyrosine kinases, initiating intracellular phosphorylation cascades after ligand-induced dimerization (37). Mice with a specific deletion of *Fgfr1* in proximal renal tubules are resistant to the FGF23-induced suppression of 1,25$(OH)_2D_3$ production (9). Therefore, FGFR1c is probably the predominant FGFR mediating the suppressive effects of FGF23 on renal tubular 1,25$(OH)_2D_3$ synthesis (**Figure 1**). However, to a lesser extent, FGFR3 and 4 may also be involved, because genetic ablation of *Fgfr3* and *Fgfr4* increases renal 1α-hydroxylase expression in *Hyp* mice, which are characterized by increased endogenous FGF23 production (38). There is good evidence that the FGF23-mediated suppression of 1α-hydroxylase transcription involves extracellular signal-regulated kinase-1 and -2 (ERK1/2) activation (39, 40), but the exact signaling pathway downstream of ERK1/2 is unknown (**Figure 1**). Interestingly, the FGF23-mediated control of the transcriptional activity of the 1α-hydroxylase gene occurs through regulatory elements located in introns of the neighboring *Mettl21b* gene (41). However, the transcription factor(s) involved in this regulation are currently unknown. It is only clear in this context that FGF23 regulates 1α-hydroxylase in a 1,25$(OH)_2D_3$ and vitamin D receptor (VDR) independent manner (42).

It is well established that parathyroid hormone (PTH) and FGF23 regulate 1α- and 24-hydroxylase (CYP24A1) expression reciprocally. FGF23 suppresses 1α-hydroxylase, but induces 24-hydroxylase expression. PTH has the opposite effects.

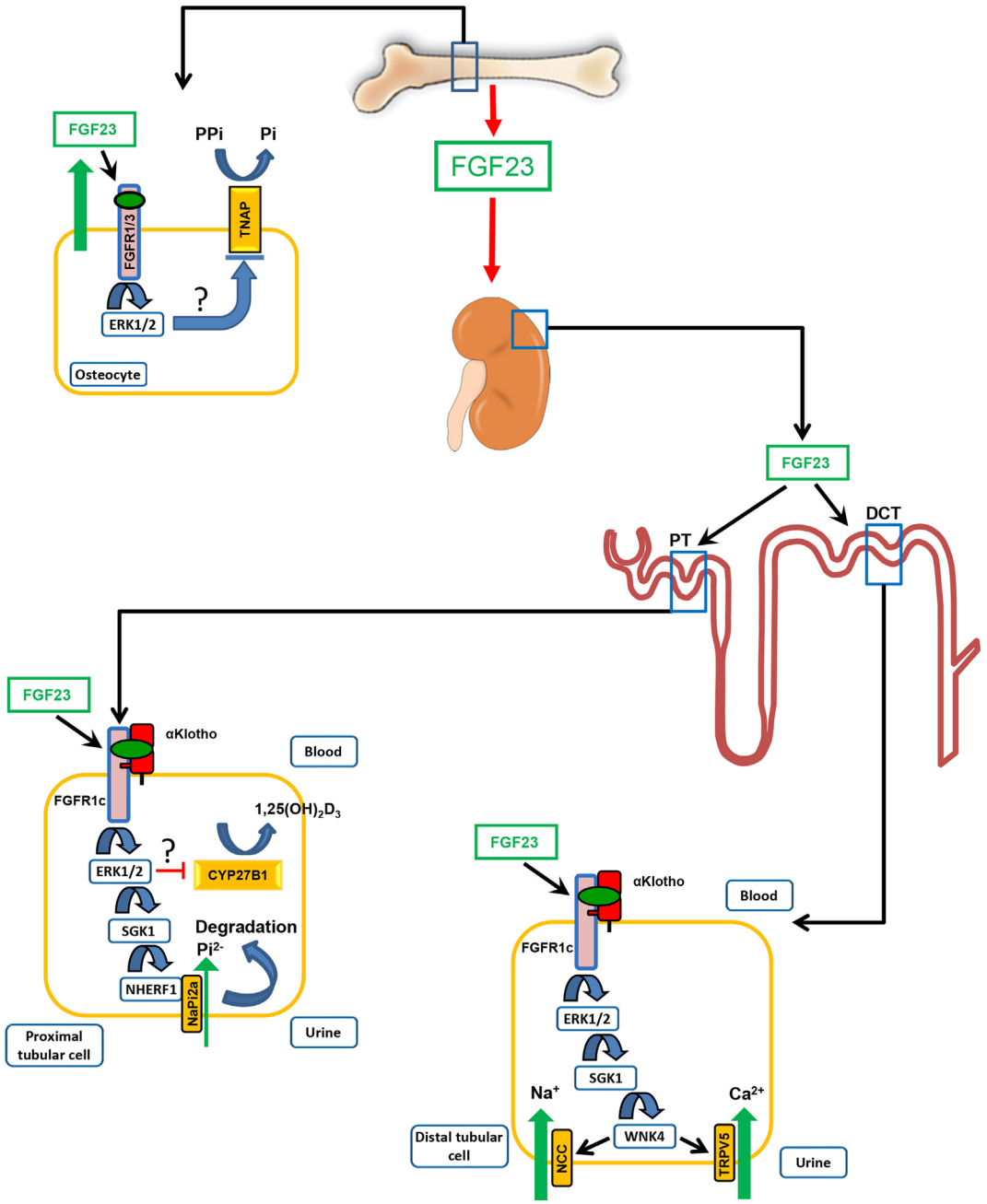

FIGURE 1 | Physiological functions of fibroblast growth factor-23 (FGF23). FGF23 is mainly produced in bone cells, osteoblasts, and osteocytes. One of the main target organs of the hormone FGF23 is the kidney. In the kidney, FGF23 acts on proximal and distal convoluted renal tubules. Binding of blood-borne FGF23 to FGF receptor-1c (FGFR1c) requires the presence of the co-receptor αKlotho. In renal proximal tubules (PT), FGF23 inhibits phosphate (Pi) re-uptake and expression of 1α-hydroxylase (CYP27B1), the rate-limiting enzyme for vitamin D hormone ($1\alpha,25(OH)_2D_3$) production. The FGF23-mediated suppression of 1α-hydroxylase transcription involves extracellular signal-regulated kinase-1 and 2 (ERK1/2) activation. However, the exact signaling pathway downstream of ERK1/2 is unknown. The inhibition of phosphate reabsorption in proximal renal tubules by FGF23 is mediated through activation of ERK1/2 and serum/glucocorticoid-regulated kinase-1 (SGK1), leading to phosphorylation of the scaffolding protein Na^+/H^+ exchange regulatory cofactor (NHERF)-1. NHERF-1 phosphorylation triggers internalization and degradation of the sodium-phosphate cotransporter NaPi-2a, so that less NaPi-2a is available in the apical membrane for phosphate uptake from urine. In distal convoluted tubules (DCT), FGF23 increases reabsorption of calcium and sodium by increasing the apical membrane abundance of the epithelial calcium channel transient receptor potential vanilloid-5 and of the sodium-chloride cotransporter NCC through a signaling cascade involving ERK1/2, SGK1, and with-no-lysine kinase-4 (WNK4). FGF23 locally produced by osteocytes is an auto-/paracrine inhibitor of bone mineralization by down-regulating tissue non-specific alkaline phosphatase (TNAP) transcription in a αKlotho-independent fashion *via* FGFR1- or FGFR3-mediated activation of ERK1/2. The signaling pathway downstream of ERK1/2, leading to suppression of TNAP transcription, is not known. TNAP is essential for normal mineralization of bone by cleaving the mineralization inhibitor pyrophosphate (PPi).

1α-hydroxylation represents metabolic conversion of the precursor 25-hydroxyvitamin D into the biologically active hormone, whereas 24-hydroxylation is an inactivation pathway (43). $1,25(OH)_2D$ is known to be a strong inducer of 24-hydroxylase (43), stimulating its own degradation. Whether FGF23 is a direct regulator of 24-hydroxylase transcription has been a controversial issue for many years. Some reports in *Fgf23*$^{-/-}$ mice (26) as well as in wild-type mice treated with recombinant FGF23 (11) suggested that FGF23 signaling may directly induce 24-hydroxylase, whereas experiments in VDR knockout mice suggested that the FGF23-mediated regulation of 24-hydroxylase is not direct, but depends on the VDR (42, 44). The latter notion has been confirmed by recent evidence showing that, in contrast to the $1,25(OH)_2D_3$-mediated induction, both the FGF23-mediated induction and the PTH-mediated suppression of 24-hydroxylase are completely lost in 1α-hydroxylase knockout mice (41). This finding strongly suggests that the FGF23 and PTH-mediated regulation of 24-hydroxylase expression are entirely indirect through altered $1,25(OH)_2D_3$ synthesis and subsequent changes in VDR-regulated promoter activity of 24-hydroxylase.

As mentioned above, FGF23 promotes renal phosphate excretion by inhibiting cellular phosphate re-uptake from the urine in proximal renal tubules (**Figure 1**). Through a signaling cascade involving the αKlotho/FGFR1c receptor complex, ERK1/2, and serum/glucocorticoid-regulated kinase-1 (SGK1), FGF23 signaling induces the phosphorylation of the scaffolding protein Na^+/H^+ exchange regulatory cofactor (NHERF)-1 which in turn leads to internalization and degradation of the sodium-phosphate cotransporters NaPi-2a and NaPi-2c (36, 45, 46). Notably, 4-week-old *Fgf23*$^{-/-}$/VDR$^{\Delta/\Delta}$ (*Fgf23*/VDR) and α*Klotho*$^{-/-}$/VDR$^{\Delta/\Delta}$ (*Klotho*/VDR) compound mutant mice lacking *Fgf23* or *Klotho* and a functioning VDR are not hyperphosphatemic (28, 29, 47). Hyperphosphatemia is only seen in older, more slowly or non-growing *Fgf23*/VDR compound mutant mice beyond 3 months of age (30, 47), suggesting that the phosphaturic effect of FGF23 is physiologically less essential compared with the 1α-hydroxylase-suppressing effect, at least in mice. Both the phosphaturic and the $1,25(OH)_2D_3$-lowering effect of FGF23 protect against hyperphosphatemia: the first effect directly through increased elimination of phosphate, and the second effect indirectly through reduced intestinal phosphate absorption. In addition, because $1,25(OH)_2D_3$ and phosphate stimulate FGF23 secretion in bone (12), the phosphaturic and $1,25(OH)_2D_3$-lowering effects of FGF23 form a negative feedback loop between bone and kidney.

In recent years, it has become clear that FGF23 is not only a regulator of vitamin D and phosphate metabolism, but also directly influences calcium and sodium handling in the distal nephron in the kidney (**Figure 1**). Skeletally mature *Fgf23*/VDR and *Klotho*/VDR compound mutant mice are characterized by renal calcium wasting (48), as well as by renal sodium wasting and subsequent hyponatremia, hypovolemia, and hypotension (49). Similar to proximal renal tubules, the FGFR1c/Klotho complex appears to be the most important receptor complex in the distal nephron, because distal tubular-specific deletion of *Fgfr1* recapitulates the renal calcium wasting seen in *Fgf23*/VDR compound mutant mice (9). In distal tubular epithelium, FGF23 regulates the apical membrane abundance of the epithelial calcium channel transient receptor potential vanilloid-5 (TRPV5) and of the sodium-chloride cotransporter NCC through a signaling cascade involving ERK1/2, SGK1, and with-no-lysine kinase-4 (48, 49). *Fgf23* and *Klotho* deficient mice are characterized by a downregulation of distal tubular TRPV5 and NCC membrane expression, leading to renal calcium and sodium wasting, despite counter-regulatory increases in circulating PTH and aldosterone (48–50). These findings indicate that the calcium- and sodium-conserving functions of FGF23 in distal renal tubules are of physiological relevance. Indeed, the increased renal conservation of calcium may help to maintain blood calcium levels despite the suppression of $1,25(OH)_2D_3$ synthesis induced by upregulated FGF23 secretion. In single *Fgf23* and *Klotho* knockout mice, the calcium-conserving function of FGF23 is masked by the profound upregulation of $1,25(OH)_2D_3$ production and subsequent hypercalcemia.

Parathyroid hormone and FGF23 have partially overlapping functions in proximal and distal renal tubules. Both hormones inhibit phosphate reabsorption in proximal renal tubules by targeting NHERF-1 phosphorylation (36, 45, 46), and increase calcium reabsorption in distal renal tubules by targeting expression and/or open probability of TRPV5 (48, 51). Albeit the signaling mechanisms are different, the proximal and distal renal target molecules of PTH and FGF23 are the same. An interesting finding in this context is that absence of FGF23 signaling in *Fgf23* deficient mice causes partial renal resistance to the phosphaturic and calcium-conserving actions of PTH (50). *Vice versa*, a reduction in PTH signaling in human patients with hypoparathyroidism has been shown to induce partial resistance to the phosphaturic actions of FGF23 (52, 53). Therefore, both hormones interact, and an important physiological function of FGF23 may be to enable normal responsiveness to PTH signaling in the kidney and also in bone (50).

Taken together, lessons learned from knockout mouse models have revealed that the most important physiological function of FGF23 is not its phosphaturic effect but the downregulation of vitamin D hormone production. It is likely that the exquisite sensitivity of the homeostatic system regulating 1α-hydroxylase transcription to lack of FGF23 signaling is caused by the absence of other suppressive hormones which might be able to effectively counter-balance FGF23 deficiency. In *Fgf23* and *Klotho* deficient mice, the suppression of PTH secretion observed in these mice is insufficient to control transcription of renal 1α-hydroxylase. In contrast, lack of the phosphaturic, as well as of the calcium- and sodium-conserving functions of FGF23 can at least partially be compensated by the phosphaturic and calcium-conserving hormone PTH, and by the sodium-conserving hormone aldosterone. Therefore, the pivotal importance of FGF23 signaling for the control of 1α-hydroxylase transcription might be considered as a systems biology problem. This problem may also have implications for the treatment of patients with antibodies against FGF23 or with small molecules blocking the FGF23 signaling pathway. The therapeutic window for these treatments is relatively narrow, and requires close monitoring of calcium and phosphorus metabolism to avoid toxic side effects (54, 55).

PHYSIOLOGICAL FUNCTIONS OF FGF23 IN BONE

Fibroblast growth factor-23 may also have physiologically relevant functions in bone on bone mineralization and on hematopoiesis. We recently reported that FGF23 is a powerful suppressor of transcription of tissue non-specific alkaline phosphatase (TNAP) mRNA in bone cells in a Klotho-independent manner (56) (**Figure 1**). TNAP is essential for the regulation of bone mineralization by cleaving the mineralization inhibitor pyrophosphate which is secreted by osteoblasts to prevent premature mineralization of osteoid (57). Based on experiments with pharmacological FGFR inhibitors, we concluded that the FGF23-induced, Klotho-independent suppression of TNAP mRNA abundance in primary murine osteoblasts is mainly mediated through FGFR3 (56). In contrast, Shalhoub et al. (58) reported that FGF23 suppresses TNAP expression in mouse osteoblast-like cells in an FGFR1-dependent manner, and that this effect could be enhanced by soluble Klotho. Thus, it awaits further clarification whether the FGF23-mediated suppression of TNAP in bone cells is mainly mediated through FGFR1, FGFR3, or both. Klotho expression in bone is very low (33, 59). Therefore, it is unlikely that Klotho expression in bone cells is sufficient to enhance FGF23 binding to FGFRs in osteoblasts and osteocytes. However, it can be assumed that due to the local production of FGF23 in osteocytes, the concentration of FGF23 within the canalicular system is high enough for auto-/paracrine, Klotho-independent signaling through FGFRs in bone. Hence, locally produced FGF23 may not only contribute to impaired mineralization under the conditions of excessive bony FGF23 secretion such as in *Hyp* mice (60), but may also serve as a physiological inhibitor of bone mineralization by downregulating TNAP expression. In line with this notion, we found an upregulation of *Tnap* mRNA abundance in *Fgf23* deficient *Fgf23*/VDR compound mutant mice compared with wild-type and VDR control mice (56). However, the relevance of this mechanism in the context of physiological ranges of FGF23 secretion remains to be shown.

Fgf23 deficient mice are characterized by increased erythropoiesis (61). Conversely, injection of recombinant FGF23 into normal mice suppresses erythropoiesis (61), and inhibition of FGF23 signaling alleviates the suppression of erythropoiesis in mice with excessive FGF23 blood levels due to renal failure (62). Therefore, FGF23 may be a physiological regulator of erythroid lineage commitment in the bone microenvironment. However, the signaling mechanisms underlying this effect are currently not known, and further studies are needed to demonstrate the relevance of this effect in relation to the physiological regulation of erythropoiesis by the renal hormone erythropoietin.

PHYSIOLOGICAL FUNCTIONS OF FGF23 IN OTHER ORGANS?

It is interesting to note that the first description of FGF23 was actually in thalamic nuclei in the murine brain (63). However, data about possible functions of FGF23 in the brain are still scarce. It was reported that high concentrations of FGF23 may interfere with neuronal ramification and may increase synaptic density in cultures of hippocampal neurons (64), but very little is known about potential physiological functions. *Fgf23*/VDR compound mutant mice do not have an overt CNS phenotype until older ages (30), but more elaborate behavioral or cognitive tests have never been done.

The parathyroid gland is one of the organs abundantly expressing αKlotho (33, 65), making it a potential target tissue for FGF23. However, conditional knockout mice with a parathyroid-specific deletion of αKlotho show normal circulating intact PTH levels (65). In addition, global *Fgf23*/VDR and α*Klotho*/VDR mutant mice at young ages do not show differences in PTH blood concentrations compared with VDR mutant mice (28, 29). Only at older ages, PTH secretion is upregulated in *Fgf23*/VDR and α*Klotho*/VDR mutant mice, relative to VDR controls, in response to chronic renal calcium wasting and partial PTH resistance (50). Hence, although high-FGF23 blood concentrations may suppress PTH secretion in a Klotho-independent fashion in rodents (65, 66), it is unlikely that FGF23 signaling has an important role in the physiological regulation of PTH secretion.

Although the heart may be an important target tissue at supraphysiological FGF23 concentrations, promoting cardiomyocyte hypertrophy in CKD patients by a Klotho-independent signaling pathway (67, 68), FGF23 is not expressed in the normal heart, and heart function is normal in *Fgf23*/VDR mutant mice (69). These findings suggest that FGF23 does not have a functional role in the heart under physiological circumstances.

Taken together, there is only little evidence that FGF23 has a role in normal physiology in organs other than kidney and bone.

CONCLUSION

The purpose of this mini-review is to highlight the current knowledge about the physiological functions of the bone-derived hormone FGF23. Excessive circulating intact FGF23 levels result in renal phosphate wasting under the conditions of a normal kidney function. However, knockout mouse models have shown that the most important physiological function of FGF23 is not the phosphaturic effect, but the suppressive effect on renal 1α-hydroxylase expression. The absence of FGF23 or of its co-receptor αKlotho results in deregulated renal 1α-hydroxylase expression and vitamin D hormone production, which cannot be compensated by other endocrine systems. Moreover, FGF23 has several additional physiological functions, which include inhibition of renal phosphate reabsorption, increased conservation of calcium and sodium in the kidney, support of a normal responsiveness of the kidney to PTH, and regulation of bone mineralization. Although excessive FGF23 may target many non-canonical tissues, there is currently little evidence for a role of FGF23 in the normal physiology of organs other than kidney and bone.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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New Insights into the Mechanism of Action of Soluble Klotho

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The *klotho* gene encodes a type I single-pass transmembrane protein that contains a large extracellular domain, a membrane spanning segment, and a short intracellular domain. Klotho protein exists in several forms including the full-length membrane form (mKl) and a soluble circulating form [soluble klotho (sKl)]. mKl complexes with fibroblast growth factor receptors to form coreceptors for FGF23, which allows it to participate in FGF23-mediated signal transduction and regulation of phosphate and calcium homeostasis. sKl is present in the blood, urine, and cerebrospinal fluid where it performs a multitude of functions including regulation of ion channels/transporters and growth factor signaling. How sKl exerts these pleiotropic functions is poorly understood. One hurdle in understanding sKl's mechanism of action as a "hormone" has been the inability to identify a receptor that mediates its effects. In the body, the kidneys are a major source of sKl and sKl levels decline during renal disease. sKl deficiency in chronic kidney disease makes the heart susceptible to stress-induced injury. Here, we summarize the current knowledge of mKl's mechanism of action, the mechanistic basis of sKl's protective, FGF23-independent effects on the heart, and provide new insights into the mechanism of action of sKl focusing on recent findings that sKl binds sialogangliosides in membrane lipid rafts to regulate growth factor signaling.

Keywords: klotho, FGF23, lipid rafts, aging, TRPC6, sialidase, IGF-1, heart disease

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DISCOVERY OF THE AGING-SUPPRESSOR GENE *klotho*

For decades, scientists have searched for genes that regulate lifespan. In 1997, one such gene was identified in a transgenic mouse strain whose mutation resulted in a syndrome resembling premature aging that included shortened lifespan, growth retardation, vascular calcification, genital atrophy, emphysema, and osteomalacia (1). The gene was named *klotho*, which in Greek mythology is one of the three goddesses of fate who spins the thread of life (1). The aging phenotypes were observed exclusively in mice that were homozygous for *SLC9A1* transgene insertion into the 5′ flanking region of the *klotho* gene, which resulted in a severe hypomorphic *klotho* allele (*kl/kl*). Since the discovery, *klotho* attracted considerable scientific interest due to its role in aging suppression. Abundant evidence has accumulated during the past two decades that supports the association between *klotho* and senescence. For instance, transgenic mice that overexpress *klotho* exhibit an extended lifespan compared with wild-type (WT) mice which has been attributed, at least partly, to *klotho*-induced resistance to insulin signaling and oxidative stress (2, 3). In humans, total Klotho protein levels decline with age in serum, while single nucleotide polymorphisms have

been identified in the *klotho* gene that correlates with reduced longevity and the pathophysiology of age-related disorders such as osteoporosis, coronary artery disease, and stroke (4–8). Finally, gene profile analyses have demonstrated that *klotho* expression is decreased in aged brain white matter in rhesus monkeys indicating a role for *klotho* as a lifespan gene in the nervous system (9).

The *klotho* gene encodes a 130 kDa type I single-pass transmembrane glycoprotein called α-Klotho that contains a short intracellular domain composed of 10 amino acids and an extracellular (EC) domain containing two internal repeats (KL1 and KL2) that are both approximately 450 amino acids long with sequence homology to family 1 β-glycosidases (1). α-Klotho differs from family I glycosidases due to the absence of two conserved glutamic acid residues in its KL1 and KL2 regions that are important for the catalytic activity of this enzyme family (1, 10–12). α-Klotho has been reported to exhibit sialidase and β-glucuronidase activities (13–16). Three primary isoforms of the α-Klotho protein have been identified as follows: (1) the full-length transmembrane form (mKl), (2) a shed soluble form [soluble klotho (sKl)], and (3) a secreted truncated form that is produced by alternative splicing of *klotho* mRNA and consists of KL1 only (17, 18). In the EC space, the secreted truncated form is presumably much less abundant relative to the shed form.

mKl associates with fibroblast growth factor receptors (FGFRs) to form coreceptors for the bone-derived phosphaturic hormone FGF23 (19, 20). sKl is produced when the mKl EC domain is shed from the cell surface into the blood, urine and cerebrospinal fluid following proteolytic cleavage of mKl near the juxtamembrane region by the metalloproteinases ADAM10 and ADAM17 (21–25). Following its release from the cell membrane, circulating sKl exerts its biological effects on distant organs or tissues. Gene and protein expression analyses show that α-Klotho is abundantly expressed in rodents and humans in the kidney and the choroid plexus of the brain, and to a lesser extent in areas such as the parathyroid gland, thyroid gland, pancreas, and sex organs (1, 26–28). Finally, the *klotho* gene family includes two additional family members β-Klotho and γ-Klotho (29, 30). Like α-Klotho, β-Klotho and γ-Klotho are type I single-pass transmembrane proteins that share sequence homology to family 1 β-glycosidases but lack dual conserved glutamic acid residues that are essential for enzymatic glycosidase activities (29, 30). β-Klotho is expressed mainly in liver, adipose tissue, and pancreas, whereas γ-Klotho is expressed in the kidney and skin (29, 30). FGF19 and FGF21 require β-Klotho as a coreceptor to bind FGFRs and activate FGF signaling pathways that regulate bile acid synthesis and energy metabolism (31).

FUNCTIONS AND MECHANISM OF ACTION OF sKl

Binding of FGF23 to mKl-FGFR coreceptors plays critical roles in vitamin D, calcium, and phosphate metabolism (19, 20, 32). Homozygous hypomorphic *kl/kl* mice have severe hypervitaminosis D, hypercalcemia, hyperphosphatemia, and extensive tissue calcification (32, 33). Dietary vitamin D or phosphate restriction rescues growth retardation and premature death in *klotho*$^{-/-}$ mice, validating that function of mKl as a coreceptor for FGF23 is critical for normal vitamin D and mineral metabolism, as well as growth and lifespan (32, 33). By contrast, the function and mechanism of action of sKl are less clear. Several recent studies have provided important information to advancing our understanding of the function and mechanism of action of sKl. In this review, we will summarize the current knowledge of pleiotropic functions of sKl and discuss recent studies that decipher the molecular mechanisms of action of sKl by identifying its receptors. Finally, we will review the cardioprotective function of sKl to illustrate an important function of sKl independently of the FGFR–FGF23 axis.

sKl Can Function As a Circulating Hormone

α-Klotho is predominantly expressed in the kidney and brain (1). However, *klotho*$^{-/-}$ mice exhibit functional defects in cells that do not express α-Klotho suggesting that circulating sKl can function as a hormone to act at a distance. Overexpression of the *klotho* gene extends lifespan in the mouse (2). The antiaging effects of α-Klotho have been attributed to inhibition of insulin-like signaling, which is an evolutionarily conserved mechanism for suppressing aging (34). *In vitro* studies have demonstrated that sKl suppresses autophosphorylation of insulin/IGF-1 receptors and downstream signaling events that include tyrosine phosphorylation of insulin receptor substrates (IRS) and phosphoinositide 3-kinase (PI3K) p85 association with IRS proteins (2). In addition, inhibition of insulin/IGF-1 signaling alleviated aging-like phenotypes in *klotho*$^{-/-}$ mice (2). sKl-mediated inhibition of insulin/IGF-1/PI3K signaling may suppress aging by inducing resistance to oxidative stress. The insulin/IGF-1/PI3K pathway is linked to oxidative stress *via* the FoxO forkhead transcription factors (FOXOs) that are downstream targets of insulin-like signaling that regulate aging (34). Inhibition of insulin-like signaling results in FOXO activation and the upregulation of genes that encode antioxidant enzymes, such as mitochondrial manganese superoxide dismutase (MnSOD), that is important for removing reactive oxygen species and reducing oxidative stress (35). Studies have revealed treatment of cultured cells with sKl reduces lipid oxidation and apoptosis induced by the superoxide-generating herbicide paraquat by blocking insulin-mediated inhibition of FOXO which promoted FOXO activation and nuclear translocation (3). Nuclear FOXO was shown to bind to the MnSOD gene promoter and increase MnSOD protein levels (3). Insulin-induced FOXO phosphorylation/inactivation was enhanced in *klotho*$^{-/-}$ mice and attenuated in transgenic mice that overexpress α-Klotho (3). Compared with WT mice, α-Klotho-overexpressing transgenic mice exhibited increased MnSOD protein levels in muscles, reduced urinary 8-OHdG levels (*in vivo* marker of oxidative DNA damage), and enhanced survival following a challenge with a lethal dose of paraquat (3).

In addition to the insulin/IGF-1 pathway, sKl has been shown to confer cytoprotective effects through other antioxidative pathways. For instance, vascular calcification is a phenotype

observed in mice homozygous for a hypomorphic *klotho* allele (*klotho*$^{-/-}$) (1). Oxidative stress contributes to the progression of vascular calcification by inducing apoptosis and senescence in vascular endothelial cells. sKl is considered to act as a hormone in the vasculature where it is continuously exposed to vascular endothelial cells. Studies have demonstrated that sKl reduces H_2O_2-induced apoptosis and senescence in human umbilical vascular endothelial cells (HUVECs) by inhibiting the caspase 3/caspase 9 and p53/p21 pathways (36). The antiapoptotic and anti-senescence effects of sKl in HUVECs may be mediated by the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, while sKl has also been shown to exert antioxidative effects in HUVECs by inducing MnSOD expression *via* activation of the cAMP/protein kinase A (PKA) pathway (37, 38). In addition to endothelial cells, *klotho* gene transfer attenuated angiotensin II-induced superoxide production, oxidative damage, and apoptosis in vascular smooth muscle cells by stimulating cAMP/PKA-mediated suppression of Nox2 NADPH oxidase protein expression (39). *In vitro* and *in vivo* studies have also demonstrated that sKl protects the lung against oxidative damage. In cultured lung epithelial cells, sKl protected the cells from hyperoxic and phosphotoxic injury by increasing cell oxidative capacity *via* induction of nuclear factor erythroid-derived 2-related factors 1 and 2 (Nrf1/2) transcriptional activity (40). In an acute hyperoxic lung injury animal model, injection of sKl-containing medium into rat peritoneum alleviated oxidative damage and interstitial edema and stimulated an increase in total antioxidant capacity (40). Finally, studies indicate α-Klotho acts as an antioxidant effector in liver and brain by modulating the reactive oxygen species-sensitive apoptosis signal-regulating kinase 1/p38 MAPK pathway (41, 42).

Elevated plasma sKl levels are independently associated with a decreased likelihood of cardiovascular disease (CVD) in humans (43). sKl may be a risk factor for CVD based on studies that have demonstrated endothelial dysfunction is inversely correlated with α-Klotho expression (1, 44). Endothelial dysfunction plays a role in the development of atherosclerosis and is characterized by reduced bioavailability of NO, impaired endothelium-dependent vasorelaxation, increased endothelial permeability, increased oxidative stress, and increased expression of adhesion molecules, pro-inflammatory, and pro-thrombotic factors (45, 46). sKl may exert vasoprotective effects on the endothelium and reduces endothelial dysfunction by regulating NO availability. Studies have shown that NO production and vasodilation are impaired in *klotho*$^{+/-}$ mice, whereas endothelial function can be restored in *klotho*$^{+/-}$ mice by parabiosis with WT mice (44, 47). In Otsuka Long-Evans Tokushima Fatty rats, an experimental animal model of atherosclerosis, adenovirus-mediated *klotho* gene delivery ameliorated vascular endothelial dysfunction, increased NO production, reduced elevated blood pressure, and prevented medial hypertrophy and perivascular fibrosis (48). Mechanistic investigations using HUVECs have demonstrated that sKl upregulates NO production *via* a cAMP-dependent pathway (37). The cAMP–PKA pathway is known to contribute to activation of endothelial NO synthase and increased NO production in coronary arteries (49–51).

Soluble klotho also prevents endothelial dysfunction by maintaining endothelial integrity and protecting against vascular permeability. In endothelial cells, calcium regulates numerous functions including proliferation, migration, and apoptosis (52). Studies report that sKl binds the transient receptor potential canonical 1 (TRPC1) calcium-permeable channel and vascular endothelial growth factor receptor 2 to strengthen their association and cause their cointernalization which regulates the expression level of TRPC1 on the plasma membrane (53). This allows sKl to tightly regulate VEGF-stimulated calcium entry and hyperactivity of calcium-dependent proteases in endothelial cells which maintains endothelial integrity (53). In support of sKl's role in maintaining endothelial integrity, the vascular endothelium is hyperpermeable in *klotho*$^{-/-}$ mice, believed due to increased TRPC1 expression and TRPC1-mediated calcium influx, hyperactivation of calcium-dependent calpain/caspase 3, and increased apoptosis and endothelial damage (53). Finally, a growing body of evidence indicates vascular inflammation plays an important role in endothelial dysfunction. Pro-inflammatory molecules, such as tumor necrosis factor-α (TNF-α), upregulate adhesion molecules on the surface of endothelial cells (54, 55). Moreover, studies have demonstrated that the expression of the adhesion molecules ICAM-1 and VCAM-1 are increased in animals with inflammation and in human atherosclerotic plaques (54). Recombinant sKl inhibited TNF-α-induced expression of ICAM-1 and VCAM-1 on HUVECs (56). In addition, sKl blocked TNF-α-induced NF-κB activation in HUVECs, which is significant because NF-κB is a transcription factor that regulates ICAM-1 and VCAM-1 expression (56). Thus, sKl may maintain endothelial integrity by regulating the expression of endothelial cell inflammatory mediators such as adhesion molecules and NF-κB.

Tumor suppressor genes regulate cell proliferation and inhibit tumor development. *Klotho* may be a tumor suppressor in a wide range of malignancies that include breast cancer, cervical cancer, pancreatic cancer, melanoma, gastric cancer, colorectal cancer, lung cancer, liver cancer, renal cell carcinoma, and ovarian cancer (57–67). In all of these cancers, *klotho* expression was reduced in tumor tissue compared with normal tissue. Epigenetic modifications, such as DNA methylation and histone modifications, often play an important role in regulating the expression of tumor suppressor genes (68). Promoter methylation and histone deacetylation have been found to be epigenetic silencing mechanisms of *klotho* expression in multiple types of cancer (58, 61–63, 65). In addition, microRNAs appear to play a role in cancer progression by targeting *klotho* and regulating its expression (68–70). The reduction of *klotho* expression in malignant tissue suggests that α-Klotho has anticancer effects. Studies by re-expression of *klotho* in cancer cells revealed that sKl acts as a tumor suppressor by inhibiting multiple signaling pathways that include the insulin/IGF-1 pathway, FGF pathway, Wnt signaling pathway, and transforming growth factor-β1 (TGF-β1) pathway.

The insulin/IGF-1 signaling pathway plays an important role in cell proliferation, apoptosis, and cancer (71, 72). Insulin and IGF-1 binding to their receptors activates IRS proteins leading to activation of PI3K/Akt or MAPK/ERK1/2 cell signaling pathways, which play a role in the normal development and

maintenance of tissues. Dysregulation of these pathways can lead to tumor development and progression. α-Klotho acts as a tumor suppressor by inhibiting insulin/IGF-1 signaling in breast cancer, lung cancer, pancreatic cancer, gastric cancer, liver cancer, colon cancer, and ovarian cancer (57, 59, 65, 73–76). Overexpression of α-Klotho or treatment with sKl inhibits insulin/IGF-1-mediated downstream effectors IRS-1, Akt1, and ERK1/2 in cancer cells (57, 59, 65, 67, 73–76). The tumor suppressive activity has been attributed to its KL1 domain (59, 76).

