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FGF23 induced left ventricular hypertrophy mediated by FGFR4 signaling in the myocardium is attenuated by soluble Klotho in mice

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Abstract

There is controversy regarding whether excess FGF23 causes left ventricular hypertrophy (LVH) directly through activation of fibroblast growth factor receptor 4 (FGFR4) in cardiomyocytes or indirectly through reductions in soluble Klotho (sK). We investigated the respective roles of myocardial FGFR4 and sKL in mediating FGF23-induced LVH using mouse genetic and pharmacological approaches. To investigate a direct role of myocardial FGFR4 in mediating the cardiotoxic effects of excess circulating FGF23, we administered rFGF23 to mice with cardiac-specific loss of FGFR4 (FGFR4 heart-cKO). We tested a model of sKL deficiency, hypertension and LVH created by the conditional deletion of FGFR1 in the renal distal tubule (FGFR1DT cKO mice). The cardioprotective effects of sKL in both mouse models was assessed by the systemic administration of recombinant sKL. We confirmed that FGF23 treatment activates PLCγ in the heart and induces LVH in the absence of membrane α-Klotho. Conditional deletion of FGFR4 in the myocardium prevented rFGF23-induced LVH in mice, establishing direct cardiotoxicity of FGF23 through activation of FGFR4. Recombinant sKL administration prevented LVH, but not HTN, in FGFR1DT cKO mice, consistent with direct cardioprotective effects. Co-administration of recombinant sKL with FGF23 in culture inhibited rFGF23-induced p-PLCγ signaling. Thus, FGF23 ability to include LVH represents a balance between FGF23 direct cardiac activation of FGFR4 and the modulating effects of circulating sKL to alter FGF23-dependent myocardial signaling pathways.

Graphical Abstract

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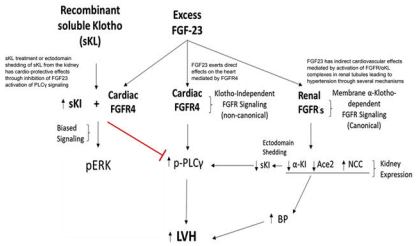
Authors contributions

XBH contributed to the experimental design, conducted and coordinated the study, analyzed data, and wrote the paper. LDQ conceived the study, reviewed and interpreted the data, led the program, and wrote the manuscript. Zhousheng Xiao performed the *in vitro* studies assessing the effects of sKL on FGF23 signaling.

Disclosures

None



Keywords

Cardiovascular; Klotho; Fibroblastic growth factors; Heart; Kidney; Fibroblastic growth factor receptors

1. Introduction

FGF23 is a bone-derived hormone whose physiological function is to regulates phosphate and calcium reabsorption and 1,25$(OH)_2$D metabolism in the kidney [1–4]. The physiological effects of circulating FGF23 are mediated by activation of FGF receptors (FGFRs) complexed with transmembrane α-Klotho (*i.e.*, canonical signaling) [5]. Structural data show that FGF23, FGFR1c, and α-Klotho for a trimeric complex necessary for FGF23 activation of FGFRs [5–9]. FGF23 co-dependence on membrane αKL is supported by the *in vitro* finding that α-KL is an obligate co-factor required for FGF23 activation of FGFR1c, 3c and 4 [5,10,11], genetic evidence that $FGF23^{-/-}$ and $\alpha KL^{-/-}$ mice are phenocopies [12,13], and pharmacological studies showing that $\alpha KL^{-/-}$ mice are refractory to FGF23 biological effects [14–16].

Recent findings implicate excess circulating FGF23 concentrations in pathological effects beyond mineral metabolism [17], including roles in cardiovascular (CV) disease and inflammation [18–28]. Indeed, multiple clinical association studies show that FGF23 is a strong predictor of left ventricular hypertrophy (LVH) and mortality in patients with renal disease, as well as the general population [19–22,29]. A causal role of FGF23 in inducing LVH is supported by genetic and pharmacological models of excess FGF23 [15,30,31]. Remarkably, rFGF23 administration for as brief as five days is sufficient to induce hypertension and LVH in mice [30–32].

Since α-KL is highly expressed in the kidney but not in the heart, it was initially thought that FGF23 may indirectly impact cardiovascular homeostasis through its canonical renal actions to induce HTN by stimulating Na reabsorption and/or suppressing Ace2 [19,22–25]. These mechanisms are respectively supported by effects of diuretics [30] to prevent FGF23-induced LVH [32], and enhanced cardiotoxic effects of angiotensin II administration [33] in *Hyp* mice with elevated FGF23.

On the other hand, an alternative hypothesis proposes that FGF23 directly causes LVH through activation of fibroblast growth factor receptor 4 (FGFR4)/PLCγ-dependent signaling in the myocardium in the absence of transmembrane α-KL [29]. Existing studies supporting this non-canonical mechanism, however, are limited by use of global $FGFR4^{-/-}$

mice, which could have effects due to loss of FGFR4 in the kidney or heart, and study designs that did not control for confounding effects hyperphosphatemia and non-specific actions imparted by high concentrations of FGF23 [29,31]. This hypothesis is further challenged by the paradoxical finding that activation of FGFRs in the heart by the related FGF21 prevents cardiac hypertrophy [34,35]. To date, the possibility that FGFR4 mediates the cardiovascular effect of FGF23 has not been tested in mice in which FGFR4 has been conditionally ablated in the heart.

In addition, FGF23 induced cardiotoxicity is most prevalent in the setting of chronic kidney disease (CKD), which is associated with reduced circulating Klotho (sKl) levels. sKL has also been shown to be protective against LVH [36], through purportedly FGF23 independent effects mediated by the downregulating TRPC6 channels in cardiomyocytes [30,36–38] or alterations lipid raft-associated PI3K signaling [39,40]. Potential interactions between FGF23 cardiotoxic and sKL cardioprotective effects have not been directly examined.

Here, we examine the effects of recombinant FGF23 (rFGF23) and sKL co-administration on LVH in mice with conditional deletion of FGFR4 in the heart (*FGFR4*$^{heart\text{-}cko}$ mice) and the effects of sKL administration in hypertensive sKL-deficient mice with LVH created by conditional deletion of FGFR1 in the renal distal tubule (*FGFR1*$^{DT\text{-}cKO}$).

