TESTING BONE CELL MODELS RESPONSIVE TO A SOLUBLE FORM OF KLOTHO

Anna Bonfitto

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Master's Thesis Committee

____

Kenneth E. White, Ph.D., Chair

____

Brittney-Shea Herbert, Ph.D.

____

Bryan P. Scheider, M.D

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Anna Bonfitto

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Fibroblast growth factor-23 (FGF23) is a hormone produced in bone that acts upon the kidney to control blood phosphate and 1,25-$(OH)_2$ vitamin D concentrations. Chronic kidney disease-mineral bone disorder (CKD-MBD) is a major public health problem, affecting 1 in 8 individuals. These patients can have markedly elevated FGF23 at end stage disease which is associated with metabolic bone anomalies, left ventricular hypertrophy, as well as increased mortality (>6-fold). The FGF23 co-receptor αKlotho (αKL) is a membrane-bound protein (mKL) that forms heteromeric complexes with FGF receptors (FGFRs) to initiate intracellular signaling. It also circulates as a cleavage product of mKL ('cleaved', or cKL). Previously, a patient with increased plasma cKL from a balanced translocation between chromosomes 9 and 13 in the *KLOTHO* gene presented with metabolic bone disease and a complex endocrine profile, despite hypophosphatemia. The lack of a reliable cell model in which to study potential FGF23-cKL interactions is a major hurdle for the field of phosphate metabolism. The goal of the present studies was to test and characterize bone cell lines that may respond to FGF23 and/or cKL, permitting study of novel aspects of phosphate handling and control of FGF23 expression. It was confirmed that stable delivery of cKL via AAV2/8 to wild type (WT) and KL-KO mice resulted in highly elevated bone FGF23 mRNA. MC3T3 (mouse) and ROS (rat) osteoblastic cell lines were tested for p-ERK1/2 responses to control FGFs, as well as FGF23 and cKL, alone or in combination. Importantly, both cell lines

demonstrated responsiveness to FGF23+cKL only, and not the individual factors. To test responsiveness at the cell level, EGR1 mRNA was tested as an index of FGFR activity and showed modest increases with the same treatments, supporting that other factors may be required for full transcriptional effects. The present studies show that MC3T3 have FGF-dependent signaling capabilities, and that the combination of FGF23+cKL is required for efficient MAPK signaling. These results demonstrated that cKL provision is permissive for efficient FGF23 signaling in bone, and revealed important implications for the regulation of FGF23 and cKL in Mendelian, and common, genetic disorders of phosphate handling and biomineralization.

Kenneth E. White, Ph.D., chair

Table of Contents

List of Tables viii

List of Figures ix

Abbreviations x

Introduction 1

Phosphate 1

Phosphate regulation 1

Metabolic disorders of phosphate dysregulation 4

Fibroblast growth factor-23 5

FGF23 structure 5

FGF23 pathway and regulation 5

FGF23 tissues and organs 6

Klotho 6

Klotho structure 6

Klotho pathway and regulation 7

Klotho tissues and organs 7

cKL and FGF23 interactions 9

Chronic kidney disease-mineral bone disorder 10

Mechanism 10

Incidence and impact 10

Hypothesis 11

Materials and Methods 12

WT and KL-KO mice 12

WT and KL-KO AAV 12

ROS (rat osteoblast) and MC3T3 (mouse osteoblast) cell lines 12
Immunoblotting 13
RNA isolation and qPCR..... 16
Results..... 18
FGF23 and cKL measurements obtained from animal studies 18
Assessing FGF23 and Klotho activity in cell model studies 25
Discussion 31
Rationale..... 31
Klotho upregulates FGF23 *in vivo*..... 31
Klotho and FGF23 interact *in vitro* 32
Future research 33
Conclusions..... 34
References 36
Curriculum Vitae

List of Tables

Table 1 Antibodies for western blot…………………………………………………………………………15

Table 2 Real-time PCR primers……………………………………………………………………………..17

List of Figures

Figure 1 Phosphate handling ……………………………………………………………………………………3

Figure 2 FGF23 and cKL pathways……………………………………………………………………………..8

Figure 3 *FGF23* fold-changes by qPCR in wild type animals……..................................…….19