Defects in the regulation of the Wnt signaling pathway also cause cancer (77). Wnt signaling is initiated when secreted Wnt ligands activate transmembrane receptors that promote the translocation of β-catenin to the nucleus where it induces the activity of transcription factors such as TCF and LEF (77). The activation of gene transcription by β-catenin leads to the synthesis of genes, such as *c-myc* and *cyclin D1*, that cause cancer cell growth and invasiveness (78). sKl is a Wnt antagonist that binds to multiple Wnt ligands and inhibits their activation of Wnt signaling (79). sKl inhibition of the Wnt signaling pathway has been shown to reduce cancer cell invasiveness, proliferation, and viability, while it increased cancer cell apoptosis (60, 64, 80). Biochemical evidence has shown that sKl reduces Wnt5A and Wnt3A expression and internalization in melanoma and lung cancer cells, which downregulates Wnt–β-catenin signaling and expression of the Wnt target genes *c-myc* and *cyclin D1* (60, 64). In melanoma cells, sKl reduced cell invasiveness by inhibiting Wnt5A stimulation of μ-calpain-mediated cleavage of Filamin A (60). TGF-β1 signaling pathway plays an important role in cancer metastasis (81), and sKl suppressed TGF-β1-induced epithelial-to-mesenchymal transition to inhibit renal fibrosis and cancer metastasis in mice (82). *In vitro* studies revealed sKl binds the type II TGF-β receptor to reduce TGF-β1 binding which inhibited receptor activation, Smad3 phosphorylation, and Smad3 transcriptional activity (82). Finally, α-Klotho acts as a tumor suppressor by modulating the FGF signaling pathway. Basic FGF-mediated ERK1/2 phosphorylation and activation of the FGF pathway inhibit colony formation in breast cancer cells (57). Overexpression of α-Klotho enhanced bFGF-mediated ERK1/2 phosphorylation and FGF pathway activation in these cells (57). In pancreatic cancer cells, overexpression of α-Klotho or the α-Klotho KL1 domain reduced bFGF-mediated phosphorylation of Akt and ERK1/2 and cancer cell growth (59).

Identification of Membrane Lipid Rafts and Gangliosides As Receptors for sKl

As a "hormone," sKl regulates multiple signaling pathways to elicit pleiotropic cellular effects. However, the mechanism of action of hormonal sKl remains poorly understood in part because membrane receptors for sKl have not been identified. Recent studies have shed light on this gap in knowledge and identified monosialogangliosides GM1 and GM3 present in lipid rafts as receptors for sKl (83). sKl co-migrated with lipid raft fractions in sucrose gradient ultracentrifugation experiments indicating sKl's affinity for lipid rafts (83). Förster resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy studies demonstrated sKl alters lipid organization and decreases membrane order within rafts (83). Studies have shown that inhibition of PI3K-dependent TRPC6 function underlies cardioprotection by sKl (84). sKl also selectively downregulated growth factor-driven PI3K/Akt signaling and TRPC6 channel function in lipid rafts, but not in non-lipid raft regions (83). *In vitro* binding assays and competition experiments using TRPC6-based functional assays identified α2,3-sialyllactose in the glycan of GM1 and GM3 gangliosides as the minimal motif required for sKl binding and regulation of TRPC6 in lipid rafts (83). Furthermore, these assays demonstrated that sKl affinity is 300-fold greater for clustered α2,3-sialyllactose compared with free α2,3-sialyllactoses which supports the notion that lipid rafts enriched in α2,3-sialyllactose-containing GM1 and GM3 gangliosides are effective targets for physiologically low circulating concentrations of sKl (~30 pM) (83). Sialylated glycans bind specifically to a number of glycan-binding proteins, but these binding interactions tend to be of low affinity. The formation of glycan clusters is a common mechanism that generates high affinity biologically relevant binding sites for multivalent glycan-binding proteins (85). Moreover, sKl is likely multivalent due to the fact that sKl forms dimers and each unit contains two highly homologous KL1 and KL2 domains with potential glycan-binding activity (86). The multimeric nature of sKl and the clustering of gangliosides likely explain why circulating sKl preferentially targets GM1 and GM3 clustered in lipid rafts rather than un-clustered GM1 and GM3 present in non-raft membranes or isolated α2,3-sialyllactose residues present in glycoproteins (**Figure 1**). The idea of sKl specifically binding lipid rafts was further supported by FRET experiments in live cells that showed sKl selectively interacts with lipid raft-associated GM1, as well as permeation experiments using hexyltriphenylphosphonium (C6TPP) showing sKl has no effect on disordered membranes (i.e., non-lipid raft membrane regions) (83). The *in vivo* relevance of these findings was confirmed by the discovery that raft-dependent PI3K signaling is upregulated in *klotho*$^{-/-}$ mouse hearts compared with WT mouse hearts (83). By contrast, PI3K signaling in non-raft membranes is not different between WT and *klotho*$^{-/-}$ mouse hearts (83).

To further support the notion that sKl binds sialogangliosides in lipid rafts to regulate TRPC6 and cardioprotection, the investigators determined a modeled structure of sKl by homology modeling and used docking protocols to examine the potential binding sites in sKl for α2,3-sialyllactose (87). It was shown that Arg^{148}, His^{246}, and the $^{465}EWHR^{468}$ motif found in the KL1 domain of sKl are important for binding α2,3-sialyllactose (87). Binding experiments using biolayer inferometry showed the KL1 domain alone indeed binds α2,3-sialyllactose with a K_d value that is similar to that reported for the entire ectodomain of sKl (83, 87). Finally, purified recombinant KL1 domain inhibits TRPC6 in cultured cells and protects against stress-induced cardiac hypertrophy in mice (87). Overall, these studies provide compelling evidence supporting that sialogangliosides GM1 and GM3 and lipid rafts can serve as membrane receptors for sKl.

sKl Functions As an Enzyme to Regulate Ion Channels/Transporters

Binding of FGF23 to FGFRs and the coreceptor mKl inhibits the synthesis of 1,25$(OH)_2$–vitamin D (32). Elevated 1,25

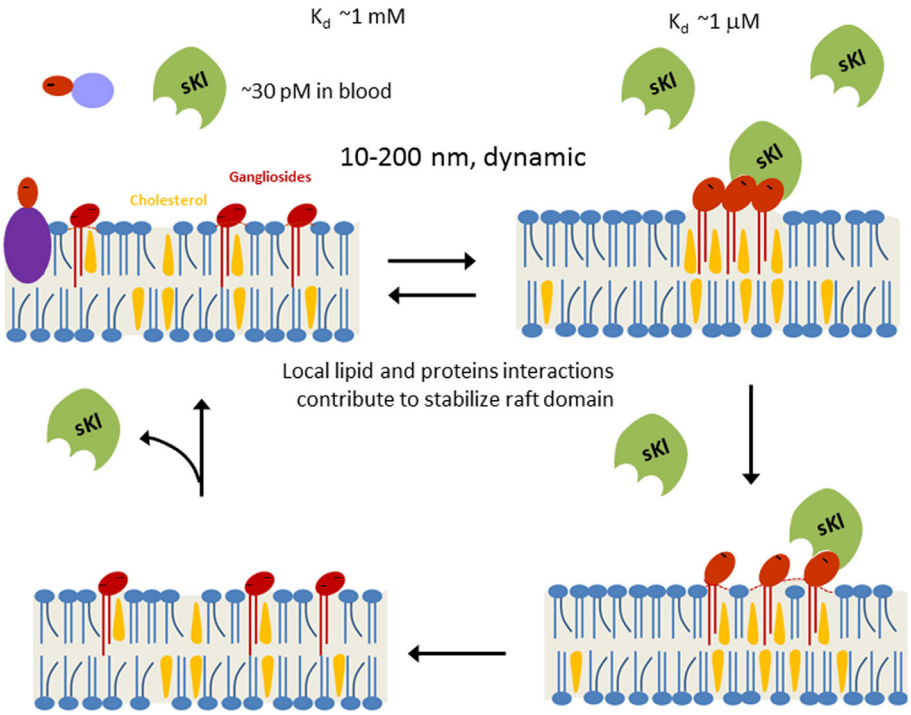

FIGURE 1 | Working model for soluble klotho (sKl) regulation of lipid rafts. Lipid rafts are highly dynamic cholesterol- and sphingolipid-rich membrane microdomains (10–200 nm in size). Formation of lipid rafts is governed by physicochemical properties of lipids and stabilized by local lipid–protein and protein–protein interactions. α2,3-Sialyllactose (dark-red ovale) is a common glycan motif present in many secreted glycoproteins, membrane glycoproteins, and glycolipids such as gangliosides. Due to low circulating concentration (~30 pM) and low binding affinity (K_d ~1 mM), sKl does not bind to isolated α2,3-sialyllactose significantly. Clustering of α2,3-sialyllactose-containing gangliosides in lipid rafts enhances the "apparent" binding affinity for the likely multivalent sKl. Binding of sKl to gangliosides decreases the formation of rafts. sKl is likely multivalent for binding sialyllactose because each sKl contains homologous KL1 and KL2 domains and it likely exists as dimers (86).

$(OH)_2$–vitamin D causes hypercalcemia in *klotho*$^{-/-}$ mice (88). In addition, sKl plays an important role in calcium homeostasis by regulating the transient receptor potential vanilloid type 5 (TRPV5) calcium channel located at the apical surface of the distal convoluted and connecting tubules that is responsible for calcium reabsorption in the distal nephron (89–91). sKl directly increases renal calcium reabsorption by enhancing cell-surface abundance of TRPV5. An early study demonstrated sKl increases TRPV5 cell-surface abundance by modifying *N*-glycan chains of TRPV5 (14). Subsequent investigations sought to identify the specific TRPV5 sugar residues that were modified by sKl and how *N*-glycan modification led to TRPV5 accumulation in the plasma membrane. Structurally, the *N*-glycan chains of TRPV5 can consist of as many as four branches (92, 93). Individual *N*-glycan branches are initiated by *N*-acetylglucosamine addition to mannose residues followed by galactose addition to form *N*-acetyllactosamine (LacNAc) (93). Galactoses can be capped with sialic acids in a reaction catalyzed by α2,3- and α2,6-sialylytransferases (94–96). sKl increases cell-surface abundance of TRPV5 by acting as a sialidase and specifically removing terminal α2,6-linked sialic acids from TRPV5 *N*-glycan chains (15). Galectins are a family of galactose-binding lectins present extracellularly on the cell surface as well as inside the cell (97, 98). Galectin-1 binds LacNAc, but not α2,6-sialylated LacNAc (99). sKl removal of terminal α2,6-sialic acids from TRPV5 *N*-glycan chains exposes LacNAc residues which bind EC galectin-1 present on the cell surface (15). The binding of galectin-1 to TRPV5 prevents endocytosis and leads to channel accumulation on the cell membrane (15). In general, the affinity for binding galectin-1 is enhanced by the polymeric structure of LacNAc in the *N*-glycan chains. Functional TRPV5 channels have a tetrameric stoichiometry which increases *N*-glycan number, polymeric LacNAc, and the affinity of TRPV5 for galectin-1 (100, 101).

In addition to TRPV5, sKl regulates other ion channels and transporters in the kidney by modifying their *N*-glycan chains. sKl increases the cell-membrane abundance of renal outer medullary potassium channel 1 (ROMK1) by removing terminal α2,6-sialic acids from *N*-glycans of the channel (16). Like TRPV5, removal of α2,6-sialic acids exposes underlying LacNAc which binds galectin-1 to prevent ROMK1 endocytosis leading to accumulation of functional channel on the plasma membrane (16). Together with the finding that sKl regulates membrane lipid rafts by binding sialogangliosides, targeting sialic acids may be a general mechanism for pleiotropic actions of sKl. How sKl appears in the urinary lumen remains unclear. Possibilities include shedding of mKl present in the apical membrane of tubular epithelial cells (if present) or *via* transcytosis from the systemic circulation across the proximal and distal renal tubules (102). Finally, it

should be noted that apically localized mKL could conceivably act on TRPV5 or ROMK1 *in situ*.

FGF23-INDEPENDENT CARDIOPROTECTION BY sKl

Cardiac hypertrophy is highly prevalent in patients with chronic kidney disease (CKD) and associated with increased mortality risk (103–106). Conventional risk factors, such as hypertension and volume overload, play important roles in the development of cardiac hypertrophy in CKD (104, 106–108). In addition, multiple CKD-specific risk factors increase the likelihood of cardiac hypertrophy including elevated circulating FGF23 levels and phosphate retention (104, 109). Circulating FGF23 concentrations increase progressively during early and intermediate stages of CKD and can reach levels that are 1,000 times above normal by late stage CKD (110–112). Elevated FGF23 levels in CKD are considered a compensatory mechanism to counteract hyperphosphatemia (113). However, chronically elevated FGF23 levels may become maladaptive to directly stimulate cardiomyocyte growth and induce cardiac hypertrophy in patients with CKD (111).

Soluble klotho levels decline during CKD, which suggests it is a biomarker for CKD diagnosis (114, 115). Studies have shown that the decline in sKl in CKD may be an independent risk factor for CKD-associated cardiac hypertrophy (109). The cardioprotective effects of sKl were investigated using a recognized model of stress-induced cardiac hypertrophy that involves overstimulation by the non-selective β-adrenoreceptor agonist isoproterenol (ISO) (84, 116, 117). Pathological heart growth was induced by ISO in WT mice as reflected by increases in heart size, heart weight indices (heart weight-to-body weight ratio or heart weight-to-tibia length ratio), cardiac fibrosis, and cardiac hypertrophic genes, and these ISO-induced increases were aggravated in *klotho*$^{-/-}$ mice (84). Additional studies revealed that klotho deficiency aggravated cardiac hypertrophy in CKD mice, in a manner completely independent of phosphate and/or FGF23 (118). Recombinant klotho ameliorated CKD-associated cardiac hypertrophy without significantly altering serum phosphate and/or FGF23 levels (118). Thus, sKl deficiency is an important risk factor for CKD-associated cardiac hypertrophy independently of the effects of hyperphosphatemia and FGF23.

Injury and stress induce pathological growth and remodeling of the heart. One important regulatory pathway in the development

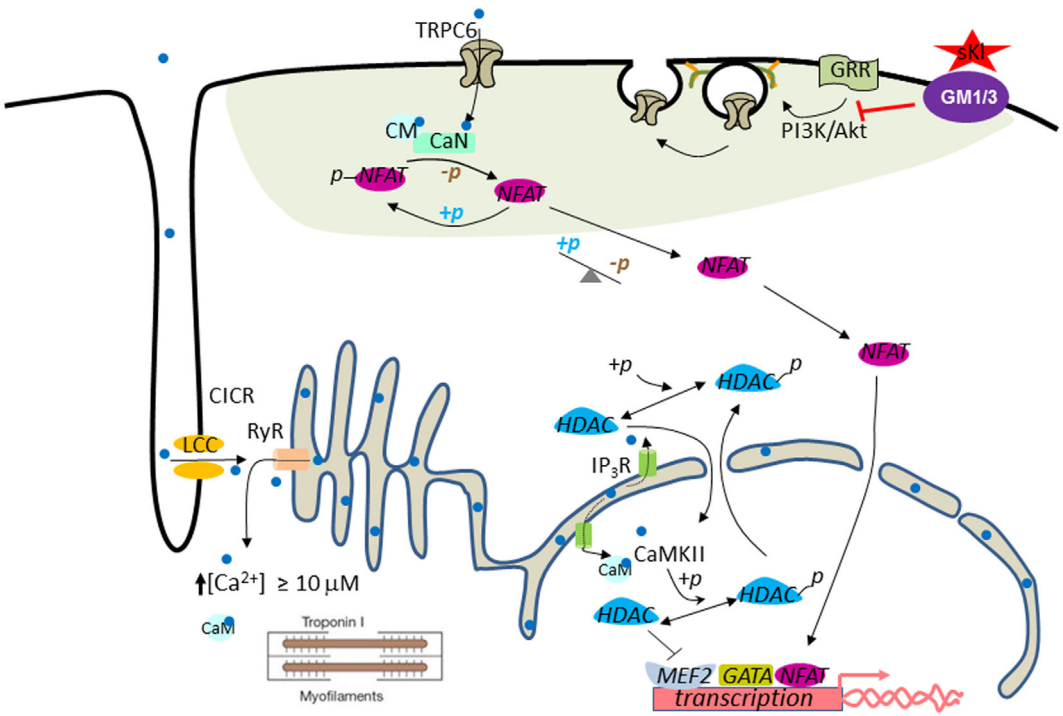

FIGURE 2 | Working model for cardioprotection by soluble klotho (sKl). In the systolic phase, Ca^{2+} (light blue dot) enters through L-type Ca^{2+} channels (LCC) in the T-tube and initiates Ca^{2+}-induced Ca^{2+} release (CICR) from ryanodine receptors (RyR). This process results in increased intracellular [Ca^{2+}] to ≥10 µM to trigger cardiac contraction (i.e., contractile Ca^{2+}). Pathological cardiac remodeling and hypertrophy are triggered by compartmentalized abnormally elevated Ca^{2+} levels, called signaling Ca^{2+}. Perinuclear/nuclear Ca^{2+} released from inositol trisphosphate receptor (IP3R) present in the nuclear envelope activates CaMKII–HDAC–MEF2 nuclear signaling cascade. CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; HDAC, histone deacetylase; MEF2, myocyte-enhancer factor-2. Activation of the TRPC6–CaN–NFAT signaling cascade originated from the sarcolemmal TRPC6 channels amplify and sustain cardiac hypertrophic gene expression through a feed-forward circuit [see text for details; calcineurin (CaN)]. Phosphoinositide 3-kinase (PI3K)–Akt signaling is important for exocytotic insertion of TRPC6-containing vesicles. sKl binds to gangliosides GM1 and GM3 (purple ovale) present in the membrane lipid rafts (green–gray shaded area) to inhibit raft-dependent PI3K signaling and TRPC6 channel function. Note that TRPC6 is localized to the lipid raft membrane microdomain.

of pathological cardiac hypertrophy involves calcium-mediated activation of the calmodulin-dependent serine-threonine protein phosphatase calcineurin (119). Activated calcineurin dephosphorylates nuclear factor of activated T cells (NFAT) and causes its translocation to the nucleus where it activates cardiac genes involved in hypertrophic growth (119). Calcium influx through multiple TRPC channels is involved in calcineurin signaling and cardiac hypertrophy (120–122). TRPC6 contains NFAT-responsive elements in its promoter, which helps to amplify and sustain cardiac hypertrophic gene expression through a feed-forward circuit (122). Cardiac TRPC channel expression is increased in stress-induced hypertrophic hearts and downregulation of TRPC channels protects against cardiac hypertrophy (123). Thus, TRPC6 is an important mediator of cardiac hypertrophy and may be a therapeutic target. Studies in both ISO-induced cardiac hypertrophy and CKD models supported that α-Klotho protects the heart by downregulating growth factor-driven PI3K-dependent exocytosis of TRPC6 (84, 118).

One important question related to sKl protection of the heart independently of the FGF23–FGF receptor axis is how sKl inhibits PI3K signaling to downregulate TRPC6 in the heart. In other words, what is the receptor that mediates sKl action to inhibit PI3K signaling? Given that FGF23 also activates the PI3K and NFAT signaling cascade and that FGF23 levels are elevated in CKD patients, questions may be raised as to whether cardioprotection observed by administration of sKl is due to binding and neutralization of circulating FGF23. The studies discussed earlier that sKl binds membrane lipid rafts and inhibits raft-dependent PI3K signaling and TRPC6 function provide further support that cardioprotection by sKl is *via* its own membrane receptors and is independent of FGF23 (**Figure 2**). Finally, other mechanisms for sKl to elicit cellular responses besides *via* binding to gangliosides in lipid rafts, such as direct interaction with IGF-1 receptors and TGF-β receptors are also possible (124, 125).

CONCLUSION AND FUTURE PERSPECTIVES

Identification of membrane lipid rafts and sialogangliosides as receptors have provided new insights into our understanding of how sKl works as a circulating hormone or local autocrine/paracrine factor to exert pleiotropic actions. As in the case of regulation of TRPV5 channels, sKl may target sialic acids to exert its action in different contexts. Other potential mechanisms also exist. Moving forward, it will be important to elucidate the crystal structure of sKl with or without its ligands, which will help with development of smaller active domains of sKl and/or klotho-mimetic for therapeutics. Further understanding of sKl secretion/shedding, regulation, and distribution, as well as handling and pharmacokinetics of endogenous and exogenously administered klotho are also important.

AUTHOR CONTRIBUTIONS

GD, JX, S-WA, and C-LH made substantial contributions to the conception and design of the manuscript, were involved in drafting of the work and critical review for important intellectual content, involved in final approval of the version of the manuscript to be published, and agreed to be accountable for all aspects of the work ensuring that all questions related to the accuracy or integrity of any part of the work will be investigated and resolved.

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REVIEW
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FGF23 Actions on Target Tissues—With and Without Klotho

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Fibroblast growth factor (FGF) 23 is a phosphaturic hormone whose physiologic actions on target tissues are mediated by FGF receptors (FGFR) and klotho, which functions as a co-receptor that increases the binding affinity of FGF23 for FGFRs. By stimulating FGFR/klotho complexes in the kidney and parathyroid gland, FGF23 reduces renal phosphate uptake and secretion of parathyroid hormone, respectively, thereby acting as a key regulator of phosphate metabolism. Recently, it has been shown that FGF23 can also target cell types that lack klotho. This unconventional signaling event occurs in an FGFR-dependent manner, but involves other downstream signaling pathways than in "classic" klotho-expressing target organs. It appears that klotho-independent signaling mechanisms are only activated in the presence of high FGF23 concentrations and result in pathologic cellular changes. Therefore, it has been postulated that massive elevations in circulating levels of FGF23, as found in patients with chronic kidney disease, contribute to associated pathologies by targeting cells and tissues that lack klotho. This includes the induction of cardiac hypertrophy and fibrosis, the elevation of inflammatory cytokine expression in the liver, and the inhibition of neutrophil recruitment. Here, we describe the signaling and cellular events that are caused by FGF23 in tissues lacking klotho, and we discuss FGF23's potential role as a hormone with widespread pathologic actions. Since the soluble form of klotho can function as a circulating co-receptor for FGF23, we also discuss the potential inhibitory effects of soluble klotho on FGF23-mediated signaling which might—at least partially—underlie the pleiotropic tissue-protective functions of klotho.

Keywords: FGF23, klotho, fibroblast growth factor receptor 4, chronic kidney disease, cardiac hypertrophy, inflammation

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FGF23—A BRIEF INTRODUCTION

The family of fibroblast growth factors (FGF) consists of 22 members in humans, with a broad range of biological functions, including the regulation of embryonic development, organogenesis, and metabolism (1). FGFs are divided into seven subfamilies based on phylogenic analyses and overlapping structures (2). Members of the FGF19 subfamily, consisting of FGF19, FGF21, and FGF23, function as circulating hormones and are, therefore, termed endocrine FGFs (3, 4). Unlike paracrine FGFs, such as FGF1 or FGF2, endocrine FGFs share a characteristic structure and lack the heparin-binding domain in their C-terminus which enables their secretion, circulation, and action on distant target organs (5, 6).

FGF23 is a bone-derived hormone that lowers serum phosphate levels (7–9). Dietary phosphate intake stimulates the production and secretion of FGF23 from osteocytes, and FGF23 directly targets the kidney to increase phosphate excretion by downregulating the cell surface expression of the sodium-dependent phosphate transporters, NaPi-2a and NaPi-2c, in the proximal tubule (10–14) (**Figure 1**). In addition, FGF23 reduces circulating levels of active vitamin D by inhibiting renal 1-α-hydroxylase (also called CYP27B1), the enzyme that converts the prehormone 25-hydroxyvitamin D into its active form, 1,25-dihydroxyvitamin D (1,25D), and by increasing the expression of 24-hydroxylase (also called CYP24A1), the enzyme that degrades 1,25D into inactive metabolites (10–14). In the parathyroid gland, FGF23 inhibits the secretion of parathyroid hormone (PTH) (15, 16) (**Figure 1**). FGF23's effects on reducing circulating levels of 1,25D and PTH further contribute to its phosphaturic actions (3, 17).

The biologically active form of FGF23 is a 32 kDa glycoprotein with a conserved N-terminus that shares homologies with the other FGF family members and contains a conserved FGF receptor (FGFR) binding site (18–20) (**Figure 2**). As the case for all endocrine FGFs, the C-terminus of FGF23 has only low binding affinity for heparin and instead is capable of interacting with alpha-klotho (termed klotho from here on) (20–24), a member of a family of three transmembrane proteins that act as FGFR co-receptors for endocrine FGFs (6, 25). The half-life of circulating FGF23 is about 45–60 min in humans (26), and appears to be much shorter in rodents, with about 20 min in mice (27) and 5 min in rats (28). Renal extraction seems to be a major contributor to FGF23 metabolism, while renal FGF23 excretion might play a minor role as FGF23 cannot be detected in urine, at least not in rodents (28). However, FGF23 can be measured in urine from patients with acute kidney injury (AKI), where elevations correlate with mortality (29). Whether in the context of AKI urinary FGF23 is derived from circulating filtered FGF23 or produced by the injured kidney is currently not clear.

FGF23—POSTTRANSLATIONAL MODIFICATIONS AND PROCESSING

FGF23 can be cleaved by subtilisin-like pro-protein convertases, such as furin, at a consensus sequence (Arg^{176}-X-X-Arg^{179}) that is not present in other FGF family members (30–32) (**Figure 2**). FGF23 is O-glycosylated at several sites (31, 33, 34), and O-glycosylation at Thr^{178} by polypeptide *N*-acetylgalactosaminyltransferase 3 (GalNT3) protects FGF23 from proteolytic cleavage (35). FGF23 is also phosphorylated at several serine residues (36), and phosphorylation at Ser^{180} *via* the secretory protein kinase family with sequence similarity-20 member C (FAM20C), also called dentin matrix protein 4, inhibits GalNT3-mediated O-glycosylation, and thereby promotes proteolytic cleavage of FGF23 (37). A tight regulation of FGF23's posttranslational modifications and processing is crucial, as mutations in modification sites and interference with processing can block or promote FGF23 cleavage, leading to elevated serum levels of intact FGF23 and hypophosphatemia (32, 38, 39) or to reduced serum levels of intact FGF23 and hyperphosphatemia (33, 34, 40–42), respectively, both associated with mineral bone disorders.

Furin-mediated cleavage of FGF23 results in the generation of two fragments and thereby separates the binding domains for FGFRs and klotho from each other (**Figure 2**). As FGF23 appears to act in concert with FGFR and klotho, it has been assumed that the two FGF23 fragments by themselves are inactive, as supported by injection studies in mice showing that both fragments lack phosphaturic activity (31). Interestingly, injections of





Cell type	Parathyroid chief cells	Renal tubular epithelial cells	Renal fibroblasts	Cardiac myocytes	Cardiac fibroblasts	Hepatocytes	Macrophages	Neutrophils
Klotho	+	+	-	-	-	-	?	-
FGFR isoform	1	1	4	4	?	4	1	2
Signal mediators	FRS2α/Ras/MAPK	FRS2α/Ras/MAPK	PLCγ/calcineurin/NFAT	PLCγ/calcineurin/NFAT	?	PLCγ/calcineurin/NFAT	FRS2α/Ras/MAPK	PKA/Rap1
Cellular effects	Decreased PTH expression	• Downregulation of NaPi-2a/c transporters • Inhibition of CYP27B1 • Activation of CYP24A1	• Increased TGFβ production • Activation	Hypertrophic growth	• Activation • Proliferation	Increased IL-6 and CRP expression	Increased TNFα production	• Decreased integrin activation • Increased rolling velocity
Organ effects	Suppression of PTH secretion	• Reduction of phosphate uptake • Reduction of vitamin D activation	Fibrosis	Hypertrophy	Fibrosis	Elevation of IL-6 and CRP secretion	-	-
Systemic effects	Reduced serum levels of PTH and calcium	Reduced serum levels of phosphate and 1,25D	Kidney failure	• Heart failure • Compensatory remodeling	Heart failure	Inflammation	Impaired immune response	• Reduced leukocyte recruitment • Impaired host defense

FIGURE 1 | The major target organs of FGF23. FGF23 can directly target different cell types in a variety of organs. The underlying molecular pathways can differ in their requirement for klotho as well as the involvement of specific FGFR isoforms and downstream signal mediators, leading to cell type-specific events and tissue-specific effects.

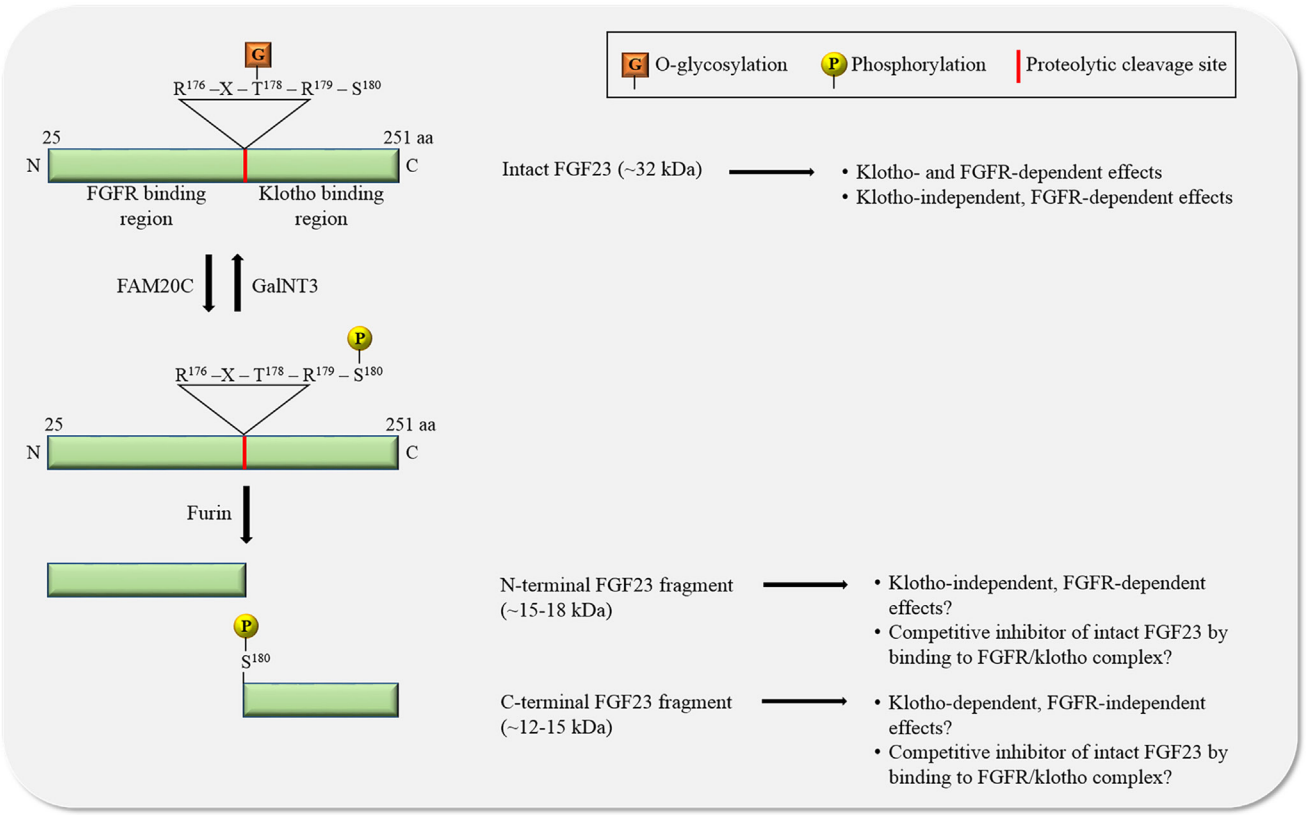

FIGURE 2 | The regulation of FGF23 by posttranslational modifications. O-glycosylation by *N*-acetylgalactosaminyltransferase 3 (GalNT3) at threonine residue 178 (T^{178}) protects FGF23 from proteolytic cleavage resulting in the generation of intact, biologically active FGF23. Phosphorylation of FGF23 on serine residue 180 (S^{180}) by family with sequence similarity-20 member C (FAM20C) prevents O-glycosylation and promotes proteolytic cleavage by furin proteases. The biological relevance of the two generated FGF23 fragments is unclear. Abbreviations: aa, amino acid; N, N-terminus; C, C-terminus.

the C-terminal fragment containing the klotho binding site in a genetic mouse model with high serum concentrations of FGF23, reduce FGF23 excess and associated renal phosphate wasting (22, 43), suggesting that FGF23 cleavage not only removes the klotho binding site from FGF23, but also generates an endogenous inhibitor of FGF23. While the mechanism underlying such an inhibitory action is not understood, it is plausible to speculate that C-terminal FGF23 can interact with klotho without binding and activating FGFRs, thereby competitively blocking access of intact FGF23 to the FGFR/klotho complex and inhibiting FGF23-induced signaling. The existence of such a mechanism is supported by an *in vitro* study, showing that the FGF23-mediated reduction of phosphate uptake in proximal tubular cells is blocked in the presence of the C-terminal FGF23 fragment (44). However, this view has been challenged by a different injection study in mice showing that C-terminal FGF23 retains phosphaturic activity (45), indicating that either the C-terminus by itself can bind FGFRs or that the fragment's cellular actions are FGFR-independent and possibly mediated by other receptors. Furthermore, cell culture studies with chimeric FGF23:FGF21 proteins have shown that the replacement of the C-terminal klotho-binding site in FGF23 does not result in a loss of FGF23's ability to activate FGFR/klotho-mediated signaling (46), suggesting that the N-terminus of FGF23 by itself can bind klotho. Nevertheless, a recent analysis of the crystal structure of the FGF23/FGFR1/klotho ternary complex clearly indicates that the N-terminus of FGF23 interacts with FGFRs, while FGF23's C-terminus is bound to klotho (47).

It is currently unclear whether FGF23 cleavage fragments are biologically active, and if so, whether this activity differs from the actions of intact FGF23. One could speculate that furin-mediated cleavage serves as a first step in further proteolysis and removal of FGF23. However, since the same bone cell not only synthesizes, but also cleaves FGF23 and is, therefore, capable of releasing intact FGF23 as well as FGF23 fragments into the circulation (48), it is reasonable to assume that the fragments have a function and are not just a proteolytic garbage product. As FGF23 synthesis and cleavage appear to be independent events, they provide two distinct levels for the regulation of FGF23 production (48). It is possible that classic factors associated with mineral metabolism [such as phosphate, calcium 1,25D, and PTH (11, 49–52)] and novel factors linked to pathologic scenarios [such as systemic elevations of inflammatory cytokines, iron deficiency, and hypoxia (53–56)] regulate FGF23 production in osteocytes and osteoblasts at different levels. The massively elevated serum levels of intact FGF23, as observed in late stages of chronic kidney

disease (CKD) (57, 58), seem to result from an increase in FGF23 synthesis (59) accompanied by an inhibition of FGF23 cleavage (53, 60). The inducers and mechanisms of FGF23 production and processing in bone are currently studied by many investigators [as reviewed in more detail elsewhere (48, 61–64)], and their characterization should provide important answers to one of the key questions in the field, i.e., why circulating FGF23 is elevated in CKD, as well as novel pharmacological targets to lower serum FGF23 levels. Furthermore, it needs to be determined whether cleavage of FGF23 only occurs in bone or also in the circulation and/or other tissues, which would suggest that the half-life of circulating FGF23 can be regulated and might be increased in CKD, and whether distant organs, such as the kidney, can control the production and processing of FGF23 in bone cells. Moreover, it is likely that changes in renal clearance of FGF23 might also contribute to CKD-associated FGF23 elevations.