2. Methods

2.1. Animal experiments

All animal research was conducted according to guidelines provided by the National Institutes of Health and the Institute of Laboratory Animal Resources, National Research Council. The University of Tennessee Health Science Center's Animal Care and Use Committee or the Institutional Animal Care and Use Committee at Genentech reviewed and approved all animal studies (Protocol number: 15–128.0).

2.1.1. Animals breeding and genotyping—Creating *FGFR4*$^{heart\text{-}cko}$ mice. The FGFR4$^{flox/flox}$ mouse was purchased from MMRRC. Mice expressing a-myosin heavy chain promoter driven, tamoxifen (Tam)–inducible heterozygous αMHC-MerCreMer (Jackson lab) were crossed for 2 generations with FGFR4$^{flox/flox}$ mice to generate FGFR4$^{flox/flox/Myh6\text{-}Cre}$ mice. All mice are on are on a C57BL/6J background. The floxed *FGFR4* mice (*FGFR4*$^{flox/flox}$) were purchased from MMRRC and are breeding in our laboratory. FGFR4$^{flox/flox}$ mice were genotyped using 41159-loxF primer 5′-gagatggcgcaacgcaattaatg-3′ and 41159-R primer 5′-ttcaggacttgtacgtaggggaaacc-3′ as recommended by MMRRC. *FGFR4* was deleted in mouse heart using *Myh6-Cre*, which we obtained from the Jackson Laboratory. Myh6-Cre targets the adult myocardium [41], and is not expressed in the kidney and is one of the most specific promoters for targeting the myocardium. All mice were moved on to the C57BL/6 J background for > 5 generations to minimize the effects of genetic variations. Conditional deletion of FGFR4 in the heart was induced by treating the *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ mice with tamoxifen (10 mg/kg for 7 days) to create FGFR4$^{heart\text{-}cko}$ mice. Deletion of the floxed FGF4 gene segment in the heart in FGFR4$^{heart\text{-}cko}$ was assessed by PCR in mice, and the specificity of *Myh6-Cre* determined by examining excision of the floxed FGFR4 segment in the kidney. *FGFR4* message and

protein in the heart from *FGFR4*$^{heart\text{-}cko}$ mice were assessed by RT-PCR and Western blot analysis as described previously [42]. Breeding of *FGFR1*$^{DT\ cKO}$ mice was described previously [32].

2.1.2. Experimental and control groups—We randomly selected and enrolled age-matched male and female littermates (6-weeks-old) from *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ and control litter-mates (*FGFR4*$^{flox/flox}$) into cohorts. We examined age-matched (6-week old) mice (including male and female) consisting of 4–5 mice per group: 1) *FGFR4*$^{flox/flox}$ + vehicle, 2) *FGFR4*$^{flox/flox}$ + rFGF23, 3) *FGFR4*$^{flox/flox}$ + Tam, 4) *FGFR4*$^{flox/flox}$ + Tam + rFGF23, 5) *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ + vehicle, 6) *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ + rFGF23, and 7) *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ + Tam, 8) *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ + Tam + rFGF23. Manipulations: At 6–8 weeks of age, mice (males and females) were injected (i.p.) with tamoxifen (Tam, 10 mg/kg/day) for 7 days, followed by injection of rFGF23 (75 ng/g/day) for 5 days. There was a 7 days gap period between Tam and rFGF23 treatment to allow the clearance of Tam from the mice. Vehicle only treated *FGFR4*$^{flox/flox}$ mice were used as negative control, while rFGF23 only treated *FGFR4*$^{flox/flox}$ mice were served as positive control. Study performed in *FGFR1*$^{DT\ cKO}$ mice was described previously [15,32]. The Institutional Animal Care and Use Committee of University of Tennessee Health Science Center approved all animal procedures and treatments. Power calculations to determine minimal sample size were performed as previously described [43].

2.1.3. Echocardiography and blood pressure measurement—Non-invasive ultrasound examination of the cardiovascular system was performed using a Vevo 2100 Ultrasound System (VisualSonics Inc., Toronto, Ontario, Canada) following standard procedures. Blood pressure was measured using mice-tail cuff technique (CODA, Kent Scientific) as described previously [32].

2.1.4. Pharmacological interventions—Recombinant human rFGF23 was a gift from Amgen. For recombinant FGF23 (rFGF23) administration, mice were injected intraperitoneally (IP) at 75 ng/g twice daily for 5 days with vehicle (PBS). To induce Myh6-Cre recombinase, Tamoxifen (10 mg/kg, Sigma) or vehicle (corn oil) was injected i.p. once a day for 0–7 days. For studies using soluble Klotho (sKL, R&D system), mice received 0.01 mg/kg i.p. injection of sKL or PBS on day 0 for 4–5 consecutive days [44].

2.1.5. Morphometric of mouse heart—Formalin fixed heart samples were embedded in paraffin and immunohistochemically staining performed as described previously [15]. To visualize cellular borders, fixed tissue was stained with wheat germ agglutinin (WGA) conjugated to Alexa Fluor555 (Invitrogen) at 1 mg/ml in PBS containing 10 mm sodium azide.

2.1.6. Real-time reverse transcriptase (RT)-qPCR and Western blot analysis of gene expression—Total RNA was isolated from whole kidney and heart of mouse at 6–8 week of age using a RNeasy Mini Kit (Qiagen, Germany). Primers for RT-PCT of FGFR4 mRNA in the heart and kidney tissue were FGFR4E6F 5′-tactggacacaccccaacgcat-3′ and FGFR4E8R 5′-gtacaccttgcagagtagctcca-3′ which generate a 381 bp DNA fragment for FGFR4 mRNA between exon 6 and 8 of FGFR4 gene. Primers

used for quantitative real-time PCR of genes including α-MHC, β-MHC, ANP, BNP, fibronectin, and trimp1 were described previously [45]. Relative expression values were evaluated with the $2^{-\Delta\Delta}$Ct or $2^{\overline{\Delta}}$Ct method using GAPDH as housekeeping gene. Western blot analysis of FGFR1, FGFR2, FGFR3, FGFR4, p-ERK, t-ERK, p-PLCϒ, t-PLCϒ, and β-actin was performed using the antibodies as described early [32,42].

