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Cardiac Fibroblasts Produce Fibroblast Growth Factor 23 in Response to Hyperphosphatemia

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Abstract

Chronic Kidney Disease (CKD) is accompanied by pathologic cardiac remodeling, including cardiac hypertrophy and fibrosis. The decline in renal function results in elevations of serum concentrations of phosphate (hyperphosphatemia) and of the bone-derived hormone Fibroblast Growth Factor (FGF) 23. Clinical studies have shown that hyperphosphatemia and high circulating levels of FGF23 are associated with cardiovascular mortality in CKD. We previously found that FGF23 can directly target cardiac myocytes via FGF Receptor (FGFR) 4 and induce cardiac hypertrophy in rodents. Furthermore, decreasing serum FGF23 levels or pharmacological blockade of FGFR4 in rodent models of hyperphosphatemia and CKD reduces cardiac hypertrophy, but not cardiac fibrosis. To directly determine whether cardiac fibroblasts are resistant to FGF23 elevations, we isolated cardiac fibroblasts from adult mice and treated them with recombinant FGF23 protein, which did not induce proliferation, pro-fibrotic gene programs or differentiation into myofibroblasts. Similarly, cardiac fibroblasts isolated from mice receiving a high-phosphate diet did also not respond to FGF23, indicating that hyperphosphatemia does not prime cardiac fibroblasts for FGF23 responsiveness. However, cardiac fibroblasts derived from mice on high-phosphate diet produced and secreted FGF23. Our study suggests that cardiac fibroblasts are not a target but a source of FGF23 and that FGF23/FGFR4 signaling is a driver of cardiac hypertrophy but not fibrosis. Elevated phosphate appears to not only induce FGF23 production in bone but also in cardiac fibroblasts which in a paracrine manner might contribute to cardiac hypertrophy in scenarios of hyperphosphatemia, such as CKD.

Keywords: Cardiac fibroblasts; FGF23; Hyperphosphatemia; Pathologic cardiac remodeling; Phosphate

Abbreviations: αSMA: Alpha Smooth Muscle Actin; AMCFs: Adult Mouse Cardiac Fibroblasts; AMCM: Adult Mouse Cardiac Myocytes; CKD: Chronic Kidney Disease; COL1A1: Collagen Type I Alpha 1 Chain; CTGF: Connective Tissue Growth Factor; FGF: Fibroblast Growth Factor; IL: Interleukin; LIF: Leukemia Inhibitory Factor; MMP: Matrix Metalloproteinase; MAPK: Mitogen-Activated Protein Kinase; NRCF: Neonatal Rat Cardiac Fibroblasts; NRVMs: Neonatal Rat Ventricular Myocytes; NFAT: Nuclear Factor of Activated T cells; PLCγ: Phospholipase C Gamma; RT-qPCR: Reverse Transcriptase Quantitative Real-time Polymerase Chain Reaction; ROS: Reactive Oxygen Species; sKL: Soluble Klotho; TGFβ: Transforming Growth Factor Bet.

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Introduction

Chronic Kidney Disease (CKD) is closely associated with pathologic cardiac remodeling, and cardiovascular disease is the leading cause of death in CKD patients [1]. Understanding the direct mechanisms that link CKD to cardiovascular pathologies is necessary to advance therapeutic options for patients, but is complicated by the variety of pathologic alterations caused by CKD that have independent associations with negative cardiovascular outcomes, including hyperphosphatemia [2], vascular calcification [3], hypertension [4], systemic inflammation [5], and elevated serum levels of Fibroblast Growth Factor (FGF) 23 [6].

FGF23 is a phosphaturic hormone that is primarily produced and released by osteocytes. FGF23 targets FGF receptor (FGFR) 1 and its co-receptor Klotho in the kidney and parathyroid gland, thereby activating Ras/Mitogen- Activated Protein Kinase (MAPK) signaling, reducing renal phosphate uptake, and lowering serum phosphate concentrations [7]. In CKD patients, serum concentrations of FGF23 are highly elevated and associated with cardiac hypertrophy and cardiovascular mortality [8,9]. Elevated FGF23 levels have also been associated with increased risk of mortality in patients experiencing heart failure with reduced ejection fraction and normal kidney function [10]. Additionally, FGF23 appears to be produced in the myocardium of rodent models of CKD [11], indicating a potential paracrine role of locally produced FGF23 in CKD. We have previously shown that at high concentrations, FGF23 can activate FGFR4 on cardiac myocytes, independently of klotho, and initiate Phospholipase Cγ (PLCγ)/Calcineurin/Nuclear Factor of Activated T cells (NFAT) signaling [12]. FGF23 induces hypertrophic growth of cultured cardiac myocytes via FGFR4 and deletion or blockade of FGFR4 in CKD rodent models with elevated FGF23 protects from cardiac hypertrophy [12]. Furthermore, FGF23 has been shown to induce myocardial fibrosis when overexpressed in the hearts of mice via the activation of Transforming Growth Factor Beta (TGFβ) and β-catenin [13]. It is likely that FGF23 affects the crosstalk between cardiac myocytes and fibroblasts that leads to pathologic changes in both cell types [14]. Interestingly, FGF23 elevations in the absence of CKD seem to induce cardiac hypertrophy that is reversible, while the associated cardiac fibrosis was not reduced [15]. These studies suggest that FGF23 can directly induce cardiac hypertrophy but does not directly drive cardiac fibrosis. Overall, it appears that FGF23's actions on the heart are dependent on the context [16].

Table 1: Primers. The following oligonucleotides shown in 5′ to 3′ orientation were used as primers in quantitative real time PCR analyses.

Gene	Orientation	Primer Sequence (5′to 3′)
Fgf23 (mouse)	Forward	CAC TGC TAG AGC CTA TCC
	Reverse	CAC TGT AGA TGG TCT GAT GG
Fgfr1 (mouse)	Forward	CAA CCG TGT GAC CAA AGT GG
	Reverse	TCC GAC AGG TCC TTC TCC G
Fgfr2 (mouse)	Forward	ATC CCC CTG CGG AGA CA
	Reverse	GAG GAC AGA CGC GTT GTT ATC C
Fgfr3 (mouse)	Forward	GTG TGC GTG TAA CAG ATG CTC
	Reverse	CGG GCG AGT CCA ATA AGG AG
Fgfr4 (mouse)	Forward	TGA AGA GTA CCT TGA CCT CCG
	Reverse	TCA TGT CGT CTG CGA GTC AG
Klotho (mouse)	Forward	TGT ATG TGA CAG CCA ATG GAA TCG
	Reverse	GAA TAC GCA AAG TAG CCA CAA AGG
Col1a1 (mouse)	Forward	GTG TTC CCT ACT CAG CCG TC
αSMA (mouse)	Reverse	ACT CGA ACG GGA ATC CAT CG
	Forward	GCC ATC TTT CAT TGG GAT GGA
	Reverse	CCC CTG ACA GGA CGT TGT TA
Ctgf (mouse)	Forward	AGA ACT GTG TAC GGA GCG TG
	Reverse	GTG CAC CAT CTT TGG CAG TG
Mmp2 (mouse)	Forward	AAC GGT CGG GAA TAC AGC AG
Mmp9 (mouse)	Reverse	GTA AAC AAG GCT TCA TGG GGG
	Forward	GCC GAC TTT TGT GGT CTT CC
	Reverse	TAC AAG TAT GCC TCT GCC AGC

In the context of CKD, FGF23 induces cardiac hypertrophy that is accompanied by pathologic alterations, while by itself FGF23/FGFR4 signaling causes cardiac hypertrophy that is not accompanied by fibrosis. Here, we aimed to determine if cardiac fibroblasts isolated from healthy wildtype mice can respond to FGF23 by undergoing pro-fibrotic activation. Furthermore, since CKD is associated with hyperphosphatemia, we determined if high phosphate levels would prime cardiac fibroblasts for responding to FGF23. To do so, we isolated cardiac fibroblasts from wildtype mice that received a high-phosphate diet for 12 weeks and treated them with recombinant FGF23. To determine if cardiac fibroblasts could be activated indirectly by cardiac myocytes that respond to FGF23, we treated isolated cardiac myocytes with FGF23 and analyzed the expression levels of factors that mediate the myocyte-fibroblast cross-communication in the heart.