KLOTHO—A PROTEIN THAT COMES IN MULTIPLE FORMS

Klotho was originally identified as an anti-aging protein, because genetically modified mice lacking klotho develop a variety of phenotypic features that are associated with premature aging, including multiple organ dysfunction and a significantly shortened life span (65). The klotho gene encodes a 130 kDa single-pass transmembrane protein that is composed of two extracellular domains, termed KL1 and KL2, a transmembrane domain and a short cytoplasmic tail (66, 67). The KL1 and KL2 domains show amino acid sequence homologies with β-glucosidase of bacteria and plants, and, therefore, klotho could potentially catalyze the release of glucose from oligosaccharides (65). However, based on the absence of two conserved glutamic acid residues that are important for enzymatic activity of this family (65), it appears that klotho has only weak glucosidase activity, if any. A recent structural analysis of the klotho ectodomain combined with an *in vitro* assay to detect glycosidase, sialidase, and β-glucuronidase activities confirmed that klotho lacks enzymatic activity (47).

Klotho is mainly expressed in the kidney, brain, and parathyroid gland (65, 68, 69). In addition to the membrane-associated full-length protein, the ectodomain of klotho can also exist in a soluble form (soluble klotho, sKL), which can be generated by proteolytic cleavage of full-length klotho *via* the α-secretases, a desintegrin and metalloproteinase (ADAM) 10 and ADAM17, leading to sKL shedding from the cell membrane (70–73). sKL cleavage can also be mediated by the β-APP cleaving enzyme 1 (BACE1), which belongs to the family of β-secretases, and the remaining membrane-associated klotho fragment is further processed and removed by the γ-secretase complex (71, 73). The kidney is the major source for sKL (73–75), but also ependymal cells of the choroid plexus in the brain might release sKL by shedding (76–78), and sKL can be detected in the blood (73, 79–84) and the cerebrospinal fluid (CSF) (83, 84). The half-life of sKL in rats is about 7 h (73), which might be shortened in CKD, where degradation of circulating sKL appears to be increased (79). It seems that sKL is cleared from the circulation by the kidney, most likely by transport across renal tubules to the apical membrane and release into the urinary lumen (73), and sKL can be measured in the urine (73, 79–82). However, other studies have failed to detect sKL in urine (85), questioning if and how sKL can enter the urinary space.

sKL—AN ENDOCRINE FACTOR WITH PLEIOTROPIC FUNCTIONS

It has been postulated that sKL acts as an endocrine factor that can target a variety of tissues (25, 86–88), but a specific receptor for sKL has not been identified to date, and the mechanisms underlying potential direct actions of sKL on target cells are only poorly understood. Several *in vitro* studies in multiple different cell types, such as fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac myocytes, pulmonary epithelial cells, oligodendrocytes, and neurons, indicate that sKL has cell-protective activities, including the inhibition of apoptosis, oxidative stress, senescence, and pathologic gene programs (80, 89–107), suggesting that sKL might protect against cellular dysfunction as well as tissue fibrosis and inflammation. It has been postulated that such protective actions of sKL also exist *in vivo*, and that a reduction in circulating sKL levels, as observed in aging or in diseases, such as CKD, contribute to widespread tissue injury (86, 87, 108). However, strong experimental evidence indicating that sKL protects tissues by directly targeting them is still missing. Furthermore, it remains unclear if pathologies associated with a global reduction in klotho expression are caused directly by the absence of sKL and its tissue-protective actions, or indirectly by the loss of membrane-associated klotho resulting in systemic alterations, such as elevations in serum levels of phosphate or FGF23 (109). To distinguish experimentally between both scenarios, animal models with preserved expression of membrane-associated klotho, but a loss of sKL production [e.g., by genetically inactivating the proteolytic cleavage site in klotho (72)] need to be generated and analyzed. Since mice with kidney-specific deletion of klotho develop the same phenotype as mice that lack klotho globally (74), sKL derived from other sources than the kidney appears to lack tissue-protective effects and might not be capable of compensating for a loss of kidney-derived sKL.

To date the molecular base for the potentially pleiotropic actions of sKL remains a mystery. Several ligand/receptor complexes, such as insulin/IGF1/IGF1R (110–113), TGFβ1/type-II TGFβ receptor (114, 115), AngII/AT1R (96, 116), and Wnt/Frizzled (115, 117–119), have been postulated to serve as direct sKL targets (88). However, it is unclear how one particular protein can inhibit various signal mediators and receptors that significantly differ in their structure, biophysical features, and mode of action. Recently, a different mechanistic explanation has been suggested for sKL's pleiotropic effects (87). The two KL domains of sKL can bind sialic acid, thereby targeting monogangliosides, such as GM1 and GM3, in cell membranes (120). Since the binding affinity of each KL domain for sialic acid is low (121), sKL preferentially interacts with lipid raft domains where gangliosides are enriched. The association with sKL might then affect overall lipid raft dynamics and composition,

thereby regulating the localization and activity of a variety of raft-associated proteins, including signaling receptors and ion channels (87). This hypothesis is supported by *in vivo* findings showing that raft-associated, but not raft-independent, phosphoinositide 3-kinase (PI3K) signaling is elevated in mice lacking klotho (120). Furthermore, by binding to sialic acid on transmembrane proteins, sKL can regulate their cell surface abundance. This has been shown for transient receptor potential vanilloid type 5 (TRPV5), renal outer medullary potassium channel 1 (ROMK1) and NaPi-2a, which are all located in renal tubular cells (81, 122–124), suggesting that sKL might regulate ion homeostasis (125). It has been reported that sKL has sialidase and β-glucoronidase activity (81, 122–124, 126), indicating that sKL not only binds, but also removes sialic acid from lipids and proteins. However, the recent structural and functional analysis of sKL described earlier refutes such a hypothesis (47). Binding sialic acids in glycolipids and glycoproteins is a plausible mechanistic explanation for sKL's pleiotropic actions (87). To further test this hypothesis, the respective binding site in sKL needs to be characterized and genetically inactivated followed by the functional characterization of the resulting sKL mutant in cell culture and animal models. Furthermore, since all eukaryotic cells contain lipids rafts (127), it is unclear how the described mechanism could ensure target specificity for sKL's action.

Based on the low binding affinity of each KL domain for sialic acid, it has been suggested that sKL acts as a multimer (87). However, strong experimental data supporting the existence of sKL oligomers is still missing. Overexpression studies in cultured cells indicate that full-length klotho is capable of forming dimers (83, 128), and sKL might exist in an oligomeric form in serum and CSF from human and mice (83). Furthermore, klotho and sKL appear to be N-glycosylated (83, 129), but to date a detailed characterization of their posttranslational modifications including functional consequences has not been conducted. Interestingly, sKL might have intracellular activity, as suggested by a recent study (129). sKL can interact with a variety of cytosolic proteins, some of which are involved in regulating cellular anti-oxidative activities or posttranslational modifications and folding of other proteins. While this mechanism needs further experimental validation, including an explanation of how sKL secretion is blocked or bypassed, it is tempting to speculate that its dysregulation in klotho-expressing tissues might contribute to aging-related injury. A potential intracellular role of klotho is also supported by the fact that the klotho-related protein, KLrP, acts as a cytosolic enzyme (130).

FGFR-MEDIATED SIGNAL TRANSDUCTION

The mammalian genome encodes four different FGFR isoforms (i.e., FGFR1-4) (1). FGFRs are receptor tyrosine kinases which are composed of an extracellular domain consisting of three immunoglobulin-like domains and containing the ligand-binding site, a transmembrane domain, and a cytoplasmic tyrosine kinase domain (131). Upon FGF binding, FGFRs form dimers and auto-phosphorylate each other at specific tyrosine residues within their cytoplasmic tails which then initiates subsequent downstream signaling events (132). The FGFR isoforms differ in their affinity for particular FGF ligands. Alternative splicing events increase the variety of FGFR1-3 isoforms, designated as b and c splice variants, thereby increasing the spectrum of FGFRs with distinct FGF binding specificities (1, 131). Although *in vivo* proof is still missing, *in vitro* studies suggest that FGFR isoforms not only form homo- but also heterodimers (133–135), which would further increase the possible combinations of FGFRs to form dimeric complexes with different FGF binding specificities.

FGFs ligands require the presence of co-receptors for efficient FGFR binding. For paracrine FGFs, this co-receptor is heparin/heparan sulfate which captures the FGF upon release and forms a stable complex with FGFRs in an isoform-dependent manner on the same or neighboring cell (131). Endocrine FGFs, such as FGF23, have significantly reduced affinity for heparin (21, 136). This feature enables FGF23 to avoid being captured by extracellular matrices and to function as a hormone, while it also reduces the capacity of heparin to promote FGF23 binding to FGFRs (137). Instead of heparin, klotho acts as an FGF23 co-receptor that promotes efficient binding of FGF23 to FGFRs (138, 139). Biochemical binding studies have shown that klotho increases the FGFR binding affinity of FGF23 by about 20-fold (23). The recent report of its atomic structure revealed that FGF23, sKL, and FGFR1c form a 1:1:1 complex, where sKL functions as a scaffold protein that brings FGFR and FGF23 in close proximity, thereby conferring stability of the ternary complex (47). Surprisingly, while heparan sulfate does not affect the formation and stabilization of the monomeric complex, it is required for complex dimerization, suggesting that the active signaling complex consists of FGF23, sKL, FGFR1, and heparan sulfate in a 2:2:2:2 stoichiometry (47). While FGFRs are widely expressed, the restricted expression of klotho to renal tubules and parathyroid gland defines FGF23 target tissues (15, 65, 75). It is plausible to assume that besides heparin and klotho, many other co-factors participate in the formation of FGF/FGFR signaling complexes, such as cell adhesion proteins of the cadherin and immunoglobulin superfamilies (140–145), and modify the binding affinity, accessibility, and activity of FGFRs.

Fibroblast growth factor receptor signaling is transduced by the cytoplasmic adaptors, phospholipase Cγ (PLCγ), and FGF receptor substrate 2α (FRS2α) (1, 132). Following ligand-induced auto-phosphorylation of FGFR, PLCγ binds directly to one specific phosphorylated tyrosine residue within the FGFR cytoplasmic tail (146, 147). Subsequent tyrosine phosphorylation of PLCγ results in PLCγ activation by the receptor (148). Downstream signal transduction is mediated by PLCγ-catalyzed production of diacylglycerol and inositol 1,4,5-triphosphate that can increase cytoplasmic calcium levels thereby inducing the activation of several calcium-sensing signal mediators, including the protein phosphatase calcineurin (131). The dephosphorylation of the transcription factor, nuclear factor of activated T cells (NFAT), by activated calcineurin causes the translocation of NFAT into the nucleus to modulate the expression of specific target genes (149). FGFR signaling can also be transduced *via* the activation of FRS2α by FGFR-mediated tyrosine phosphorylation (131). In contrast to PLCγ, FRS2α is constitutively

bound to FGFR independently of the receptor's activation state (150). FRS2α-mediated signaling results in the activation of Ras/mitogen-activated protein kinase (MAPK) and PI3K/Akt signaling (131).

Although FGFRs can stimulate a variety of downstream signaling branches (132), most FGF and FGFR isoforms have been shown to employ the FRS2α/Ras/MAPK pathway to mediate their cellular effects. This is also the case for FGF23. In the presence of klotho, FGF23 can bind to the c splice variants of FGFR1-3 as well as to FGFR4 which results in the activation of MAPK signaling (47, 138, 139). In FGF23's physiologic target organs (15, 16, 151–154), FGFR1 appears to be the main FGF23 receptor which acts in concert with klotho (**Figure 3**, I). It has been shown in HEK293 cells, which express different FGFR isoforms, including FGFR1, but lack klotho, that the forced overexpression of full-length klotho followed by FGF23 treatment results in FRS2α/Ras/MAPK signaling (47, 128, 155, 156). Interestingly, the same effect was observed when HEK293 cells were co-treated with FGF23 and sKL (47, 139). These *in vitro* studies show that the introduction of klotho is sufficient to make FGFR expressing cells responsive to FGF23 resulting in an activation of FRS2α/Ras/MAPK signaling. Furthermore, they indicate that membrane-associated klotho and sKL share a common function, which is that both mediate FGF23-induced FRS2α/Ras/MAPK signaling.

FGF23 AND KLOTHO CO-REGULATE FGFR-MEDIATED SIGNALING

Overall, one could speculate that sKL acts as a circulating FGF23 co-receptor that promotes an interaction between FGF23 and membrane-bound FGFRs, thereby facilitating FGF23 binding to cell types that *per se* do not express klotho and mediating temporary responsiveness to FGF23. Such a mechanism has been reported in fibroblast and myoblast cell lines in which the combined treatment with FGF23 and sKL activates FGFR1/MAPK signaling leading to increased cell survival (157). Furthermore, combined treatment with FGF23 and sKL induces Ras/MAPK signaling in cultured osteoblasts, which is FGFR1-dependent and results in increased FGF23 production (158). FGF23 and sKL together also induce the phosphorylation of FRS2α and MAPK in cultured chondrocytes, which seems to be mediated by FGFR3 (159). Interestingly, elevating serum sKL levels in mice by viral overexpression of sKL in the liver or by injection of recombinant sKL protein causes increased Ras/MAPK signaling and reduces NaPi-2a expression in the kidney resulting in increased renal phosphate excretion and hypophosphatemia (47, 157). When conducted in mouse models with CKD or genetic klotho deficiency, such sKL elevations reduce renal NaPi-2a expression as well as the increases in serum phosphate levels (158). Combined, these animal studies suggest that sKL can compensate for the reduction or loss of klotho, and that

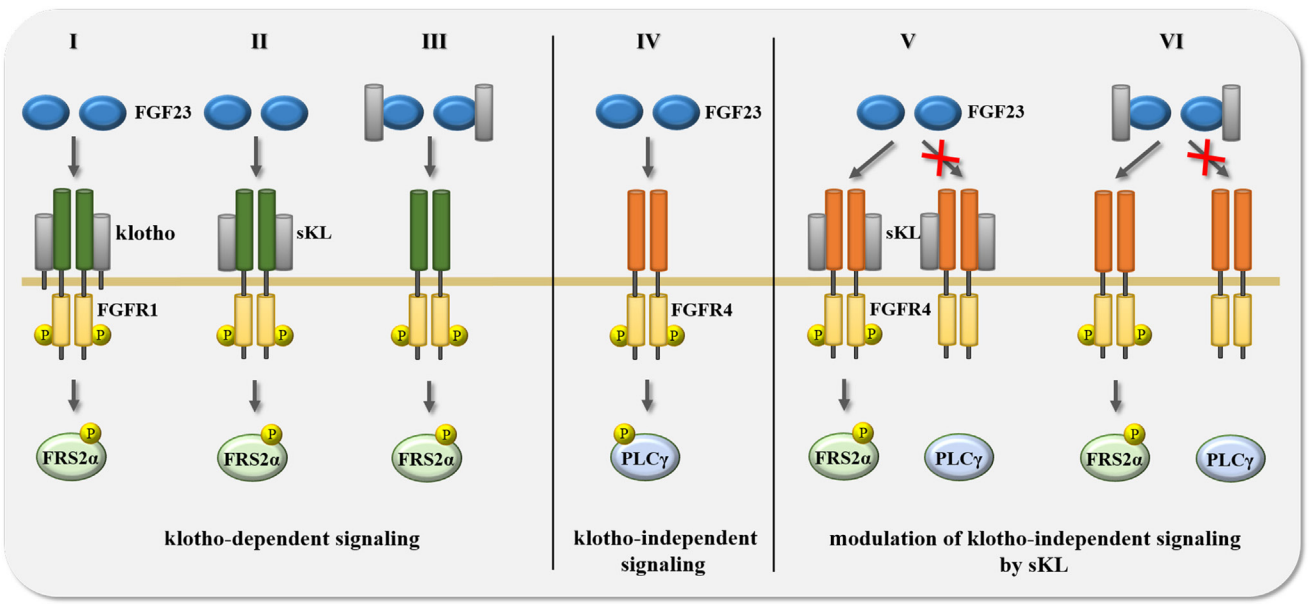

FIGURE 3 | Summary of potential scenarios for the crosstalk between FGF23 and klotho in the regulation of signal transduction. FGF23-activated signaling events are mediated and modified by the availability of either membrane-associated full-length klotho or soluble klotho (sKL). (I–III) Transmembrane klotho as well as sKL mediate FGF23 binding to FGFR1 resulting in the phosphorylation of FGF receptor substrate 2α (FRS2α) and the subsequent activation of Ras/mitogen-activated protein kinase (MAPK) signaling. (IV) In the absence of klotho and sKL, FGF23 binds FGFR4 which induces the phosphorylation of phospholipase Cγ (PLCγ) and the activation of calcium-regulated signal pathways, such as calcineurin/NFAT. (V–VI) Membrane-associated klotho and sKL can modulate klotho-independent FGF23/FGFR4 signaling in two different ways. First, in the presence of klotho or sKL, FGF23 cannot bind to FGFR4, and thereby not activate the PLCγ-driven signaling cascade. Second, binding of FGF23 to FGFR4 can also occur in the presence of transmembrane klotho or sKL, but causes different downstream events, switching from PLCγ to FRS2α phosphorylation, thereby activating Ras/MAPK instead of calcineurin/NFAT signaling. Abbreviation: P, protein phosphorylation.

by targeting the kidney, sKL might increase FGF23-mediated FGFR1/Ras/MAPK signaling in tubular cells, thereby reducing renal phosphate uptake and lowering serum phosphate levels. One could speculate that sKL acts as a circulating factor that elevates FGF23 production in the bone and mediates FGF23 signaling in target organs by promoting FGFR1 binding (160). This view is supported by the recent finding that sKL lacking the FGFR binding domain does not show phosphaturic activity when injected into mice and cannot mediate FGF23-induced Ras/MAPK signaling in HEK293 cells. Similarly, mutant forms of sKL and FGF23 which disrupt sKL-FGF23 binding fail to activate Ras/MAPK signaling (47).

As described before, a recent structural analysis shows that sKL acts as a scaffold protein for FGF23 and FGFRs that promotes FGF23/FGFR-mediated signaling, suggesting that all effects of sKL are FGF23-dependent and thereby challenging the concept that sKL can function as an FGF23-independent hormone (47). A crystal structure can only provide a snap shot of one particular state within a multi-step signaling process and the precise order of binding events among the signal inducers remains unknown. Two scenarios seem to be possible. First, sKL binds a membrane-associated FGFR which then enables FGF23 binding to the receptor complex (**Figure 3**, II). It has been shown that sKL has high affinity for FGFR1c (22), and experiments in cell culture overexpression systems show that full-length klotho can bind FGFRs in the absence of FGF23 (139), suggesting that sKL might have similar abilities. It remains to be established whether endogenous sKL binding and effects are FGFR isoform-specific. As mentioned before, studies in HEK293 cells with overexpressed full-length klotho or with sKL incubations suggest that klotho preferably binds to FGFR1c, FGFR2c, FGFR3c, and FGFR4 (47, 139). Second, FGF23 and sKL bind to each other in solution and then together interact with an FGFR on the cell surface (**Figure 3**, III). Although the sKL/FGF23 complex has been detected in the circulation of rodents (161) and in extracts and supernatants from transfected HEK293 cells (159), surface plasmon resonance spectroscopy studies have shown that sKL binds FGF23 only with very low affinity in the absence of FGFR (22). Clearly, more experimental work is required in order to determine whether FGF23 and sKL can interact with each other in solutions, such as cell culture supernatants or blood.

Studies in HEK293 cells and in primary cardiac myocytes showed for the first time that FGF23 can also affect cell types that do not express klotho (162), implying that the presence of klotho is not a prerequisite for FGF23 responsiveness. Whereas klotho-expressing cells respond to FGF23 by activating the FRS2α/Ras/MAPK cascade (138), FGF23 stimulates PLCγ/calcineurin/NFAT in cells that lack klotho (155, 162, 163). Furthermore, while FGFR1 acts as the main FGF23 receptor in "classic" klotho-expressing FGF23 target organs (138, 139), klotho-independent effects of FGF23 appear to be mediated by FGFR4 (**Figure 3**, IV) (155, 163, 164). In HEK293 cells, FGF23 only induces PLCγ binding to FGFR4, but to none of the other FGFR isoforms (155). Furthermore, deletion of FGFR4 or co-treatment with FGFR4-specific blocking agents inhibits FGF23-induced PLCγ/calcineurin/NFAT signaling in cardiac myocytes (155). Finally, FGF23 can activate the PLCγ/calcineurin/NFAT pathway in cultured hepatocytes, which of all FGFR isoforms only express FGFR4 (163). Overall, it appears that depending on the FGFR isoform and the presence or absence of klotho, FGF23 activates distinct downstream signaling pathways. The FGFR4/PLCγ/calcineurin/NFAT cascade seems to be a major mediator of klotho-independent FGF23 signaling (109), whose tissue-specific effects are discussed in detail below.

Since elevations in serum FGF23 levels, as observed in aging and in CKD, correlate with decreases in renal expression of klotho and in circulating levels of sKL (80, 165), it is interesting to speculate that associated pathologies are caused by both, high FGF23 and low sKL. If true, one would assume that sKL might act as an inhibitor of klotho-independent pathologic actions of FGF23. The existence of such a mechanism is supported by a study in vascular smooth muscle cells and endothelial cells, where sKL counterbalances the effects of FGF23 (98). As discussed earlier for the stimulating effects of sKL on FGF23/FGFR1 signaling, it is possible that sKL inhibits FGF23/FGFR4 signaling by either binding first to FGFR4 (**Figure 3**, V), or *via* an initial interaction with FGF23 (**Figure 3**, VI). sKL could then block the interaction between FGF23 and FGFR4 and thereby inhibit PLCγ/calcineurin/NFAT signaling. It is possible that sKL acts as a circulating FGF23 decoy receptor, as described for other ligands and truncated forms of their transmembrane receptors, such as vascular endothelial growth factor (VEGF) and the soluble form of VEGF receptor-1 (called sFlt-1) generated by alternative splicing (166). It is also possible that sKL can bind FGFR4 and block the access or affinity of FGFR4 for FGF23. A similar mechanism has been reported in cultured proximal tubular epithelial cells, where sKL binding to FGFR1 inhibits FGF2/FGFR1 signaling, most likely by competing with FGF2 for FGFR1 binding (101). Such a mechanism might also underlie sKL's inhibitory actions toward FGF2-induced Ras/MAPK signaling and proliferation in tumor cells (112). As an alternative mechanism, FGFR4 and FGF23 might still be able to form a complex in the presence of sKL, but activate FRS2α/Ras/MAPK rather than PLCγ/calcineurin/NFAT downstream signaling, resulting in significant differences in cellular effects (**Figure 3**, VI).

Shedded sKL consists of the KL1 and KL2 domains (70–73). Furthermore, it has been reported that also the KL1 domain alone can be generated by proteolytic cleavage from membrane-associated klotho (70) or by alternative splicing and subsequent secretion into the extracellular environment (66, 67). However, a more recent study indicates that alternative splicing is not involved in the generation of any soluble forms of klotho, as alternative klotho mRNA transcripts are primed for nonsense-mediated mRNA decay and are not translated into protein (167). Whether soluble KL1 actually exists in animals and in humans, and if so, is present in the circulation and can act as a hormone, needs to be established. Since the KL1 domain seems to be sufficient for sialoganglioside binding (121), and KL1 has tumor suppressor activity *in vitro* and *in vivo* (112, 156), one could speculate that KL1 by itself has biological activity. However, studies with recombinant proteins have shown that KL1 cannot mediate FGF23-induced Ras/MAPK signaling in cultured cells (156), and when injected into mice, KL1 does not lower serum phosphate levels (112). A recent structural study reporting that

sKL binding to FGF23 and FGFR1 is mediated by KL2 and the linker region connecting KL2 with KL1 supports the idea that KL1 by itself might not have biological activity, at least no activity that requires FGF23 and FGFRs (47).

EFFECTS OF FGF23 ON THE HEART

The heart was the first organ that was shown to respond to circulating FGF23 in a klotho-independent fashion (162). FGF23 directly targets cardiac myocytes *via* the described FGFR4/PLCγ/calcineurin/NFAT signaling pathway and induces cardiac hypertrophy, and potentially other changes in cardiac remodeling, including cardiac fibrosis and altered cardiac metabolism, eventually resulting in reduced heart function (155, 162, 164) (**Figure 1**). Animal models with elevated serum FGF23 levels, induced by CKD, genetic deletion of klotho, injection of recombinant FGF23 protein, or a high phosphate diet, develop cardiac hypertrophy, which can be blocked by administration of specific inhibitors for FGFR4 or calcineurin (155, 164, 168). Since only some of these animal models develop kidney injury, hyperphosphatemia, or hypertension, while all of them have elevated FGF23 and cardiac hypertrophy, one can assume that by directly targeting the myocardium, FGF23 is a major driver of cardiac remodeling that acts independently of other pro-hypertrophic factors, such as high blood pressure or uremic toxins. However, these animal models also show low serum sKL levels, and it seems that in all scenarios studied to date, FGF23 elevations are always accompanied by a decrease in sKL concentrations (109). Since animal experiments indicate that sKL reductions *per se* can contribute to cardiac injury (80, 107, 165, 169), it would be important to conduct studies in the absence of FGF23 in order to determine whether in the context of CKD, FGF23 elevations are required for the induction of cardiac injury. However, such loss-of-function studies are not feasible, as FGF23 deletion or inhibition in rodents elevates serum phosphate levels and causes severe cardiovascular injury resulting in premature death (12, 170). Instead, the cardiac myocyte-specific deletion of the FGF23 receptor, i.e., FGFR4, followed by the elevation of circulating FGF23 should help to determine whether direct cardiac actions of FGF23/FGFR4 contribute to cardiac hypertrophy.

Several studies have shown that repetitive intravenous and intraperitoneal injections of recombinant FGF23 protein in wild-type mice induce cardiac hypertrophy within 5 days, indicating that FGF23 has potent hypertrophic effects on the heart (162, 171–173). However, it remains unknown whether FGF23 elevations are also sufficient to impair cardiac function and cause heart failure. Future experiments in animal models with increased serum FGF23 levels need to study the observed changes in cardiac structure and function in more detail, and put them into context with particular FGF23 exposure concentrations and times, which is experimentally challenging. It has been hypothesized that long-term exposure at very high FGF23 concentrations, as the case in patients with late stages of CKD, who can develop up to 1,000-fold elevations for months (174, 175), causes pathological cardiac remodeling and contributes to uremic cardiomyopathy (109, 176–178). Such a hypothesis is supported by a recent study in mice lacking the alpha 3 chain of type IV collagen (Col4a3), a genetic animal model for CKD (179). Dependent on the genetic background, these mice either develop fast-progressing kidney injury and die at around 10 weeks of age, or the development of severe kidney injury takes longer resulting in extended survival until about 20 weeks (180, 181). Although both mouse lines show the same degree in blood pressure elevations, only slow-progressing Col4a3 knockout mice develop cardiac hypertrophy and fibrosis which is accompanied by increased cardiac expression levels of FGFR4 (179). At 10 weeks of age, fast-progressing Col4a3 knockout mice show significantly higher elevations in intact FGF23 concentrations than slow progressors. Although serum FGF23 levels in slow-progressing mice further increase between 10 and 20 weeks, they never reach the levels of those detected in fast progressors. Overall, this study indicates that the duration of the exposure to increased circulating FGF23, rather than the degree of FGF23 elevation *per se*, is an important determinant in the development of pathologic cardiac remodeling in this model of CKD. Future studies should determine the impact of cardiac FGF23/FGFR4 signaling in comparison to other potential factors, such as hyperphosphatemia or hypertension, which based on the increased duration of impact might also have more damaging effects in slow-progressing Col4a3 knockout mice. Furthermore, detailed cardiac analyses of the rodent model for adenine-induced tubulointerstitial nephropathy, where serum FGF23 levels are highly elevated (182) and appear to positively correlate with cardiac hypertrophy (183), should help to further determine a causative role of FGF23 in uremic cardiomyopathy.

Elevations in cardiac FGFR4 expression and calcineurin activity have not only been reported in animal models of CKD (155, 168, 184), but also in patients with CKD (185). A retrospective study with autopsy samples from heart tissue of 24 deceased pediatric patients showed that only individuals who had developed cardiac hypertrophy also showed significant elevations of FGFR4 in the heart, but not of FGFR1, as well as of calcineurin and NFAT (185). In these patients, FGFR4 expression positively correlated with the size of individual cardiac myocytes suggesting a causative relationship between FGFR4/PLCγ/calcineurin/NFAT activation and the induction of cardiac hypertrophy in humans. This is further supported by the finding that compared to dialysis patients who remained high FGF23 concentrations, patients who received a kidney transplant and had reduced FGF23 levels also showed lower cardiac expression of FGFR4, calcineurin, and NFAT (185). In the same study, CKD patients also showed the presence of sKL protein in the heart which must be derived from the circulation, as no increase in cardiac klotho mRNA could be detected. Interestingly, in this study declining sKL protein levels in the heart in combination with elevated FGF23 production correlated with cardiac hypertrophy, indicating that a reduction in sKL's inhibitory actions toward FGF23's pro-hypertrophic effects might contribute to uremic cardiomyopathy (185), thereby supporting the hypothesis that sKL can act as a decoy receptor for FGF23, as discussed above.

Several cell culture studies indicate that cardiac myocytes serve as a direct target for FGF23 (155, 162, 164, 186–188). Besides the activation of pro-hypertrophic gene programs (162, 188), FGF23 might also induce the expression of pro-fibrotic factors

and inflammatory cytokines in cardiac myocytes, thereby contributing to cardiac injury (188). Furthermore, it has been recently shown that FGF23 can activate cardiac fibroblasts isolated from adult mice and newborn rats (188, 189), and that the experimental elevation of FGF23 expression in an animal model for myocardial infarct further increases cardiac fibrosis (189). Therefore, it is possible that by directly targeting cardiac fibroblasts, FGF23 contributes to cardiac fibrosis and pathologic cardiac remodeling (**Figure 1**). The underlying mechanism is not clear, but since cardiac cells, including fibroblasts, in humans and rodents do not express klotho (75, 162, 184, 185), it would be worth to study a potential involvement of the FGFR4/PLCγ/calcineurin/NFAT signaling cascade. It has been recently shown that fibroblasts isolated from an injured mouse kidney can respond to FGF23 resulting in the activation of pro-fibrotic gene programs (190), as discussed below. Interestingly, this FGF23 effect on injury-primed renal fibroblasts was mediated by FGFR4 and PLCγ/calcineurin/NFAT signaling and resulted in the activation of the pro-fibrotic transforming growth factor β (TGFβ) signaling cascade (191). Based on these studies, one could hypothesize that FGF23 responsiveness is also increased in injured cardiac fibroblasts. It would be interesting to determine effects of FGF23 on cardiac fibroblasts isolated from uremic hearts. Although they do not provide evidence for direct and causative actions of FGF23 on the heart, some animal studies have indicated that FGF23 might require a fibrotic (189) or inflammatory milieu (192, 193) in order to contribute to cardiac injury. As suggested for the kidney (190, 191), FGF23's pro-fibrotic effect on the heart might be also mediated by FGF23-induced TGFβ signaling (189). However, a recent study has reported that FGF23 does not elevate TGFβ in isolated neonatal rat cardiac myocytes or fibroblasts, and that other mediators, such as the renin–angiotensin–aldosterone system, connective tissue growth factor, or endothelin-1, might be involved in FGF23-induced alterations in the myocyte-fibroblast crosstalk that may contribute to cardiac fibrosis (188).

The concept of priming cardiac cells (and possibly of other cell types in the body) by tissue injury resulting in increased FGF23 sensitivity appears to be plausible. Although cause and mechanism of the priming event are not known, it is possible that CKD-related stressors, such as elevations in serum levels of phosphate or uremic toxins, increase the cell surface expression of FGFR4 which then senses FGF23 elevations. Since fibroblasts do not express FGFR4, or only at very low levels (155), this mechanism would fit with the concept of injury-induced FGF23 responsiveness of fibroblasts resulting in pro-fibrotic actions of FGF23. The hypothesis that FGF23 mainly contributes to cardiac injury in the context of CKD is also supported by two other findings. First, although serum FGF23 levels are elevated following pressure overload *via* transaortic constriction in mice, FGF23 does not appear to be required for the development of pathologic cardiac hypertrophy in this animal model (171). Second, patients with X-linked hypophosphatemia (XLH), genetic forms of hypophosphatemic rickets, have high serum levels of FGF23 but do not develop cardiac hypertrophy (194). However, another clinical study challenges this view (195). Furthermore, it has been shown that genetic mouse models for XLH and autosomal recessive hypophosphatemic rickets (ARHR) show an increase in cardiac mass, while cardiac functions seems to be normal (172, 173, 196). Overall, primary forms of pathologic cardiac remodeling might not require FGF23, and not every scenario of FGF23 elevations might result in cardiac hypertrophy. In the context of secondary CKD-associated cardiac damage, the type and severity of kidney injury might also affect FGF23's actions on the heart. A recent study in a genetic mouse model with primary podocyte injury leading to a CKD-mineral bone disease-like phenotype shows that although serum levels of phosphate and FGF23 were elevated, mice showed no signs of pathologic cardiac remodeling (197). It is possible that the absence of a cardiac phenotype was based on the relatively short exposure time, as mice died within 8–10 weeks. Of note, although mice developed significant kidney injury, they did not have renal fibrosis. Therefore, one could speculate that mice did not develop cardiac hypertrophy, since they lack pathologic stimuli released by activated renal fibroblasts and/or associated with kidney fibrosis that act in synergy with FGF23 to harm the heart.