2.1.7. Cell culture studies—HEK-293 T cells were treated with or without sKL(3 μM) and FGF23 (1 μM) alone and in combination for 2 h in presence of heparin (10 μg/ml). Then the cells were lysed with 150 μl of M-PER Cell Protein Extraction reagent (Pierce Biotechnology, Rockford, IL, USA) with 1 × Halt protease inhibitor and 1 mM phenylmethanesulfonyl fluoride (PMSF) per well. After three of 30-s sonication, total cell lysates were centrifuged at 13,000 xg for 10 min and supernatants were stored at −80 °C until use. Protein concentrations of the supernatant were determined with a total protein assay kit (Bio-Rad, Hercules, CA). Equal quantities of protein were subjected to 4–12% Bis-Tris gradient Gels (Invitrogen, Carlsbad, CA) and were analyzed with standard western blot protocols (HRP-conjugated secondary antibodies from Santa Cruz Biotechnology and ECL chemiluminescent immunodetection system from GE Healthcare Bio-Sciences). Anti-Phospho-ERK1/2 (Thr202/Tyr204) (D13.14.4E, #4370), anti-ERK1/2 (#9102), anti-Phospho-PLCγ1 (D25A9, #8713), and anti-PLCγ1 (#2822) were purchased from Cell Signaling Technologies (Danvers, MA). Anti-Klotho (KAL-KO604) was purchased from Cosmo Bio USA, Inc. (Carlsbad, CA). Anti-β-actin (sc-47778) antibody was obtained from Santa Cruz Biotechnology (Paso Robles, CA). The intensity of bands was quantified using Image J software (http://rsb.info.nih.gov/ij/).

2.2. Statistics

We evaluated differences between two groups by unpaired t-test and multiple groups by one-way analysis of variance with a Tukey post-hoc test. All values are expressed as means ± SD. All statistical tests are performed with an alpha of 0.05 as the significance threshold. All computations were performed using GraphPad Prism7 (GraphPad Software Inc. La Jolla, CA, USA). We observed no differences in the responses of male and female mice, and the results represent combined data from both sexes.

3. Results

3.1. Conditional deletion of FGFR4 in the cardiomyocytes protects mouse from FGF23-induced LVH

Studies have demonstrated that FGF23 induced LVH in mice through activation of cardiac FGFR4 in a FGFR4 global knockout mouse model [31]. To test if cardiomyocytes-specific deletion of FGFR4 protects against FGF23-induced LVH, we created a conditional deletion of FGFR4 in the heart ($FGFR4^{heart\text{-}cko}$) by treating the tamoxifen inducible $Myh6\text{-}Cre/FGFR4^{flox/flox}$ ($FGFR4^{flox/flox/Myh6\text{-}Cre}$) mice with tamoxifen [46]. Based on a pilot study, we chose to use a low dose of tamoxifen of 10 mg/kg/day to test tamoxifen-induced deletion of cardiac FGFR4 by Myh6-Cre and effects of rFGF23-induced LVH in these $FGFR4^{heart\text{-}cKO}$ mice.

First, we performed the experiment using *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ mice as shown in Fig. 1. In this study, we pre-treated *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ mice with tamoxifen (10 mg/kg/day) for 7 days to create *FGFR4*$^{heart\text{-}cKO}$ mice, or vehicle treated the mice as control. Using this protocol, we demonstrated that expression of FGFR4 mRNA was barely detected in the heart of *FGFR4*$^{heart\text{-}cKO}$ mice but found abundant FGFR4 mRNA in the kidney compared to control mice, consistent with cardiac specific deletion of *FGFR4* (Fig. 1. 1A). Deletion of cardiac FGFR4 was confirmed by comparing Western blot analysis of FGFR4 protein in heart tissues of wild type and *FGFR4*$^{heart\text{-}cKO}$ mice (Fig. 1B).

To study if complete deletion of cardiac FGFR4 prevents FGF23-mediated LVH, we grouped *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ mice into 1) vehicle control (veh), 2) rFGF23 injected, 3) Tamoxifen treated (Tam + Veh), and 4) Tamoxifen treated+rFGF23 (Tam + rFGF23). Seven days after last tamoxifen treatment to achieve totally deletion of cardiac FGFR4 in *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ mice, then rFGF23 (75 ng/g body weight) was given by intraperitoneally (i.p.) twice daily to induce LVH. rFGF23 or vehicle alone treated *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ mice was used as positive or negative control, respectively. To eliminate the confounding effect of tamoxifen, *FGFR4*$^{flox/flox}$ mice was also treated with tamoxifen in this study. As shown in Fig. 1C and D, deletion of cardiac FGFR4 prevented rFGF23-induced LVH as determined by heart weight/body weight ratio (Fig. 1C), cross section of heart (Fig. 1D), and cardiac hypertrophic (ANP, BNP, αMHC) and fibrosis (timp1, fibronectin) markers (Fig. 1E).

To show that tamoxifen per se does not impact rFGF23-induced LVH, we injected tamoxifen to control (*FGFR4*$^{flox/flox}$) and *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ mice for 0–7 days, respectively, followed by administration of rFGF23 (75 ng/g body weight) for 5 days. We found that tamoxifen treatment had no effect on cardiac FGFR4 expression level in control (*FGFR4*$^{flox/flox}$) mice as determined by quantitative real-time PCR (data not shown). rFGF23 treatment caused LVH in tamoxifen treated control mice, as determined by cardiac echography and heart weight/body weight ratio (Fig. 2A and B). Thus, the dosage of tamoxifen used in this study had no visible effect on rFGF23-induced LVH in FGFR4$^{flox/flox}$ control mice.