Materials and Methods

Study Approval

All animal protocols and experimental procedures for mouse diet conditioning studies and for primary cell isolations from adult mice and from newborn rats were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Alabama Birmingham, School of Medicine. All protocols adhered to the Guide for Care and Use of Laboratory Animals to minimize pain and suffering.

Recombinant Proteins and Antibodies

Recombinant mouse proteins from R&D Systems are FGF23 (2629-FG/CF), IL-1β (401-ML), and TGFβ (7666-MB). Primary antibodies used are anti-COL1A1 (84336S, Cell Signaling), anti-αSMA (14968S, Cell Signaling), anti-CTGF (ab6992, Abcam), and anti-GAPDH (CB1001 Millipore Sigma). Secondary antibodies for Western blotting are HRP- conjugated goat-anti-mouse (W4021, Promega), and goat-anti-rabbit (W4011, Promega).

Mouse Experiments

Two-month-old male C57BL/6J mice purchased from the Jackson Laboratories were switched from normal chow (Teklad TD.200407) to a 2% phosphate diet (Teklad TD.08020) for 12 weeks. Mice on a standard 0.7% phosphate diet (Teklad TD.180287) served as negative controls. High-phosphate chow contained additional monobasic potassium phosphate and monobasic and monohydrate sodium phosphate.

Mineral content of calcium (1.0%), sodium (0.2%), potassium (0.6%), chloride (0.4%), and magnesium (0.2%) were similar between the normal and high-phosphate chows. After 12 weeks of diet conditioning, animals were sacrificed, and blood was collected for serum chemistry analysis. Hearts were isolated and either freshly used for fibroblast isolation or perfused *ex vivo* (0.5 M KCl in 0.9% NaCl) and snap-frozen for storage and further analyses. All animals were maintained in temperature-controlled environments with a 12-hour light/dark cycle and allowed *ad libitum* access to food and water.

Isolation and Treatment of Adult Mouse Cardiac Fibroblasts (AMCFs)

Male C57BL/6J mice at 9-12 weeks of age or male C57BL/6J mice receiving a 2% or 0.7% phosphate diet for 12 weeks were used for AMCF isolations. Two mouse hearts were combined per isolation. The chest cavity was opened, and hearts were rapidly excised and washed twice in perfusion buffer (113 nM NaCl, 4.7 mM KCl, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 10 mM Na-HEPES, 12 mM $NaHCO_3$, 10 mM $KHCO_3$, 30 mM taurine, 10 mM 2,3-Butanedione Monoxime [BDM], 5.5 mM glucose [17]. The perfusion buffer was aspired, hearts were minced and mixed with 20 mL digestion buffer (300 U/mL collagenase type 2 [LS004176, Worthington Biochemical Corporation] in perfusion buffer) in a 50-mL tube. The minced hearts were triturated in the suspension 10 times with a standard 10 mL-plastic serological pipette, and the tissue was digested under slow rotation (35 rpm) at 37°C for 1 hour. Cells were released by triturating the suspension 10 times with a standard 10 mL-plastic serological pipette and stop buffer (10% v/v fetal bovine serum [FBS; 26140079, Gibco] in perfusion buffer) was added. Cells were filtered through a cell strainer (70 µm, BD Falcon) and centrifuged at 5 g for 5 minutes to remove myocytes and cell debris. The supernatant with remaining cardiac fibroblasts was centrifuged again at 250 g for 10 minutes. The cell pellet was resolved in 10 mL culture medium (Dulbecco's Modified Eagle Medium [DMEM; Corning] with 10% FBS [Gibco]) and 1% penicillin/streptomycin solution (P/S; Gibco)] and plated in a 10 cm-culture dish. Two hours after plating, the culture dish was washed 3 times with PBS to remove non-fibroblasts, and the culture medium was replaced. After 7 days, cardiac fibroblasts (Passage 0) were harvest by trypsinization and counted with a hemocytometer. For expression studies, 1 x 10^5 cells were seeded per well in 6-well plates. For proliferation assays, 1 x 10^4 cells were seeded per well in 96-well plates. Cells (Passage 1) were left undisturbed for 24 hours, followed by a switch to starvation medium (DMEM with 1% P/S) for another 24 hours. The isolated cardiac fibroblasts were cultured in FBS-free medium in the presence of recombinant FGF23 (50 ng/mL), IL-1β (10 ng/mL), TGFβ (10 ng/mL), or vehicle (PBS) for 24 hours. Experiments and analysis were conducted with isolated fibroblasts from Passage 1.

Serum and AMCF Culture Supernatant FGF23 Content

At endpoint, blood was collected from mice via cardiac puncture, transferred into microvette serum gel tubes (20.1344, Sarstedt) and centrifuged at 10,000 g for 5 minutes. Serum supernatants were collected and stored at -80°C. Serum levels of total FGF23 were assessed using an ELISA from Quidel (60-6300). Using the same ELISA, FGF23 was measured in cell culture supernatants collected from AMCFs in Passage 1 at either 24 or 48 hours after changing the media to starvation medium Medium was centrifuged at 1,000 g for 5 minutes, and supernatants were diluted 1:3 in diluent solution (Quidel) prior ELISA.

Isolation and Treatment of Neonatal Rat Ventricular Myocytes (NRVMs)

NRVMs were isolated using a standard isolation kit (LK003300, Worthington Biochemical Corporation), as previously described [18]. Hearts from 1-2 days old Sprague Dawley rats (Envigo) were harvested, minced in calcium- and magnesium-free Hank's Balanced Salt Solution (HBSS), and the tissue was digested with 50 µg/mL trypsin at 4°C for 16-20 hours. 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 1 hour. Cells were released by triturating the suspension 20 times with a standard 10 mL-plastic serological pipette and by filtering through a cell strainer (70 µm, BD Falcon). Cells were incubated at room temperature for 20 minutes and spun at 50 g for 5 minutes. After resuspension in plating medium [DMEM with 17% Media 199 (12350039, Gibco]), 15% FBS (26140079, Gibco), and 1% penicillin/streptomycin solution (P/S; 15140122; Gibco)], cells were counted using a hemocytometer. 3 x 10^5 cells were seeded on laminin-coated (10 µg/mL in PBS; 23017015, Gibco) glass cover slips (CLS1760012, Chemglass) in 24-well plates. Cells were left undisturbed in plating medium at 37°C for 72 hours and then cultured in maintenance medium [DMEM (10013CV, Corning) with 20% Media 199 (12350039, Gibco), 1% insulin-transferrin-sodium selenite solution (I18841VL; Sigma-Aldrich) and 1% P/S] in the presence of 100 µM 5-bromo-2'- deoxyuridine (BrdU; B9285, Sigma-Aldrich) for 4 additional days. Isolated cardiac myocytes were then cultured in BrdU-containing FBS-free maintenance medium for 24 hours in the presence of recombinant FGF23 (50 ng/mL), IL- 1β (10 ng/mL), TGFβ (10 ng/mL), or vehicle. Cell lysates were collected after treatment was completed.