Cell culture and animals studies have shown that FGF23/FGFR4-induced hypertrophy is reversible upon removal or inhibition of the FGF23 stimulus (164). Furthermore, studies in a rat model of CKD have shown that administration of a FGFR4-specific blocking antibody (anti-FGFR4) not only prevents the induction of cardiac hypertrophy (155), but also blocks the progression of cardiac injury in animals with already established cardiac hypertrophy (164). Therefore, FGF23/FGFR4 might serve as a pathomechanism of uremic cardiomyopathy that could be tackled pharmacologically. Since FGF23 mediates its physiologic functions mainly *via* FGFR1, while its klotho-independent actions on the heart and other organs seem to be mediated by FGFR4 (155, 163, 191), a therapeutic approach to block FGFR4 should only interfere with FGF23's pathologic actions, while leaving its role as an important regulator of phosphate metabolism unaffected. Since global FGFR4 knockout mice are viable and do not develop any significant phenotypic alterations (198), and delivery of anti-FGFR4 does not show toxic effects in rats (155), FGFR4 appears to be an appropriate drug target and its systemic blockade might only result in minor side effects. However, other findings dampen the excitement for FGFR4 blocking therapies. First, although anti-FGFR4 treatment of CKD rats as well as lowering elevated FGF23 levels by taking mice off a high phosphate diet, reduce cardiac hypertrophy, these interventions seem to have little or no effect on cardiac fibrosis (164). Compared to CKD patients who received a kidney transplant, dialysis patients have higher serum FGF23 levels and develop cardiac fibrosis, but cardiac FGFR4 expression levels do not correlate with fibrosis (188), further supporting the notion that cardiac FGF23/FGFR4 signaling might not directly contribute to fibrosis. If cardiac fibrosis persists following FGFR4 blockade, one would assume that although hypertrophy is halted, cardiac injury will progress and heart function will further decline, eventually resulting in heart failure. Clearly, future *in vitro* and *in vivo* studies need to determine cardiac effects of FGF23 in a cell type-specific manner to elucidate precise cardiac actions of FGF23 and its potential for drug development. Second, it has been shown that FGF23 also

has acute effects on the myocardium. Within seconds, FGF23 increases cytoplasmic calcium levels in an FGFR-dependent manner in cultured cardiac myocytes (186). Furthermore, within minutes, FGF23 elevates the contractile force of isolated murine ventricular muscle strips, which does not occur in the presence of an FGFR4 inhibitor (164). Therefore, FGF23/FGFR4 might have beneficial effects on the heart which involve an increase in cardiac function, and its blockade might result in adverse outcomes. Whether acute effects of FGF23 on calcium homeostasis and contractility are indeed beneficial, or result in arrhythmia, as suggested by a different study (187), requires further analyses. Furthermore, it would be interesting to determine a potential involvement of FGF23 in the development of physiological hypertrophy, as observed during pregnancy and in athletes (199). However, to date it is still unknown whether under these two conditions of extreme physiologic and metabolic alterations, serum FGF23 levels are even elevated. Only one study has reported an increase of circulating FGF23 by about twofold in pregnant mice (200), and a different study showed that in professional cyclists, serum FGF23 levels increase by about 50% during a 3-week race (201). An elevation of FGF23 in these scenarios appears to be plausible, as they should both involve significant changes in calcium/phosphate homeostasis. However, analyses of larger populations are required to draw meaningful conclusions. Furthermore, to determine whether circulating FGF23 might contribute to physiologic cardiac hypertrophy requires studies in animals with genetic modifications of the FGF23/FGFR4 signaling pathway in the heart.

It appears that FGF23 can hit the heart in several ways: directly, by targeting cells in the myocardium, and indirectly, by contributing to traditional as well as non-traditional or CKD-specific cardiovascular risk factors (63, 125, 202). It has been shown that by targeting kidney distal tubules *via* FGFR1 and klotho, FGF23 elevates the expression of the sodium chloride co-transporter (NCC) and reduces levels of angiotensin-converting enzyme 2 (172, 173). By doing so, FGF23 increases sodium retention and activates the renin–angiotensin system, respectively. Combined, these renal effects lead to hypertension which is an established inducer of pathologic cardiac remodeling (199). FGF23 has been shown to be also involved in the regulation of iron metabolism by affecting erythropoiesis (62, 203). FGF23 reduces renal production of erythropoietin and might thereby contribute to CKD-associated anemia (204). Furthermore, FGF23 promotes expression of inflammatory cytokines in the liver (as discussed below) (163), and possibly in other tissues. Since *vice versa*, iron deficiency and inflammatory cytokines increase FGF23 production in bone (53), FGF23 could be part of a vicious cycle that contributes to FGF23-driven pathologies associated with CKD, such as anemia and systemic inflammation (62, 203), which are also potent inducers of pathologic cardiac remodeling and injury (199).

EFFECTS OF FGF23 ON THE LIVER

In mammals, the liver is among the organs with highest FGFR4 expression levels (205), raising the question if FGF23 has direct hepatic actions. Indeed, it has been shown that FGF23 can activate FGFR4/PLCγ/calcineurin/NFAT signaling in cultured hepatocytes which lack klotho, and thereby induce the expression of the inflammatory cytokines, interleukin-6 (IL-6) and C-reactive protein (CRP) (163). As already described for the associated cardiac remodeling, animal models with elevated serum FGF23 levels, induced by renal ablation, deletion of klotho, injection of FGF23, or administration of a high phosphate diet, show increased hepatic and serum levels of IL-6 and CRP, which are reduced when FGFR4 is deleted or pharmacologically inhibited (163). As discussed for the heart, hepatocyte-specific deletion of FGFR4 will be necessary to determine whether direct actions of the FGF23/FGFR4 signaling system on the liver contribute to systemic inflammation, and thereby to inflammatory injury in different tissues. Since studies in CKD patients have shown that higher serum FGF23 levels are associated with increased circulating concentrations of inflammatory cytokines, such as CRP, IL-6, IL-12, and tumor necrosis factor α (TNFα) (206–209), and that an elevation in these cytokines is a strong predictor of poor clinical outcome (210–213), the direct hepatic actions of FGF23/FGFR4 might serve as a novel pathomechanism that links CKD with systemic inflammation and contributes to morbidity and mortality (**Figure 1**). If hepatocytes and/or other cell types of the liver directly respond to FGF23 needs to be further investigated. Primary hepatocyte cultures are not absolutely pure and contain other cell types, such as Kupffer cells, which are specialized macrophages that release cytokines, such as IL-6 and TNFα, to communicate with hepatocytes (214). Since it has been shown that FGF23 can directly target other macrophage populations (215, 216), it is possible that FGF23 induces IL-6 production in Kupffer cells, which then indirectly affects hepatocytes.

In the described animal models with FGF23 excess, elevations in the hepatic production of inflammatory cytokines occur in the absence of increased liver enzymes (163). To date, no cell culture or animal studies have reported damaging actions of FGF23 on liver cells, which is consistent with the clinical observation that the presence of CKD *per se* does not promote liver injury despite marked increases in serum FGF23 levels. As discussed for cardiac hypertrophy, it is possible that FGF23-induced expression of inflammatory cytokines is not pathological, at least not initially, but has protective functions. Kupffer cell-derived IL-6 is a major regulator of hepatocyte proliferation and survival and thereby promotes liver regeneration (214). The hypothesis that FGF23/FGFR4 might act as a hepato-protective signaling pathway is supported by the finding that mice lacking FGFR4 are more sensitive to carbon tetrachloride-induced liver injury (217) and fail to restore liver mass after partial hepatectomy (218). Other FGF family members, such as paracrine FGF7 and FGF9, have been shown to mediate repair in response to liver injury (219). Further research is needed to determine whether physiologic concentrations or only high levels of FGF23, as observed in advanced CKD, can stimulate hepatic cytokine expression, and whether FGF23-induced cytokines have physiologic functions or mediate global tissue injury.

It is likely that FGF23 can stimulate inflammatory cytokine expression and secretion from other known reservoirs. This is supported by a genome-wide analysis of FGF23-regulated genes

in a mouse model of CKD that suggested inflammatory cytokine genes as general FGF23 targets (220), and by studies in which FGF23 stimulated TNFα expression in macrophages (215, 216) and in the spleen (221). Furthermore, NFAT activation induces the expression of a variety of cytokines, such as IL-2, IL-4, IL-6, and TNFα, in different cells types, including T cells and mast cells (222). Therefore, it is possible that by activating klotho-independent calcineurin/NFAT signaling in other cell types, FGF23 induces the production of inflammatory cytokines in multiple tissues contributing to systemic elevations of inflammatory mediators as well as tissue injury (223).

Several factors that are dysregulated in CKD associate with elevations in inflammatory cytokines and might contribute to inflammatory tissue damage, including increased serum phosphate levels (224, 225). However, since only some of the studied animal models with elevated FGF23 develop kidney injury or hyperphosphatemia, while all of them show increases in serum concentrations of inflammatory cytokines (163), it is possible that FGF23 acts as a major driver of inflammation in CKD. Associations between serum levels of FGF23 and inflammatory cytokines have been also reported in adults without CKD and in the elderly, despite their significantly lower FGF23 levels relative to patients with CKD (226–228). This suggests a general pro-inflammatory role of FGF23 that is independent of reduced kidney function, and that FGFR4 blockade might be effective in reducing chronic inflammation. In CKD, several liver-controlled mechanisms, such as iron and lipid metabolism or detoxification, are out of order. Whether FGF23-mediated activation of FGFR4 in hepatocytes can also regulate these facets of liver function needs to be investigated. Klotho is not expressed in the liver (75, 163), and to date direct effects of sKL on the liver have not been reported. However, since a reduction in global klotho expression in rodents (163, 221, 229, 230) and humans (209) has been associated with systemic elevations of inflammatory cytokines, it is tempting to speculate that sKL's anti-inflammatory effects are—at least partially—due to its inhibitory actions toward the FGF23-mediated production of inflammatory cytokines. While such a mechanism has been described in cultured endothelial cells where FGF23-induced expression of IL-1 is inhibited by sKL (231), it was not found in cultured spleen cells, where the FGF23-induced production of TNFα is not reduced in the presence of sKL (221).

EFFECTS OF FGF23 ON LEUKOCYTES

Chronic kidney disease is a state of acquired immune deficiency involving cellular and humoral immunity (232). The incidence of bacterial infections in CKD patients is higher than in the general population, and acute infections with bacteria, viruses, and fungi substantially contribute to the high hospitalization rates and mortality (233–235). As described above, the pro-inflammatory CKD environment is most likely caused by a variety of sources, including activated immune cells, and *vice versa* systemic inflammation is associated with an impaired function of the immune system. Since in CKD patients elevated serum FGF23 levels are independently associated with the incidence of infections (175, 236, 237), a role of FGF23 not only in the regulation of the inflammatory response, but also in the associated host defense is plausible (223).

The pathomechanism underlying the impaired host defense in CKD is not well understood. The innate immune response requires the recruitment and activation of immune cells to the site of infection, and neutrophils are among the most prominent leukocyte subsets during this process. Neutrophils from CKD patients are unresponsive to further stimulation and activation, indicating that CKD-associated factors might contribute to defects in the host defense by inducing neutrophil dysfunction (238–240). A recent experimental study has shown that by directly targeting neutrophils, FGF23 can inhibit neutrophil recruitment and thereby impair the host defense (241) (**Figure 1**). Mechanistically, FGF23 blocks chemokine- and selectin-mediated β_2-integrin activation on neutrophils, thereby preventing the interaction between β_2-integrin and intracellular adhesion molecule-1 on endothelial cells, neutrophil arrest on the endothelium and trans-endothelial neutrophil migration. This FGF23 effect appears to be klotho-independent and requires FGFR activity. Since neutrophils only express FGFR2 on the cell surface, while FGFR1 and FGFR4 are localized in the cytoplasm (242), one can assume that FGF23 targets neutrophils *via* FGFR2. FGF23 actions on neutrophils seem to be dose-dependent and only occur at high FGF23 concentrations, which result in the activation of protein kinase A (PKA), subsequent inhibition of the signal mediator Rap1, and eventually deactivation of β_2-integrin. This pathomechanism can be translated into humans, as leukocytes isolated from CKD patients show increased rolling velocity which can be reduced by pharmacologic FGFR inhibition, and *vice versa*, FGF23 treatment of leukocytes isolated from healthy subjects elevates rolling velocity (241). Furthermore, two more recent studies show that FGF23 treatment of isolated human leukocytes reduces expression levels of CD11b integrin (208) and weakens chemotaxis (243). Combined, these findings suggest direct inhibitory actions of FGF23 on integrin activation in leukocytes, resulting in reduced leukocyte adhesion and migration which might serve as a pathomechanism that causatively links increase in serum FGF23 levels with impaired host defense in CKD. Since integrin inactivation occurs rapidly following FGF23 treatment (241), this effect appears to be direct. However, the analysis of rodent models with cell type-specific deletion of FGFR2 will be required to determine whether FGF23 can indeed directly target neutrophils.

Direct effects of FGF23 have been also described for other leukocyte populations suggesting a broader role of FGF23 in the regulation of the immunological and inflammatory response. For example, FGF23 might alter monocyte function by inhibiting 1-α-hydroxylase and thereby 1,25D synthesis (244, 245). Furthermore, FGF23 can stimulate TNFα expression in macrophages (215, 216) (**Figure 1**). FGF23 effects on monocytes and macrophages seem to be mediated by Ras/MAPK signaling and occur in the absence of klotho (215, 244). FGFR1 is expressed at highest levels in these cells (216, 244) and, therefore, FGFR1 might mediate FGF23 effects. However, animal studies with monocyte/macrophage-specific deletion of FGFR1 will be necessary in order to determine if FGF23 can directly target these leukocyte populations. Since 1,25D can inhibit FGF23-induced TNFα in

macrophages (215), it is interesting to speculate that FGF23's inhibitory actions on the innate immunity can be counter-regulated by 1,25D (246), which would explain the stimulating effects of 1,25D on antibacterial macrophage response (247).

INDICATIONS THAT FGF23 MIGHT AFFECT A VARIETY OF OTHER TISSUES AND CELL TYPES

While the effects of klotho and sKL on the central nervous system have been extensively studied, as reviewed elsewhere (77, 248), it is less clear whether FGF23 can directly target neurons. A study in hippocampal neuron cultures isolated from mice shows that FGF23 treatment reduces the complex cell morphology and enhances synaptic density (106). This effect occurs in the absence of klotho that is not expressed in hippocampal neurons, requires FGFR activity and involves PLCγ signaling. Co-treatment with sKL inhibits the FGF23 effect and causes an activation of Akt signaling (106). As Akt can be activated *via* FRS2α, this finding supports the hypothesis described earlier, that sKL might modify FGF23-induced downstream signaling (**Figure 3**). By directly targeting hippocampal neurons, FGF23 might contribute to learning and memory deficits which are observed in many patients with CKD, especially in children (249–251). Indeed, it has been reported that elevations in serum FGF23 levels are associated with cognitive impairment in CKD patients (252). Furthermore, transgenic mice overexpressing cleavage-resistant FGF23 resulting in elevated serum FGF23 levels show reduced long-term potentiation in the hippocampus and impaired spatial learning and memory (253). However, since administration of a high phosphate diet ameliorates this phenotype, FGF23-induced hypophosphatemia rather than direct FGF23 actions on the brain might be involved. Clearly, more detailed *in vitro* and *in vivo* experiments are necessary to test the existence of direct pathologic actions of FGF23 on neurons as well as other cell types in the central nervous systems.

Since FGF23 can directly induce injury of the heart muscle, it is tempting to speculate that FGF23 might also contribute to skeletal muscle dysfunction and atrophy that is found in many patients with CKD (254). Interestingly, animal models for ARHR and XLH with primary FGF23 elevations show deficiencies in skeletal muscle fiber contraction and develop muscle weakness, which is ameliorated after injections of an FGF23-blocking antibody (196, 255). FGF23 might also have beneficial effects on skeletal muscle, as elevations of circulating FGF23 by intraperitoneal injections of recombinant FGF23 in wild-type mice extends exercise performance (256). Similar to cardiac myocytes, skeletal muscle cells express FGFR4, but lack klotho (205), and it has been shown that FGFR4 is a key regulator of myogenic differentiation and muscle regeneration after injury (257, 258). Furthermore, activating FGFR4 mutations contribute to rhabdomyosarcoma, a childhood cancer originating from skeletal muscle (259). However, a recent study shows that acute and prolonged FGF23 treatments have no effect on the function of isolated mouse skeletal muscle fibers or on an established cell culture model for myoblasts and myotubes (260).

Several types of lung injury are associated with CKD (261, 262), raising the question whether serum FGF23 can directly target and damage the lung. Since kidney, lung, and heart are in close interactions with each other (263), it is possible that FGF23 serves as one of the many pathophysiologic factors that can impact the balance between the three organs. The lung expresses all four FGFR isoforms (198), and like the liver, the lung has high levels of FGFR4 (264). However, as global FGFR4 knockout mice do not develop a lung phenotype (198), the role of FGFR4 in the regulation of lung development and function is unclear. Furthermore, analyses of klotho expression in the lung have provided conflicting results (65, 75, 205, 265), and, therefore, it is unclear whether direct FGF23 actions would be klotho-dependent and/or klotho-independent. So far only one mechanistic study has aimed to analyze potential direct effects of FGF23 on lung cells (265). FGF23 can target bronchial epithelial cells *via* FGFR1 and klotho to induce Ras/MAPK signaling and the expression of the inflammatory cytokine IL-8, which occurs in concert with TGFβ signaling that potentiates FGF23 effects by elevating FGFR1 expression. Addition of sKL attenuates FGF23 actions (265), indicating that membrane-bound klotho and sKL have opposite effects in this scenario and again pointing toward counterbalancing protective effects of sKL in regards to FGF23's pathologic actions. Interestingly, the FGF23 effect only occurs in bronchial epithelial cells isolated from patients with cystic fibrosis (265), suggesting that the priming of cells by disease-specific stimuli might be necessary for FGF23 responsiveness. Since patients with cystic fibrosis have elevated serum FGF23 levels (265), it is possible that by targeting the lung and other tissues, such as the liver (163), FGF23 contributes to the systemic inflammation that is common in this disease (266). It has also been speculated that FGF23 might serve as a biomarker for chronic obstructive pulmonary disease (267), and smokers have elevated serum FGF23 levels (268, 269), indicating that FGF23 might be involved in a wider spectrum of chronic lung disorders. Of note, mice with global FGF23 deletion develop lung emphysema, which can be partially rescued by the deletion of NaPi-2a resulting in a normalization of phosphate metabolism (270). This finding indicates that in this animal model lung injury might be caused by hyperphosphatemia and FGF23, suggesting important physiologic actions of FGF23 on the lung. While to date only little is known about potential effects of FGF23 on the lung, several human, and experimental studies have reported reductions in levels of klotho and sKL in a variety of lung disease, indicating cell-protective effects of sKL, as reviewed elsewhere (271, 272). Furthermore, mice globally lacking klotho develop pulmonary emphysema (65, 273, 274).

Whether the endothelium expresses klotho, or not, is currently under debate (275), and, therefore, it is unclear if FGF23 could have klotho-independent actions on blood vessels. Studies in cultured endothelial cells have shown that FGF23 can promote oxidative stress (92) and induce the expression of cell adhesion proteins (231), suggesting that FGF23 might contribute to endothelial dysfunction. However, clinical and experimental studies have shown that FGF23 does not associate with and contribute to vascular calcification in CKD (276), where rather

elevations in serum phosphate levels act as the major culprit (177). Whether FGF23 affects other aspects of vascular injury associated with CKD and might directly target different cell types in blood vessels, such as vascular smooth muscle cells, needs to be determined. Similarly, it is currently investigated, whether FGF23 can directly affect bone cells in a paracrine manner, and if so, whether this effect requires klotho or not. Interestingly, it has been shown that FGF23 can directly target chondrocytes which lack klotho, and thereby suppress proliferation and induce hypertrophy and differentiation (159, 277, 278). As briefly mentioned before, also macrophages and monocytes (215, 216, 244, 245) as well as cells in the spleen (221) can respond to FGF23. Furthermore, FGF23 has been shown to increase proliferation of prostate cancer cell lines (279).

It has been shown that "classic" FGF23 target organs which express klotho under normal conditions can also respond to FGF23 in a klotho-independent manner. For example, FGF23 effects in the parathyroid gland are mediated by FGFR1 and klotho, resulting in FRS2α/Ras/MAPK signaling and reduced PTH secretion (15, 154) (**Figure 1**). Interestingly, in mice lacking klotho specifically in the parathyroid gland, FGF23 retains its inhibitory actions on PTH secretion, but activates calcineurin/NFAT instead of Ras/MAPK signaling (16). This animal model supports the hypothesis discussed above, that klotho determines the branch of FGF23-mediated downstream signaling (**Figure 3**). However, since both, klotho-dependent and klotho-independent signaling, have the same physiologic effect, i.e., to reduce PTH secretion, the biological relevance of klotho-independent signaling is unclear, but it might act as back-up mechanism to ensure FGF23 responsiveness of the parathyroid gland in situations of klotho deficiency. FGF23 seems to also affect the kidney in a klotho-independent manner, at least in the scenario of kidney injury. It has been shown in an animal model of acute tubulointerstitial injury that FGF23 contributes to fibrosis (190, 280) (**Figure 1**). However, FGF23 can only directly target fibroblasts that are derived from an injured kidney (190), indicating that pathologic actions of FGF23 depend on the context and the presence of other pathologic stimuli. FGF23 effects on injury-primed fibroblasts are mediated by FGFR4 and calcineurin/NFAT signaling and result in an upregulation of TGFβ production, which then carries the pro-fibrotic signal forward to induce extensive fibrosis and tissue injury (191). Since neither healthy nor injured renal fibroblast express klotho (190), this pathologic effect of FGF23 appears to be klotho-independent. Interestingly, the introduction of klotho expression or treatment with sKL causes a switch from calcineurin/NFAT to Ras/MAPK signaling in isolated injured renal fibroblasts and attenuates FGF23's pro-fibrotic actions (191). Overall, this study supports the hypothesis that sKL can counterbalance FGF23's pathologic effects by inducing a switch in FGF23-induced downstream signaling toward the FRS2α/Ras/MAPK cascade (**Figure 3**). Whether FGF23 can induce renal injury by targeting other cell types than fibroblasts as well as the spectrum of renal diseases that might involve FGF23 as a causative kidney-damaging factor remains to be established. Furthermore, it needs to be studied whether in "classic" FGF23 target organs, klotho-independent signaling only occurs in the situation of klotho reduction or absence, or can also co-exist in the same tissue or even cell in parallel to klotho-mediated signaling events and might, therefore, have physiologically relevant functions.

CONCLUSION AND OUTLOOK

FGF23 can directly affect several cell types and tissues. Depending on the target, FGF23 actions occur in the presence or absence of klotho, require different FGFR isoforms and are mediated by different signal transduction cascades. The variety of molecular pathways that can be activated by FGF23 in a cell type-specific context ensures that FGF23 has numerous effects that differ among tissues, and can include changes in the cellular uptake and secretion of other factors as well as modifications of cell growth and migration. Klotho-independent FGF23 actions might be more widespread than originally thought. However, based on the diverse nature of FGF23-induced signaling events and effects, traditional read-outs to detect FGF23 responsiveness are not adaptable to every potential target tissue, thereby complicating the identification of novel FGF23 actions. Furthermore, *in vitro* studies that are necessary to determine potential direct effects of FGF23 on a defined cell type, are hindered by the fact that FGF23 requires specific signaling receptors and mediators whose expression levels, localization, or activity might change when cells are isolated and kept in culture over time. Current findings indicate that klotho-independent actions of FGF23 might be exclusively pathological. However, more animal experiments analyzing FGF23 effects in a concentration- and time-dependent manner are needed to confirm this view. It is also possible that such studies will reveal that initial FGF23 effects are cell protective and meant to compensate for tissue injury. Most likely, FGF23's pathologic actions occur in combination with other injury and stress stimuli, whose presence might alter the "molecular make-up" of cells and thereby increase their FGF23 responsiveness. However, it will be challenging to mimic such a multifactorial scenario in cultured cells in order to test this hypothesis.

Besides osteocytes in the bone, several other tissues and cell types have been reported to produce FGF23. The original studies reporting the cloning of FGF23 include FGF23 expression analyses of selected human and mouse tissues by reverse transcription PCR, showing signals for heart, liver, brain, small intestine, lymph node, and thymus (10, 18). This outcome overlaps with more comprehensive qPCR-based tissue screens in mice, reporting that besides these tissues also spleen, lung, skeletal muscle, and stomach contain FGF23 mRNA (205, 281). More detailed analyses of tissues and cultured cells by qPCR, immunoblotting, and immunohistochemistry, have confirmed and extended the findings of these screening studies, showing FGF23 expression in the heart (184, 185, 192, 193, 282), liver (283, 284), kidney (190, 285–287), spleen (216, 288, 289), brain (18), skeletal muscle (256), bone marrow (216, 290–293), and macrophages (215, 294). Also certain types of tumors have been shown to produce FGF23 (10, 279). Combined with the various FGF23 effects, as described above, these findings indicate that FGF23 might not only act as a hormone, but also functions as a paracrine factor. Furthermore, FGF23 might play important roles during

embryonic development. In zebrafish embryos, FGF23 expression is confined to the cells of the corpuscles of Stannius which regulate mineral ion homeostasis in advanced bony fish, also called teleosts (295). During embryonic mouse development, FGF23 is predominantly expressed in somites, heart, and liver (296). Although global FGF23 knockout mice are born at normal Mendelian ratios and do not develop an obvious phenotype before 10 days after birth (296), indicating that FGF23 is not essential for embryonic development, it is still possible that FGF23 contributes to proper organogenesis.

FGF23 expression often seems to occur in the context of pathologic stimuli or tissue damage, suggesting that either FGF23 contributes to the injury process or is induced to protect from injury. Tissue-specific knockout studies will be required to analyze the role of locally produced FGF23 in comparison to bone-derived, circulating FGF23. Furthermore, it needs to be determined whether the mechanisms that regulate FGF23 synthesis as well as posttranslational modifications and processing in the bone are also active in other FGF23 producing organs, or whether FGF23's precise form and modifications and, therefore, bioactivity varies and depends on the source. Clearly, a key open question that needs to be answered is whether the N- and C-terminal FGF23 cleavage fragments have a biological function, and if so, whether this is different from the role of intact FGF23.

AUTHOR CONTRIBUTIONS

BR designed and generated the three figures. CF wrote the majority of the text, which then was edited by BR.

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REVIEW
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Paracrine Effects of FGF23 on the Heart

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Fibroblast growth factor (FGF) 23 is a phosphaturic hormone primarily secreted by osteocytes to maintain phosphate and mineral homeostasis. In patients with and without chronic kidney disease, enhanced circulating FGF23 levels associate with pathologic cardiac remodeling, i.e., left ventricular hypertrophy (LVH) and myocardial fibrosis and increased cardiovascular mortality. Experimental studies demonstrate that FGF23 promotes hypertrophic growth of cardiac myocytes *via* FGF receptor 4-dependent activation of phospholipase Cγ/calcineurin/nuclear factor of activated T cell signaling independent of its co-receptor klotho. Recent studies indicate that FGF23 is also expressed in the heart, and markedly enhanced in various clinical and experimental settings of cardiac remodeling and heart failure independent of preserved or reduced renal function. On a cellular level, FGF23 is expressed in cardiac myocytes and in other non-cardiac myocytes, including cardiac fibroblasts, vascular smooth muscle and endothelial cells in coronary arteries, and in inflammatory macrophages. Current data suggest that secreted by cardiac myocytes, FGF23 can stimulate pro-fibrotic factors in myocytes to induce fibrosis-related pathways in fibroblasts and consequently cardiac fibrosis in a paracrine manner. While acting on cardiac myocytes, FGF23 directly induces pro-hypertrophic genes and promotes the progression of LVH in an autocrine and paracrine fashion. Thus, enhanced FGF23 may promote cardiac injury in various clinical settings not only by endocrine but also *via* paracrine/autocrine mechanisms. In this review, we discuss recent clinical and experimental data regarding molecular mechanisms of FGF23's paracrine action on the heart with respect to pathological cardiac remodeling.

Keywords: fibroblast growth factor 23, cardiac remodeling, left ventricular hypertrophy, cardiac fibrosis, endothelial dysfunction, autocrine, paracrine

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FIBROBLAST GROWTH FACTORS (FGFs) AND THEIR RECEPTORS

The FGF family consists of 22 members classified into seven subfamilies due to their phylogenetic relationship (1). Based on their mechanism of function, FGFs are further divided into paracrine, endocrine, and intracrine FGFs (**Table 1**) (2–5). Paracrine FGFs exhibit a heparan sulfate-binding site at their C-terminal region and bind to FGF receptors (FGFR) on the cell surface using heparan sulfate as a co-factor to mediate local biological activities (6). In contrast to paracrine FGFs, endocrine FGFs display a klotho-binding site at their C-termini, and due to their very low heparan sulfate-binding affinity, endocrine FGFs are directly secreted into the blood and exert their functions as endocrine hormones in various target tissues using α-klotho or β-klotho as co-receptors for

TABLE 1 | Classification of fibroblast growth factor (FGF) family members according to their mechanistic function.

Function	FGF
Paracrine	FGF1, **FGF2**, **FGF3**, FGF4, FGF5, FGF6, FGF7, **FGF8**, **FGF9**, **FGF10**, **FGF16**, FGF17, FGF18, FGF20, FGF22
Endocrine	**FGF15/19**, **FGF21**, **FGF23**
Intracrine	FGF11, FGF12, FGF13, FGF14

Bold printed FGFs play a role in heart development, health, or disease conditions.

FGFRs (7–10). Intracrine FGFs are not secreted and exert their biological functions within the same cell (11). While not all FGFs are endogenously expressed in the heart of humans and rodents (12), it was shown that FGF2, FGF3, FGF8, FGF9, FGF10, FGF16, FGF15/19, FGF21, and FGF23 all act on the heart in a paracrine or endocrine manner to induce physiological or pathological pathways in heart development, health, and disease (**Table 1**) (5, 13). The specific role of these FGFs and their respective FGFRs in the heart are nicely discussed in more detail in two reviews by Itoh and colleagues (5, 13).

In mammals, due to alternative splicing, seven FGFRs are generated from four different FGFR genes (*FGFR1*, *FGFR2*, *FGFR3*, *FGFR4*) with different FGF-binding affinity (14). Paracrine FGFs bind heparan sulfate to promote an FGF/FGFR/heparan sulfate complex while endocrine FGFs bind α-klotho or β-klotho to induce a complex consisting of FGF/FGFR/klotho, which both directly phosphorylate the FGFR intracellular tyrosine kinase domain. After FGFR activation, FGFR substrate 2α (FRS2α) is activated downstream, thereby inducing rat sarcoma protein (RAS)-mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)-AKT serine/threonine kinase (AKT), or signal transducer and activator of transcription (STAT) signaling within the cell, respectively (3, 4, 15, 16). In addition, phospholipase Cγ (PLCγ) can also be phosphorylated due to FGFR activation leading to activation of calcium-dependent signaling mediating cell motility (15). In the cardiovascular system, FGFR1c, FGFR2b, FGFR2c, FGFR3c, and β-klotho are physiologically expressed at very high levels while FGFR1b, FGFR3b, FGFR4, and α-klotho are only moderately expressed (12).

FIBROBLAST GROWTH FACTOR 23

FGF23 belongs to the endocrine FGF family and mainly functions as a hormone-like FGF. It is mainly expressed and secreted by osteocytes in the bone as a 32-kDa phosphaturic and calcitriol inhibitory protein regulating phosphate homeostasis (17). FGF23 exists in a full-length biologically active form and can be cleaved into N- and C-terminal fragments. The classic target organs of FGF23 are the kidney and the parathyroid glands where it exerts its physiological function through FGFRs with α-klotho as a co-factor (9, 18). The FGFR-binding site is located in the N-terminus while the α-klotho binding site is located in the C-terminus. The proprotein convertase cleavage site is stabilized by O-linked glycosylation *via* Galnt3 (19). FGF23 acts on the kidney *via* FGFR1c/α-klotho/MAPK signaling to regulate phosphate and vitamin D metabolism. It reduces renal phosphate reabsorption by suppression of sodium phosphate co-transporters NaPi-2a and NaPi-2c, thereby lowering serum phosphate levels (17, 20). Moreover, FGF23 decreases active vitamin D synthesis by down-regulation of 1α-hypdroxylase and upregulation of 24-hydroxylase resulting in low levels of $1,25(OH)_2D_3$ in serum (17). In the parathyroid gland, FGF23 inhibits the secretion of parathyroid hormone (21).

FGF23 expression is almost absent in the central nervous system, endocrine non-reproductive system, and metabolic system, and only minimally expressed in the gastrointestinal system, immune system, reproductive system, and cardiovascular system in healthy adults (12). In pathological conditions, FGF23 was shown to be excessively enhanced in bone (22), heart (23–26), liver (27), and kidneys (28, 29–32).

FGF23 AND THE CARDIOVASCULAR SYSTEM

Pathological cardiac remodeling, i.e., left ventricular hypertrophy (LVH), myocardial fibrosis, and vascular calcification are the major cardiovascular pathologies in the general population and in patients with chronic kidney disease (CKD) presenting with 15–21% and up to 90% LVH, respectively (33). Since α-klotho as a specific co-receptor for FGF23 is not expressed in human and rodent hearts (12, 25, 34), direct effects of endocrine-acting FGF23 on the cardiovascular system were not supposed for a long time. However, the causes of cardiovascular disease pathologies are multifactorial. In 2008, FGF23 was discussed for the first time as a new mediator for the progression of LVH in CKD (35). In recent years, recent clinical and experimental studies demonstrated positive associations between endocrine-acting FGF23, cardiac remodeling, and endothelial dysfunction in pathological conditions in humans and rodents. In 2011, Faul and colleagues presented for the first time that administration of FGF23 directly induces hypertrophic growth of isolated neonatal rat cardiomyocytes *in vitro* and LVH *in vivo* in an FGFR-dependent but α-klotho-independent manner (34). In contrast to the established FGF23/FGFR/α-klotho signaling complex primarily mediating the induction of RAS/MAPK pathway (9), FGF23/FGFR activates PLCγ in cardiac myocytes and induces hypertrophic cell growth using calcineurin/nuclear factor of activated T cell (NFAT) signaling in the absence of α-klotho (34). Interestingly, these effects were independent of blood pressure levels. Furthermore, it was shown that FGF23 increases intracellular calcium levels in cardiac myocytes *in vitro* and promotes contractility of murine cardiac myocytes and ventricular muscle strips *ex vivo* α-klotho independently (36). Thus, the α-klotho-independent action of FGF23 on the heart became more and more likely. In 2015, Grabner et al. identified the FGFR4 isoform as specific FGFR in the heart mediating FGF23's pro-hypertrophic action without α-klotho (37). In recent years, the importance of the role of enhanced *circulating* FGF23 on the development of cardiac remodeling was thoroughly demonstrated in patients with CKD (34, 38–43) and end-stage renal disease (ESRD) (34, 35, 44). Reaching serum levels of up to 1,000-fold higher than in healthy individuals, FGF23, and associated alterations in

mineral metabolism, including hyperphosphatemia, hypercalcemia, secondary hyperparathyroidism, vitamin D, and klotho deficiency, are associated with uremic cardiomyopathy, LVH, premature death, and all cause mortality in CKD (35, 38, 41, 45–48).

Clinical studies further indicate that circulating levels of FGF23 are also elevated in patients with dilated cardiomyopathy, ischemic heart disease, acute decompensated and chronic heart failure (HF), atrial fibrillation, and cardiogenic shock, despite normal renal function, suggesting that enhanced cardiac synthesis of FGF23 may cause elevated circulating FGF23 levels and thereby promote cardiac injury (49–61). The endocrine action of FGF23 on the heart and other organ systems was previously described in detail (62–69).

PARACRINE ACTION OF FGF23 ON THE HEART

It was suggested that FGF23 is not just a biomarker of increased risk but also a direct inducer of cardiac injury in the settings of CKD or HF (34, 70–72). Although FGF23 was discovered in 2001 as an endocrine hormone (73), cardiac FGF23 may be upregulated in certain clinical settings and promote cardiac remodeling *via* autocrine and/or paracrine mechanisms.