Next, we examined the time-course of tamoxifen exposure on FGFR4 expression in the heart. We observed that tamoxifen induced cardiac specific deletion of FGFR4 in a time-dependent manner in *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ mice, such that deletion of cardiac FGFR4 was incrementally achieved during the 7d of tamoxifen treatment (Fig. 2C). There was incomplete deletion of FGFR4 prior to day 5, but by day 7, expression of cardiac FGFR4 was barely detected in the heart of *FGFR4*$^{heart\text{-}cKO}$ mice. Consistent with the incomplete deletion of cardiac FGFR4, rFGF23 induced LVH in 0–5d tamoxifen treated *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ mice (Fig. 2A), whereas 7d tamoxifen treated *FGFR4*$^{flox/flox/Myh6\text{-}Cre}$ mice showing more complete reductions in *FGFR4* where refractory to FGF23-induced LVH, as determined by heart weight/body weight ratio (Fig. 2D, cardiac echography (Fig. 2E), left ventricular mass (LV mass) (Fig. 2F), left ventricular anterior (LVAWs) and posterior (LVPWs) wall thickness, and cardiac output (Fig. 2G–I), indicating that rFGF23 caused LVH by directing targeting cardiac FGFR4 in the heart. Interestingly, rFGF23 treatment caused hypertension (HTN) in both control and *FGFR4*$^{heart\text{-}cKO}$ mice

(Fig. 2J). rFGF23 induced HTN has been shown to be mediated by FGFR/α-Klotho in the kidney through multiple mechanisms, including stimulating Na reabsorption and/or suppressing Ace2 and α-Kl expression [19,22–25,30] [15,32],. This finding suggests that rFGF23 induced HTN does not contribute to LVH under the condition in this study.

3.2. sKL rescues FGF23-induced LVH

To test if sKL can attenuate FGF23-mediated LVH, we treated the wild type mice with recombinant FGF23, and sKL, separately and in combination. Like the above studies, rFGF23 treatment intraperitoneally twice daily for 5 days at a dose of 75 ng/g body weight to wild type mice induces hypertension (Fig. 3A) and LVH (Fig. 3B–E), as measured by significant increases in HW/BW, cross-section of heart, LV mass, LV wall thickness (LVAWs). Interestingly, concomitant treatment with sKL (10 ng/g body weight, i.p) with rFGF23 prevented rFGF23 induced HTN and LVH, including normalization of LV mass and LVAW (Fig. 3A–D). sKL treatment alone decreased blood pressure but had no effect on heart size in wild type mice (Fig. 3A–E). Our study also showed that FGF23 impaired cardiovascular function by reducing the cardiac output, stroke volume, systolic volume, and diastolic volume of the heart, while treatment of sKL protected mice from FGF23 triggered adverse effects on the heart functions (Fig. 3F–I).

3.3. sKL rescues renal distal tubule FGFR1 loss of function mediated LVH

Next, to test the effect of sKL in another model of FGF23-independent LVH, we used the FGFR1 distal tubule conditional knockout mouse model ($FGFR1^{DT\,cKO}$), which has suppressed renal α-Klotho expression, hypertension and LVH [32]. We treated 3-month old $FGFR1^{DT\,cKO}$ mice with sKL by IP injection at a dose of 10 ng/g body weight for 5 days. As previously reported, $FGFR1^{DT\,cKO}$ mice developed LVH compared to wild type mice [15], and treatment with sKL corrected the LVH in $FGFR1^{DT\,cKO}$ mice (Fig. 4A and B). LVH reoccurred in $FGFR1^{DT\,cKO}$ mice within 10 days after stopping sKL treatment (Fig. 4C). We observed that sKL administration lowered blood pressure in wild-type mice (Fig. 4E). sKL treatment, however, did not lower systolic blood pressure in $FGFR1^{DT\,cKO}$ mice (Fig. 4E). These finding suggests that in sKL can correct LVH in $FGFR1^{DT\,cKO}$ mice independent of its potential effects on blood pressure [47,48].

3.4. sKL biases FGF23/FGFR4 signaling pathways in heart toward cardioprotection

In vitro studies indicate that FGF23 selectively activates FGFR1/ERK signaling in the presence of αKL [5], but preferentially activates FGFR4/PLCγ signaling in the absence of co-expression of αKL [29]. To explore the possible that sKL effects to prevent FGF23-induced LVH similarly biases signaling *in vivo*, we measured PLCϒ and ERK signaling in the heart of rFGF23 treated mice in the presence and absence of concomitant sKL administration (Fig. 5). We found that rFGF23 administration increased abundance of cardiac p-PLCϒ in wild type but not in $FGFR4^{heart\text{-}cKO}$ mice induced by tamoxifen (Fig. 5A and B). In contrast to tissues that co-express FGFRs and α-KL [5], treatment with rFGF23 did not induce p-ERK activation in the hearts of wild-type mice, consistent with the absence of cardiac FGFR/α-KL canonical signaling (Fig. 5A and C). sKL co-administration blocked rFGF23-induced activation of cardiac p-PLCϒ (Fig. 5D and E), but imparted FGF23-mediated increases in p-ERK in the heart of in wild-type mice, suggesting that the presence

of sKL biases the signaling pathways activated by FGF23 from putative cardiotoxic p-PLCγ to cardioprotective ERK signaling. Consistent with this interpretation, sKL treatment resulted in reductions of rFGF23-induced cardiac hypertrophic gene and fibrosis markers (Fig. 5H). Thus, the presence of sKL may reconstitute a FGF23/FGFR1/ERK signaling pathways that abrogates the FGF23/FGFR4/PLCγ cardiotoxic signaling in the heart *in vivo*. Since dietary phosphate restriction and vitamin D administration are associated with increased serum sKL [49,50], FGF23 cardiac toxicity may be further modified by nutrient and environmental factors.

3.5. Soluble klotho bias FGF-23 signaling in vitro

HEK-293 T cells where stimulated by rFGF23 in the presence or absence of co-transfected s-KL In the absence of s-KL, FGF23 had no effect on ERK activity, but stimulated both PLCγ and Akt pathways (Fig. 6 A and B). In contrast, in the presence of s-KL, FGF23 stimulates ERK activity but the activation of PLCγ and Akt pathways are lost (Fig. 6 A and B).

4. Conclusions

Patients with chronic kidney disease (CKD) have high cardiovascular mortality associated with elevated FGF23 [19,23,51–55] that is purported to cause left ventricular hypertrophy (LVH) through direct noncanonical activation of FGFR4 and PLCγ pathways in cardiomyocytes in the absence of α-KL [22,31,45]. Elevations of FGF23, however, are also associated with reductions in sKL, which can modify the development of LVH. Indeed, reductions in sKL are associated with increased cardiovascular mortality [56], and elevations of sKL can have FGF23 independent cardioprotective effects through alterations of TRPC6 signaling in the myocardium [39]. In the present study, we investigated the interactions between FGF23 and sKL on the heart.