RNA Isolation and Quantitative Real-time PCR

For RNA isolation from snap-frozen mouse tissue the RNeasy Plus Mini Kit (74136, Qiagen) was used, according to the manufacturer's protocol. The same kit and protocol were used for RNA isolations from cultured AMCFs and NRVMs. Employing a two-step reaction method, 1 µg of total RNA was Reverse Transcribed (RT) into cDNA using iScript Reverse Transcription Supermix (1708840, BioRad). Quantitative real-time PCR (RT-qPCR) was performed with 100 ng of cDNA, SsoAdvanced Universal SYBR Green Supermix (172-5272, BioRad), and sequence specific primers (Table 1). Samples were run in triplicate on a CFX96 Touch Real-Time Detection Instrument (1855196, Bio-Rad). Amplification was performed in 40 cycles (95°C, 30s; 98°C, 15s; 60°C, 30s; 65°C, 5s). The generated amplicon was systematically double-checked by its melting curve. Relative gene expression was normalized to expression levels of the housekeeping gene *Gapdh*. Results were evaluated using the $2^{-\Delta\Delta Ct}$ method and expressed as mean ± Standard Error of the Mean (SEM).

Protein Isolation and Immunoblotting

AMCF cells were scraped from 6-well plates in 100 µL RIPA extraction buffer (50 mM Tris base, 200 mM NaCl, 1% Triton X-100, 0.25% DOC, 1 mM EDTA, 1 mM EGTA, pH 7.5 with protease inhibitor cocktail [11873580001, Roche], protein phosphatase inhibitors [P5726, Sigma-Aldrich]) and incubated on ice for 30 minutes. Cell lysate was centrifuged at 14,000 rpm and 4°C for 30 minutes, and the supernatant was boiled in Laemmli sample buffer (1610737, BioRad) with β-mercaptoethanol (1610710, Bio-Rad) at 95°C for 5 minutes and stored at -80°C. Protein samples were loaded onto 8% or 10% SDS polyacrylamide gels and separated by SDS-PAGE. Gels were run in Tris/Glycine/SDS buffer (1610732, Bio-Rad) at 20 mA per gel. Proteins were electroblotted onto PVDF membranes (IPVH00010, Merck Millipore) via a semi-dry cassette (1703940, Bio-Rad) in Tris/Glycine Buffer (1610734, Bio-Rad) with 20% methanol at 20 V for 1 hour. Membranes were then blocked in 5% BSA in tris-buffered saline with 0.1% Tween20 (TBST) for 1 hour and probed with antibodies against COL1A1 (1:1,000), αSMA (1:1,000), CTGF (1:1,000), and GAPDH (1:10,000) in BSA/TBST at 4°C overnight. Horseradish peroxidase-conjugated goat-anti-mouse and goat-anti-rabbit (1:10,000) were used as secondary

antibodies in BSA/TBST at 4°C for 1 hour. Membranes were activated using Clarity Western ECL Substrate (1705060, BioRad), and images were taken and quantified using a BioRad Chemi-Doc Imaging System (Software Version: 2.1.0.32 BETA CL19032).

Proliferation

The proliferation of AMCF was analyzed by assaying with AlamarBlue cell viability reagent (A50100, Invitrogen) according to the manufacturer's protocol. In brief, 1 x 10^4 cells per well were seeded into black 96-well flat-bottom cell culture plates and allowed to attach overnight. After cell starvation overnight followed by treatment with FGF23, TGFβ or vehicle in triplicates for 48 hours, 10 µL AlamarBlue cell viability reagent was added per well. Absorbance at 570 nm and 600 nm was recorded with a plate reader (BioTEK SYNERGY) after 6 hours and FGF23- and TGFβ-stimulated proliferation of AMCF was quantified in relation to vehicle-treated control cells.

Statistics

Data organization, scientific graphing, and statistical significance of differences between experimental groups were performed by using GraphPad Prism (version 9.0.0). All results are expressed as mean ± SEM. Comparisons between 3 or more groups were performed by one-way ANOVA followed by post-hoc Tukey test. Comparisons between 2 groups were performed by two-tailed t-tests. A significance level of P≤0.05 was accepted as statistically significant. Sample size was determined on the basis of sample availability, prior experimental studies performed in our laboratory and from prior literature [12,15]. No formal randomization was used in any experiment. For *in vivo* experiments, animals were unbiasedly assigned into different experimental groups. Group allocation was not performed in a blinded manner. Whenever possible, investigators were blinded to experimental groups.

Results

FGF23 Does Not Activate Isolated Cardiac Fibroblasts

To test if FGF23 can activate cardiac fibroblasts and induce their differentiation into myofibroblasts, we isolated Adult Mouse Cardiac Fibroblasts (AMCFs) from C57BL/6J mice and treated them with recombinant mouse FGF23 (50 ng/mL) or vehicle control for 24 hours. Proliferation of AMCF cultures, measured via AlamarBlue assays, was unaffected by FGF23 treatment (Figure 1A). Furthermore, gene expression of *Alpha Smooth Muscle Actin* (*αSMA*) and *Connective tissue growth factor* (*Ctgf*), key genes elevated in activated myofibroblasts [19-24], was unaltered by FGF23 treatment as determined by RT-qPCR (Figure 1B, C). This finding was further supported by Western blot analysis of cell culture lysates showing that FGF23 does not elevate αSMA, CTGF, and Collagen Type I Alpha 1 Chain (COL1A1) on protein level (Figure 1D-G). In contrast, TGFβ (10 ng/mL) used as a positive control (19,24-28), increased AMCF proliferation and the expression of markers on mRNA and protein level. Additionally, we found that FGF23 did not induce gene expression of the Extracellular Matrix (ECM) regulatory proteins *Matrix metalloproteinase* (*Mmp*) *2* and *Mmp9*, while IL-1β (10 ng/mL), used as a positive control [24,29], elevated the expression of both genes (Figure 1H, I). We confirmed the identity of our cell culture model as AMCFs by analyzing the expression of key markers for cardiac fibroblast, myofibroblasts and endothelial cells, confirming that prior treatments our cells contained cardiac fibroblasts and not already activated myofibroblasts or endothelial cells (Figure 1J). The expression of *Fgfr 1-4*, *Klotho*, and *Fgf23* were also analyzed via RT-qPCR, showing that *Fgfr1* had the highest expression levels, followed by *Fgfr2* (Figure 1K), while *Fgfr4* was only expressed at low levels. *Fgfr3, Klotho,* and *Fgf23* expression was not detectable.