Cardiac FGF23 and LVH

Recently, we analyzed myocardial autopsy samples of deceased patients with childhood-onset ESRD and investigated the well-established FGF23/FGFR4 signaling cascade mediating cardiac hypertrophy. We demonstrated that FGF23 is expressed by cardiac myocytes in humans as the full-length biologically active protein (25). Moreover, cardiac FGF23 and FGFR4 are strongly enhanced in heart tissue of CKD patients when compared to age- and sex-matched controls and both positively associate with the development of LVH. As expected, klotho is not expressed in the human heart on mRNA level. However, soluble klotho protein can be clearly detected in myocardial tissue of CKD patients by Western blot and immunohistochemical analysis. The amount of soluble klotho in myocardial tissue is found to be reduced in CKD patients, and negatively associated with the duration of ESRD, dialysis vintage, and cardiac myocyte cross-sectional area. Interestingly, cardiac expression of FGF23, FGFR4, and the amount of soluble klotho in cardiac tissue are found to be dysregulated in dialysis patients and ameliorated in patients with functioning renal allografts. Similarly, FGF23/FGFR4-associated calcineurin/NFAT pathway and the expression of pro-hypertrophic markers are normalized after kidney transplantation. Multiple linear regression analysis points out that cardiac FGF23 expression and the amount of soluble klotho protein in cardiac tissue are independent predictors of cardiac myocyte cross-sectional area while the mode of renal replacement therapy (RRT) at time of death is found to be the only independent predictor for the induction of cardiac FGF23 synthesis. The amount of soluble klotho protein in the cardiac tissue of CKD patients correlates with the duration of ESRD and the mode of RRT at time of death, and finally, cardiac FGFR4 expression correlates with estimated glomerular filtration rate, mode of RRT at time of death, cumulative time spent on dialysis, and cardiac FGF23 expression levels. The above-mentioned findings are supported by a recent study investigating myocardial tissue of 5/6 nephrectomized (Nx) rats, which is a well-established model of experimental uremia (26). Indeed, cardiac *Fgf23* gene expression is up-regulated in myocardial tissue of 5/6Nx rats compared with sham operated animals. Furthermore, experimental uremia induces full-length cardiac Fgf23 protein demonstrated by Western blot, which positively correlates with the heart weight to body weight ratio, and enhanced β*MHC* and *BNP* expression levels. Although these association studies do not prove causality, data suggests the existence of a *paracrine* FGF23 mechanism in the heart mediating LVH in uremic cardiomyopathy.

Studies in patients with acute decompensated heart failure (ADHF) point out that circulating FGF23 levels are significantly enhanced in ADHF compared to controls, whereas myocardial *FGF23* gene expression is not consistently altered (60). However, the investigated study cohort is rather small and *FGF23* mRNA levels greatly vary within both groups, which may have biased the results of this study. Since ADHF is defined as sudden or gradual onset of HF symptoms (74), the degree of HF progression may also define the strength of cardiac FGF23 induction. Nevertheless, FGF23 is detected in myocardium of both HF and normal patients by quantitative real-time PCR confirming the presence of FGF23 in the human myocardium. In addition, immunohistochemical analysis detects FGF23 expression in the cardiac myocytes of normal myocardial tissue and in failing hearts.

A recent study by Slavic and colleagues investigated cardiac and circulating FGF23 in experimental heart hypertrophy (75). They showed that increased afterload due to transverse aortic constriction (TAC) in mice induces an increase in circulating intact Fgf23 serum levels within 24 h after TAC, which maintains for at least 4 weeks post-surgery. TAC significantly increases cardiac *Fgf23* gene transcripts while bone *Fgf23* production is higher overall compared to the heart but not significantly stimulated by TAC. Furthermore, pressure overload dramatically reduces the cleavage of Fgf23 in serum. Interestingly, expression of *Galnt3*, which O-glycosylates and thereby stabilizes Fgf23 protein, is increased in the heart post-TAC but unchanged in bone. By contrast, expression of *Fam20c* and *Furin*, promoting Fgf23 cleavage, remain unchanged in heart tissue of mice after TAC. Taken together, these data suggest that cardiac FGF23 synthesis is induced due to pressure overload and reduced FGF23 cleavage further contributes to enhanced intact FGF23 in the heart. Importantly, the up-regulation of cardiac and serum intact Fgf23 is not due to alteration of mineral metabolism as serum levels of phosphate, calcium, sodium, potassium, and iron remain unchanged in mice after TAC compared to sham-operated animals. Instead, serum aldosterone levels, shown to stimulate FGF23 expression *in vitro* (76), are induced in TAC-operated mice. To confirm whether induction of renin–angiotensin–aldosterone system (RAAS) triggers pressure overload-induced up-regulation of cardiac Fgf23, the authors treated wild-type mice with spironolactone for 2 weeks post-TAC surgery (75). Administration of spironolactone inhibits the induction of circulating intact Fgf23 levels in TAC mice and reduces the mRNA expression of *Fgf23* in bone but not in the heart, suggesting that a pressure overload-induced

increase in bone *Fgf23* synthesis is driven by aldosterone secretion in TAC mice. Interestingly, heart weight to body weight ratio, cardiac myocyte size, end-diastolic left ventricular wall thickness, fractional shortening, and mean blood pressure after TAC are not improved by aldosterone inhibition, indicating that increased systemic Fgf23 may be less important in the development of pressure overload-induced cardiac hypertrophy. However, TAC-induced expression of cardiac *Fgfr1*, *Fgfr3*, and *Fgfr4*, nuclear localization of NFAT protein within the cardiac myocytes, *Rcan1* induction, and consequently cardiac hypertrophy and fibrosis are also present in global homozygous *Fgf23*$^{-/-}$/vitamin D receptor (VDR)$^{\Delta/\Delta}$ or *Klotho*$^{-/-}$/VDR$^{\Delta/\Delta}$ double knockout mice compared to respective wild-type mice. Neither deletion of Fgf23 protected against pressure overload-induced cardiac hypertrophy and fibrosis, nor klotho deficiency exacerbated TAC-induced pathological hypertrophic phenotype in mice. Thus, FGF23 would appear to be a consequence of pressure overload-induced hypertrophy and perhaps not the initial inducer, suggesting that additional factors could mediate hypertrophic responses during pressure overload independent of FGF23.

FGF2 is expressed in cardiac myocytes and fibroblasts, and angiotensin II or adrenergic stimulation induces FGF2 synthesis in cardiac myocytes, which in turn stimulates itself in a feed-forward loop (77). High blood pressure induces LVH *via* up-regulation of FGF2, which is shown to be necessary for hypertrophic development in the TAC model (78). Neonatal and adult cardiac fibroblasts mainly express the high-molecular weight isoform of FGF2, which is stimulated and secreted through angiotensin II treatment, thereby acting on cardiac myocytes *via* induction of a fetal gene program resulting in cardiac hypertrophy (79). However, neither spironolactone treatment nor FGF23 knockout alters the induction of FGF2 in the high blood pressure-induced cardiac hypertrophy through TAC (75). Interestingly, it was shown that high-molecular weight FGF2 isoform directly stimulates FGF23 synthesis in the bone (80, 81). Whether up-regulation of FGF2 in the heart due to pressure overload-induced cardiac hypertrophy after TAC is also responsible for the induction of cardiac FGF23, and if so, the role of cardiac FGF23 in this setting has to be further elucidated.

However, these conclusive findings coincide with clinical and experimental studies showing that enhanced FGF23 serum levels and/or the induction of cardiac FGF23 synthesis are not associated with high blood pressure-induced LVH (25, 34, 37). In addition, the fact that cardiac FGF23 is still enhanced after mineralocorticoid receptor (MR) blockade while bone FGF23 and systemic FGF23 are reduced supports our hypothesis that induction of cardiac FGF23 may have additional blood pressure-independent paracrine effects on the heart, which needs to be addressed in future studies.

Cardiac FGF23 and Myocardial Fibrosis

The role of circulating and/or cardiac FGF23 in the progression of cardiac fibrosis and its potential effects on myocardial fibroblasts promoting cardiac diastolic function is largely unknown. Initial experimental data in rodents demonstrated that FGF23 stimulates the renal expression and production of transforming growth factor β (TGF-β) (82, 83). TGF-β is a classic cytokine involved in different signaling pathways, which is shown to induce fibrosis in various tissues.

Recently, Smith et al. reported that FGF23 is synthesized locally by renal tubules and activates injury-primed fibroblasts (31). Moreover, FGF23 stimulates gene expressions involved in TGF-β signaling pathways *via* FGFR4-dependent activation of NFAT and enhancement of TGF-β autoinduction in injury-primed renal fibroblasts (84). These data suggest a paracrine mechanism of FGF23 in the kidney acting on renal fibroblasts to induce pro-fibrotic signaling pathways.

In the heart, cardiac FGF23 expression is upregulated in post-myocardial infarction (MI) in rodents and mediates cardiac fibrosis through the activation of β-catenin (24). β-catenin is a pro-fibrotic factor cross talking with TGF-β signaling (83, 85, 86). Hao and colleagues demonstrated that FGF23 is endogenously expressed in neonatal rat cardiac myocytes and fibroblasts, adult mouse cardiac fibroblasts (AMCF), and the adult mouse heart (24). They also confirm the findings of Slavic et al. mentioned above (75), showing an increase in cardiac FGF23 mRNA and protein levels in pressure overload-induced failing mouse hearts after TAC. In addition, FGF23 plasma levels are significantly induced post-MI compared to sham-operated mice. Angiotensin II and phenylephrine, both pro-fibrotic and pro-hypertrophic stimulators, clearly up-regulate *FGF23* expression in AMCF. Furthermore, stimulation with FGF23 promotes proliferation, collagen I and III synthesis, and β-catenin activation in AMCF. Cardiac-specific overexpression of FGF23 using adeno-associated virus carrying FGF23 (AAV-FGF23) in mice followed by MI or ischemia reperfusion significantly enhances the activation of cardiac β-catenin, TGF-β, collagen I and III, and cardiac fibrosis in mice injected with AAV-FGF23 compared to negative controls. It is important to note that the injection of AAV-FGF23 does not result in enhanced β-catenin, TGF-β, or collagen I and III expression levels in sham-operated mice compared with respective control animals. Thus, endogenous cardiac FGF23 synthesis may only impair myocardial fibrosis after MI or ischemia reperfusion through induction of paracrine signaling pathways, including activation of β-catenin and TGF-β. However, the activation of β-catenin-mediated induction of TGF-β and collagen synthesis by FGF23 under healthy conditions remains controversial.

Andrukhova and colleagues demonstrated that both skeletal and cardiac Fgf23 expression are increased in mice and rats after MI independent of changes in serum soluble klotho levels (23). In addition, they found increasing serum intact Fgf23 levels in mice and rats after experimental MI, and immunohistochemical staining of heart tissue clearly reveals expression of Fgf23 in left ventricular cardiac myocytes after MI in mice and rats but not in infiltrating leukocytes within the infarct scar, suggesting that cardiac myocytes are the major cellular source of increased cardiac Fgf23 expression post-MI. Importantly, despite the induction of Fgf23, serum levels of PTH, phosphate, calcium, and sodium, and urinary excretion of phosphate, calcium, and sodium remain unchanged post-MI. In addition, biochemical markers of bone metabolism, including urinary excretion of collagen cross-link deoxypyridinoline, serum alkaline phosphatase, Dickkopf-1, and osteoprotegerin, do not differ between mouse and rats post-MI compared with sham control-operated animals. Although the

molecular link between cardiac injury and enhanced Fgf23 levels remains to be further elucidated, these data strongly support the hypothesis that cardiac FGF23 expression is linked to pathological cardiac changes and may exert paracrine mechanisms in the heart.

In an extension to experimental studies in rats and mice, we recently analyzed the myocardial tissue of CKD patients and investigated cardiac FGF23 expression and myocardial fibrosis (87). Cardiac FGF23 synthesis and accumulation of fibrillar collagen fibers are strongly enhanced in CKD patients on dialysis treatment compared to controls. Cardiac fibrosis correlates with duration of dialysis and soluble klotho deficiency detected by immunohistochemical staining of klotho protein in heart tissue sections. Interestingly, no significant correlations between cardiac *FGF23* expression and fibrosis could be detected in CKD patients. By performing human fibrosis RT profiler PCR array analyses of myocardial tissue in these patients, angiotensinogen (*AGT*), and angiotensin II-driven pathways known to promote fibrotic response, including collagen remodeling, activation of TGF-β/TGF-β receptor/Smad complexes, and induction of pro-hypertrophic and pro-fibrotic growth factors endothelin-1 and connective tissue growth factor (*CTGF*), are significantly enhanced in patients on dialysis treatment. Although cardiac fibrosis positively correlates with the induction of *AGT* in the heart, soluble klotho deficiency but not endogenous FGF23 expression is shown to be correlated with enhanced cardiac TGF-β/TGF-β receptor pathway. We performed further *in vitro* studies using neonatal rat ventricular myocytes (NRVM) and fibroblasts (NRCF) in order to discriminate which cell type express FGF23 in the heart and may induce pro-fibrotic signaling. Hereby, we could clearly detect endogenous *Fgf23* mRNA expression in NRVM but not in NRCF, which is in contrast to Hao et al. also demonstrating FGF23 production in cardiac fibroblasts of neonatal rats and adult mice (24). However, treatment of NRCF with recombinant FGF23 significantly stimulates collagen remodeling, promotes the induction of pro-fibrotic TGF-β/TGF-β receptor/Smad complexes, and induces *Ctgf* and *endothelin-1* expression *in vitro* (87). Importantly and in line with the Hao et al. study mentioned above, FGF23 significantly stimulates proliferation and migration of NRCF. In addition, FGF23 induces angiotensinogen expression in cardiac myocytes, promotes extracellular matrix remodeling, and induces pro-fibrotic and pro-hypertrophic growth factors in NRVM while TGF-β/TGF-β receptor signaling unchanged. Taken together, our data suggest that secreted by cardiac myocytes, FGF23 may directly target cardiac fibroblasts and promote pro-fibrotic mechanisms in a paracrine manner. This mechanism in concert with its autocrine induction of fibrotic and hypertrophic-related factors in cardiac myocytes, cardiac FGF23 may act on both cardiac cell types and thereby promote cardiac hypertrophy and myocardial fibrosis in a paracrine/autocrine fashion.

Cardiac FGF23 and Endothelial Dysfunction

Clinical and experimental studies demonstrated that high FGF23 serum levels, hyperphosphatemia, and klotho deficiency are strongly associated with cardiovascular complications in CKD patients (71). Elevated FGF23 serum levels associate with endothelial dysfunction and small vessel disease, stroke, and brain infarction in patients with CKD stage 3–4 (40) and in the general population (58, 61, 88), respectively. Whether enhanced circulating levels of FGF23 also impact on vascular calcification is still an open question (39, 42). Voigt and colleagues identified FGF23 accumulation in calcified carotid atherosclerotic lesions from subjects with normal renal function (89). In addition, a recently published study by van Venrooij et al. evaluated the relationship between kidney function, vascular calcification, and FGF23 expression in patients with heart disease (90). They detected FGF23 protein expression by immunohistochemistry in coronary arteries of patients undergoing heart transplantation, which associates with declining renal function. Vascular FGF23 expression and FGFR1, FGFR3, and α-klotho are observed in both tunica intima and tunica media in areas of calcification. Interestingly, co-immunostaining for FGF23 and CD68 demonstrates co-localization of FGF23 with inflammatory macrophages. Quantitative real-time PCR analysis confirms the gene expression of FGF23 in human coronary artery tissue, also suggesting a potential paracrine effect of FGF23 in vessels.

We performed *in vitro* studies using human coronary artery endothelial cells (HCAEC) in order to investigate the underlying molecular pathways of FGF23-mediated endothelial cell function in the presence and absence of its co-receptor α-klotho (91). First, we confirmed the previously reported findings (90) that FGFR1 and α-klotho are present in the intima of human coronary arteries. In addition, cultured HCAECs express FGFRs and α-klotho, and FGF23 specifically stimulates phosphorylation of FGFR1 and thereby activates the receptor, suggesting that vascular endothelial cells are a target for paracrine FGF23 signaling. Although α-klotho is not altered by FGF23 treatment on mRNA or protein level, FGF23 increases the expression of ADAM17, responsible for cleavage of membrane bound α-klotho, and consequently the secretion of soluble α-klotho. Investigating endothelial cell function targeted by FGF23, we showed that FGF23 activates intracellular signaling to induce the release of nitric oxide (NO) *via* activation of AKT/endothelial NO synthase (eNOS) pathway *in vitro* in the presence of α-klotho. Furthermore, FGF23 treatment enhances the formation of reactive oxygen species (ROS) through induction of NADPH oxidase 2 (Nox2)/p67phox/p47phox/Rac1 signaling complex. In addition, FGF23 stimulates the expression of ROS detoxifying enzymes manganese superoxide dismutase and catalase by activating Foxo forkhead transcription factor 3a (Foxo3a) in the presence of α-klotho, indicating that FGF23-induced ROS production is being neutralized by antioxidant mechanisms. Co-treatment with FGF23 and pan-FGFR inhibitor blunts all NO synthesis, ROS formation, and ROS degradation. Taken together, these data suggest that FGF23 effects on HCAEC mediating endothelial function in the presence of α-klotho are FGFR dependent. Inhibition of α-klotho using a neutralizing antibody specifically blocks FGF23-mediated activation of AKT/eNOS and consequently the release of NO. Most importantly, blocking of α-klotho further inhibits the expression of SOD2 and catalase induced by FGF23 but does not affect FGF23 stimulated Nox2 signaling and ROS formation. Our data suggest that in states of Klotho deficiency, e.g., CKD, FGF23-induced NO synthesis is blunted and ROS formation overrules ROS degradation.

Consequently, enhanced circulating as well as locally synthesized FGF23 may promote endothelial dysfunction and further impact on the progression of cardiovascular disease in CKD. However, the hypothesized paracrine signaling mechanism of FGF23 mediating functional response in vessels has to be further proven in experimental animal models.

FGF23 and Chronic Inflammation

Cardiac hypertrophy and myocardial fibrosis can be promoted by pro-inflammatory cytokines, such as TNF-α or IL-6, and pro-fibrotic molecules including TGF-β and angiotensin II (92). Inflammatory markers, including IL-6, CRP, TNF-α, and fibrinogen, are independently associated with circulating levels of FGF23 in patients with CKD, and FGF23 associates with a greater odds ratio of severe inflammation in these patients (93). Furthermore, FGF23 directly stimulates hepatic secretion of pro-inflammatory cytokines in wild-type mice and in cultured hepatocytes by activating FGFR4 independent of α-klotho (94), indicating a novel mechanism of FGF23 and chronic inflammation. Furthermore, chronic inflammation in cardiac fibroblasts was shown to induce the expression of FGF23 in the heart (95), suggesting a paracrine signaling mechanism of chronic inflammation inducing cardiac FGF23. However, hard evidence from experimental studies is lacking.

The cytokine oncostatin M (OSM) belongs to the IL-6 family and is involved in various biological processes, including inflammation and ischemic heart disease (96). OSM is further shown to be a major mediator of cardiac remodeling (97). After binding to a receptor heterodimer consisting of gp130 and OSM receptor β, OSM induces a Janus Kinase/STAT, MAPK, or PI3K/AKT signaling pathway, respectively (98–100), also all known to be induced by FGF/FGFR complexes. Richter and colleagues find that FGF23 is present in cardiac myocytes of patients with ischemic cardiomyopathy, myocarditis, and dilated cardiomyopathy (101). In addition, adult rat cardiac myocytes clearly expresses *Fgf23* on the mRNA level, which is excessively induced by OSM treatment *in vitro* (102). Moreover, cultured OSM-treated non-cardiac myocytes, mainly fibroblasts, also synthesize FGF23. However, FGF23-expressing non-cardiac myocytes are only rarely observed in diseased human heart tissue (101). ELISA quantifications of cardiac myocyte cell supernatant demonstrates significantly increased C-term FGF23 concentrations due to OSM, and Western blot analysis revealed that OSM stimulates both unmodified full-length FGF23 protein and cleaved FGF23 fragments in cell lysates of adult rat cardiac myocytes (102). Similarly, in corresponding cell supernatant C- and N-terminal FGF23 fragments are clearly detected. Inflammatory HF in mice caused by macrophages infiltration of the myocardium shows a strong increase in FGF23 expression and secretion in cardiac myocytes, which is associated with macrophage infiltration and activation of OSM receptor β cascade leading to cardiac myocyte remodeling with enhanced α-smooth muscle actinin expression, STAT3 phosphorylation, induction of atrial natriuretic peptide (ANP), and destrin. These findings suggest that FGF23 is endogenously expressed in cardiac myocytes in inflammatory HF of humans and rodents and associates with macrophage infiltration and OSM receptor activation.

FGF23 is also shown to be expressed in pro-inflammatory M1 macrophages and specifically up-regulated by lipopolysaccharide and interferon gamma (IFN-γ) *via* NF-κB and JAK/STAT1 pathways, respectively (103), indicating that FGF23 produced by M1 macrophages may modulate pro-inflammatory functions *in vitro* and may support innate immune response to tissue injury. Therefore, it is possible that endogenous expression and secretion of FGF23 by infiltrating macrophages induce cardiovascular and kidney disease progression through induction of inflammation and fibrosis.

Cardiac FGF23 and RAAS

The activation of RAAS was shown to have direct hypertensive effects and stimulates pro-inflammatory, pro-hypertrophic, and pro-fibrotic pathways in cardiac cells, which are pharmacologically suppressed by angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARB), and MR antagonists (92). The initial RAAS factor is the induction of angiotensinogen expression. Renin converts angiotensinogen into angiotensin I, and angiotensin I is further metabolized to angiotensin II by ACE, finally affecting myocardial hypertrophy and fibrosis. In contrast, angiotensin-converting enzyme 2 (ACE2) degrades angiotensin I and II into angiotensin 1–9 and 1–7, respectively, acting as vasodilatory and hypotensive reagents. It was shown that FGF23 directly stimulates RAAS through inhibition of ACE2 (82) and recent studies indicate that activation of RAAS in turn can also induce FGF23 synthesis (104). In addition, renin is suppressed by $1,25(OH_2)D_3$ thereby avoiding RAAS activation (66). Since FGF23 suppresses $1,25(OH_2)D_3$ metabolism in the kidney, one may speculate that FGF23 also alters RAAS indirectly through inhibition of $1,25(OH_2)D_3$. However, the precise mechanisms of how cardiac FGF23 interacts with the local RAAS in the heart and thereby induce cardiac remodeling and impair diastolic function, remains to be further elucidated.

Angiotensin II and aldosterone, as active RAAS components, promote vascular and myocardial fibrosis as well as cardiac hypertrophy also independent of high blood pressure through pathologic signaling mechanisms involving cardiac myocytes and immune cells (92). However, the role of angiotensin II in health and disease is multifactorial, cardiac myocytes and fibroblasts express angiotensin II-type 1 and 2 receptors (AT1R, AT2R). Additionally, as we recently demonstrated, isolated cardiac myocytes and fibroblasts of neonatal rats clearly express angiotensinogen (*Agt*), which was affected by FGF23 treatment *in vitro*, although *Agt* mRNA levels are frequently higher in cardiac myocytes than in fibroblasts (87). In addition, pro-fibrotic and pro-hypertrophic molecules, i.e., TGF-β, collagen 1, CTGF, and endothelin-1, known to be induced by AngII and/or aldosterone, are stimulated by FGF23 treatment in both cardiac myocytes and fibroblasts. Importantly, both AngII and aldosterone significantly up-regulate endogenous FGF23 expression in cardiac myocytes and induce hypertrophic cell growth, *βMHC*, *ANP*, and *BNP*. Whether these pathologies promoting cardiac hypertrophy and fibrosis are directly due to RAAS-mediated up-regulation of FGF23 in the heart or indirectly through the FGF23-mediated stimulation of RAAS is not clear. However, cardiac myocytes and fibroblasts may be potent target cells for local, non-canonical

RAAS activation and paracrine FGF23 signaling (**Figure 1**). Taken these findings together, RAAS is an important regulator and key player in cardiovascular remodeling, resulting in HF that promotes sympathetic activation, systemic inflammation, and pathological cardiac hypertrophy. In turn, AngII and aldosterone induce FGF23 expression in cardiac myocytes in a feed-forward loop, although the underlying molecular mechanisms remain to be identified.

FUTURE PERSPECTIVES AND POTENTIAL THERAPEUTIC OPTIONS TO PREVENT FGF23-ASSSOCIATED CARDIOVASCULAR PATHOLOGIES

The possibilities of a therapeutic intervention to reduce or even prevent FGF23-associated cardiovascular diseases are certainly more diverse. Direct targeting of the FGF23/FGFR signaling complex is theoretically a straightforward option. It was shown that treatment with a monoclonal anti-FGF23 neutralizing antibody improved secondary hyperparathyroidism with increased vitamin D synthesis and normalization of bone markers in a rat model of experimental uremia (105). Nevertheless, chronic inhibition of FGF23 resulted in increased calcium and phosphate load, enhanced aortic calcification, and higher mortality of CKD rats compared to controls. In contrast, anti-FGF23 antibody therapy was proven to be effective and safe to cure bone abnormalities in hereditary causes of elevated FGF23 levels but reduced serum phosphate levels, e.g., X-linked hypophosphatemic rickets (106). Taken together, in states of high phosphate, general blocking of FGF23 is not suitable. Regarding the heart, the physiological relevance of locally produced FGF23 in the heart and the specific (side) effects of cardiac FGF23 suppression in health and disease are yet not clear and has to be elucidated in the future.

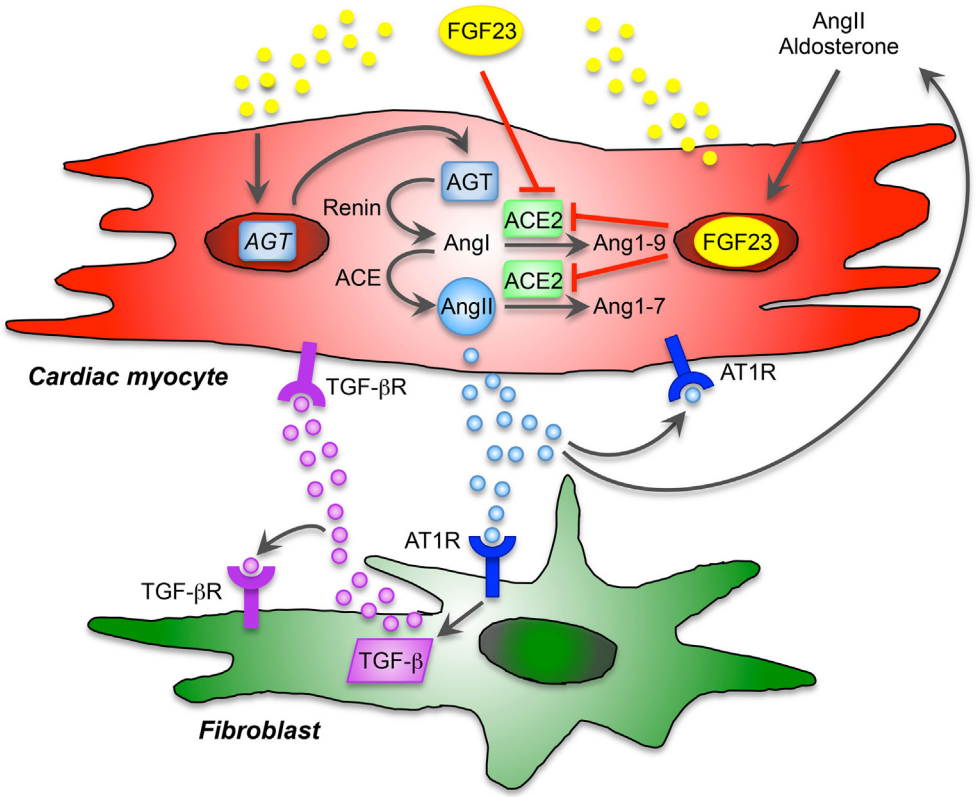

FIGURE 1 | Proposed model of cardiac FGF23 and local renin-angiotensin-aldosterone system (RAAS) in the heart. The local, non-canonical activation of RAAS directly contributes to cardiac remodeling and hypertrophy and affects different cardiac cell types. Angiotensin II (AngII) type 1 receptor (AT1R) is expressed by cardiac myocytes and fibroblasts and thereby displays an important role in maladaptive remodeling. AngII and aldosterone are stimulators of FGF23 expression and secretion, and cardiac FGF23 directly induces the expression of angiotensinogen (*AGT*) mRNA in cardiac myocytes. After activation, AGT is converted into AngI by renin and further to AngII through angiotensin-converting enzyme (ACE). Angiotensin-converting enzyme 2 (ACE2), which physiologically cleaves AngI and AngII counteracting the pathological effects of AngII, is inhibited by FGF23. Thus, FGF23 directly contributes to RAAS activation *via* induction of AGT and suppression of ACE2 in cardiac myocytes resulting in enhanced AngII and aldosterone synthesis. AngII binds to AT1R and promotes hypertrophic response in cardiac myocytes in an autocrine/paracrine manner and stimulates cardiac FGF23 in a feed-forward loop. In addition, FGF23-mediated activation of RAAS and consequently increased AngII contribute to cardiac fibrosis *via* binding of AngII to AT1R on cardiac fibroblasts, which directly promotes differentiation and matrix production in cardiac fibroblasts. In addition, AngII/AT1R induces the expression of transforming growth factor β (TGF-β), which in turns binds to TGF-β receptor (TGF-βR) on cardiac fibroblasts to induce itself or stimulate hypertrophic molecules and signaling in cardiac myocytes. Taken together, endogenous expression of FGF23 in cardiac myocytes is directly involved in the activation of local RAAS contributing in pathological hypertrophy and fibrosis in a paracrine manner.

Targeting specific FGFRs may be another efficient strategy for therapeutic interventions with lesser side effects. An overview of FGFR small-molecule compounds, monoclonal antibodies, and FGFR analogs currently under development for the use as pro- or anti-FGF signaling therapeutics among others for the treatment of cardiovascular diseases in humans, is nicely summarized in a Review by Katoh (107). Regarding the treatment of LVH, Grabner and colleagues impressively demonstrated that targeting FGFR4 specifically prevents FGF23-induced cardiac hypertrophy *in vitro* and *in vivo* in CKD and non-CKD models (37). However, the specific FGFRs mediating the FGF23-induced signaling pathways in cardiac fibroblasts and other non-cardiac myocyte cells, such as endothelial cells and macrophages, is not finally discovered so fare. Moreover, whether blocking of FGFR4 also ameliorates cardiac fibrosis, improve endothelial function or reduce inflammatory processes is not known. In addition, the translation of

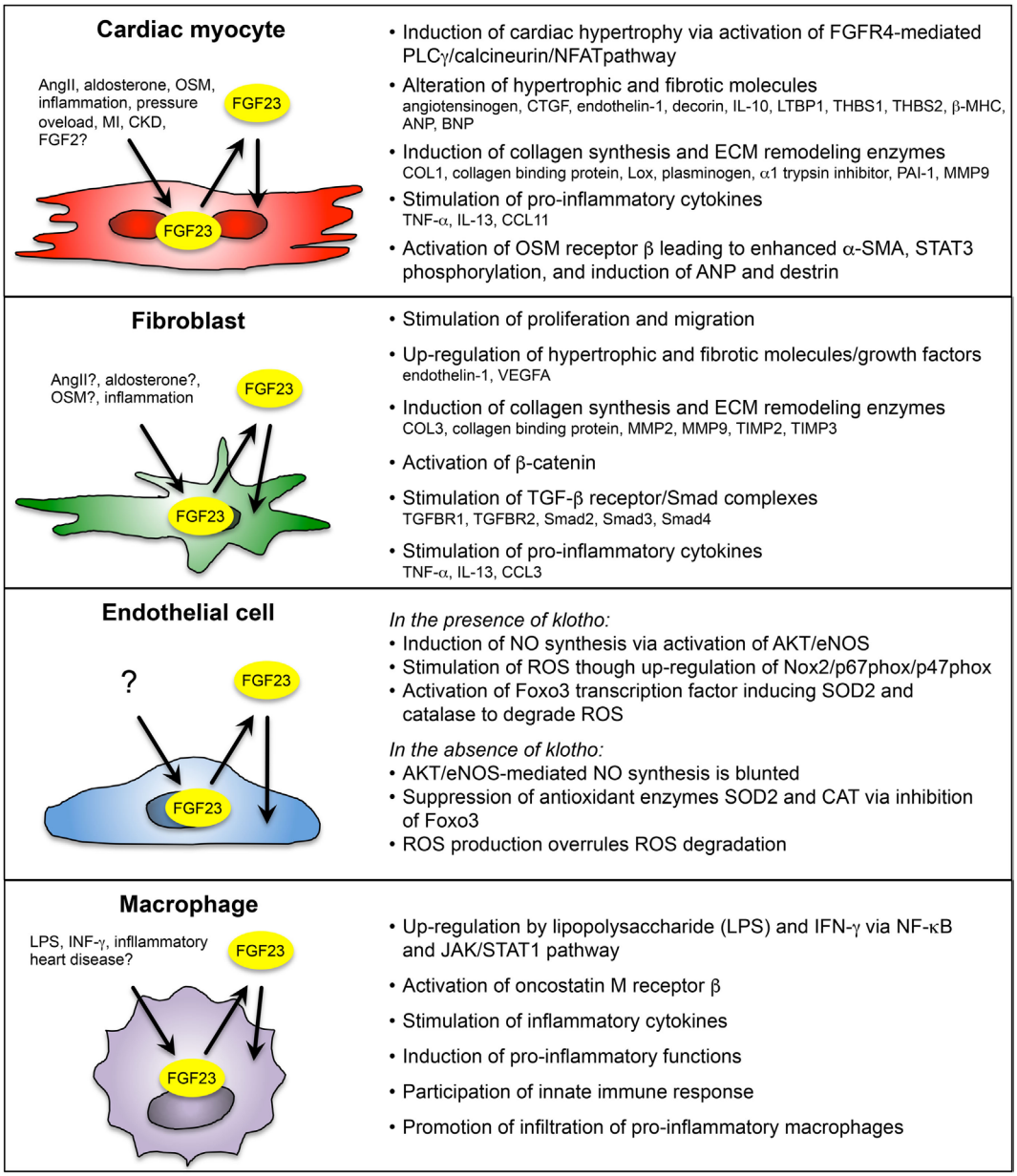

FIGURE 2 | Overview of paracrine/autocrine acting FGF23 on cardiac myocyte and non-cardiac myocyte during cardiac remodeling and heart failure (HF). FGF23 is endogenously expressed in cardiac myocytes, fibroblasts, coronary artery endothelial cells, and macrophages in the heart. Depending on the cell type, FGF23 is induced by various pathogenic stimuli, including angiotensin II (AngII), aldosterone, oncostatin M (OSM), interferon γ (IFN- γ), lipopolysaccharide (LPS), inflammation, pressure overload, myocardial infarction (MI), and chronic kidney disease (CKD). Cardiac FGF23 exerts pro-hypertrophic, pro-fibrotic, and pro-inflammatory response in different cardiac cell types, which in turn activates pathologically phenotypic changes in the same or nearby cells through stimulation and secretion of growth factors, inflammatory cytokines, and pro-hypertrophic and pro-fibrotic molecules. [References for FGF23 in cardiac myocytes (23, 25, 26, 34, 37, 87, 102); cardiac fibroblasts (24, 87, 101); endothelial cells (90, 91); and macrophages (90, 95, 103).]

this approach into clinical settings has to be investigated in future clinical studies.