We confirmed that FGF23 activated cardiotoxic PLCγ signaling pathways in the heart *in vivo*, and for the first time showed that FGF23-induced LVH can be blocked by conditional deletion of FGFR4 in the heart. Prior studies had inferred the role of cardiac FGFR4 in mediating FGF23 effects based on observations in global FGFR4 null mice and complementary *in vitro* studies [29]. Our studies are limited by the fact that we did not include controls consisting of Tamoxifen treated $FGFR4^{+/+,Myh6\text{-}Cre}$ mice to test for potential confounding effects of Cre on the heart [57–59]. The expression of Cre and the deletion of FGFR4 in the heart, however, did not by themselves result in cardiotoxicity, as evidenced by the normal heart size or alterations in cardiac markers. Thus, the prevention of FGF23 induced LVH is likely mediated by cardiac specific loss of FGFR4, rather than the cardiac effects of Cre overexpression.

Conditional deletion of FGFR4 in the heart did not prevent FGF23 induced hypertension (HTN), suggesting that FGF23 activation of canonical FGFRs/αKl in renal tubules is mediating the hypertensive response to FGF23. In this regard, FGF23 administration to mice induces hypertension through activation of RAAS by suppression of 1,25D, which would increase renin expression [60], and/or reduction of ACE2 expression [38,61,62], and by

stimulation of sodium-chloride co-transporter (NCC) in the renal distal tubule (DT) leading to sodium retention [30].

The most important new findings are the ability of sKL administration to prevent FGF23-induced LVH and effects of sKL to reduce FGF23/FGFR/PLCγ signaling both *in vivo* and *in vitro*. Indeed, our study showed that co-injection of sKL with rFGF23 rescued mice from FGF23-induced LVH, as measured by heart weight/body weight ratio, LV wall thickness, cardiac output, stroke volume, systolic volume, and diastolic volume of the heart. Actions of sKL released into the circulation from ectodomain shedding may similarly block FGF23-dependent FGFR activation of PLCγ signaling. This inhibition of PLCγ may provide additional cardioprotective effects [5,45] to those mediated by sKL downregulating TRPC6 channels in cardiomyocytes [37,63].

In addition, Klotho also regulates blood pressure, as evidenced by the fact that kidney-specific deletion of αKl causes salt-sensitive HTN in mice, and administration of sKL inhibits RAAS and normalizes blood pressure in CKD mouse models [47]. Our observation that sKL administration attenuated FGF23-mediated hypertension is consistent with these observations and could have contributed to the effects of sKL to attenuate LVH. Thus, FGF23 effects to suppress kidney expression of α-Kl [15,64] may lead to LVH through loss of sKL's cardioprotective effect [64] as well as other effects of Klotho to regulate blood pressure. Moreover, since increased dietary phosphate is associated with decreased serum sKL [65,66], variations in sKL might account for the effects of hyperphosphatemia to impact FGF23-induced LVH in CKD.

Additional studies in a hypertensive mouse model cause by distal tubular deletion of FGFR1 ($FGFR1^{DT\ cKO}$), however, dissociate sKL's effects on the heart and blood pressure control. We found that sKL administration can prevent LVH in $FGFR1^{DT\ cKO}$ that develops renal mediated hypertension and LVH in the setting of reduce Klotho but without elevations of FGF23 [36] [32]. sKL attenuated the severity of LVH but not hypertension in $FGFR1^{DT\ cKO}$ mice, suggesting that suppression of renal expression of αKL and reduced circulating sKL levels are mediating LVH in this model [32]. In contrast, sKL did not correct the hypertension in $FGFR1^{DT\ cKO}$ mice, suggesting that loss of FGFR1 causes HTN through mechanisms independent of sKL. Interestingly, LVH relapsed in these mice after stopping sKL treatment, indicating that the levels of circulating sKL reversibly attenuate cardiotoxic signaling leading to LVH *in vivo*.

In summary, we show for the first time that cardiac specific deletion of FGFR4 in the heart prevents FGF23-induced LVH in mice. These findings confirm the direct effects of FGF23 to activate non-canonical FGFR4 leading to increased PLCγ signaling and the development of LVH, as originally proposed by Faul's laboratory [29,31]. However, we also discovered that the administration of recombinant sKL prevents FGF23-induced LVH, and biases myocardial FGFR signaling from PLCγ toward ERK activation. Finally, we confirm that FGF23 and sKL regulate blood pressure, likely through renal actions. We propose a new schema (See Graphical Abstract), where FGF23 exerts cardiovascular effects through renal effects mediated activation of FGFR/α-KL complexes in renal tubules, and direct cardiotoxic effects mediated by non-canonical activation of FGFR4 in the myocardium that

can be attenuated by increased circulating levels of sKL. This novel renal-cardiac axis is controlled by α-KL dependent activation of FGFRs in the kidney that regulate blood pressure, as well as sKL released from the kidney by ectodomain shedding that has both FGF23 independent effects mediated by TRPC6 and other mechanism, and FGF23-dependent effects to alter coupling to FGFR signaling pathways in the myocardium. If so, the hormonal administration of sKL may be an alternative approach to blocking FGF23 for treating FGF23- induced LVH and HTN in clinical settings.