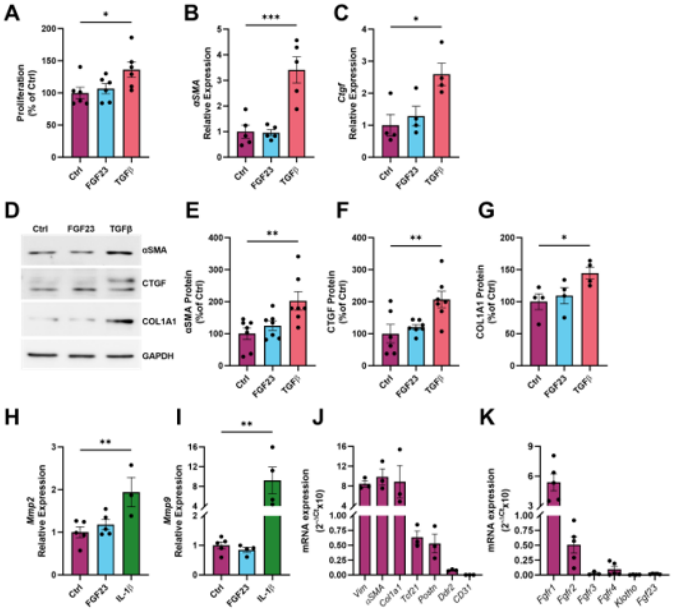

Figure 1: FGF23 does not activate cardiac fibroblasts isolated from wildtype mice.
Primary Adult Mouse Cardiac Fibroblasts (AMCFs) isolated from C57BL/6J mice were treated with vehicle, recombinant Fibroblast Growth Factor 23 (FGF23, 50 ng/mL), or Transforming Growth Factor-β (TGF-β, 10 ng/mL) for 24 and 48 hours. (**A**) Cells were analyzed for proliferation via AlamarBlue assay. (**B, C**) RT-qPCR analysis of *Alpha Smooth Muscle Actin* (*αSMA*) and *Connective tissue growth factor* (*Ctgf*) using total RNA from cell culture lysates. (**D**) Representative Western blot analysis of AMCFs for αSMA, CTGF, and Collagen Type I Alpha 1 Chain (COL1A1) protein. (**E-G**) Quantification of Western blot signals for αSMA, CTGF, and COL1A1 by densitometry. Data is presented as a percentage relative to control protein levels. (**H, I**) RT-qPCR analysis of *Matrix metallopeptidase* (*Mmp*) *2* and *Mmp9* from AMCFs treated with vehicle, recombinant FGF23 (50 ng/mL), or Interleukin 1β (Il-1β, 10 ng/mL) for 24 hours. (**J**) RT-qPCR analysis of AMCFs for markers of cardiac fibroblasts (*Vim1*, *αSMA*, *Col1a1*), myofibroblasts (*Tcf21*, *Postn*) and endothelial cells (*Ddr2*, *CD31*). (**K**) AMCFs were characterized for the presence of the FGF23/FGFR signaling complex by RT-qPCR analysis of *Fibroblast growth factor receptors* (*Fgfr*) *1-4*, *Klotho*, and *Fgf23*. All RT-qPCR analyses were conducted using *Gapdh* as a house keeping gene. All values are shown as ±SEM. n=3-7; *P≤0.05; **P≤0.01; ***P≤0.001.

It has been shown that fibroblasts in the kidney only respond to FGF23 following kidney injury, which induces *Fgfr4* expression in renal fibroblasts [30,31]. Thus, FGF23 might act as a profibrotic factor only in situations of injury-priming and rather aggravate but not initiate fibrosis. To investigate a potential role of hyperphosphatemia in inducing FGF23 responsiveness in cardiac fibroblasts, we placed C57BL/6J mice on a high-phosphate (2%) diet or a chow with normal phosphate levels (0.7%) for 12 weeks before isolating AMCFs. We observed that regardless of the diet, FGF23 treatment did not affect AMCF proliferation (Figure 2A). mRNA expression levels of *αSMA* and *Ctgf* (Figure 2B,C), as well as protein levels of αSMA, CTGF, and COL1A1 (Figure 2D-G) were also unaltered by FGF23 treatment. Additionally, diet-conditioning did not affect the expression levels of *Fgfr1* or *Fgfr4* in AMCFs (Figure 2H). Together, these findings indicate that FGF23 does not activate cardiac fibroblasts. Furthermore, exposure of the mouse heart to elevations in systemic phosphate levels does not change the expression levels of FGFRs and does not induce FGF23-responsiveness in cardiac fibroblasts.

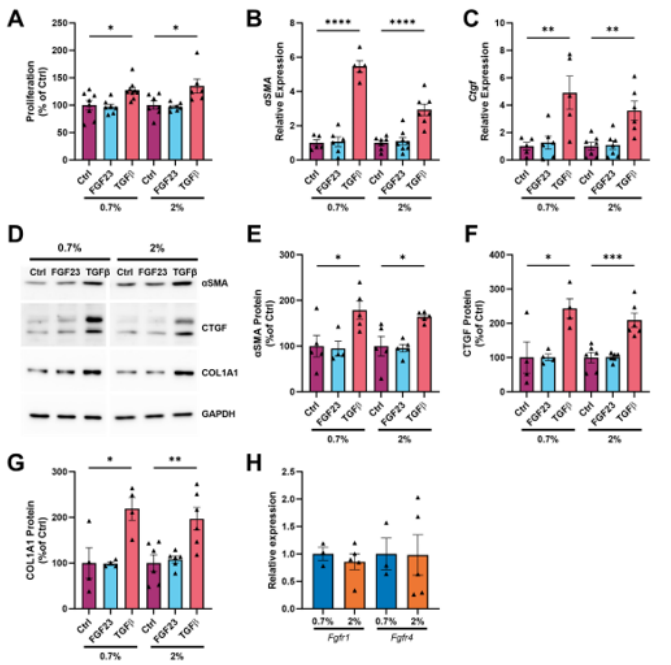

Figure 2: FGF23 does not activate cardiac fibroblasts isolated from wildtype mice on a high-phosphate diet.

Primary Adult Mouse Cardiac Fibroblasts (AMCFs) were isolated from C57BL/6J mice that were fed a normal phosphate (0.7%) or high-phosphate (2%) diet for 12 weeks. AMCFs were treated with vehicle, recombinant Fibroblast Growth Factor 23 (FGF23, 50ng/mL), or recombinant Transforming Growth Factor-β (TGF-β, 10ng/mL) for 24 and 48 hours. (**A**) Cells were analyzed for proliferation via AlamarBlue assay. (**B, C**) RT-qPCR for *Alpha Smooth Muscle Actin* (*αSMA*) and *Connective tissue growth factor* (*Ctgf*) mRNA using total RNA from cell culture lysates. (**D**) Representative Western blot analysis of AMCF cultures for αSMA, CTGF, and Collagen Type I Alpha 1 Chain (COL1A1) protein. (**E-G**) Quantification of Western blot signals for αSMA, CTGF, and COL1A1 by densitometry. Data is presented as a percentage relative to control protein levels. (**H**) RT-qPCR for FGF receptors, *Fgfr1* and *Fgfr4*, from AMCF cell lysates. RT-qPCR analysis was conducted using *Gapdh* as a house keeping gene. All values are shown as ±SEM. n=4-9; *P≤0.05; **P≤0.01; ***P≤0.001; ****P≤0.0001.