The involvement of FGF23 in the RAAS activation is of particular interest and might be another promising target pathway for the therapeutic intervention of FGF23-mediated cardiac pathology. In patients with stable ischemic heart disease (SIHD) within the Prevention of Events With Angiotensin-Converting Enzyme (PEACE) trial, high FGF23 levels were independently associated with risk of HF or cardiovascular mortality (108). Furthermore, ACE inhibitor (ACEi) therapy reduced the incident of both events only in the patient subgroup with the highest FGF23 quartile. A clinical study in patients with advanced chronic systolic HF showed that the absence of ACEi therapy independently associated with increased circulating FGF23 levels (59). Interestingly, in this study, ACEi therapy was further associated with a lower risk of death, urgent heart transplantation, and ventricular assist device implantation only in HF patients without CKD in the highest FGF23 tertile, but not in those with CKD. The authors conclude that the association of FGF23 with adverse events reflects the activation of RAAS. In a recently published large multicentric European prospective observational study in patients with new onset and worsening HF (BIOSTAT-CHF trail), patients with the highest FGF23 quintile presented with worse renal function, more severe HF, increased congestion, and enhanced renin and aldosterone levels suggesting pronounced RAAS activation (109). Furthermore, higher plasma levels of FGF23 were independently associated with higher aldosterone levels and less successful uptitration of ACEi and ARB in addition to increased risk of mortality and HF hospitalization. In contrast, the use of beta-blocker was not significantly associated with high FGF23 concentrations. In CKD patients, it was shown that high FGF23 levels correlated with impaired ACE inhibition (110). In addition, CKD patients with the highest FGF23 responded with less effective ACEi therapy.

However, all of these studies hypothesized that increased FGF23 levels may provide additional information regarding the tolerability and effectiveness of RAAS blockade and may identify a subset of patients with SIHD or HF with or without CKD which may benefiting from an ACEi therapy. Nevertheless, only the effects of systemic RAAS inhibition on circulating FGF23 levels were investigated and very little is known about the impact of the local RAAS/FGF23 system in the heart. It is unknown if systemic treatment with RAAS inhibitors effectively target cardiac FGF23. A small cohort study in pediatric CKD patients on dialysis treatment or with functioning renal allograft failed to associate the expression of cardiac FGF23 with RAAS medications (25). One can speculate that the impaired efficiency of ACEi therapy in renal insufficiency is caused by additional overexpression of FGF23 in the heart, which may be resistant to systemic RAAS inhibition. This hypothesis is supported by the recently reported interactions of cardiac FGF23 synthesis and RAAS (87). AngII and aldosterone induce endogenous FGF23 in the heart and FGF23 stimulates *AGT* expression as well as the activation of RAAS in a feed forward loop, which may result in a resistance of therapeutic intervention.

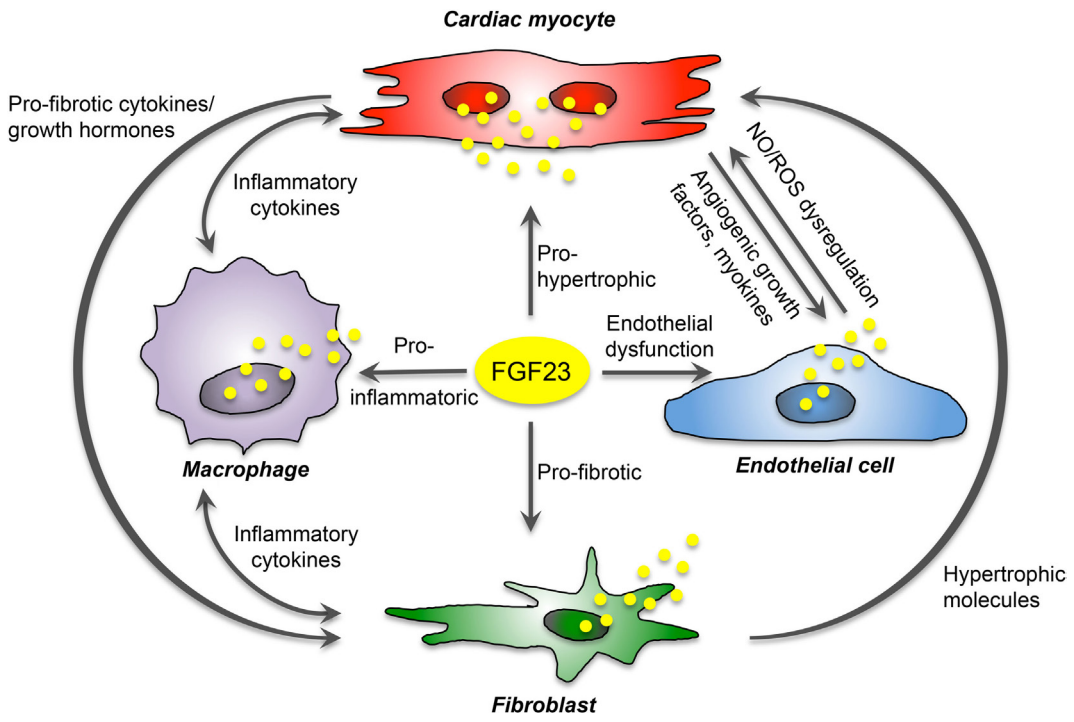

FIGURE 3 | Proposed FGF23-mediated crosstalk between cardiac cells. FGF23 is expressed in cardiac myocytes, fibroblasts, endothelial cells, and macrophages, and directly induces pro-hypertrophic, pro-fibrotic, and pro-inflammatory signaling in a paracrine manner. Moreover, it promotes endothelial dysfunction in states of klotho deficiency. In addition, cardiac FGF23 stimulates different pathologic factors in each cardiac cell type and thereby participate indirectly in the crosstalk between cardiac myocytes and non-cardiac myocytes mediating cardiac hypertrophy and fibrosis.

Another important point is that most clinical trials determine the circulating levels of C-term FGF23. What is about the intact FGF23 protein in HF patients, which is discussed to be more important to mediate signaling events, and do RAAS inhibitory therapeutics impact on the posttranslational modification and/or cleavage of circulating and cardiac FGF23? It was shown in patients with autosomal dominant hypophosphatemic rickets, hyperphosphatemic familial tumoral calcinosis, and CKD, that the fraction of total FGF23 species representing intact biologically active hormone can markedly vary between different clinical settings (111). The cleavage of FGF23 in the bone is very well established, but less is known about the local processing of FGF23 in the heart and different cardiac cell types. The measurement of both biologically active and total FGF23 protein would represent an important advance for the understanding of its role also in different cardiovascular diseases. Moreover, future studies should provide more insights in the impact of therapeutic medications on the synthesis and cleavage of circulating and cardiac FGF23.

Important to note, treatment with ACEi or vitamin D was shown to improve soluble klotho levels in pediatric patients presenting with mild to moderate CKD (112). Taken together, these observations support the hypothesis of a direct cross talk between RAAS, vitamin D, FGF23, and klotho pathways, which can be targeted by RAAS inhibitor treatment. Normalization of soluble klotho may result in further modification of the specific FGFR for FGF23-mediated signaling in the heart though the interaction of FGF23 with klotho (65). It remains to be proven whether this will result in prevention of FGF23-driven pathological cardiac remodeling and counterregulate FGF23 cardiac toxicity.

SUMMARY

Enhanced cardiac FGF23 is reported in various clinical and experimental settings of cardiac remodeling or failure, e.g., CKD, ADHF, ischemic cardiomyopathy, myocarditis, dilated cardiomyopathy, inflammatory HF, TAC-induced pressure overload, experimental MI, and ischemia reperfusion in rodents. On a cellular level, FGF23 is expressed in cardiac myocytes and in other non-cardiac myocytes, including cardiac fibroblasts, vascular smooth muscle, and endothelial cells in coronary arteries, and in inflammatory macrophages (**Figure 2**).

The communication of cardiac myocytes and non-cardiac myocytes is mediated by intracellular signaling promoting pathologic ventricular remodeling and hypertrophy. Thereby, cardiac myocytes respond to stress stimuli with secretion of inflammatory cytokines, and damage-associated molecular patterns to impact on local non-cardiac myocytes. This activates resident macrophages and fibroblasts, which secrete pro-hypertrophic and pro-fibrotic cytokines to promote cardiac myocyte hypertrophy, fibroblast differentiation, matrix deposition, and finally interstitial myocardial fibrosis. Current data suggest that secreted by cardiac myocytes, FGF23 can stimulate pro-fibrotic factors in myocytes to induce fibrosis-related pathways in fibroblasts and consequently cardiac fibrosis in a paracrine manner, and while acting on cardiac myocytes, FGF23 directly induces pro-hypertrophic genes and promotes the progression of LVH in an autocrine fashion. In addition, *in vitro* studies suggest that FGF23 can induce endothelial dysfunction in the setting of klotho deficiency *via* enhanced ROS production and suppression of antioxidant enzymes resulting in increased oxidative stress, which in turn may contribute to the progression of cardiovascular disease in humans. Finally, endogenous FGF23 expression and secretion in infiltrating macrophages in the heart induce cardiovascular disease progression through induction of inflammation and fibrosis. Overall, despite the known expression in bone, FGF23 is now established as also synthesized and secreted in the heart of humans and rodents by different cardiac cell types. Therefore, cardiac FGF23 is involved in the complex interaction between cardiac myocytes, fibroblasts, endothelial cell, and macrophages to induce signaling pathways and phenotypic changes within the same or nearby cells in an autocrine or paracrine manner, respectively (**Figure 3**).

Nevertheless, recent studies suggest that FGF23 is involved in the activation of local RAAS and, in turn, AngII and aldosterone as active RAAS components induce the expression of FGF23 in the heart in a feed-forward loop. It is known that RAAS activation promotes pro-inflammatory, pro-fibrotic, and pro-hypertrophic processes in cardiac cells. Since AT1R is present in cardiac myocytes, fibroblasts, and macrophages, these cell types are potent target cells for local, non-canonical RAAS activation and paracrine FGF23 signaling.

However, hard evidence showing that enhanced cardiac FGF23 directly induces pathologic cardiac remodeling and hypertrophy in a paracrine manner is lacking. The induction of cardiac FGF23 may also be a consequence of the failing heart. Whether cardiac FGF23 alone is sufficient to induce spontaneous cardiac hypertrophy and fibrosis and whether high cardiac synthesis of FGF23 always leads to the progression of HF must be investigated in further studies. Finally, which factor directly stimulates cardiac FGF23 expression and under which conditions cardiac FGF23 leads to pathological changes of the heart must also be clarified in suitable experimental studies and in specific animal models.

AUTHOR CONTRIBUTIONS

ML-N and DH equally contributed to conception and design of the manuscript; ML-N performed extensive literature search and wrote the first draft of the review; DH critically reviewed the manuscript. Both authors read and approved the final manuscript.

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REVIEW
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FGF23 in Cardiovascular Disease: Innocent Bystander or Active Mediator?

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Fibroblast growth factor−23 (FGF23) is a mainly osteocytic hormone which increases renal phosphate excretion and reduces calcitriol synthesis. These renal actions are mediated via alpha-klotho as the obligate co-receptor. Beyond these canonical "mineral metabolism" actions, FGF23 has been identified as an independent marker for cardiovascular risk in various patient populations. Previous research has linked elevated FGF23 predominantly to left-ventricular dysfunction and consecutive morbidity and mortality. Moreover, some experimental data suggest FGF23 as a direct and causal stimulator for cardiac hypertrophy via specific myocardial FGF23-receptor activation, independent from alpha-klotho. This hypothesis offers fascinating prospects in terms of therapeutic interventions, specifically in patients with chronic kidney disease (CKD) in whom the FGF23 system is strongly stimulated and in whom left-ventricular dysfunction is a major disease burden. However, novel data challenges the previous stand-alone hypothesis about a one-way road which guides unidirectionally skeletal FGF23 toward cardiotoxic effects. In fact, recent data point toward local myocardial production and release of FGF23 in cases where (acute) myocardial damage occurs. The effects of this local production and the physiological meaning are under current examination. Moreover, epidemiologic studies suggest that high FGF-23 may follow, rather than induce, myocardial disease in certain conditions. In summary, while FGF23 is an interesting link between mineral metabolism and cardiac function underlining the meaning of the bone-heart axis, more research is needed before therapeutic interventions may be considered.

Keywords: FGF23, cardiovascular diseases, heart failure, hypertrophy, left ventricular, myocardial infarction

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INTRODUCTION

Despite relevant progress in diagnosis and therapy, mortality in patients with chronic kidney disease (CKD) and end-stage renal disease (ESRD) remains high, with an overall 65% 5-year mortality (1). Mortality and morbidity associated with CKD are mainly driven by a vast increase in the rates of cardiovascular events. Indeed, the risk of a patient with early CKD to develop cardiovascular disease (CVD) is ∼20 times higher than to finally require renal replacement therapy (2). Although traditional cardiovascular risk factors including hyperlipidemia, diabetes, and hypertension are highly prevalent in patients with CKD (3), they cannot alone explain the high cardiovascular disease burden in CKD patients. Several "non-Framingham" or "non-traditional"

risk factors have been proposed to contribute to this exploding cardiovascular risk, among which parameters of the so-called CKD—mineral and bone disorder (CKD-MBD) may be of particular importance (4). Many of these parameters link all three entities of the CKD-MBD syndrome, i.e., renal, skeletal and cardiovascular disease. Some of the "non-traditional" cardiovascular risk factors within the spectrum of CKD-MBD such as hyperphosphataemia (5), hypo-and hypercalcaemia (6) as well as secondary hyperparathyroidism (7) have been known for a long time to predict cardiovascular mortality in CKD patients (4). Fibroblast growth factor-23, FGF23 is a rather novel player within the broad spectrum of the CKD-MBD syndrome (8).

THE SPECIFIC CARDIOVASCULAR PATHOLOGY OF CKD PATIENTS

The cardiovascular disease spectrum of CKD and ESRD patients is specific in various aspects. In addition to accelerated cardiovascular calcification the central characteristic feature of CKD-associated CVD is the development of left-ventricular hypertrophy (LVH). The pathophysiology of CKD-associated LVH is complex and multifactorial and LVH occurs even in the absence of severe and long-standing uncontrolled arterial hypertension or aortic valvular disease in CKD patients (9). Instead, increased vascular stiffness, anemia, hypervolaemia, activation of the renin angiotensin, and of the sympathetic system as well as the toxic effects of uremia-associated circulating factors also contribute to the development of LVH in the setting of CKD (9). Among the latter, FGF23 is a candidate undergoing an intense debate.

THE EVOLUTION OF FGF23 RESEARCH OVER TIME

An important milestone in our understanding of the bone-heart axis was the discovery of the profound metabolic, specifically mineral effects of FGF23 (10). FGF23 is a 32 kDa bone-derived potent regulator of vitamin D and phosphate metabolism. It was originally identified only some 15 years ago in the osteological community as a phosphaturic hormone in renal phosphate wasting syndromes such as oncogenic osteomalacia (11). Shortly thereafter, it was found that plasma concentration of FGF23 rises dramatically with increasing severity of CKD (12), and moreover, is independently associated with poorer outcome among non-dialysis CKD patients (13, 14) as well as dialysis patients (15). Soon, FGF23 left the nephrology niche and proved to be of particular interest for the cardiology community, since the dismal association between high FGF23 levels and poor prognosis is also detectable in patients selected primarily via cardiac disorders (16–18). Abundant cohort studies followed, all pointing toward the same direction—a strong association between elevated FGF23 levels, cardiovascular morbidity and mortality (19). Of note, FGF-23 turned out to be a stronger predictor of heart failure decompensation rather than of acute atherosclerotic cardiovascular events (14, 20).

The next evolutionary step in our FGF23 understanding was the transfer from association toward causality: Recent experimental data established a causal pathway and linked extra-cardiac FGF-23 directly to the development of cardiovascular pathologies, specifically cardiomyopathy (21). However, as discussed below, even this point of view is again moving forward as evidence grows that the cardiovascular system itself may be able to affect FGF23 levels with potential, yet to be determined, local and systemic effects.

PHYSIOLOGY OF FGF23

Circulating FGF23 is mainly produced by osteocytes and osteoblasts. FGF23 primarily targets the renal tubular and parathyroid cells. These canonical effects of FGF23 (i.e., regulating phosphate, vitamin D metabolism and PTH) depend upon its interaction with FGF23 receptors and its obligate renal co-receptor alpha-klotho (22). In renal tubular cells, upon binding to the FGF-receptor and alpha-klotho—FGF23 (1) stimulates the excretion of phosphate, (2) reduces the activation of calcidiol to calcitriol, and (3) increases the degradation of the latter (23) Hypothetical non-canonical renal effects of FGF23 include the stimulation of distal tubular sodium and calcium absorption (24, 25) as well as suppression of angiotensin converting enzyme 2 transcription in the kidney (26). Furthermore, *in vitro*, FGF23 treatment of bovine parathyroid cells inhibits PTH secretion (27) (summarized in **Figure 1**).

There is a striking association between decreasing glomerular filtration rate (GFR) and raising FGF23 levels in humans. In fact, the magnitude of FGF23 increase with the decline in kidney function is unique among the various biomarkers of the CKD-MBD syndrome. While a PTH increase by a factor of 20–30 may already represent severe, uncontrolled hyperparathyroidism, FGF23 levels over 1,000-fold higher than in healthy controls have been described in CKD (28).

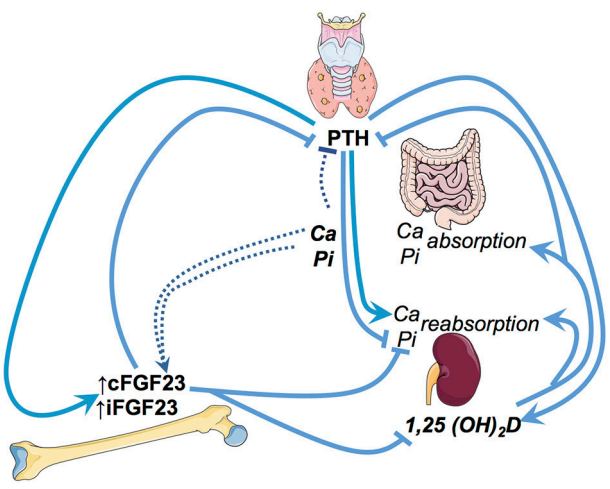

FIGURE 1 | Illustration of the key regulatory pathways of FGF23 in phosphate and vitamin D metabolism. Ca, Calcium; Pi, Phosphate; iFGF23, intact FGF23; cFGF23, c-terminal FGF23; PTH, parathyroid hormone.

FGF23 REGULATION: PHOSPHATE AND BEYOND

The underlying mechanisms of the tremendous rise of FGF23 in renal insufficiency remain partly unclear. Various endogenous, but also external factors contribute to FGF23 regulation such as the phosphorus load and active Vitamin D [1,25 (OH) $_2$D3] which appear to be main stimulators of FGF-23 synthesis (29, 30), while other factors including calcium, parathyroid hormone, inflammation, and iron are also involved (31).

Indeed, the exact mechanistical evidence through which decreasing GFR and/or high serum phosphate can increase FGF23 concentrations is still lacking. In fact, it appears an oversimplification to establish a smooth and direct link between phosphate load and/or retention leading to FGF23 increase or vice versa, phosphate restriction, and/or: excretion to FGF23 decrease. Animal and *in vitro* experiments have to date yielded conflicting results. In mice, feeding a high phosphate diet has been shown to increase circulating FGF23 (32), and FGF23 levels correlated positively with levels of circulating phosphate in patients with ESRD (10). However, dietary phosphate modulation has some corresponding effects on circulating FGF23 levels in some studies (33, 34), while other studies found no effect (12). *In vitro*, cultured osteoblast increased FGF23 production in response to 1,25 (OH) $_2$D and PTH (35) but not to phosphate (36). In a mouse model of progressive renal failure, Zhang et al. found that severe phosphate restriction (0.2 and 0.02%) did not modulate serum levels of FGF23 (37) despite the expected severe reduction in renal phosphate excretion.

Beyond factors that affect osteoblastic and osteocytic FGF23 production, FGF23 levels and actions undergo modifications via changes in its release (38), cleavage (39), distribution, and allocation of receptors and co-receptors. Cleavage of FGF23 by a furin pro-protein convertase into its N- and C-terminal fragments appears to be the main regulator of its biological effects (40). To date, only intact FGF23 has clearly been shown to have a physiological effect, while the function of the C-terminal fragments remains more controversial (41). Goetz and coworkers have shown that c-terminal FGF23 fragments actually inhibit the FGF23-FGF-R-klotho-interaction (41) and thus antagonize phosphaturic effects of intact FGF23. Hence, FGF23 cleavage is a relevant metabolic step regulating FGF23 activity.

The distribution of FGF-receptors is a major determinant of FGF23 function and is responsible for mediating canonical (renal and phosphaturic) and (e.g., cardiac) effects. FGF-Receptor 1 (FGF-R1) is thought to be the major renal effector of FGF23, mediating primarily phosphaturic and calcitriol-regulatory effects of FGF23 (42). Klotho as obligate co-receptor is of major importance regarding renal mode of action of FGF23 (22).

CARDIOVASCULAR EFFECTS OF RAISED FGF23

As already mentioned, over the last years many studies have shown significant and independent associations between increased FGF23 and dismal outcomes in humans. Remarkably, this association is not limited to CKD patients (non-dialysis CKD (13, 14) and ESRD (15, 43)), but also detectable in patients without overt CKD (16–18, 44) in whom no primary significant FGF23 excess is to be expected. While there appears to be a strong and independent association between FGF23 levels and increased mortality as well as heart failure, the association to the development of atherosclerosis is much less pronounced (14, 20) suggesting that occlusive atherosclerotic disease may not be the primary link between increased FGF23 levels and mortality (45). Numerous additional explanations have been proposed as basis for the prognostic impact of FGF23 on (cardiovascular) mortality, which comprise a contribution of raised FGF23 to endothelial dysfunction (46), stimulation of the renin-angiotensin system (47), arterial stiffness (46), vascular calcification (48), inflammation (31), and left-ventricular hypertrophy.

SPECIFIC FOCUS UPON FGF23 AS THE DRIVING FORCE FOR LVH

Left-ventricular dysfunction has gained specific interest regarding its association with increasing levels of FGF23 now representing an outstanding research field in cardiorenal medicine, since LVH is highly prevalent in CKD and is presumably among the major driving forces for sudden cardiac death (SCD) (49). SCD in turn is among the leading causes of death in CKD patients and the single most prevalent cause for CV death—more prevalent event than classical atherosclerotic cardiovascular events in these patients (50).

Indeed, LVH is a very common finding in patients with CKD and ESRD with up to 74% of patients showing some form of this pathological cardiac remodeling (51–53). The exact prevalence of LVH most likely varies with duration and severity of CKD as well as with the sensitivity of the detection method. Actually, virtually all patients with severe non-dialysis CKD or ESRD will have at least subtle—if not substantial—changes of left ventricular function, geometry, and structure. Evolving and more sophisticated echocardiography and magnetic resonance imaging techniques will make detection of subclinical myocardial changes more sensitive in future. LVH not only predisposes to sudden cardiac death (49), but also increases the risk of developing heart failure: heart failure with preserved ejection fraction (HFPEF) as well as heart failure with reduced ejection fraction (HFREF).

Recent research has attributed a substantial role in LVH pathogenesis to FGF23: As early as 1997 Nehme et al. hinted at it by showing that children with X-linked hypophospataemic rickets (XLH), an X-linked dominant disease with FGF23 overexpression and consecutive hypophosphataemia, to have signs of LVH (54).

In much less selected patients, evidence for an association between FGF23 and LVH was first published by Mirza et al. who showed that in the PIVUS cohort, a Swedish cohort with around 800 patients aged 70 or more, intact FGF23 correlated with the presence of LVH, as determined by echocardiography (55). Noteworthy, this relationship held true, even for FGF23 levels within the normal range. The effects of increased FGF23 were then prospectively analyzed by Faul et al. in a large cohort of 3070

non-dialysis CKD patients. The authors found baseline levels of c-terminal FGF23 to positively correlate with the presence of LVH. Increased FGF23 baseline levels were also associated with an up to 7-fold increased incidence of LVH development in the following 3 years (56). However, there are also studies in hemodialysis patients that have found no association between FGF23 and ventricular mass (57, 58).

In continued research, mainly driven by Faul and coworkers (21, 56, 59–61), several experimental settings were used to determine whether the FGF23 association with LVH was causative or purely associative. The authors concluded that FGF23 is within the causal pathway of CKD-associated LVH development. *In vitro*, the treatment of neonatal rat ventricular myocytes for 48 h with different concentrations of FGF23 induced morphometric hypertrophy similar in extent to treatment with FGF2, a known strong inducer of LVH. These histological changes accompanied a change in the gene expression profile reflecting pathological hypertrophy. The effect was dose dependent, with no further increase seen after 15 ng/ml FGF23 (56). Interestingly, the authors concluded that the cardiac effects of FGF23 were Klotho independent since Klotho was not expressed by cardiac myocytes.

In-vivo experiments using intravenous and intramyocardial injection of FGF23 also showed induction of LVH in non-CKD mice (56). Again, Klotho did not appear to be necessary for the cardiac effects of FGF23. Using Klotho-null mice, Faul et al. showed that even in the absence of Klotho, treatment with FGF23 induces LVH to a similar manner as seen in wild type mice. Interestingly, mice heterozygous for Klotho, in which FGF23 raises less pronouncedly than in Klotho null mice, also develop LVH, though to a lesser extent than klotho null mice. While the authors state that this reflects a dose dependent effect of FGF23, it may also reflect the total loss of protective klotho (56).

To further examine the interplay between FGF23 and its cardiac receptors Faul et al. also investigated the effect of FGF23 on FGFR4 null mice and found that abrogation of the FGFR4 prevented the development of LVH *in vitro* as well as *in vivo* while overexpression of FGFR4 induced LVH in non-CKD mice (21). These data raise the hypothesis that the myocardial FGF23 effects are mediated predominantly through the FGFR4 activation of PLC/calcineurin/NFAT pathway. However, it remains unclear if the proposed cardiac effects of FGF23 are all direct effects upon the myocardium or in part reflect systemic FGF23 effects since administration of FGF23 has been suggest to raise blood pressure (24), potentially contributing the induction of LVH (56). Also, XLH-children do not universally exhibit LVH despite chronically raised FGF23 levels (54, 62). Hence, all these findings warrant critical reappraisal and indeed, very recent data point into a different direction.

FGF23 AND MYOCARDIAL DISEASE: CHICKEN OR EGG?

The postulated absence of klotho from the myocardium has been the subject of some debate (23), since klotho is essentially required for FGF23 to exert its renal effects (47). Its role in the setting of cardiomyocyte FGF23 toxicity thus remains controversial: While Faul reported a Klotho-independent effect of FGF-23 on cardiomyocytes, other groups suggested that klotho deficiency rather than a FGF23 excess causes cardiac hypertrophy (63): In heterozygous klotho-deficient CKD mice, the development of LVH was not modified by interventions normalizing FGF23 and phosphate levels, but only via exogenous klotho application. In humans, it is currently impossible to clearly separate the effects of CKD-induced klotho deficiency, phosphate load, and FGF23 raise, particularly as no reliable assays for soluble klotho measurements are available so far.

FGF23 has traditionally been believed to be mainly of skeletal origin from which it affects systemically its canonical kidney and parathyroid gland as well as non-canonical targets such as cardiomyocytes (64). While above-mentioned epidemiological data and experimental findings accuse FGF23 (presumably from skeletal origin) as being directly noxious to myocardial cells and being a causative agent for LVH induction and myocardial damage, some data point toward a different or even opposite direction (**Figure 2**). Two major unsolved issues emerge in this respect: First, does the FGF23, which may finally act upon the myocardium, only originate from the bone or also directly from the myocardium and second, are these actions only deleterious or are there some (dose- and time-dependent) beneficial effects detectable?

Indeed, some beneficial cardiovascular effects have been attributed to FGF23: Remarkably, acute elevations of FGF23 were shown to be positively inotropic: FGF23 induced acute elevations of intracellular calcium of primary cardiomyocytes, an effect that could be abrogated by the calcium channel blocker verapamil. FGF23 treatment of a ventricular muscle strip led to increased contractility, which was blocked by inhibition of FGF receptors (65). Hypothetically, this finding allows speculations about a potential physiological role of FGF23 elevations in situations of acute cardiomyocyte stress. If these effects are (patho) physiologically relevant, it appears counterintuitive that skeletal FGF23 is the sole source for such cardiac actions, because this would require a fast myocardial-skeletal messenger.

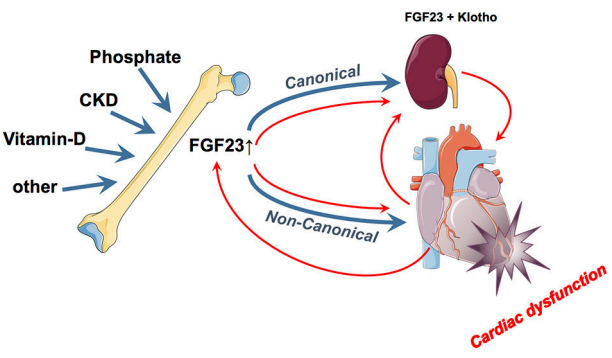

FIGURE 2 | Summary of the currently hypothesized canonical and non-canonical effects of FGF23 on the myocardium and vice-versa. In blue is illustrated the "bone damages heart via FGF23 model" while the red pathways illustrate the "heart damage induces FGF23 alterations model".

In fact, recently Richter el al found cultured cardiomyocytes to express FGF23 (66). In subsequent clinical studies, FGF23 was shown to be present in the explanted hearts of patients with ischaemic or dilated cardiomyopathy undergoing heart transplantation, but not in healthy hearts (67). In another set of experiments, Andhrukova et al. found that, in the setting of experimental myocardial infarction in mice, circulating FGF23 is increased in the circulation with a concomitant reduction of $1,25(OH)_2D3$ (68). Additionally to increased FGF23 production in the bone, myocardial FGF23 was also increased on a protein and mRNA level suggesting that increased circulating FGF23 post-myocardial infarction is at least partly derived from the myocardium itself (68). To add to the complexity of the FGF23-myocardium interaction experiments with the pressure overload model of transverse aortic constriction (TAC) demonstrated that LVH profoundly increased serum levels of intact FGF23, augmented cardiac mRNA and protein expression of FGF23, and increased FGF23 transcription in bone by an aldosterone-driven mechanism (69).

These experimental data are supported by some clinical data fueling the hypothesis that FGF23 release and myocardial damage is not a one-way route from the skeleton to the myocardium: Cross-sectional data derived from a cohort study and *post-hoc* subgroup analyses from a randomized trial on mechanical assist device implantation support the hypothesis that the heart is an active player—not only a recipient—in FGF23 metabolism (70, 71): these patients had much higher FGF-23 than healthy individuals or patients with myocardial infarction not complicated by heart failure. Moreover, among these patients with cardiogenic shock, increased levels of FGF23 on admission correlated with increased mortality at 30 days and 1 year (70). Considering the speed and the magnitude of the FGF-23 rise we speculate that the myocardial damage *per se* induces FGF23 (be it heart-derived and/or bone derived). Hence, this challenged the mono-directional hypothesis that external factors stimulate bone FGF23 release which in turn induces myocardial damage.

Recent data by Anderson et al. underlines how much this topic is currently still up for debate. The authors found that, in a group of patients with acute heart failure, FGF23 is sharply elevated in the circulation (72). In contrary, this does not appear to hold true for chronic cardiac damage as Richter et al. found no upregulation of FGF23 in explanted hearts of patients undergoing heart transplantation for chronic severe heart failure (66).

IS MODIFICATION OF FGF23 A PROMISING THERAPEUTIC OPTION IN CARDIAC DISEASE?

Since many data and researchers suggest that FGF23 excess negatively impacts the cardiovascular system, it appears a potential treatment modality to reduce the circulating amount or to block the FGF23-target organ interaction. However, there are at least two relevant arguments that pharmacological FGF23 blockade is not as straightforward as one could assume. First, as mentioned above, some (early) rise in FGF23 (presumably specifically FGF23 produced and released from the myocardium itself) might have stabilizing effects in the setting of acute myocardial damage. Second, the optimal level and the most appropriate tool of intervention to lower (systemic) FGF23 are unclear.

Clear warning signals against an unreflected (unspecific) blockade emerge from *in vivo* experiments by Shalhoub et al. The authors showed that pharmacological blockade of FGF23 using a pan-blocking antibody failed to prevent the development of LVH in uraemic rats (73). Not only were these results in conflict with the results obtained by Faul et al. (56) but worryingly, FGF23 blockade led to increased aortic calcification and even mortality in the animals (73). The authors attributed this detrimental finding to the raise in serum phosphorus levels which occurred with the blockade of the potent phosphaturic actions of FGF23. Similar results were found when inhibiting FGF23 signaling by a pan-FGFR inhibitor which resulted in increased phosphorus and FGF23 levels leading to multifocal, multiorgan soft tissue mineralization (74). These data clearly remind us that some (systemic) FGF23 actions are beneficial—e.g., the avoidance of phosphate toxicity. Hence, a more specific mode of action such as blocking chronically stimulated FGF23-receptor interactions at the myocardium might hold promise in this respect. Hypothetically, restoration of klotho levels might help to re-direct FGF23 actions toward canonical (renal) signaling pathways and block off-target signaling. Potential effects of FGF23 lowering in humans were shown in the EVOLVE trial in which the use of the PTH-lowering agent Cinacalcet was compared to placebo in patients with terminal renal failure on haemodialysis. Noteworthy, the aim of the intervention was not directly to influence FGF23 synthesis or release but primarily targeted hyperparathyroidism. In this trial, patients receiving cinacalcet showed a significant reduction in mean FGF23 levels (75). Furthermore, patients that responded with a prominent FGF23 decrease of $> 30\%$ showed a pronounced reduction in cardiovascular mortality in the 20 week follow-up (75). Against a direct pathophysiological contribution of FGF23 stands that within the placebo group, patients who had a reduction of FGF23 during the study period had no better prognosis than patients who did not have such FGF-23 reduction. Currently, a fully human monoclonal IgG1 antibody, Burosumab, is under investigation for rare diseases with primary FGF23 excess and the clinical picture of phosphate-losing osteomalacia (X-linked hypophosphatemia). In such diseases, where a primary FGF23 excess hits a physiological kidney function, the approach really holds promise and allows to normalize hypophosphatemia. However, a broader application is currently not appealing since chronic effects of FGF23 blockade in conditions with reactive, secondary FGF23 excess are unpredictable.