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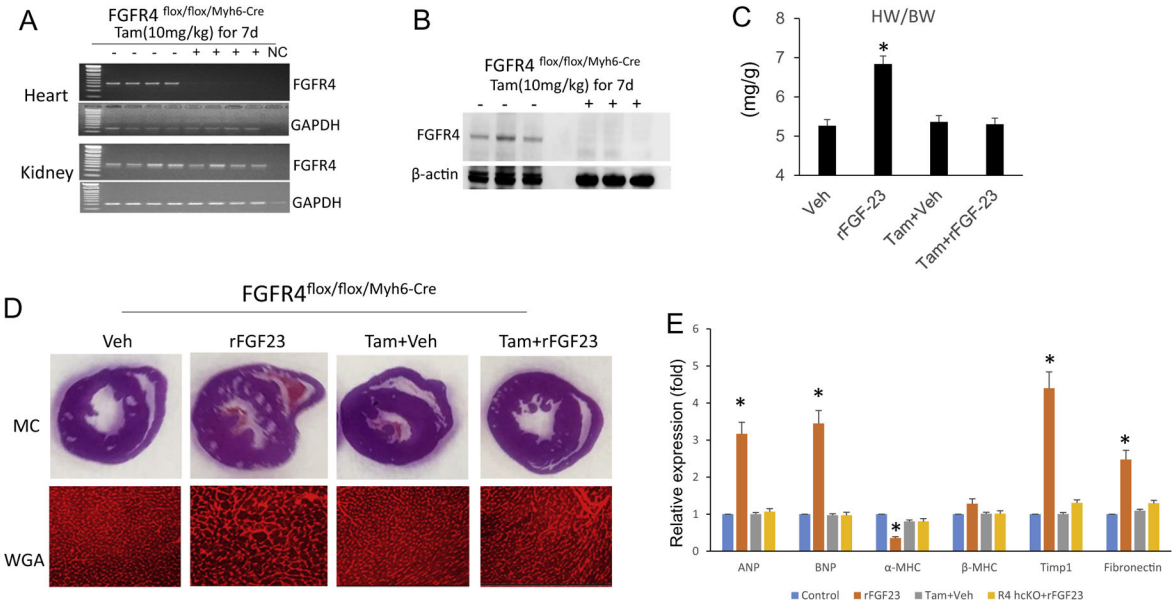

Fig. 1.

Conditional deletion of cardiac FGFR4 blocks rFGF23 induced LVH. Cardiac deletion of FGFR4 was achieved by injection of tamoxifen (Tam, 10 mg/kg) to FGFR4$^{flox/flox/Myh6\text{-}Cre}$ mice for 7 days as determined by RT-PCR (A) and Western blot analysis (B). Injection of rFGF23 (75 ng/g body weight, i.p.) for 5 days induced LVH, which was rescued in FGFR4$^{heart\text{-}cKO}$ mice confirmed by heart weight/body weight ratio (C), cross section of heart and WGA (D), and cardiac hypertrophic markers and fibrosis markers (E). $n = 4–5$/group, $*p < .05$ *vs* controls. All values are shown as mean ± S.D.

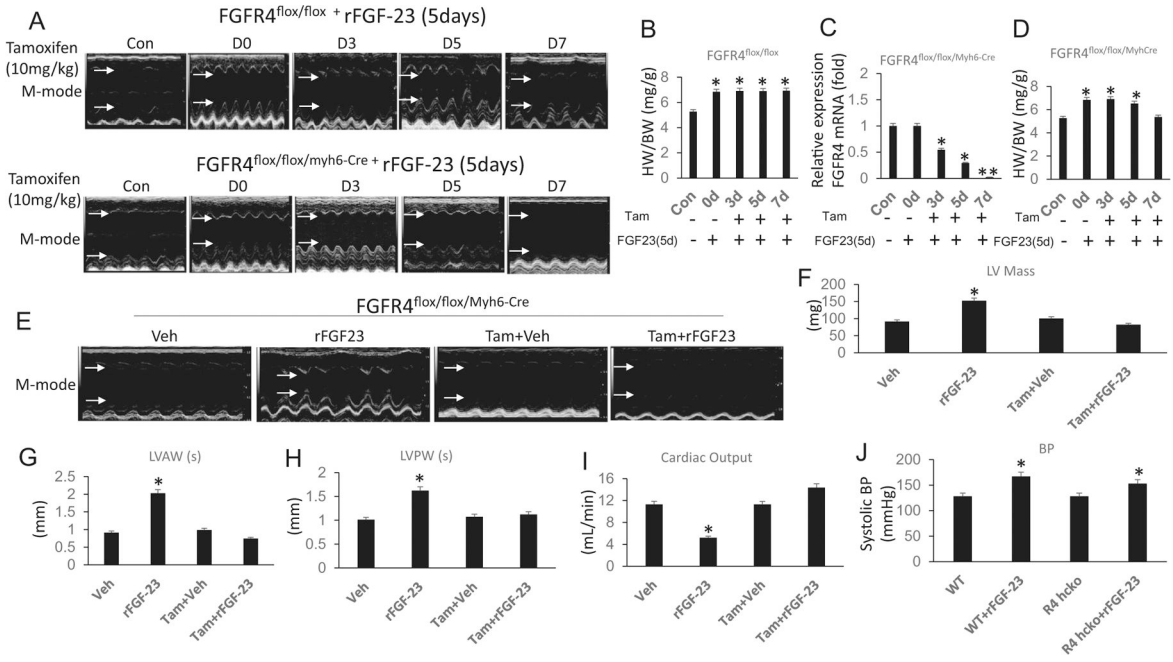

Fig. 2.

Echocardiographic evaluation of the role of FGFR4 in FGF23-mediated LVH. FGFR4 $^{flox/flox}$ and Myhe-Cre;FGFR4$^{flox/flox}$ mice were treated with tamoxifen for 7 days to create control and conditional cardiac FGFR4 knock mice. Both groups were treated with FGF23 for 5 days to induce LVH. Injection of rFGF23 (75 ng/g body weight, i.p.) for 5 days induced LVH in tamoxifen treated control mice with FGFR4 expression in the heart (A, top and B), but not in FGFR4$^{heart\text{-}cKO}$ mice created by tamoxifen treatment for 7 days to delete FGFR4 in the heart (A, bottom, and D). D0 indicates analysis at baseline and D3, D5 and D7 reflect 3, 5 and 7 of Tamoxifen treatment. Tamoxifen induced cardiac FGFR4 specific deletion in a time-dependent manner, with FGFR4 progressively decreasing by 50 and 75% after tamoxifen treatment for 3 and 5 days, respectively (C). FGFR4 expression was reduce by over 90% after 7 days of tamoxifen treatment. rFGF23 induced LVH in mice treated with tamoxifen for up to 5 days when FGFR4 could still be detected, but not after 7d of tamoxifen treatment *FGFR4$^{flox/flox/Myh6\text{-}Cre}$* mice that deleted FGFR4 in the heart. Effects of deletion of cardiac FGFR4 to attenuate rFGF23-mediated LVH was further confirmed by echocardiography (*E*-I). FGFR4 deficiency in the heart had no effect on rFGF23 induced hypertension (J). $n = 4–5$/group, $*p < .05$ *vs* controls. All values are shown as mean ± S.E.