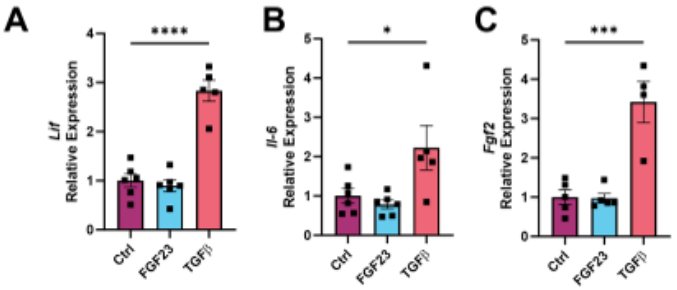

Figure 3: FGF23 does not alter expression levels of common intramyocardial communication factors in primary cardiac myocytes cultures.

Neonatal Rat Ventricular Myocytes (NRVMs) were treated with vehicle, recombinant Fibroblast Growth Factor 23 (FGF23, 50ng/mL), or recombinant Transforming Growth Factor-β (TGF-β, 10ng/mL) for 24 hours, followed by RT- qPCR expression analysis of (**A**) *Leukemia Inhibitory Factor interleukin 6 family cytokine* (*Lif*), (**B**) *Interleukin 6* (*Il-6*), and (**C**) *Fgf2*. RT- qPCR analysis was conducted using *Gapdh* as a house keeping gene. All values are shown as ±SEM. n=4-6; *P≤0.05; ***P≤0.001; ****P≤0.0001.

FGF23 Does Not Induce Expression of Intramyocardial Communication Factors in Isolated Cardiac Myocytes

To investigate whether FGF23 could indirectly activate cardiac fibroblasts by inducing the expression of factors in cardiac myocytes that are known to target fibroblasts in a paracrine manner, we treated primary Neonatal Rat Ventricular Myocytes (NRVMs) with recombinant FGF23 or vehicle control. We conducted a RT-qPCR expression analysis of *Leukemia inhibitory factor* (*Lif*), *Interleukin 6* (*Il-6*), and *Fgf2* which are known to mediate the bidirectional communication between cardiac fibroblasts and cardiac myocytes and drive fibroblast activation [32] and myocyte hypertrophy [33]. FGF23 did not induce any changes, while TGFβ used as a positive control [24,34-36] elevated expression levels of the three factors (Figure 3A-C). This finding suggests that FGF23 does not activate cardiac fibroblasts by targeting cardiac myocytes and does not induce paracrine crosstalk between both cell types.

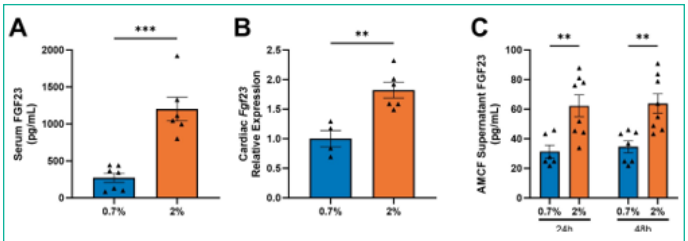

Figure 4: The administration of a high-phosphate diet induces the production and secretion of FGF23 by cardiac fibroblasts in mice.

C57BL/6J mice that were fed a normal phosphate diet (0.7%) or high-phosphate diet (2%) for 12 weeks were analyzed for (**A**) serum Fibroblast Growth Factor 23 (FGF23) concentration and (**B**) cardiac tissue *Fgf23* mRNA expression. (**C**) Supernatants from cultured primary Adult Mouse Cardiac Fibroblasts (AMCFs) isolated from 0.7% diet and 2% diet mice were analyzed for FGF23 protein after 24 and 48 hours. FGF23 protein was analyzed via ELISA. RT-qPCR analysis was conducted using *Gapdh* as a house keeping gene. All values are shown as ±SEM. n=4-8; **P≤0.01; ***P≤0.001.

High-Phosphate Diet Stimulates the Production of FGF23 in Cardiac Fibroblasts

Elevated phosphate levels are a major inducer of FGF23 expression in bone, where osteoblasts and osteocytes serve as the main source of FGF23 [24,37]. Previous studies have shown that also the heart can produce FGF23, at least in the context of CKD [24,38]. However, it remains unclear what cardiac cell types can express FGF23. To determine whether cardiac fibroblasts serve as a source of FGF23 and whether hyperphosphatemia can affect FGF23 production by cardiac fibroblasts, we isolated AMCFs from mice on normal chow with 0.7% phosphate content and from mice receiving a 2% phosphate diet for 12 weeks. As expected, [15,24], the high-phosphate diet significantly elevated serum FGF23 concentrations, compared to mice on control chow (Figure 4A). RT-qPCR analysis of total heart tissue showed a significant increase in the cardiac expression levels of *Fgf23* in mice on high-phosphate diet (Figure 4B). Furthermore, supernatants of isolated AMCFs from mice on both diets contained FGF23 protein, with significantly higher FGF23 levels in AMCFs from mice on high-phosphate diet when analyzed 24 and 48 hours after cell isolation (Figure 4C). These findings demonstrate that cardiac fibroblasts serve as a source for cardiac-derived FGF23, especially in the presence of elevated serum phosphate concentrations.

Discussion

Our study demonstrates that FGF23 does not activate cardiac fibroblasts that were isolated from adult wildtype mice. We found that FGF23 does not modify proliferation or induce the expression of pro-fibrotic proteins in these cells. Cardiac fibroblasts isolated from mice with diet-induced hyperphosphatemia also do not respond to FGF23, indicating that high-phosphate concentrations do not prime cardiac fibroblasts for FGF23-re-

sponsiveness. While we have previously shown that FGF23 induces hypertrophy in isolated cardiac myocytes [15,24], here we found that FGF23 does not induce the expression of factors in cardiac myocytes that could potentially activate fibroblasts in a paracrine manner. However, we found that the administration of a high-phosphate diet does not only cause elevations in circulating FGF23 levels, but also induce FGF23 expression in the heart and FGF23 secretion by cardiac fibroblasts. Combined our study suggests that cardiac fibroblasts act as source but not as a target of FGF23.

Hyperphosphatemia is closely associated with cardiovascular remodeling, including cardiac hypertrophy and fibrosis [18,39-41]. Elevated serum phosphate induces a significant increase in circulating FGF23 levels, and elevations in serum FGF23 have been linked to cardiac hypertrophy and all-cause mortality [8,39,41-44]. Previously, we have shown that FGF23 can activate FGFR4 on cardiac myocytes, inducing PLCγ/calcineurin/NFAT signaling and subsequent cardiac hypertrophy. However, whether cardiac fibroblasts are also a target of FGF23 and FGF23's involvement in the cardiac fibrosis observed in animal models of hyperphosphatemia is not well understood [12,15]. We have previously demonstrated that the administration of a high-phosphate diet for three months induces significant elevations of systemic FGF23 levels as well as cardiac hypertrophy and fibrosis in mice [15,24]. Most notably, we observed that elevations in serum FGF23, left ventricular wall thickness, and individual myocyte cross-sectional area were reversible by placing mice back on a control diet with normal phosphate content for three additional months. However, there was no significant reduction in cardiac fibrosis in these mice [15]. Furthermore, the blockade of FGFR4 by intraperitoneal injections of an FGFR4-specific antibody (U3-11; U3Pharma) in 5/6 nephrectomized rats inhibited myocyte hypertrophy but did not affect the development of cardiac fibrosis [15]. In CKD patients, it has been observed that cardiac fibrosis correlates with decreased soluble klotho (sKL), implicating increased cardiac FGF23/FGFR4 signaling in the progression of fibrosis, but there was no significant correlation between cardiac FGF23 concentrations and fibrosis [45]. These findings combined with our new data suggest that FGF23 may not be a driver of hyperphosphatemia-induced cardiac fibrosis.