CARDIAC FGF23 RESEARCH: THE NEXT LEVEL

In summary, the jury is still out on the exact role of FGF23 in the development of cardiovascular abnormalities. The FGF23-klotho axis and pathophysiological cardiac effects are a constantly

evolving field. Each players' role (klotho, local and systemic FGF23, FGF23-receptors) needs to be exactly determined. Facing the massive threat induced by left-ventricular dysfunction to patients with CKD and ESRD and the potential direct and indirect involvement of low klotho, high FGF23 levels, together with other features of CKD-MBD in this setting an urgent roadmap for further research emerges.

Prior to any therapeutic intervention with the aim to minimize potentially negative FGF23 effects upon cardiac structure and function, research needs to focus on and clarify relevant unsolved issues. Just to name a few, the community needs to prove how cardiac disease induces (rather than follows) FGF-23 secretion, to what degree cardiomyocytes may themselves produce FGF-23 in health and disease, whether such locally produced FGF-23 has a physiological role in (acute) myocardial damage; and whether or not (systemic) FGF23 excess itself directly drives the development of myocardial damage. Only when these questions have been answered, can we try to discuss whether and how to intervene on serum FGF-23 level.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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CORRECTION
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Corrigendum: FGF23 in Cardiovascular Disease: Innocent Bystander or Active Mediator?

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In the original article, there was an error. The last paragraph contained several mistakes carried on from a previous version.

A correction has been made to the section Cardiac FGF23 Research: The Next Level, paragraph 2 and should read:

"Prior to any therapeutic intervention with the aim to minimize potentially negative FGF23 effects upon cardiac structure and function, research needs to focus on and clarify relevant unsolved issues. Just to name a few, the community needs to prove how cardiac disease induces (rather than follows) FGF-23 secretion, to what degree cardiomyocytes may themselves produce FGF-23 in health and disease, whether such locally produced FGF-23 has a physiological role in (acute) myocardial damage; and whether or not (systemic) FGF23 excess itself directly drives the development of myocardial damage. Only when these questions have been answered, can we try to discuss whether and how to intervene on serum FGF-23 level."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way.

The original article has been updated.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
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Therapeutic Interference With Vascular Calcification—Lessons From Klotho-Hypomorphic Mice and Beyond

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Medial vascular calcification, a major pathophysiological process associated with cardiovascular disease and mortality, involves osteo-/chondrogenic transdifferentiation of vascular smooth muscle cells (VSMCs). In chronic kidney disease (CKD), osteo-/chondrogenic transdifferentiation of VSMCs and, thus, vascular calcification is mainly driven by hyperphosphatemia, resulting from impaired elimination of phosphate by the diseased kidneys. Hyperphosphatemia with subsequent vascular calcification is a hallmark of klotho-hypomorphic mice, which are characterized by rapid development of multiple age-related disorders and early death. In those animals, hyperphosphatemia results from unrestrained formation of $1,25(OH)_2D_3$ with subsequent retention of calcium and phosphate. Analysis of klotho-hypomorphic mice and mice with vitamin D_3 overload uncovered several pathophysiological mechanisms participating in the orchestration of vascular calcification and several therapeutic opportunities to delay or even halt vascular calcification. The present brief review addresses the beneficial effects of bicarbonate, carbonic anhydrase inhibition, magnesium supplementation, mineralocorticoid receptor (MR) blockage, and ammonium salts. The case is made that bicarbonate is mainly effective by decreasing intestinal phosphate absorption, and that carbonic anhydrase inhibition leads to metabolic acidosis, which counteracts calcium-phosphate precipitation and VSMC transdifferentiation. Magnesium supplementation, MR blockage and ammonium salts are mainly effective by interference with osteo-/chondrogenic signaling in VSMCs. It should be pointed out that the, by far, most efficient substances are ammonium salts, which may virtually prevent vascular calcification. Future research will probably uncover further therapeutic options and, most importantly, reveal whether these observations in mice can be translated into treatment of patients suffering from vascular calcification, such as patients with CKD.

Keywords: vascular calcification, bicarbonate, carbonic anhydrase inhibitors, magnesium, mineralocorticoid receptor, ammonium salts, osteogenic signaling, phosphate

INTRODUCTION

Medial vascular calcification is a key pathophysiological process associated with the risk of cardiovascular events in a variety of clinical conditions such as aging, diabetes, and chronic kidney disease (CKD) (1, 2). Accordingly, vascular calcification is a powerful predictor of cardiovascular and all-cause mortality (3–5). Vascular calcification in CKD results mainly from impaired renal phosphate elimination with subsequent hyperphosphatemia and precipitation of calcium-phosphate (6). Accordingly, plasma phosphate concentrations are correlated with the incidence of cardiovascular events, heart failure, and death (7, 8).

Vascular calcification results, at least in part, from an active process in vascular smooth muscle cells (VSMCs) (6). Exposure of VSMCs to enhanced extracellular phosphate concentrations is followed by osteo-/chondrogenic transdifferentiation *via* complex intracellular signaling pathways (9). Phosphate complexes with calcium to form pro-inflammatory calcium-phosphate nanoparticles (10, 11). Calcium-phosphate crystals are further involved in the formation of protein–mineral complexes, the calciprotein particles (CPPs) (12). These can transform into more toxic secondary CPPs containing crystalline calcium-phosphate (13). Osteo-/chondrogenic signaling cascades in VSMCs can be triggered by calcium-phosphate nanoparticles and/or secondary CPPs (14–20).

Osteo-/chondrogenic signaling involves upregulation of the type III sodium-dependent phosphate transporter PIT1 (also known as SLC20A1) (21, 22). The transdifferentiated VSMCs express osteogenic transcription factors, such as MSH homeobox 2 (MSX2) and core-binding factor alpha 1 (CBFA1, also known as runt-related transcription factor 2, RUNX2) as well as chondrogenic transcription factors such as SRY-Box 9 (SOX9) (23–25) to facilitate, *via* various complex mechanisms, vascular tissue mineralization (1). Vascular calcification can be prevented by inhibition of CBFA1 (26). The transcription factor NFAT5 (nuclear factor of activated T-cells 5) upregulates CBFA1 expression, an effect mediated by the transcription factor SOX9 (27). Osteo-/chondrogenic reprogramming ultimately upregulates the expression and activity of tissue non-specific alkaline phosphatase (ALPL), an enzyme hydrolyzing the calcification inhibitor pyrophosphate (28). Transdifferentiated VSMCs are also able to secrete matrix vesicles to actively promote tissue mineralization (29). Vascular osteo-/chondrogenic transdifferentiation precedes vascular calcification (30) and has been observed in vasculature of CKD patients (31). Accordingly, osteo-/chondrogenic transdifferentiation predisposes vascular tissue in CKD patients to vascular calcification (32). The orchestration of vascular calcification is, however, still incompletely understood (33).

Valuable insight into mechanisms of vascular calcification was gained by analysis of the klotho-hypomorphic mice (34). Klotho is a transmembrane protein with highest expression in kidney, but also found in parathyroid glands and choroid plexus (34). The extracellular domain of klotho is cleaved off and released into blood (35). Soluble klotho confers protection of kidneys (36) and cardiovascular system (37). Klotho counteracts tissue fibrosis (38, 39), progression of CKD (38), cardiomyopathy (38), vascular calcification (38), and tumor growth (39). Klotho is in part effective by interference with TGFβ1 signaling (39).

Klotho is required for the negative regulation of 25-hydroxyvitamin D3 1-α-hydroxylase (1-α-hydroxylase) by FGF23 and thus for inhibition of 1,25-dihydroxyvitamin D3 ($1,25(OH)_2D_3$) production (35, 40). Contrary to CKD patients, production of $1,25(OH)_2D_3$ is excessive in klotho-hypomorphic mice, resulting in elevated phosphate levels (35). Therefore, the mice suffer from severe tissue calcification, mimicking the findings in mice with renal failure (41). These mice further display a wide variety of age-related disorders and early death (34, 35). Conversely, overexpression of klotho increases the life span of mice (42). Apparently, klotho may similarly influence the life span of humans (43). Although $1,25(OH)_2D_3$ may exhibit protective effects during calcification (44), its excessive formation in klotho-hypomorphic mice increases intestinal calcium and phosphate uptake and renal phosphate retention, thus driving the phenotype and tissue calcification (35, 45). The life span of klotho-hypomorphic mice is substantially increased by vitamin D_3-deficient diet (45). Moreover, klotho stimulates Na^+/K^+-ATPase activity (46) and lack of klotho leads to extracellular volume depletion with secondary increase of ADH and aldosterone release (40). Dehydration, in turn, downregulates klotho expression (47). Although the origin of hyperphosphatemia differs between CKD and klotho-hypomorphic mice, both lead to comparable sequelae of vascular calcification (**Figure 1**).

The present brief review addresses attempts to interfere with vascular calcification, premature aging and early death of klotho-hypomorphic mice and similar models. We anticipate that insights from maneuvers successful in klotho-hypomorphic mice may improve our understanding of the mechanisms underlying calcifications in patients with CKD.

BICARBONATE

Most CKD patients and klotho-hypomorphic mice suffer from acidosis (50, 51), which may further enhance plasma phosphate concentrations (52) and aggravate CKD (53–57). Conversely, alkali administration may slow the progression of CKD (53–56). In contrast to rats, in which metabolic acidosis has been shown to slow the progression of renal disease (58–60), in CKD patients, the deterioration of renal function is accelerated by acidosis and slowed by bicarbonate treatment (56, 61, 62).

Bicarbonate treatment of klotho-hypomorphic mice decreased tissue calcification and increased the average life span of those mice (63). Bicarbonate treatment did not significantly modify plasma concentrations of $1,25(OH)_2D_3$ and calcium, but significantly decreased plasma phosphate concentrations and plasma aldosterone concentrations (63). Bicarbonate treatment was presumably primarily effective by decreasing intestinal phosphate absorption and renal phosphate reabsorption (63). Alkalinization of the intestinal lumen is expected to compromise phosphate solubility and absorption.

CARBONIC ANHYDRASE INHIBITION

Extracellular pH can be modified by treatment with carbonic anhydrase inhibitors, such as acetazolamide (64). The diuretic interferes with proximal tubular bicarbonate reabsorption and, thus, leads to renal bicarbonate loss and acidosis (64). Extracellular

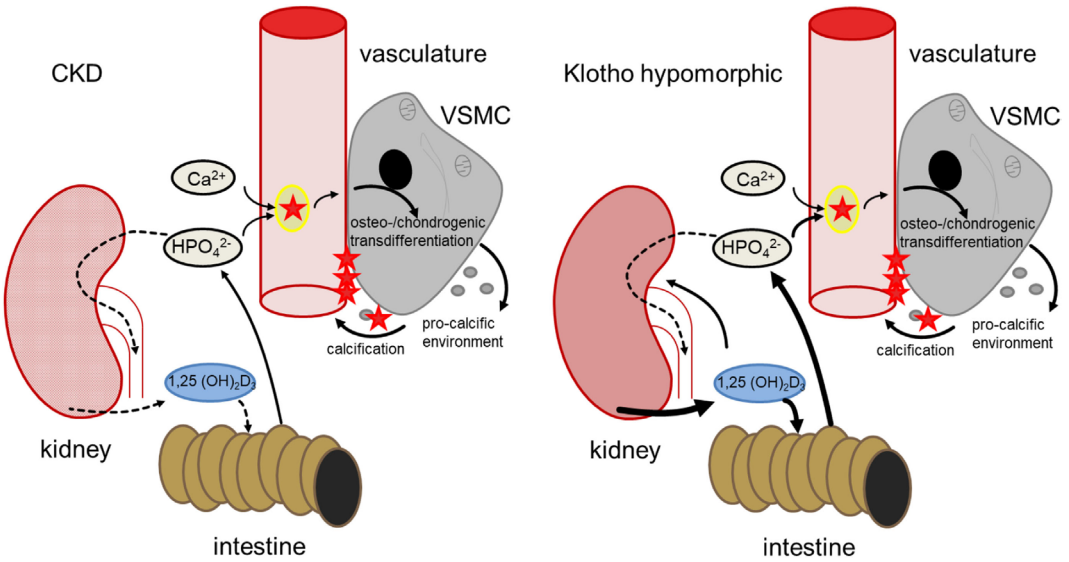

FIGURE 1 | Comparison of hyperphosphatemia due to chronic kidney disease (CKD) (left) and klotho deficiency (right). In CKD, renal failure leads to impaired phosphate elimination and reduced formation of calcitriol [1,25$(OH)_2D_3$]. Phosphate overload and formation of secondary calciprotein particles (CPPs) (48) induce an osteogenic remodeling of vascular smooth muscle cells (VSMCs), causing matrix-vesicle release and a pro-calcific environment and subsequent vascular mineralization. In klotho-hypomorphic mice, klotho deficiency causes an unrestrained calcitriol formation. This causes increased intestinal and renal phosphate reabsorption, increasing phosphate levels. Therefore, despite the potential anti-calcific effects of calcitriol (49), this phosphate overload exceeds the capabilities of anti-calcific mechanisms in the body and may induce a comparable osteo-/chondrogenic transdifferentiation of VSMCs. Although the origin of hyperphosphatemia differs between CKD and kl/kl mice, both presumably suffer from excessive phosphate concentrations and increased formation of secondary CPPs to induce a similar VSMC-mediated calcification.

pH has a profound effect on calcium and phosphate solubility, which is enhanced by acidification and decreased by alkalinization (65). Moreover, acidosis counteracts vascular calcification by downregulation of PIT1 expression (58, 65, 66) and inhibition of renal tubular phosphate reabsorption with increase of renal phosphate elimination (67).

Acetazolamide treatment of klotho-hypomorphic mice blunted the calcifications in trachea, lung, kidney, stomach, intestine, and vascular tissues, reversed the excessive aortic *Alpl* transcript levels as a marker of aortic osteo-/chondrogenic signaling, increased the plasma concentrations of the calcification counteracting proteins osteoprotegerin, osteopontin as well as fetuin-A (68–70) and, thus, tripled the life span despite unaltered plasma concentrations of FGF23, 1,25$(OH)_2D_3$, calcium and phosphate (64). *In vitro*, acidic medium prevented the phosphate-induced upregulation of *ALPL* mRNA expression in primary human aortic smooth muscle cells, indicating that extracellular acidosis interferes with osteo-/chondrogenic transdifferentiation of VSMCs (64). Acidic conditions may impair the formation of small calcium-phosphate complexes during hyperphosphatemia and, thus, hinder VSMC osteo-/chondrogenic transdifferentiation.

It should be kept in mind that the bicarbonaturia and, thus, systemic acidosis following carboanhydrase inhibitor treatment depends on renal function and may, thus, be lacking in CKD patients.

MAGNESIUM

In CKD patients, lower serum magnesium levels are associated with vascular calcification (71) and are predictive for increased arterial stiffness and mortality (72). Previous *in vitro* studies have shown that magnesium treatment is able inhibit phosphate-induced VSMCs calcification (73–75). Magnesium is able to interfere with hydroxyapatite formation (76). Also, magnesium interferes with osteo-/chondrogenic reprogramming of VSMCs.

Experiments in mice treated with excessive levels of vitamin D_3, mimicking excessive vitamin D receptor activation during klotho deficiency, revealed magnesium supplementation as a further potential treatment to reduce the progression of vascular calcification (77). Vitamin D_3 overload was followed by extensive vascular calcification and upregulation of aortic osteoinduction as shown by expression of the osteogenic markers *Msx2*, *Cbfa1*, and *Alpl* (77). Those effects were blunted by additional treatment with $MgCl_2$. Vitamin D_3 overload upregulated the aortic expression of calcium-sensing receptor (CASR), an effect augmented by additional $MgCl_2$ supplementation (77). Magnesium can activate CASR (78) and CASR activation in VSMCs inhibits osteo-/chondrogenic remodeling and calcification (79).

Those *in vivo* observations were supported by *in vitro* experiments using primary human aortic VSMCs. Addition of $MgCl_2$ to the VSMCs cell culture medium reversed the phosphate-induced calcification and osteo-/chondrogenic signaling, effects paralleled by upregulation of CASR expression. The protective effects of $MgCl_2$ were virtually abrogated by the CASR antagonist NPS-2143 or by silencing of the CASR gene (77). Thus, magnesium supplementation may reduce the progression of vascular calcification at least in part by activating CASR. Magnesium

supplementation may thus be beneficial in CKD patients (80). Recently, a first pilot trial indicated that magnesium supplementation is safe in CKD patients and is able to reduce serum calcification propensity (81).

MINERALOCORTICOID RECEPTOR (MR) INHIBITION

Vascular smooth muscle cells express the MR (82) and MR stimulation by aldosterone triggers the osteo-/chondrogenic signaling (82–87) by upregulation of PIT1 expression (87, 88), leading to expression of osteogenic transcription factors and enzymes and subsequent mineralization (87). Klotho-hypomorphic mice develop renal sodium loss and hyperaldosteronism (89). Hyperaldosteronism presumably contributes to the stimulation of vascular calcification in klotho-hypomorphic mice (40, 87, 90) and CKD patients (91). Accordingly, treatment with the MR antagonist spironolactone reduces the extent of vascular calcification in klotho-hypomorphic mice and rats with adenine-induced renal failure (88) and reduces cardio-/cerebrovascular mortality in dialysis patients (92). Spironolactone treatment of klotho-hypomorphic mice reduced aortic PIT1-dependent osteoinductive signaling, but increased cystatin-C levels (87). MR blockade with spironolactone may particularly suppress the progression of vascular calcification in patients with hyperaldosteronism.

Spironolactone may be effective even at normal levels of circulating aldosterone (93, 94). Aldosterone is produced not only in adrenal glands, but in diverse tissues (95–98) including the vasculature (99). Aldosterone synthase (also known as CYP11B2) is expressed during calcifying conditions and, thus, aldosterone may be produced in VSMCs (99, 100). Vascular aldosterone production is particularly important under pathological conditions (97). Vascular aldosterone may foster development of hypertension (101). CYP11B2 is upregulated in atheroma-plaques (102) and contributes to oxidative stress (103). In accordance, high-phosphate treatment increased aldosterone synthase expression in VSMCs (90) and silencing of aldosterone synthase attenuated the phosphate-induced osteo-/chondrogenic transdifferentiation and calcification *in vitro*. Similarly, aldosterone synthase expression is higher in coronary arteries from patients with impaired renal function and correlated with *CBFA1* expression. Aldosterone synthase expression in VSMCs is upregulated by disruption of APEX1-dependent gene suppression (90). Accordingly, APEX1 is protective against VSMC calcification (90, 104).

Aldosterone synthase expression is similarly enhanced in klotho-hypomorphic mice (90). In those mice, aortic osteo-/chondrogenic signaling is decreased by spironolactone, but not by adrenalectomy and in adrenalectomized klotho-hypomorphic mice, spironolactone treatment still significantly blunts aortic osteoinductive reprogramming (90).

Mineralocorticoid receptor antagonism may, thus, be a therapeutic option for hyperphosphatemic patients even in the absence of hyperaldosteronism (86). Spironolactone may further protect VSMCs in diabetes (105), which may lead to upregulation of vascular aldosterone synthase (100).

The effects of spironolactone in CKD patients are under study (106, 107). Clinical trials indicate that spironolactone treatment reduces morbidity and mortality in hemodialysis patients (92). MR inhibition may cause a transient reduction of renal function and promote hyperkalemia, but has been shown to be relatively safe in CKD patients (92, 108).

AMMONIUM SALTS

Besides its acidifying effect on extracellular pH (109, 110), NH_4^+ may dissociate to H^+ and NH_3 which easily crosses membranes, thus entering cells and cellular compartments (111). In acidic intracellular compartments NH_3 binds H^+ and is trapped as NH_4^+ (112). The binding of H^+ alkalinizes acidic cellular compartments (113) and the intracellular/intra-compartimental accumulation of NH_4^+ swells cells and acidic intracellular compartments (114–116). Cell swelling may downregulate the cell volume sensitive transcription factor NFAT5 (117, 118). Moreover, alkalinization of acidic cellular compartments may interfere with the maturation of several proteins including TGFβ1 (119), a key factor in the regulation of osteo-/chondrogenic signaling of VSMCs (120–122).

Treatment of klotho-hypomorphic mice with NH_4Cl containing drinking water prevented soft tissue and vascular calcifications and increased their life span more than 12- (♂) or 4-fold (♀) without significantly affecting extracellular pH or plasma concentrations of $1,25(OH)_2D_3$, calcium, and phosphate (123). Tissue calcification and aging were further delayed in klotho-hypomorphic mice by NH_4NO_3 (124).

NH_4Cl prevents vascular calcification apparently not by inducing acidosis. Untreated klotho-hypomorphic mice suffer from respiratory acidosis resulting from severe lung emphysema (123). NH_4Cl treatment prevents the development of lung emphysema and, thus, respiratory acidosis (123). Instead, NH_4Cl induces a metabolic acidosis of similar extracellular pH as in untreated mice (123).

NH_4Cl treatment prevents development of extracellular volume depletion, thus normalizing ADH release and plasma aldosterone levels (40). The decrease of plasma aldosterone concentrations following NH_4Cl treatment presumably contributes to the decrease of vascular calcification. However, the effect of NH_4Cl on survival and calcification (123) is, by far, larger than that of aldosterone receptor blockade (87).

NH_4Cl treatment is presumably mainly effective by interference with osteo-/chondrogenic transdifferentiation of VSMCs (123). In aortic tissue of klotho-hypomorphic mice and in phosphate treated VSMCs *in vitro*, NH_4Cl disrupted the increased expression of osteogenic and chondrogenic markers *CBFA1* and *SOX9* and of ALPL (123). Osteo-/chondrogenic reprogramming is paralleled by VSMCs senescence (125), and thus vascular aging (126). NH_4Cl treatment reversed the upregulation of *PAI-1*, *p21*, and *GLB1*, key elements in the orchestration of senescence (127). *TGFβ1* expression was upregulated in aortic tissue of klotho-hypomorphic mice and in phosphate treated VSMCs, which in turn triggers cellular senescence, osteo-/chondrogenic reprogramming and aging (128) and is decreased by NH_4Cl treatment (123). NH_4Cl further impairs maturation of TGFβ1 (119). TGFβ1 is a stimulator of NFAT5

expression (129). NH_4Cl treatment reverses the enhanced expression of *NFAT5* in klotho-hypomorphic mice and in phosphate treated VSMCs (123). Addition of exogenous TGFβ1 protein or NFAT5 overexpression triggers osteo-/chondrogenic reprogramming of VSMCs *in vitro*, which cannot be reversed by NH_4Cl, indicating that NH_4Cl is effective upstream of mature TGFβ1 (123). NH_4Cl treatment is, at least in part, effective by disrupting TGFβ1-dependent osteo-/chondrogenic signaling (123) and, thus, vascular calcification (130).

Further *in vitro* experiments revealed that inhibition of the vacuolar H^+ ATPase with bafilomycin A1 or following dissipation of the pH gradient across the membranes of acidic cellular compartments with methylamine similarly disrupted phosphate-induced TGFβ1-dependent osteo-/chondrogenic signaling in VSMCs (131), supporting the hypothesis that vascular acidic cellular compartments are necessary for promoting vascular calcification.

A concern of NH_4Cl treatment is the cerebral ammonia toxicity (114–116). However, the employed NH_4Cl dosage does apparently not lead to toxic ammonia concentrations as NH_4Cl treated male klotho-hypomorphic mice reached a life span close to that of untreated wild-type mice, and behavioral studies did not reveal any defect in NH_4Cl treated wild-type mice (123). Needless to say, NH_4Cl treatment may be hazardous in patients with hepatic failure.

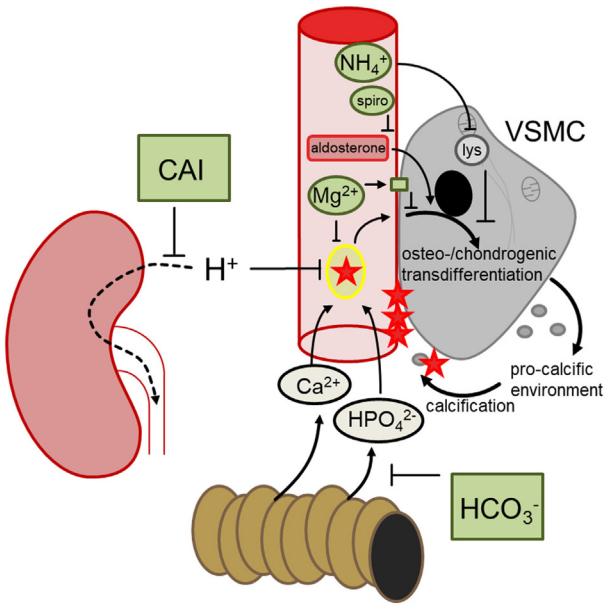

FIGURE 2 | Hypothetical mechanisms involved in the discussed treatments preventing vascular calcification. During hyperphosphatemia, phosphate complexes with calcium and forms secondary calciprotein particles, inducing osteo-/chondrogenic transdifferentiation of vascular smooth muscle cells (VSMCs), which in turn generates a pro-calcific environment and subsequent active tissue mineralization. Oral bicarbonate treatment may impair intestinal phosphate reabsorption, ameliorating hyperphosphatemia. Renal carboanhydrase inhibition causes proton retention, which may reduce the formation of calcium-phosphate nanoparticles. Magnesium may similarly directly prevent calcium-phosphate complexation and stimulates the calcium sensing receptor in VSMCs, blunting osteo-chondrogenic transdifferentiation. This transdifferentiation is also directly stimulated by aldosterone, which may be blunted by spironolactone (spiro). Ammoniumchloride leads to lysosomal (lys) alkalinization which appears to dissipate the osteo-chondrogenic transdifferentiation of VSMCs. These mechanisms may provide the basis to develop a therapeutic approach to reduce the burden of vascular calcification.

CONCLUSION

Klotho-hypomorphic mice and mice with vitamin D_3 overload suffer from severe vascular calcification. Experiments in those animals shed novel light on the mechanisms orchestrating vascular calcification and led to the discovery of several powerful therapeutic opportunities (**Figure 2**). The most effective treatment turned out to be NH_4Cl, but also acetazolamide, spironolactone, bicarbonate, or magnesium supplementation were able to reduce the progression of vascular calcification *in vivo*. Further studies are required to fully define advantages and disadvantages of those treatments, to possibly uncover additional therapeutic options and—most importantly—to clarify whether the successful treatments in mice can be translated into avoidance of vascular calcification in human disease, such as CKD, diabetes, and (premature) aging.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Role of Fibroblast Growth Factor-23 in Innate Immune Responses

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Fibroblast growth factor-23 (FGF-23) is a bone-derived hormone that activates FGFR/α-Klotho binary complexes in the kidney renal tubules to regulate phosphate reabsorption and vitamin D metabolism. The objective of this review is to discuss the emerging data that show that FGF-23 has functions beyond regulation of mineral metabolism, including roles in innate immune and hemodynamic responses. Excess FGF-23 is associated with inflammation and adverse infectious outcomes, as well as increased morbidity and mortality, particularly in patients with chronic kidney disease. Enhancer elements in the FGF-23 promoter have been identified that mediate the effects of inflammatory cytokines to stimulate FGF-23 gene transcription in bone. In addition, inflammation induces ectopic expression of FGF-23 and α-Klotho in macrophages that do not normally express FGF-23 or its binary receptor complexes. These observations suggest that FGF-23 may play an important role in regulating innate immunity through multiple potential mechanisms. Circulating FGF-23 acts as a counter-regulatory hormone to suppress 1,25D production in the proximal tubule of the kidney. Since vitamin D deficiency may predispose infectious and cardiovascular diseases, FGF-23 effects on innate immune responses may be due to suppression of 1,25D production. Alternatively, systemic and locally produced FGF-23 may modulate immune functions through direct interactions with myeloid cells, including macrophages and polymorphonuclear leukocytes to impair immune cell functions. Short-acting small molecules that reversibly inhibit FGF-23 offer the potential to block pro-inflammatory and cardiotoxic effects of FGF-23 with less side effects compared with FGF-23 blocking antibodies that have the potential to cause hyperphosphatemia and soft tissue calcifications in animal models. In conclusion, there are several mechanisms by which FGF-23 impacts the innate immune system and further investigation is critical for the development of therapies to treat diseases associated with elevated FGF-23.

Keywords: fibroblast growth factor-23, innate immunity, klotho, macrophage activation, PMNs, LPS stimulation, infection risk

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Fibroblast growth factor-23 (FGF-23) is a bone-derived hormone that participates in a bone–kidney endocrine network regulating mineral metabolism. FGF-23 is produced by osteoblasts and osteocytes in bone and enters the circulation to inhibit phosphate reabsorption and reduce serum 1,25D levels through the activation of canonical FGFR/α-Klotho receptor complexes in renal tubules (1–3). The major physiological functions of FGF-23 are to (1) coordinate bone mineralization with renal handling of phosphate (1, 4) and (2) act as a counter-regulatory hormone for 1,25D (**Figure 1A**). Elevated levels of FGF-23 are associated with several hereditary and acquired hypophosphatemic disorders including X-linked hypophosphatemic rickets (Hyp mice homolog)—caused by inactivating mutations of *Phex* (5–7)—autosomal recessive hypophosphatemic rickets 1—caused by

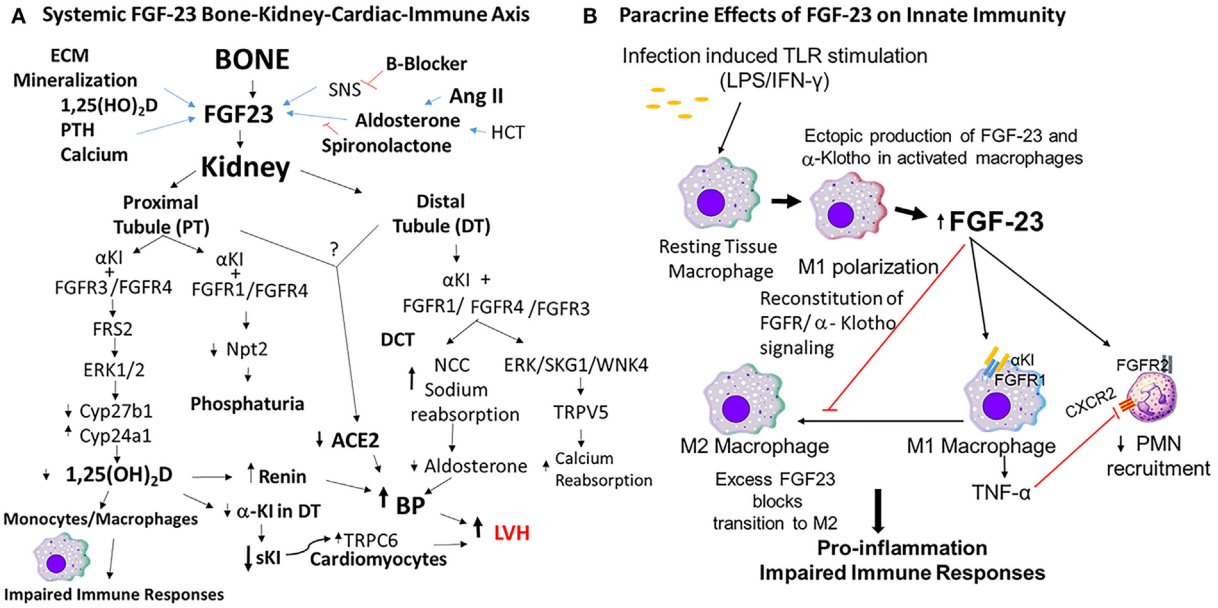

FIGURE 1 | Mechanisms of fibroblast growth factor-23 (FGF-23) effects to impair innate immune responses. **(A)** FGF-23 bone–kidney–cardiac–immune axis. FGF-23 is produced by osteoblasts/osteocytes in bone in response to local and systemic factors and targets the kidney to create multiple endocrine networks (4, 13) in bone, including (1) an FGF-23 vitamin D counter-regulatory loop (4, 14); (2) a calcium-FGF-23 endocrine loop, where calcium stimulates FGF-23 in bone and FGF-23 stimulates calcium reabsorption in the DT (15, 16); (3) a bone–kidney axis, where FGF-23 is regulated by factors involved in extracellular matrix mineralization to coordinate renal phosphate handling (1). In addition, there is a bone–renal–cardiac axis that augments hemodynamic responses through a feed forward bone–cardio–renal loop, where angiotensin II (Ang II) stimulates FGF-23 production in bone (17) and FGF-23 suppresses Ace2 in the kidney (18), a volume regulatory loop where diuretics and aldosterone stimulate FGF-23 production by bone and FGF-23 targets the distal tubule to increase sodium reabsorption, and a FGF-23 soluble Klotho (sKl) regulatory axis, where FGF-23 suppresses α-Kl expression in the distal tubule leading to reduction in sKl and loss of the hormonal effects of this antiaging hormone. In this schema, FGF-23 regulates innate immune response through suppression of 1,25(OH)D2 production by the kidney. **(B)** Paracrine effects of FGF-23 on innate immune responses. Macrophages do not normally express FGF-23 or α-Klotho, but in the setting of infection, LPS/IFNγ induces the ectopic expression of both FGF-23 and its co-receptor α-Klotho that reconstitutes paracrine FGF-23 signaling in macrophages. FGF-23 stimulates pro-inflammatory responses in M1 macrophages and blocks the transition to M2 macrophages (3). In addition, FGF-23 is proposed to directly activate FGFR2 in PMNs to decrease recruitment (19).

inactivating mutations of *Dmp1* (5, 7)—ARHR2—caused by inactivating mutations in *Enpp1* (6–10)—and Raine syndrome—caused by inactivating mutations in FAM20C (11, 12). Increased FGF-23 is the result of either increased gene transcription or diminished cleavage of FGF-23 in these disorders.

ROLE OF FGF-23 IN INFLAMMATION

There are emerging data that FGF-23 may have effects on immune responses. Vitamin D, which is regulated by FGF-23, has well-described effects on both innate and adaptive immunity (20–22). Vitamin D has an overall effect to enhance innate immune responses and exert anti-inflammatory effects through local and systemic effects. Evidence that FGF-23 may have an overall impact on immunity comes from the association between elevated FGF-23 levels and inflammation. FGF-23 is increased in inflammatory bowel disease (23) and chronic kidney disease (CKD) (4). In CKD, elevated FGF-23 initially functions to maintain mineral homeostasis, but persistent elevations are maladaptive and associated with increased morbidity and mortality (24–26), cardiovascular disease (26–30), inflammation, and infections (31, 32). Infections, most commonly caused by infected catheters and pneumonia (33), are second to cardiovascular disease in causing death in CKD (34) and are >100-fold higher than the general population. Recent clinical association studies suggest that elevated FGF-23 contributes to an increase in susceptibility or severity of infections in CKD. Moreover, elevated FGF-23 levels correlated with increased IL-6, TNFα, CRP, fibrinogen, and severe inflammation in CKD patients (31, 35). In a CKD mouse model of bacterial pneumonia, FGF-23 administration exacerbated disease severity and its inhibition improved outcomes (19). Although these studies suggest that FGF-23 interacts with the immune system, they do not reveal whether FGF-23 directly regulates immune cell functions or indirectly affects immune responses through FGF-23 regulation of 1,25D.