Figure 4 *FGF23* percent changes in KL KO mice………………………………………………….………………20

Figure 5 *EGR1* mRNA changes in KL KO mice………………………………………………………….…….21

Figure 6 *EGR1* and *C-fos* mRNA from KL KO mice……………………………………………..…………….23

Figure 7 *EGR1* mRNA fold-changes in ROS cells…………………………………………………………….25

Figure 8 *EGR1* mRNA fold-changes in MC3T3 cells……………………………………………………..……26

Figure 9 Western blot of treated ROS cells……………………………………………………….…………28

Figure 10 Western blot of treated MC3T3 cells……………………………………………………….……….29

Abbreviations

AAV	adeno-associated virus
ADHR	autosomal dominant hypophosphatemic rickets
ARHR	autosomal recessive hypophosphatemic rickets
CKD	chronic kidney disease
CKD-MBD	chronic kidney disease-mineral bone disorder
cKL	cleaved/soluble Klotho
DMP1	dentin matrix protein 1
EGR1	early growth response protein 1
ENPP1	ectonucleotide pyrophosphatase/phosphodiesterase 1
FGF2	fibroblast growth factor-2
FGF8	fibroblast growth factor-8
FGF23	fibroblast growth factor-23
FGFR	fibroblast growth factor receptor
KL	klotho
KO	knockout
mKL	membrane-bound klotho
Npt2a	type II sodium phosphate co-transporter a
Npt2c	type II sodium phosphate co-transporter c

p-Erk	phospho-Erk
PHEX	phosphate-regulating gene with homologies to endopeptidases on the X chromosome
Pi	inorganic phosphorous
PTH	parathyroid hormone
TIO	tumor-induced osteomalacia
WT	wild type
XLH	x-linked hypophosphatemic rickets
$1,25\text{-}(OH)_2D$	active vitamin D
$1\alpha(OH)$ase	1-α hydroxylase
βME	beta mercaptoethanol

Introduction

Phosphate

Phosphate regulation

Phosphorous is vital both structurally and functionally in mammals. Inorganic phosphorus (Pi), the most common form utilized in biological processes such as development, bone formation, and cellular pathways, is present as an ion (PO_4) [1]. In addition to being an integral part of the mammalian skeleton, phosphorus is also a crucial component of the backbone of nucleic acids. A typical adult has a total phosphate serum level of 11-12 mg/dL, with 3-4 mg/dL being inorganic phosphate. Eighty-five percent of phosphorus is present in the form of hydroxyapatite in the skeleton and teeth [2, 3].

Phosphorus homeostasis is maintained by a balance of absorption through the gastrointestinal tract and excretion by the kidneys [1], as well as retention and release of phosphorus in bones [4] (Figure 1). The main source of phosphorus is dietary, and averages 20 mg/kg daily [5]; the majority of ingested phosphorus is absorbed through the intestine. Further absorption is induced by the active form of vitamin D, 1,25-$(OH)_2D$. The amount of 1,25-$(OH)_2D$ is determined in part by the enzymatic conversion from 25-hydroxy vitamin D by 1-α-hydroxylase (1α(OH)ase), which is produced by the kidney [6].

Phosphorus is excreted and reabsorbed in the kidneys according to levels of various factors, including vitamin D, parathyroid hormone (PTH) and fibroblast growth factor-23 (FGF23). Feedback loops ensure that phosphate levels are regulated in healthy individuals. PTH increases the amount of vitamin D by upregulating 1-α hydroxylase, which causes more absorption of phosphate in the intestine [6]. The increase in 1,25-$(OH)_2D$ then decreases the amount of PTH. Higher vitamin D levels also upregulate FGF23 production to complete the feedback loop (Figure 1).