The function of cardiac FGF23 has been widely debated and has been further complicated by studies that contradict the findings of others, as well as a predominant focus on models of cardiovascular injury and CKD. In the present study, we observed that FGF23 treatments do not activate AMCF proliferation, transition to myofibroblasts, or extracellular matrix remodeling, with or without priming the mice with a high-phosphate diet. This data contradicts the findings of other studies that observed AMCFs responses to treatments with recombinant FGF23 by increasing procollagen production [13], and that Neonatal Rat Cardiac Fibroblasts (NRCF) respond by increasing collagen remodeling, *Ctgf* expression, and activating TGFβ/TGFβ receptor/SMAD complexes [45], indicating a role in fibrosis. One possible source for this contradiction may be derived by our different methods of isolation, where trypsin is used as a digestive enzyme in their study while our protocol uses collagenase. Trypsin usage in the isolation of primary cells is known to cause collateral damage to cells and, to preserve the physiological properties of the primary cells, collagenase has been recommended for digestion [46-49]. Additionally, co-isolation of NRVMs and NRCFs may add stress to the cells in suspension, though the protocol used to specifically isolate NRCFs is not well described, so additional methods and materials used to isolate NRCFs from the myocytes are unknown to us and therefore cannot be scrutinized for possible cell stressors [45].

A common thread in the connection of FGF23 and fibrosis has been initial systemic or tissue stress, while FGF23 interactions with cardiac fibroblasts in hyperphosphatemia models without additional organ injury has largely been left uninvestigated. It has been shown that FGF23 does not activate fibroblasts from normal kidneys. However, obstructed kidneys show elevations in *Fgf23, Fgfr1,* and *Fgfr4* expression, and FGF23 can activate fibroblasts from obstructed kidneys without increasing proliferation [31]. Injury-primed renal fibroblasts respond to FGF23 by enhancing TGFβ signaling and initiating two pro-fibrotic signaling pathways: FGFR4-PLCγ-TRPC6-Ca^{2+}-calcineurin/NFAT and FGFR4-PLCγ-TRPC6-Ca^{2+}-Reactive Oxygen Species (ROS)-JNK-TGFβ [30]. Additionally, *Ffg23* expression is increased in the hearts of rodents after 5/6 nephrectomy [11], Transverse Aortic Constriction (TAC) [50], and Myocardial Infarction (MI) [13,51], and *Fgfr4* expression is increased in rodent hearts after MI [13], suggesting that such a pathway may also be present in the heart to drive myocardial fibrosis. This is further supported by data demonstrating that patients with atrial fibrillation had significant increases in FGF23, FGFR4, αSMA, and COL1A1, and cultured human primary fibroblasts that were isolated with trypsin showed increases in ROS production with subsequent STAT3 and SMAD3 signaling in response to FGF23 treatment [52]. This data strongly supports the presence of an FGF23/FGFR4 mediated signaling mechanism to induce fibrosis in cardiac fibroblasts in models with injury. Though our high-phosphate diet model shows increased serum FGF23 and cardiac *Fgf23* expression, we do not observe an increase in *Fgfr1* or *Fgfr4* expression, a step that appears to be crucial for fibroblast activation by FGF23, indicating that administration of a high-phosphate diet does not sufficiently prime cardiac fibroblasts for FGF23 responsiveness.

It is possible that FGF23 can indirectly activate cardiac fibroblasts by inducing the expression of factors in myocytes that mediate the cardiac myocyte-fibroblast crosstalk. However, here we found that FGF23 does not increase the expression of such factors in NRVMs, indicating that it is unlikely that FGF23 is targeting cardiac myocytes to induce cardiac fibroblast activation. Several studies have reported that the heart can produce FGF23 [13,45,53], though the specific cell type(s) that serve as the source of cardiac FGF23 are not clear. *In vitro* studies using different models for cardiac myocytes and fibroblasts provided conflicting outcomes. One study reported *Fgf23* expression in NRVMs, NRCFs (>10 fold higher than NRVM mRNA), and AMCFs (>30 fold higher than NRVM mRNA) [13]. Another study observed *Fgf23* expression in NRVMs, but not in NRCFs [45]. We found that AMCFs produce and secrete FGF23 protein at levels detectable by ELISA, which was further elevated when cardiac fibroblasts were isolated from mice receiving a high-phosphate diet. It is well established that FGF23 can induce signaling in cardiac myocytes via activation of FGFR4 [12,18,54], and FGF23 can interact with FGFR1 in the presence of sKL [55]. FGFR1 and FGFR2 play a pro-survival and protective role after cardiovascular injury, and treatment with sKL alleviates cardiac fibrosis in mice [56,57]. Elevated plasma phosphate downregulates sKL levels [58], and though the intake of a high-phosphate diet increases FGF23 production in heart tissue and systemically, the serum phosphate levels are maintained, indicating that sKL may not be downregulated in this model. It is interesting to speculate that cardiac fibroblasts may secrete FGF23 that interacts with FGFR1 in the presence of sKL to initiate cardio-protective

mechanisms. Future studies will need to determine if a high-phosphate diet is sufficient to lower sKL levels to determine if an increase in FGF23/FGFR1 signaling in the heart is feasible, or if the local FGF23 elevation is more likely to activate FGFR4. Vitamin D Receptor (VDR) activity upregulates sKL [58], and high levels of FGF23 downregulates the synthesis of active vitamin D [59], therefore FGF23 may downregulate sKL indirectly via decreased VDR activity.

Future studies should determine a potential role of a high-phosphate diet not only in the priming of cardiac fibroblasts but also of cardiac myocytes and whether Adult Mouse Cardiac Myocytes (AMCM) respond to FGF23 differently than NRVMs, for example by secreting pro-fibrotic factors. Furthermore, the role of cardiac fibroblast-derived FGF23 needs to be determined, including potential pro-hypertrophic actions that could serve as a paracrine FGF23-driven mechanisms that contributes to hyperphosphatemia-associated cardiac hypertrophy, in addition to the pro-hypertrophic effects of bone-derived circulating FGF23. A mouse model with cardiac fibroblast-specific deletion of *Fgf23* will be needed to address this important question. Additionally, it should be determined if CKD can prime cardiac fibroblasts for FGF23 responsiveness. Various other CKD-associated pathologic changes, other than hyperphosphatemia, could serve as such priming events, such as systemic inflammation or increased serum concentrations of uremic toxins.

Conclusion

FGF23 on its own is a potent inducer of cardiac hypertrophy but not fibrosis. Hyperphosphatemia, which causes cardiac hypertrophy and fibrosis, does not induce FGF23 responsiveness in cardiac fibroblasts, suggesting that other factors than FGF23 drive cardiac fibrosis in hyperphosphatemia. Whether in the context of CKD cardiac fibroblasts respond to FGF23 needs to be determined. If it turns out that also in CKD FGF23 only targets cardiac myocytes and not cardiac fibroblasts, and that FGF23 promotes cardiac hypertrophy but not fibrosis, pharmacological blockade of FGFR4, which is the cardiac FGF23 receptor, would not serve as a therapeutic approach to prevent pathological cardiac remodeling and increase survival in CKD patients.

Author Statements

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C.F. and D.K. have served as consultants for Bayer. C.F. also consulted Calico Labs and U3Pharma GmbH, Germany.