INFLAMMATORY STIMULI INDUCE FGF-23 EXPRESSION IN OSTEOBLASTS/OSTEOCYTES AND IMMUNE CELLS

Inflammatory stimuli upregulate FGF-23 expression in bone, where FGF-23 is usually expressed, but also in immune cells and tissues that do not normally express FGF-23. Inflammatory cytokines have been shown to induce FGF-23 expression *in vivo*

and *in vitro*. Inflammation increases circulating FGF-23 levels in both animal models and humans in response to infection, inflammation, and oxidative stress (36–38). For example, mouse serum FGF-23 levels are significantly increased following inoculation with Gram-negative (*E. coli*) or Gram-positive (*S. aureus*) bacteria, or LPS administration in mice (37). Numerous studies have demonstrated that FGF-23 transcription in osteoblasts/osteocytes is regulated by LPS, IL-1β, and TNFα (39). An enhancer residing in the −16 kb region of FGF-23 was identified and following deletion using CRISPR/Cas9 technology was demonstrated to be responsible for the stimulation of FGF-23 transcription by inflammatory stimuli (40). Bacterial components stimulate immune cells through toll-like receptors (TLRs) and stimulation of TLRs 2, and 4 on bone marrow-derived dendritic cells increased FGF-23 mRNA expression. TLR4 recognizes the bacterial cell wall component LPS, and TLR2 recognizes lipotechoic acids present in bacterial membranes leading to the activation of NF-κB pathway. Inhibition of NF-κB activation prevented the upregulation of FGF-23 mRNA in response to LPS stimulation. Hif1α is another transcription factor that stimulates FGF-23 in osteoblasts (41), indicating that oxidative stress/inflammation stress may induce expression of FGF-23.

Macrophages may be central to FGF-23 regulation and function. Resting RAW264.7 macrophages, which do not normally express FGF-23, when stimulated with LPS/IFN-γ to induce M1 polarization express significant increases in FGF-23. The increase is also mediated by NF-κB-dependent activation of the FGF-23 promoter (3). In addition, the M1 macrophages upregulate Klotho transcription, including the full-length α-Kl message and the alternatively spliced s-Kl message and protein levels. By contrast, IL-4 induction of M2 macrophages results in only modest increases in FGF-23 expression and no upregulation of α-Klotho transcription. Not only do macrophages make FGF-23 and respond to FGF-23 (see below), but make other cytokines, such as OSM, that induce tissue expression of FGF-23, such as in cardiomyocytes (42). In contrast to M1 macrophages, Masuda et al. (37) did not observe upregulation of FGF-23 mRNA in T cells stimulated with the polyclonal stimulators PMA and ionomycin. Lymphocytes also express TLRs 2 and 4, and it is not known whether stimulation through these receptors will upregulate FGF-23 mRNA in these cells.

These observations suggest that inflammatory mediators stimulate FGF-23 gene transcription in osteoblasts/osteocytes that normally express FGF-23 and in macrophages and tissues that do not normally express FGF-23. The resulting local and systemic production of FGF-23 may play a critical role in the ensuing immune responses through targeting FGFR/α-Klotho receptor complexes in the kidney or reconstituted receptor complexes in the local inflammatory tissue environment.

POTENTIAL MECHANISMS FOR FGF-23 IMPAIRMENT OF HOST RESPONSES

Indirect Effects Mediated by FGF-23 Suppression of 1,25D

Under physiological conditions, changes in FGF-23 results in reciprocal changes in 1,25D, and *vice versa*. FGF-23 may affect immune responses through activation of FGFR/α-Klotho complexes in the proximal renal tubule leading to suppression of 1,25D by the kidney (3, 19, 43–46). The association between low vitamin D and high FGF-23 serum levels with infectious and cardiac deaths in a large cohort of patients with end-stage renal disease (47, 48) may be due to the loss of the anti-inflammatory effects of 1,25D (49, 50). The loss of 1,25D may result in the amplification of inflammatory response and subsequently increased tissue pathology.

Direct Effects of FGF-23 on Macrophages and PMNs

Another mechanism is direct pro-inflammatory actions of FGF-23 on immune cells. There is emerging evidence that FGF-23 directly interacts with immune cells, such as PMNs and/or macrophages through binding of FGFR/α-Kl receptors (**Figure 1B**).

Rossaint et al. (19) used a murine model of CKD, induced by 5/6 nephrectomy, to measure the effect of excess FGF23 on *E. coli* pneumonia. The authors demonstrate that mice with CKD had decreased recruitment of PMNs into the lungs and increased disease severity. Neutralization of FGF23 with anti-FGF23 antibody restored PMN recruitment and the host response in these mice. The authors proposed that elevated circulating levels of FGF-23 directly activated FGFR2 in PMNs to impair the host response to infection.

Direct effects of FGF-23 on PMNs are controversial for several reasons. First, PMNs lack the obligate FGF-23 co-receptor α-Klotho. To deal with this inconsistency, this hypothesis proposes that FGF-23 targets PMNs through the non-canonical FGFR2 pathway (2). Second, FGFR2, which is the only FGFR expressed in PMNs, is not a target for FGF-23 in multiple functional studies and target engagement assays (2, 51–53). Third, experimental designs in existing studies are not sufficient to establish the role of FGFR2 or PMNs in mediating FGF-23's adverse effects on host responses. In this regard, a single intravenous injection of 200 ng of rFGF-23 to mice was the only *in vivo* evidence that elevated FGF-23 impairs PMN responses to sepsis. Essential studies to ablate FGFR2 in PMNs or to test the effects of chronic elevations of FGF-23 in the absence of confounding effects of CKD (19, 54) have not been performed. Finally, effects of FGF-23 inhibition to improve outcomes with a blocking antibody have not controlled for confounding effects of reciprocal increases in 1,25D production in response to FGF-23 inhibition.

There is compelling data that FGF-23 targets macrophages. Several studies have demonstrated that macrophages and DCs express FGFR1 and inflammatory stimuli upregulates α-Klotho expression in macrophages to reconstitute FGFR–α-Klotho signaling (3, 37). Stimulation of these cells with FGF-23 resulted in induction of TNF-α mRNA and protein expression in primary macrophages as well as macrophage cell lines through activation of binary FGFR/α-Klotho complexes (3, 37). In addition, the ability of FGFR inhibitors to block FGF-23 signaling in macrophages confirms that the canonical FGFR/Kl signaling pathway is active in macrophages (3).

If FGF-23 is directly affecting innate immune responses, animal models with elevated FGF-23 should exhibit abnormal host responses even in the absence of CKD. Indeed, a sterile inflammation model to induce the infiltration of peritoneal macrophages

with thioglycolate shows that Hyp mice have increased FGF-23 expression in macrophages (3). Macrophages isolated from Hyp mice expressed higher levels of FGF-23 and Klotho compared with WT controls. Hyp macrophages also had increased basal ERK activation and increased TNF-α mRNA, consistent with activation of FGFR/αKl signaling. Serum levels of TNF-α were increased consistent with the pro-inflammatory phenotype [i.e., kidney inflammation (18) and cardiovascular abnormalities in Hyp mice (55)]. These data support the hypothesis that locally produced and circulating FGF-23 activate immune cells in the inflammatory milieu (3, 43), leading to adverse outcomes (56). In this scenario, infection with bacteria stimulates the production of FGF-23 locally in M1 activated macrophages which also upregulate FGFR/α-Kl complexes (3). Autocrine stimulation of the M1 cells with FGF23 amplifies TNF production and inhibits the transition to a wound healing M2 phenotype, resulting in excess tissue damage and increased morbidity and mortality (**Figure 1B**).

Based on these findings, a new hypothesis proposes that FGF-23 actions on innate immunity are mediated by activation of reconstituted canonical FGFR/α-Kl receptors in tissue macrophages during infections by both systemic and local production of FGF-23 (**Figure 1B**) (3, 19, 37, 43). Although the full effects of FGF-23 on immune cell function remain to be defined, in this schema, FGF-23 is proposed to have pro-inflammatory effects mediated by activated macrophages. Additional studies are needed to characterize the impact of FGF-23 on immune responses. In particular, experiments that conditional delete FGF-23, FGFRs, and α-Klotho in different myeloid cells are needed to define the role of FGF-23/FGFR/α-Klotho signaling in mediating immune responses.

ROLE OF α-KLOTHO AND SOLUBLE KLOTHO (sKl) IN INFLAMMATION

α-Klotho gene has two transcripts that encode a long type I transmembrane (TM) protein containing KL1 and KL2 domains and a short secreted protein containing only a single KL domain (57). The ~130-kDa TM protein is an obligate co-receptor for binding of FGF-23 to FGFRs (2, 58). Ectodomain shedding by ADAM10 and ADAM17 generates a circulating α-Klotho isoform that lacks the TM domain (59). The short secreted ~60-kDa isoform (s-KL) is generated by the alternative spliced transcript. Similar sized as soluble KL1 and KL2 fragments are also generated by additional post-translational cleavage of the shed isoform (57, 60). The ~60-kDa s-KL gene product emerged during evolution before FGF-23 and likely has FGF-23 independent functions, including antiaging, anti-inflammatory, and anti-fibrotic effects due to actions of secreted forms of KL to inhibit Wnt, IGF-1, and TGF-β signaling (61–65). Since excess FGF-23 is associated with decreased expression of α-Klotho in the kidney and circulating Kl, it is difficult to determine if the adverse effects attributed to excess FGF-23 are actually caused by Klotho deficiency. Indeed, Klotho depletion is associated with increased inflammation in multiple experimental models.

A recent study showed that renal Klotho mRNA and protein were significantly decreased leading to increased inflammation in kidney of the *db/db* mouse model of diabetes (66). Addition of sKl or overexpression of α-Klotho suppressed NF-κB activation and subsequent production of inflammatory cytokines in response to TNF-α stimulation *in vitro*. Klotho serves as an anti-inflammatory modulator, which negatively regulates the production of NF-κB-linked inflammatory proteins *via* a mechanism that involves phosphorylation of Ser^{536} in the transactivation domain of RelA (66). Thus, distinguishing between FGF-23 and α-Klotho-dependent effects are important, because Klotho administration would correct abnormalities caused by FGF-23 suppression of Klotho.

FGF-23 REGULATES HEMODYNAMIC RESPONSES TO COUNTER THE HYPOTENSIVE EFFECTS OF INFLAMMATION

Could FGF-23 effects regulate renal process that have cardiovascular effects, be linked to inflammatory responses (53, 67)? For example, FGF-23 upregulates NCC in the DT leading to hypertension and suppression of aldosterone levels (15, 16, 55). FGF-23 administration suppresses ACE2 and αKl expression in the kidney, which could potentially cause left ventricular hypertrophy by enhancing the response to Ang II and/or decreasing circulating sKl (68). From a teleological perspective, FGF-23 control of blood pressure may serve to attenuate the hypotensive effects of inflammation, or alternatively account for the link between inflammation and hypertension (69).

THERAPEUTIC POTENTIAL OF FGF-23 ANTAGONIST

If elevations of FGF-23 are causally linked to adverse outcomes, as emerging data are suggesting, then efforts to inhibit the end-organ effects of excess FGF-23 may improve outcomes in clinical conditions of FGF-23 excess. Advancements in pharmacological tools to block FGF-23 have been made, including blocking antibodies, which show efficacy and safety in treating hypophosphatemic rickets in hereditary disorders of FGF-23 excess (70). Blocking antibodies, however, have a narrow safety window and long half-life (16, 70), which may limit their use in CKD. Indeed, FGF-23-blocking antibody has not been shown to safely reduce FGF-23 in CKD without worsening hyperphosphatemia. Use of an FGF-23-blocking antibody also induced aortic calcification associated with increased risk of mortality in a rodent model of CKD (16). A novel FGF-23 antagonist, Zinc13407541, has been developed, which is short-acting and effectively antagonizes FGF-23 actions *in vitro* and *in vivo* without elevating serum phosphate (71). Unlike FGF-23-blocking antibodies, this small molecule compound can specifically block FGF-23/FGFR/Klotho signaling and increase serum $1,25(OH)_2D$ levels (71) and suppresses TNF-α expression in activated macrophages. With further lead optimization, derivatives of Zinc13407541 could potentially be a titratable pharmacological tool to block FGF-23-related mortality in CKD.

In conclusion, a new understanding of FGF-23 functions proposes functions beyond mineral metabolism that includes indirect and direct effects on components of the myeloid lineage that may account for the association between elevated FGF-23

and impaired host responses to infections. Distinguishing between the proposed mechanisms, namely, FGF-23 suppression of 1,25D, activation of FGFR2 in PMNs in the absence of α-Klotho, and FGF-23 activation of canonical FGFR/α-Kl signaling in macrophages, is important, if we are to make progress in preventing and treating complications of excess FGF-23. Additional studies are needed to determine the relative importance of FGF-23 direct effect on different components of the myeloid lineage in regulating innate immune function and the clinical significance of FGF-23 actions to counteract the systemic and local immune effects of Vitamin D. Distinguishing between these direct and indirect effects will help establish whether pharmacological inhibition of FGF-23 or administration of 1,25D or sKl can prevent the adverse outcomes associated with FGF-23 excess.

AUTHOR CONTRIBUTIONS

XH performed the *in vitro* studies characterizing the ectopic expression of FGF-23 in activated macrophages. EF performed investigations evaluating impact of FGF-23 on the susceptibility to infection. ZX performed the studies characterizing regulation of FGF-23. LQ supervised all research studies related to FGF-23 effects in macrophages. All the authors contributed to the literature review and writing of this manuscript.

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REVIEW
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Targeting Fibroblast Growth Factor 23 Signaling with Antibodies and Inhibitors, Is There a Rationale?

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Fibroblast growth factor 23 (FGF23) is a phosphotropic hormone mainly produced by bone. FGF23 reduces serum phosphate by suppressing intestinal phosphate absorption through reducing 1,25-dihydroxyvitamin D and proximal tubular phosphate reabsorption. Excessive actions of FG23 result in several kinds of hypophosphatemic rickets/osteomalacia including X-linked hypophosphatemic rickets (XLH) and tumor-induced osteomalacia. While neutral phosphate and active vitamin D are standard therapies for child patients with XLH, these medications have several limitations both in their effects and adverse events. Several approaches that inhibit FGF23 actions including anti-FGF23 antibodies and inhibitors of FGF signaling have been shown to improve phenotypes of model mice for FG23-related hypophosphatemic diseases. In addition, clinical trials indicated that a humanized anti-FGF23 antibody increased serum phosphate and improved quality of life in patients with XLH. Furthermore, circulatory FGF23 is high in patients with chronic kidney disease (CKD). Many epidemiological studies indicated the association between high FGF23 levels and various adverse events especially in patients with CKD. However, it is not known whether the inhibition of FGF23 activities in patients with CKD is beneficial for these patients. In this review, recent findings concerning the modulation of FGF23 activities are discussed.

Keywords: hypophosphatemia, rickets, osteomalacia, antibody, chronic kidney disease-mineral and bone disorder (CKD-MBD)

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INTRODUCTION

Fibroblast growth factor 23 (*FGF23*) was identified as a responsible gene for autosomal dominant hypophosphatemic rickets (ADHR) in 2000 by positional cloning (1). FGF23 was also cloned as a responsible humoral factor for tumor-induced osteomalacia (TIO), a rare paraneoplastic syndrome (2). ADHR and TIO are diseases characterized by hypophosphatemia associated with impaired renal tubular phosphate reabsorption. Subsequent studies established that FGF23 is a hormone mainly produced by bone and regulates phosphate and vitamin D metabolism by binding to Klotho–FGF receptor complex (3–7). Klotho is a single membrane spanning protein. While there is also soluble Klotho, its role in FGF23 signaling is not clear (8). FGF23 was shown to transduce signals only in tissues which express membrane form Klotho (6). FGF23 reduces serum phosphate by inhibiting intestinal phosphate absorption through decreasing serum 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] level and proximal tubular phosphate reabsorption (3). Therefore, ADHR and TIO have been considered to be caused by excessive actions of FGF23. These results lead to develop a concept that the inhibition of FGF23 activities may be beneficial for hypophosphatemic patients caused by excessive actions of FGF23. Furthermore, it has been shown that FGF23 is high in patients with chronic kidney

disease (CKD) and can be extremely high in some subjects with end-stage renal disease (ESRD) (9, 10). Circulatory FGF23 levels have been shown to be associated with various adverse events such as higher mortality, cardiovascular events, and left ventricular hypertrophy, especially in subjects with CKD. However, it is not known whether these events are direct consequences of FGF23 activities (11). In this review, the significance of inhibiting FGF23 activities in various diseases is discussed.

FGF23-RELATED HYPOPHOSPHATEMIC DISEASES

Chronic hypophosphatemia is an important cause for rickets and osteomalacia characterized by impaired mineralization of bone matrix. Rickets develops in children before the closure of epiphyseal plate and results in growth retardation and bone deformities. In contrast, osteomalacia in adults can cause severe muscle weakness and bone pain. Serum phosphate level is maintained by intestinal phosphate absorption, renal tubular phosphate handling and equilibrium between extracellular phosphate and phosphate in bone or intracellular fluid. Of these, renal phosphate handling is the main determinant of chronic serum phosphate level. Most phosphate filtered through glomeruli is reabsorbed in proximal tubules by type 2a and 2c sodium-phosphate cotransporters. FGF23 reduces the expression of these sodium-phosphate cotransporters and suppresses phosphate reabsorption (3). At the same time, FGF23 reduces serum $1,25(OH)_2D$ by modulating the expression of vitamin D-metabolizing enzymes (3). FGF23 suppresses the expression of *CYP27B1* that encodes a protein responsible for the production of $1,25(OH)_2D$. FGF23 also enhances *CYP24A1* expression that encodes an enzyme that works to reduce $1,25(OH)_2D$ level.

After the identification of FGF23, several kinds of enzyme-linked immunosorbent assay for FGF23 have been established (12, 13). A part of FGF23 protein is proteolytically cleaved into inactive N-terminal and C-terminal fragments before or during the process of secretion. FGF23 level can be regulated by both *FGF23* transcription and this posttranslational processing of FGF23 protein. For example, iron deficiency seems to enhance *FGF23* production and also the processing of FGF23 protein (14). Therefore, FGF23 level does not always reflect the amount of *FGF23* transcription. Intact assay using two kinds of antibodies that recognize N-terminal and C-terminal portions of the processing site of FGF23 detects only full-length biologically active FGF23 (12). In contrast, C-terminal assay using antibodies against the C-terminal part of FGF23 measures both full-length and processed inactive C-terminal fragment of FGF23 (13). FGF23 level measured by C-terminal assay seems to correlate with the amount of *FGF23* transcription. Intravenous iron preparations inhibit gene expression of *FGF23*. While iron dextrane does not affect the cleavage of FGF23, several iron preparations such as iron polymaltose and iron carboxymaltose may block the cleavage, resulting in paradoxical hypophosphatemia (14). This two step regulation of FGF23 needs to be kept in mind when interpreting results of FGF23 levels by intact and C-terminal FGF23 assays. Measurement of FGF23 levels in patients with chronic hypophosphatemia indicated that FGF23 levels are high in hypophosphatemic patients with ADHR and TIO by both assays (12, 13, 15). In contrast, FGF23 levels are low in patients with chronic hypophosphatemia from other causes such as vitamin D deficiency and Fanconi syndrome indicating that circulatory FGF23 level is suppressed in these patients by hypophosphatemia or other accompanying metabolic changes (16). From these results, high FGF23 in patients with chronic hypophosphatemia seemed to indicate that this hypophosphatemia is caused by excessive actions of FGF23.

In addition to patients with ADHR and TIO, FGF23 levels have been shown to be high in several kinds of hypophosphatemic diseases (**Table 1**). Of these, X-linked hypophosphatemic rickets (XLH) caused by inactivating mutations of *phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX)* is the most prevalent cause of genetic hypophosphatemic disease. More than three hundred kinds of mutations in *PHEX* have been assembled in a database.[1] PHEX is a single membrane spanning protein mainly expressed in bone and teeth (17). There is a murine model of XLH called *Hyp*. *Hyp* mice show similar biochemical features to those of patients with XLH. Genetic analysis indicated that there is a deletion in 3′ region of *Phex* gene in *Hyp* mice (18). It has been shown that *Fgf23* is overexpressed in bone and circulatory Fgf23 is high in *Hyp* mice (19). Therefore, it is

[1]http://www.phexdb.mcgill.ca/.

TABLE 1 | FGF23-related hypophosphatemic diseases.

	Responsible gene	Mutations	Reference
X-linked hypophosphatemic rickets: XLH	*PHEX*	Inactivating	(20)
Autosomal dominant hypophosphatemic rickets: ADHR	*FGF23*	Activating	(1)
Autosomal recessive hypophosphatemic rickets 1: ARHR1	*DMP1*	Inactivating	(21, 22)
Autosomal recessive hypophosphatemic rickets 2: ARHR2	*ENPP1*	Inactivating	(23, 24)
Osteoglophonic dysplasia	*FGFR1*	Activating	(25)
Jansen-type metaphyseal chondrodysplasia	*PTH1R*	Activating	(26)
Hypophosphatemia with dental abnormality and ectopic calcification	*FAM20C*	Inactivating	(27)
McCune-Albright syndrome	*GNAS1*	Activating	(28)
Epidermal nevus syndrome: ENS	*HRAS, KRAS, NRAS*	Activating	(29)
Tumor-induced osteomalacia: TIO	*FN-FGF1, FN-FGFR1*	Activating	(30)
Hypophosphatemia after infusion of saccharated ferric oxide, iron polymaltose, or ferric carboxymaltose			
Biliary atresia			

PHEX, phosphate-regulating gene with homologies to endopeptidases on the X chromosome; FGF23, fibroblast growth factor 23; DMP1, dentin matrix protein 1; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase 1; FGFR1, fibroblast growth factor receptor 1; PTH1R, parathyroid hormone 1 receptor; FAM20C, family with sequence similarity 20, member C; GNAS1, guanine nucleotide binding protein, alpha-stimulating activity polypeptide 1; HRAS, Harvey rat sarcoma viral oncogene homolog; KRAS, Kirsten rat sarcoma rival oncogene homolog: NRAS, neuroblastoma RAS viral oncogene homolog; FN, fibronectin; FGFR, FGF receptor.

believed that inactivating mutations in *PHEX* somehow induce enhanced expression of *FGF23* in bone and cause excessive actions of FGF23 in patients with XLH. Signals from FGF receptor was reported to be involved in the overproduction of FGF23 production in *Hyp* mice (7). However, the precise detailed role of PHEX in the regulation of *FGF23* expression needs to be established.

Mutations in *dentin matrix protein 1 (DMP1)* and *ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1)* results in autosomal recessive hypophosphatemic rickets 1 and 2, respectively (21–24). Furthermore, mutations in several other genes have been shown to cause hypophosphatemic diseases with high FGF23 levels (31). Inactivating mutations in *FAM20C* was reported in Raine syndrome, a usually lethal osteosclerotic disease (32). However, hypophosphatemia with high FGF23 was later reported in some surviving patients (27). Osteoglophonic dysplasia is caused activating mutations in *FGF receptor 1*. Hypophosphatemia with high FGF23 in this disease again suggests the involvement of signals from FGF receptor in FGF23 production (25). Epidermal nevus syndrome is caused by somatic mutations in *RAS* oncogenes and is characterized by sebaceous nevi and skeletal defects (29). These oncogene products can transduce signals from receptor tyrosine kinases including FGF receptor. Jansen-type metaphyseal chodrodysplasia and McCune-Albright syndrome are caused by activating mutations in *parathyroid hormone* (*PTH*) *1 receptor* and *GNAS1*, respectively (26, 28). These results suggest that cyclic AMP pathway is involved in FGF23 production. In some patients with TIO, *FN (fibronectin)-FGF receptor 1* or *FN-FGF1* fusion gene was reported in responsible tumors (30). It is likely that these genes activate some intracellular signaling pathway to enhance FGF23 production.

In addition to diseases with known genetic causes, hypophosphatemia with high FGF23 has been reported in patients receiving some intravenous iron preparations (33, 34). Recently, it has been reported that biliary atresia can be associated with hypophosphatemia with high FGF23 (35). In most of these FGF23-related hypophosphatemic diseases, FGF23 is considered to be overexpressed in bone while the detailed mechanism of this overproduction is not clear. On the contrary, in patients with TIO, the responsible tumors produce FGF23 and FGF23 is shown to be expressed in liver in a patient with biliary atresia. Collectively, these results indicate that excessive production and actions of FGF23 can cause several kinds of hypophosphatemic diseases.

THE INHIBITION OF FGF23 ACTIVITY AS A NEW THERAPEUTIC MANEUVER FOR FGF23-RELATED HYPOPHOSPHATEMIC DISEASES

Direct FGF23 Targeting

Tumor-induced osteomalacia is a paraneoplastic syndrome and can be cured by complete resection of the responsible tumors. However, it is sometimes difficult to find the responsible tumors in patients with TIO. Even when the responsible tumors can be found, it is not always possible to completely remove the lesions. For those patients with TIO whose responsible tumors cannot be removed, neutral phosphate and active vitamin D are usually prescribed. For patients with most other FGF23-related hypophosphatemic diseases including XLH, the same drugs are also used. However, these medications are not drugs based on the pathophysiology of these diseases. In addition, these medications may not able to correct impaired growth completely in patients with hypophosphatemic rickets (36). Furthermore, administration of phosphate and active vitamin D can be associated with several adverse events such as hypercalcemia, hypercalciuria, nephrocalcinosis, and gastrointestinal symptoms (37).

It has been shown that excessive activities of FGF23 underlie the pathogenesis of FGF23-related hypophosphatemic diseases as mentioned above. Therefore, the suppression of FGF23 activities has been considered as a novel therapy for patients with these diseases. Murine monoclonal antibodies against N-terminal and C-terminal portions of human FGF23 were obtained. These antibodies inhibited *in vitro* FGF23 activity and synergistically increased serum phosphate and $1,25(OH)_2D$ levels in normal mice (38). These results as well as phenotypes of hyperphosphatemia and high $1,25(OH)_2D$ in *Fgf23* null mice confirmed that FGF23 is a physiological regulator of phosphate and vitamin D metabolism (4, 38). Then, these antibodies were tested in *Hyp* mice and were shown to increase serum phosphate and $1,25(OH)_2D$ (39). In addition, repeated administration of these antibodies enhanced longitudinal growth of long bones, increased bone mineral density and corrected impaired mineralization of *Hyp* mice (39). Furthermore, these antibodies increased grip power and spontaneous movement of *Hyp* mice (40). These results suggested that the inhibition of FGF23 activities by FGF23 antibodies can ameliorate biochemical, morphological, histological and clinical abnormalities of patients with FGF23-related hypophosphatemic rickets/osteomalacia.

Based on the results of these preclinical studies, humanized anti-FGF23 monoclonal antibody (burosumab) was developed and tested in several clinical trials. The initial phase I study involving 38 adult patients with XLH showed that burosumab increased serum phosphate, $1,25(OH)_2D$ and tubular maximum transport of phosphate per glomerular filtration rate (TmP/GFR), an index of proximal tubular phosphate reabsorption, in a dose-dependent manner (41). Subsequent study with 28 adult patients with XLH showed that subcutaneous injections of burosumab every 4 weeks increased serum phosphate, $1,25(OH)_2D$ and TmP/GFR after each administration (42). Furthermore, burosumab was shown to significantly improve patient perception of physical functioning and stiffness (43). These results indicated that burosumab can improve biochemical abnormalities of adult patients with XLH and improve quality of life. However, it was unclear from these studies whether burosumab can also work in children and ameliorate rickets/osteomalacia.

While the results have not yet been published as articles so far, several clinical trials with burosumab are ongoing. Results presented in several scientific meetings and available on the web suggest that burosumab can improve biochemical abnormalities in child patients with XLH and also in patients with TIO.[2,3] In addition, burosumab seems to improve roentgenological signs of

[2]http://www.ultragenyx.com/pipeline/krn23-xlh/.
[3]http://www.ultragenyx.com/pipeline/krn23-tio/.

rickets in children. Based on these results, the US Food and Drug Administration granted a Breakthrough Therapy Designation for burosumab in 2016 (see footnote 2). However, there are several questions to be answered in the future studies. Long-term safety should be carefully monitored. In addition, it has not yet been shown whether burosumab can heal rickets/osteomalacia and normalize height in child patients with XLH. Furthermore, it has not been established how long burosumab should be used for child patients with XLH. Theoretically, burosumab seems to be effective in patients with other FGF23-related hypophosphatemic diseases than XLH and TIO. This also needs to be proved in the future trials. Collectively, burosumab has been shown to inhibit FGF23 actions in human and seems to be promising as a novel therapy for patients with FGF23-related hypophosphatemic diseases.

In addition to anti-FGF23 antibody, a computationally identified compound binding to FGF23 was also shown to increase serum phosphate and $1,25(OH)_2D$ in a model mouse of FGF23-related hypophosphatemic diseases (44). These compounds can be cost-effective compared to the antibody. However, this small molecule has not been used in clinical trials.

Klotho–FGF Receptor Complex Targeting

Another approach is to inhibit the binding of FGF23 to Klotho–FGF receptor complex. Purified C-terminal fragment of FGF23 protein was shown to compete with full-length FGF23 for the binding to Klotho–FGF receptor complex (45). Administration of this C-terminal fragment of FGF23 temporally increased serum phosphate of *Hyp* mice (45). The C-terminal portion of FGF23 protein was fused to Fc portion of IgG1 to increase the stability of the C-terminal fragment (46). This FGF23 c-tail-Fc fusion molecule also increased serum phosphate and improved mineralization of *Hyp* mice when repeatedly injected (46). In addition to this C-terminal fragment of FGF23, several small compounds were shown to impair the interaction between FGF23 and Klotho–FGF receptor complex (47).

Targeting FGF Receptor

In addition, the inhibition of FGF receptor was shown to inhibit FGF23 activities (48, 49). Several reports suggest that signals from FGF receptor stimulate FGF23 production in bone (7, 48). Therefore, it is possible that the inhibition of FGF receptor suppresses both the production and the actions of FGF23. NVP-BGJ398, a pan FGF receptor inhibitor, increased serum phosphate, enhanced bone growth, increased mineralization, and corrected the disturbed growth plate structure in *Hyp* mice (49).

Targeting of Downstream Signals from FGF Receptor

The inhibition of mitogen-activated protein kinase (MAPK) pathway was also shown to impair FGF23 actions while FGF receptor can activate several signal transduction pathways. PD0325901, an inhibitor of MAPK pathway, was shown to increase serum phosphate and $1,25(OH)_2D$, and correct impaired mineralization in *Hyp* mice (50). These preclinical studies indicated that there are several ways to inhibit excessive FGF23 activities causing FGF23-related hypophosphatemic diseases.

However, FGF receptor-MAPK pathway is involved in numerous biological processes in addition to mediating FGF23 actions suggesting that the inhibition of this pathway can be associated with various adverse events. NVP-BGJ398 and PD0325901 have been tested in several clinical trials mainly for patients with solid cancers.[4] NVP-BGJ398 was shown to inhibit FGF23 secretion in patients with TIO caused by malignant tumors (51). However, these inhibitors have not yet tested for patients with genetic FGF23-related hypophosphatemic diseases or TIO caused by benign tumors. FGF23 c-tail-Fc fusion molecule and other small compounds that impair the binding of FGF23 to Klotho–FGF receptor complex have not been tested in clinical trials, either.

FGF23 AND CKD-MBD

Circulatory FGF23 level is high in patients with CKD. Several studies indicated that FGF23 starts to increase in the early phase of CKD (10, 52). This increase of FGF23 precedes those of PTH and phosphate. While the detailed regulatory mechanisms of FGF23 production and circulatory FGF23 levels remain to be clarified, $1,25(OH)_2D$ and high phosphate diet were shown to enhance FGF23 levels (53). However, in patients with early CKD with high FGF23 level, $1,25(OH)_2D$ and phosphate are not high. Recent studies suggest that inflammation and iron deficiency are involved in this high FGF23 in patients with CKD (54).

In a model rat of early CKD, the inhibition of Fgf23 activity by anti-FGF23 antibodies were shown to enhance proximal tubular phosphate reabsorption and increase serum phosphate and $1,25(OH)_2D$ (55). These results suggest that high FGF23 in patients with early CKD suppresses phosphate reabsorption and works to prevent the development of hyperphosphatemia. The inhibition of Fgf23 activities by anti-FGF23 antibody in a model rat of CKD was also shown to cause higher mortality by increasing serum phosphate and promoting ectopic calcification (56). Therefore, this increase of FGF23 in patients with early CKD is considered to be one of adaptive responses to maintain mineral homeostasis.

In contrast, there are now many epidemiological studies that indicate the association between high FGF23 levels and various adverse events especially in patients with CKD or ESRD (11). For example, high FGF23 levels in patients who were beginning hemodialysis were shown to be associated with higher mortality during the first year of hemodialysis (57). Adverse events associated with high FGF23 are quite diverse including cardiovascular events, left ventricular hypertrophy, progression of CKD, fractures, higher mortality, frailty, insulin resistance, and so on. These associations suggest that the inhibition of FGF23 activity may be beneficial in patients with CKD under certain conditions. Actually, secondary analysis of Evaluation Of Cinacalcet Hydrochloride Therapy to Lower CardioVascular Events indicated that cinacalcet-induced reductions in serum FGF23 were associated with lower rates of cardiovascular death and major cardiovascular events (58). However, there has been no study that examined effects of

[4]https://clinicaltrials.gov/ct2/home.

inhibiting FGF23 activities by anti-FGF23 antibody or inhibitors in patients with ESRD. In addition, the reason for the association between high FGF23 levels and various adverse events are not clear enough. While it was reported that FGF23 signals in a Klotho-independent fashion through FGFR4/nuclear factor of activated T cells/calcineurin, which may cause left ventricular hypertrophy (59, 60), it has not been shown how FGF23 can activate FGF receptor without Klotho. In addition, it is not clear either whether FGF23 induces various adverse events directly working on several tissues like heart, bone and kidney. Further studies are necessary to establish the usefulness of inhibiting FGF23 activities in patients with CKD.

CONCLUSION

Fibroblast growth factor 23 is a hormone that reduces serum phosphate and $1,25(OH)_2D$ levels. For patients with excessive hormone actions, therapeutic measures to suppress the activities of that hormone are used. Therefore, it is reasonable to develop methods to inhibit FGF23 actions for patients with FGF23-related hypophosphatemic diseases. Patients with hypophosphatemic disorders by FGF23 excess may benefit from FGF23 blocking antibodies or inhibitors of FGF23 signaling. However, these therapies need careful monitoring because deficient actions of FGF23 result in hyperphosphatemic disease. On the contrary, FGF23 seems to be high in response to changes of mineral homeostasis or other metabolic process in patients with CKD. Patients with CKD may benefit from these novel therapies to the extent that FGF23 secretion is excessively stimulated and beyond what's needed in compensation. However, there is not enough evidence that indicates the inhibition of FGF23 activities is useful for patients with CKD.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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