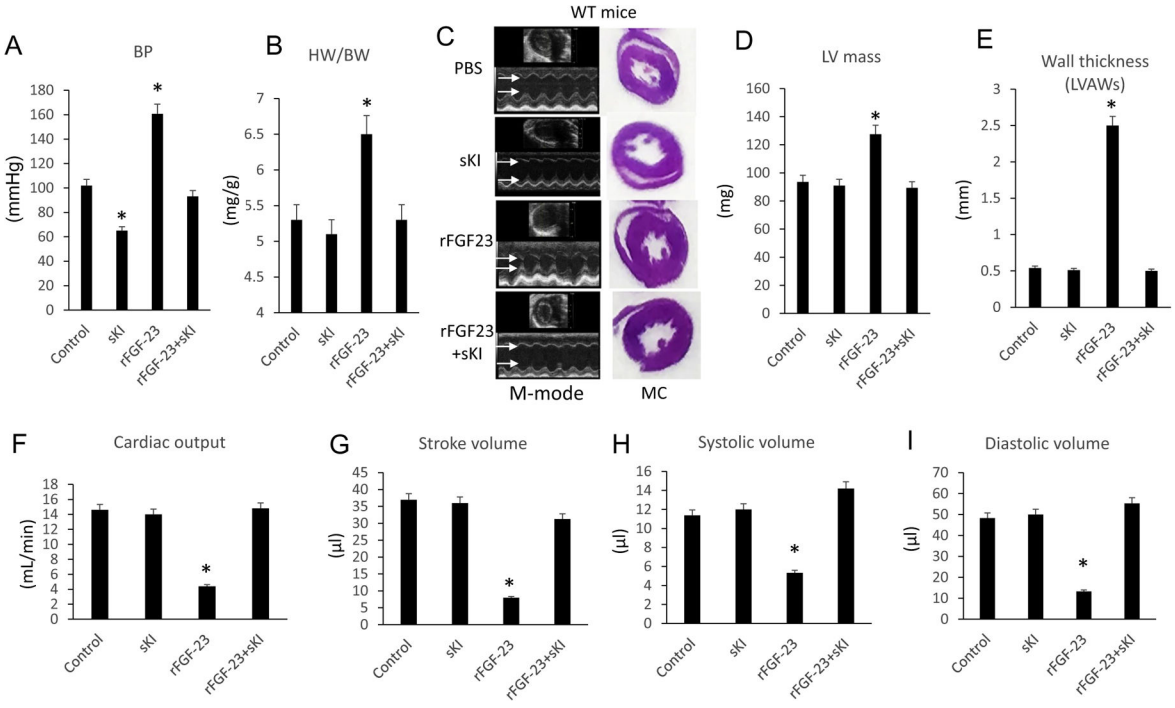

Fig. 3.

sKL protects rFGF23 administration induced LVH in mice. rFGF23 administered at the dose of 75 ng/g body weight intraperitoneally twice daily for 5 days to wild-type (WT) mice induces hypertension (A) and LVH (B-D), as measured by, heart weight/body weight ratio (B), echocardiography (C), LV mass (D), and wall thickness (E). sKL treatment at the dose of 10 ng/g body weight (i.p) at the same time with rFGF23 prevented rFGF23 induced HTN (A) and LVH (B-E). rFGF23 treatment impaired cardiovascular function by reducing the cardiac output (F), stroke volume (G), systolic volume (H), and diastolic volume (I) of the heart, while treatment of sKL reversed the effects of FGF23 on these parameters (FeI). n = 4–5/group, *$p < .05$ *vs* controls. All values are shown as mean ± S.E.

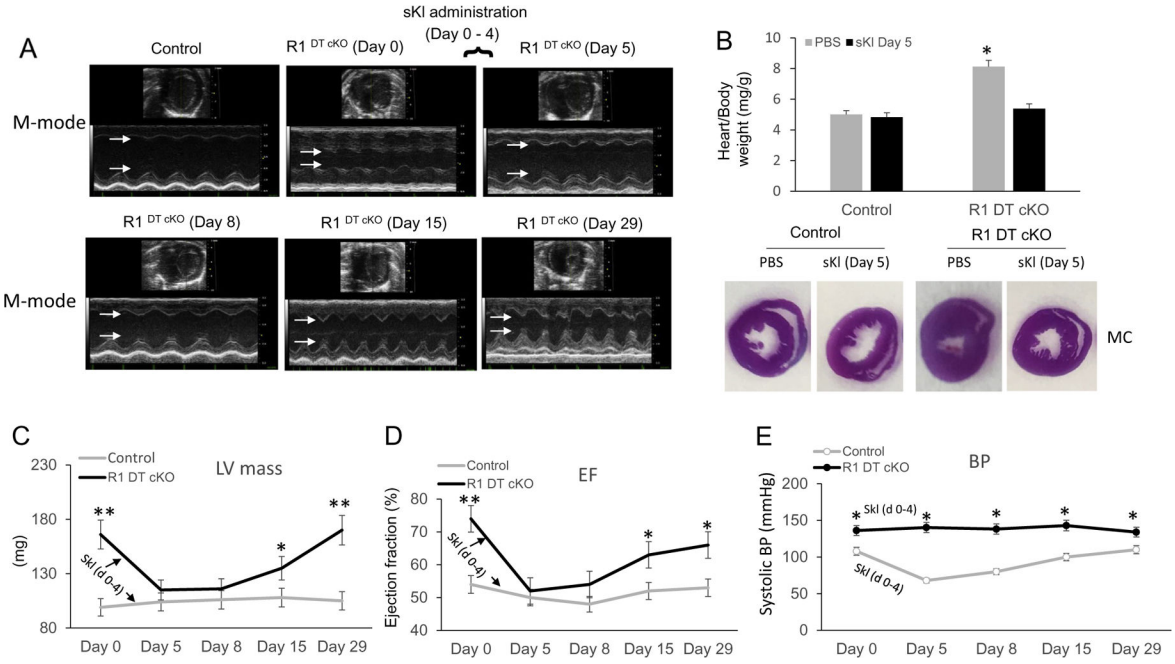

Fig. 4.

sKL corrects LVH in distal tubular FGFR1 deficient mice. Treatment with recombinant sKL prevents the development of LVH in *FGFR1*$^{DT\ cKO}$ mice by echocardiogram (A), heart/body weight ratio and echocardiography (B), LV mass measurements (C), and ejection fractions (D). LVH developed in these mice after stopping sKL treatment (A and C). Interestingly, sKL treatment lowered blood pressure in wild-type mice by not *FGFR1*$^{DT\ cKO}$ mice (E). n = 4–5/group, *$p < .05$ vs controls. All values are shown as mean ± S.E.