C.Y. and C.F. are inventors on two pending patents (PCT/US2019/049211; PCT/US19/49161) and they are co- founders of a startup biotech company (Alpha Young LLC). Currently C.F. is the CSO of Alpha Young, and C.Y. is employed as the lead-scientist by Alpha Young. C.F. has a patent on FGFR inhibition (European Patent No. 2723391), and he received honoraria for publishing a book ("FGF23", Elsevier, ISBN9780128180365).

Author Contributions

I.C., B.R., and C.F. conceived and designed the research; I.C., B.R, and S.M.T. performed all experiments; I.C. and B.R. analyzed the data; I.C., B.R. and C.F. interpreted the results of experiments; S.M.T., D.K., C.Y., B.C., K.H., A.F. and Q.L. assisted with data interpretation and troubleshooting; I.C. prepared the figures; I.C. and C.F. drafted the manuscript; all authors edited and revised the manuscript and approved the final version of the manuscript.

References

1. Rai NK, Wang Z, Drawz PE, Connett J, Murphy DP. CKD Progression Risk and Subsequent Cause of Death: A Population-Based Cohort Study. Kidney Med. 2023; 5: 100604.
2. Zhou C, Shi Z, Ouyang N, Ruan X. Hyperphosphatemia and Cardiovascular Disease. Front Cell Dev Biol. 2021; 9: 644363.
3. Rennenberg RJ, Kessels AG, Schurgers LJ, van Engelshoven JM, de Leeuw PW, Kroon AA. Vascular calcifications as a marker of increased cardiovascular risk: a meta-analysis. Vasc Health Risk Manag. 2009; 5: 185- 97.
4. Kjeldsen SE. Hypertension and cardiovascular risk: General aspects. Pharmacol Res. 2018; 129: 95-9.
5. Wang Y, Gao L. Inflammation and Cardiovascular Disease Associated with Hemodialysis for End-Stage Renal Disease. Front Pharmacol. 2022; 13: 800950.
6. Heine GH, Seiler S, Fliser D. FGF-23: the rise of a novel cardiovascular risk marker in CKD. Nephrol Dial Transplant. 2012; 27: 3072-81.
7. Quarles LD. Endocrine functions of bone in mineral metabolism regulation. J Clin Invest. 2008; 118: 3820-8.
8. Gutierrez OM, Mannstadt M, Isakova T, Rauh-Hain JA, Tamez H, Shah A, et al. Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. N Engl J Med. 2008; 359: 584-92.
9. Scialla JJ, Parekh RS, Eustace JA, Astor BC, Plantinga L, Jaar BG, et al. Race, Mineral Homeostasis and Mortality in Patients with End-Stage Renal Disease on Dialysis. Am J Nephrol. 2015; 42: 25-34.
10. Koller L, Kleber ME, Brandenburg VM, Goliasch G, Richter B, Sulzgruber P, et al. Fibroblast Growth Factor 23 Is an Independent and Specific Predictor of Mortality in Patients with Heart Failure and Reduced Ejection Fraction. Circ Heart Fail. 2015; 8: 1059-67.
11. Leifheit-Nestler M, Grabner A, Hermann L, Richter B, Schmitz K, Fischer DC, et al. Vitamin D treatment attenuates cardiac FGF23/FGFR4 signaling and hypertrophy in uremic rats. Nephrol Dial Transplant. 2017; 32: 1493-503.
12. Grabner A, Amaral AP, Schramm K, Singh S, Sloan A, Yanucil C, et al. Activation of Cardiac Fibroblast Growth Factor Receptor 4 Causes Left Ventricular Hypertrophy. Cell Metab. 2015; 22: 1020-32.
13. Hao H, Li X, Li Q, Lin H, Chen Z, Xie J, et al. FGF23 promotes myocardial fibrosis in mice through activation of beta-catenin. Oncotarget. 2016; 7: 64649-64.
14. Leifheit-Nestler M, Haffner D. Paracrine Effects of FGF23 on the Heart. Front Endocrinol (Lausanne). 2018; 9: 278.

15. Grabner A, Schramm K, Silswal N, Hendrix M, Yanucil C, Czaya B, et al. FGF23/FGFR4-mediated left ventricular hypertrophy is reversible. Sci Rep. 2017; 7: 1993.

16. Faul C. FGF23 effects on the heart-levels, time, source, and context matter. Kidney Int. 2018; 94: 7-11.

17. Li D, Wu J, Bai Y, Zhao X, Liu L. Isolation and culture of adult mouse cardiomyocytes for cell signaling and in vitro cardiac hypertrophy. J Vis Exp. 2014: 51357.

18. Faul C, Amaral AP, Oskouei B, Hu MC, Sloan A, Isakova T, et al. FGF23 induces left ventricular hypertrophy. J Clin Invest. 2011; 121: 4393-408.

19. Tarbit E, Singh I, Peart JN, Rose'Meyer RB. Biomarkers for the identification of cardiac fibroblast and myofibroblast cells. Heart Fail Rev. 2019; 24: 1-15.

20. Roy SG, Nozaki Y, Phan SH. Regulation of alpha-smooth muscle actin gene expression in myofibroblast differentiation from rat lung fibroblasts. Int J Biochem Cell Biol. 2001; 33: 723-34.

21. Ehrlich HP, Allison GM, Leggett M. The myofibroblast, cadherin, alpha smooth muscle actin and the collagen effect. Cell Biochem Funct. 2006; 24: 63-70.

22. Folger PA, Zekaria D, Grotendorst G, Masur SK. Transforming growth factor-beta-stimulated connective tissue growth factor expression during corneal myofibroblast differentiation. Invest Ophthalmol Vis Sci. 2001; 42: 2534-41.

23. Zhang H, Tian L, Shen M, Tu C, Wu H, Gu M, et al. Generation of Quiescent Cardiac Fibroblasts From Human Induced Pluripotent Stem Cells for In Vitro Modeling of Cardiac Fibrosis. Circ Res. 2019; 125: 552-66.

24. Clinkenbeard EL, Noonan ML, Thomas JC, Ni P, Hum JM, Aref M, et al. Increased FGF23 protects against detrimental cardio-renal consequences during elevated blood phosphate in CKD. JCI Insight. 2019; 4: e123817.

25. Swaney JS, Roth DM, Olson ER, Naugle JE, Meszaros JG, Insel PA. Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. Proc Natl Acad Sci USA. 2005; 102: 437-42.

26. Lijnen P, Petrov V. Transforming growth factor-beta 1-induced collagen production in cultures of cardiac fibroblasts is the result of the appearance of myofibroblasts. Methods Find Exp Clin Pharmacol. 2002; 24: 333-44.

27. Santiago JJ, Dangerfield AL, Rattan SG, Bathe KL, Cunnington RH, Raizman JE, et al. Cardiac fibroblast to myofibroblast differentiation in vivo and in vitro: expression of focal adhesion components in neonatal and adult rat ventricular myofibroblasts. Dev Dyn. 2010; 239: 1573-84.

28. Moita MR, Silva MM, Diniz C, Serra M, Hoet RM, Barbas A, et al. Transcriptome and proteome profiling of activated cardiac fibroblasts supports target prioritization in cardiac fibrosis. Front Cardiovasc Med. 2022; 9: 1015473.