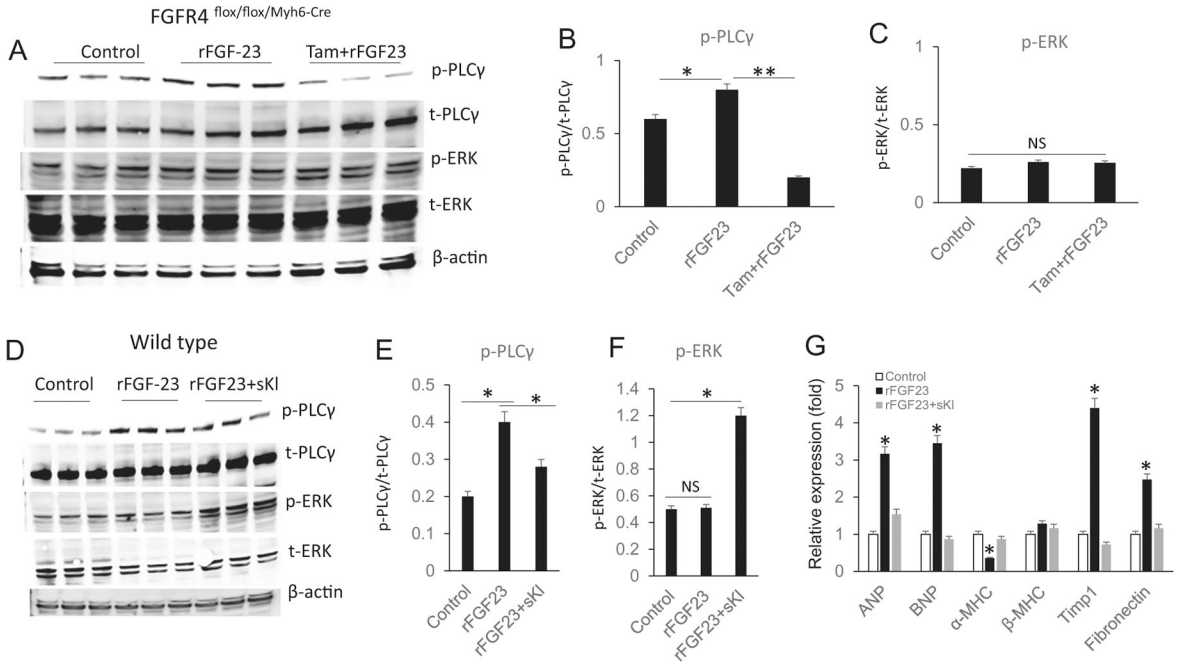

Fig. 5.

sKL administration converts FGF23/p-PLCγ signaling into FGF23/p-ERK signaling in heart. rFGF23 injection increased p-PLCγ in the heart of wild-type mice, which was abolished in the heart of FGFR4$^{heart\text{-}cKO}$ mice (A and B). rFGF23 administration had no effect on cardiac ERK activity (C). Treatment of recombinant sKL attenuated rFGF23 induced p-PLCγ activation (D and E), and imparted FGF23-dependent p-ERK activation (D and F). Treatment with sKL blocked rFGF23-induced hypertrophic (ANP, BNP, αMHC, βMHC) and fibrosis markers (timp1 and fibronectin) in the heart of mice (G). n = 4–5/group, *$p < .05$ vs controls. All values are shown as mean ± S.E.

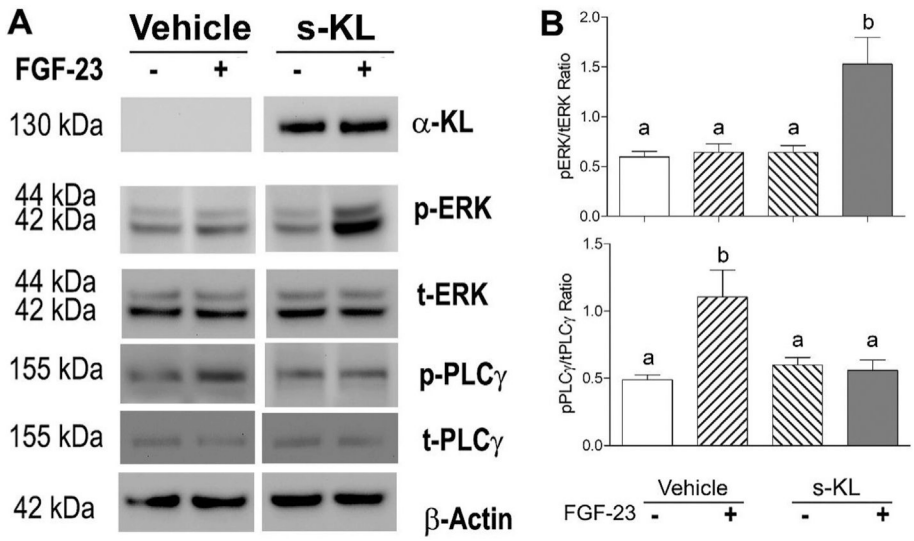

Fig. 6.

The effects of the FGF-23 on FGF23-induced signaling in HEK-293 T cells in the presence or absence of human soluble α-KL (s-KL). (**A**) Western blot analysis of α-KL from conditioned medium and FGF-23/ERK and FGF-23/PLCγ signaling from cell ly-sates; (**B**) Quantification of the pERK/tERK and pPLCγ1/tPLCγ ratio. Data are expressed as the mean ± S.D. from three independent experiments. Values sharing the same superscript in different groups are not significantly different at $P < .05$.