29. Huang Q, Lan F, Wang X, Yu Y, Ouyang X, Zheng F, et al. IL-1beta-induced activation of p38 promotes metastasis in gastric adenocarcinoma via upregulation of AP-1/c-fos, MMP2 and MMP9. Mol Cancer. 2014; 13: 18.

30. Smith ER, Holt SG, Hewitson TD. FGF23 activates injury-primed renal fibroblasts via FGFR4-dependent signalling and enhancement of TGF-beta autoinduction. Int J Biochem Cell Biol. 2017; 92: 63-78.

31. Smith ER, Tan SJ, Holt SG, Hewitson TD. FGF23 is synthesised locally by renal tubules and activates injury- primed fibroblasts. Sci Rep. 2017; 7: 3345.

32. Pellman J, Zhang J, Sheikh F. Myocyte-fibroblast communication in cardiac fibrosis and arrhythmias: Mechanisms and model systems. J Mol Cell Cardiol. 2016; 94: 22-31.

33. Kakkar R, Lee RT. Intramyocardial fibroblast myocyte communication. Circ Res. 2010; 106: 47-57.

34. Matsuoka I, Nakane A, Kurihara K. Induction of LIF-mRNA by TGF-beta 1 in Schwann cells. Brain Res. 1997; 776: 170-80.

35. Yao Z, Fenoglio S, Gao DC, Camiolo M, Stiles B, Lindsted T, et al. TGF-beta IL-6 axis mediates selective and adaptive mechanisms of resistance to molecular targeted therapy in lung cancer. Proc Natl Acad Sci USA. 2010; 107: 15535-40.

36. Strand DW, Liang YY, Yang F, Barron DA, Ressler SJ, Schauer IG, et al. TGF-beta induction of FGF-2 expression in stromal cells requires integrated smad3 and MAPK pathways. Am J Clin Exp Urol. 2014; 2: 239-48.

37. Yoshiko Y, Wang H, Minamizaki T, Ijuin C, Yamamoto R, Suemune S, et al. Mineralized tissue cells are a principal source of FGF23. Bone. 2007; 40: 1565-73.

38. Leifheit-Nestler M, Grosse Siemer R, Flasbart K, Richter B, Kirchhoff F, Ziegler WH, et al. Induction of cardiac FGF23/FGFR4 expression is associated with left ventricular hypertrophy in patients with chronic kidney disease. Nephrol Dial Transplant. 2016; 31: 1088-99.

39. Gutierrez OM, Januzzi JL, Isakova T, Laliberte K, Smith K, Collerone G, et al. Fibroblast growth factor 23 and left ventricular hypertrophy in chronic kidney disease. Circulation. 2009; 119: 2545-52.

40. Yilmaz MI, Sonmez A, Saglam M, Yaman H, Kilic S, Demirkaya E, et al. FGF-23 and vascular dysfunction in patients with stage 3 and 4 chronic kidney disease. Kidney Int. 2010; 78: 679-85.

41. Seeherunvong W, Abitbol CL, Chandar J, Rusconi P, Zilleruelo GE, Freundlich M. Fibroblast growth factor 23 and left ventricular hypertrophy in children on dialysis. Pediatr Nephrol. 2012; 27: 2129-36.

42. Hsu HJ, Wu MS. Fibroblast growth factor 23: a possible cause of left ventricular hypertrophy in hemodialysis patients. Am J Med Sci. 2009; 337: 116-22.

43. Seiler S, Reichart B, Roth D, Seibert E, Fliser D, Heine GH. FGF-23 and future cardiovascular events in patients with chronic kidney disease before initiation of dialysis treatment. Nephrol Dial Transplant. 2010; 25: 3983-9.

44. Kirkpantur A, Balci M, Gurbuz OA, Afsar B, Canbakan B, Akdemir R, et al. Serum fibroblast growth factor- 23 (FGF-23) levels are independently associated with left ventricular mass and myocardial performance index in maintenance haemodialysis patients. Nephrol Dial Transplant. 2011; 26: 1346-54.

45. Leifheit-Nestler M, Kirchhoff F, Nespor J, Richter B, Soetje B, Klintschar M, et al. Fibroblast growth factor 23 is induced by an activated renin-angiotensin-aldosterone system in cardiac myocytes and promotes the pro-fibrotic crosstalk between cardiac myocytes and fibroblasts. Nephrol Dial Transplant. 2018; 33: 1722-34.

46. Hu Z, Chen Y, Gao M, Chi X, He Y, Zhang C, et al. Novel strategy for primary epithelial cell isolation: Combination of hyaluronidase and collagenase I. Cell Prolif. 2023; 56: e13320.

47. Daniels JT, Kearney JN, Ingham E. Human keratinocyte isolation and cell culture: a survey of current practices in the UK. Burns. 1996; 22: 35-9.

48. Chen RH, Zhu J, Zhang RZ, Wang SY, Li Y. The tolerance of human epidermal cells to trypsinization in vitro. Cell Tissue Bank. 2020; 21: 257-64.

49. Xie Z, Shi J, Zong M, Xu Q, Liu C, Wen J, et al. Isolation and Culture of Primary Human Gingival Epithelial Cells using Y-27632. J Vis Exp. 2021.

50. Slavic S, Ford K, Modert M, Becirovic A, Handschuh S, Baierl A, et al. Genetic Ablation of Fgf23 or Klotho Does not Modulate Experimental Heart Hypertrophy Induced by Pressure Overload. Sci Rep. 2017; 7: 11298.

51. Andrukhova O, Slavic S, Odorfer KI, Erben RG. Experimental Myocardial Infarction Upregulates Circulating Fibroblast Growth Factor-23. J Bone Miner Res. 2015; 30: 1831-9.

52. Dong Q, Li S, Wang W, Han L, Xia Z, Wu Y, et al. FGF23 regulates atrial fibrosis in atrial fibrillation by mediating the STAT3 and SMAD3 pathways. J Cell Physiol. 2019; 234: 19502-10.

53. Gheorghiade M, Zannad F, Sopko G, Klein L, Pina IL, Konstam MA, et al. Acute heart failure syndromes: current state and framework for future research. Circulation. 2005; 112: 3958-68.

54. Han X, Cai C, Xiao Z, Quarles LD. FGF23 induced left ventricular hypertrophy mediated by FGFR4 signaling in the myocardium is attenuated by soluble Klotho in mice. J Mol Cell Cardiol. 2020; 138: 66-74.

55. Chen G, Liu Y, Goetz R, Fu L, Jayaraman S, Hu MC, et al. alpha-Klotho is a non-enzymatic molecular scaffold for FGF23 hormone signalling. Nature. 2018; 553: 461-6.

56. Matsiukevich D, House SL, Weinheimer C, Kovacs A, Ornitz DM. Fibroblast growth factor receptor signaling in cardiomyocytes is protective in the acute phase following ischemia-reperfusion injury. Front Cardiovasc Med. 2022; 9: 1011167.

57. Chen WY. Soluble Alpha-Klotho Alleviates Cardiac Fibrosis without Altering Cardiomyocytes Renewal. Int J Mol Sci. 2020; 21: 2186.

58. Li SS, Sheng MJ, Sun ZY, Liang Y, Yu LX, Liu QF. Upstream and downstream regulators of Klotho expression in chronic kidney disease. Metabolism. 2023; 142: 155530.

59. Blau JE, Collins MT. The PTH-Vitamin D-FGF23 axis. Rev Endocr Metab Disord. 2015; 16: 165-74.