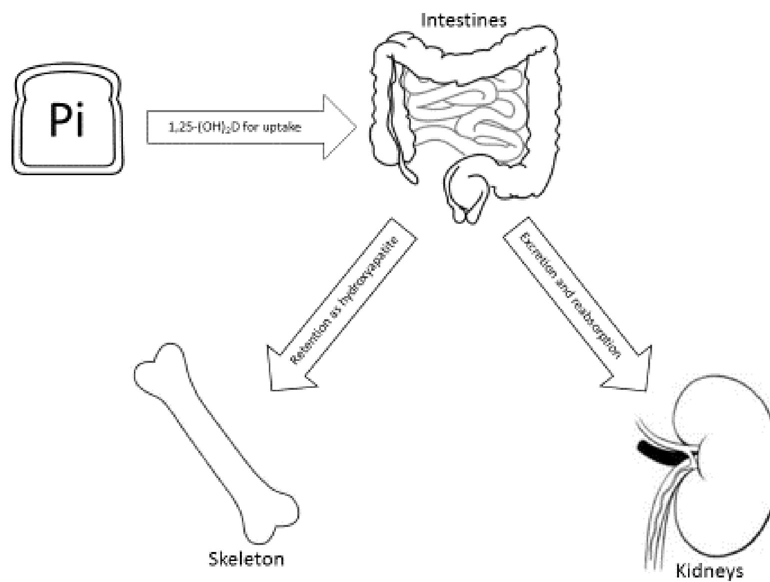

Figure 1 Phosphate handling
Dietary phosphorus is absorbed by the intestines with the help of 1,25-$(OH)_2D$, and is stored in the skeleton. Phosphorus is also filtered by the kidneys; some is excreted in the urine and the amount needed to maintain appropriate blood phosphate levels is reabsorbed.

Metabolic disorders of phosphate dysregulation

Autosomal dominant hypophosphatemic rickets (ADHR) is a disorder caused by a gain of function mutation of the gene *FGF23* [7]. Mutations stabilize a cleavage site at amino acids 176-179 and the resulting increase in full-length FGF23 contributes to renal phosphate wasting. Other symptoms of ADHR include rickets, osteomalacia, and inappropriately normal serum levels of 1,25-$(OH)_2D$ along with hypophosphatemia [1]. In addition, it has been shown that a deficiency of iron upregulates expression of *FGF23* and contributes to the ADHR phenotype [8, 9].

Autosomal recessive hypophosphatemic rickets (ARHR) manifests similar phenotypes as ADHR, including deformities of the lower extremities, phosphate wasting, and normal 1,25-$(OH)_2D$ serum levels along with hypophosphatemia [1]. Two genes have been associated with ARHR; mutations in both *DMP1* and *ENPP1* cause the disorder by loss of function [10-12]. A DMP1 null mouse model also showed severe skeletal muscle deficits [13]. Despite the underlying mutation, ARHR is characterized by high levels of FGF23 as in ADHR, but the mechanism is not as well understood [1].

X-linked hypophosphatemic rickets (XLH) shares features of ADHR and ARHR such as rickets, lower limb deformities, osteomalacia, hypophosphatemia, and inappropriately normal 1,25-$(OH)_2D$ levels in the serum [1]. FGF23 is also elevated in some patients with XLH, but the primary cause of phosphate wasting is excessive urinary excretion. The

mutated gene for XLH is *PHEX*, located at Xp22.1; over 350 mutations have been found to cause disease [1, 14]. Although the specific mechanism for the loss of function of *PHEX* has not been elucidated, the loss does increase FGF23 levels and a PHEX transgene has been used to partially reverse mineralization defects in bones of a mouse model of XLH [15]. Also, the same phenomenon of iron upregulating FGF23 in ADHR has been observed in XLH [16].

Fibroblast Growth Factor-23

FGF23 structure

Fibroblast growth factor 23 (FGF23) is a hormone that was found to be the phosphaturic factor active in ADHR [7]. The structure of FGF23 is that of a glycoprotein; there are 251 amino acids, and the C-terminal end binds to its co-receptor Klotho [17]. FGF23 is the product of three exons located at 12p13.3 [7]. High levels of serum phosphate and 1,25-$(OH)_2D$ upregulate FGF23, which is primarily made by osteocytes and osteoblasts [17]. It has been determined that in the murine FGF23 promoter, the region from -200 and -400 base pairs contains the element that responds to 1,25-$(OH)_2D$ [18].

FGF23 pathway and regulation

Investigations have found that FGF23 is also a key factor in the phosphate wasting of several other hypophosphatemic disorders, including tumor-induced osteomalacia (TIO),

XLH, and ARHR [19]. FGF23 decreases levels of phosphate through inhibition of Npt2a and Npt2c, two renal phosphate cotransporters. In addition to phosphate levels and 1,25-$(OH)_2D$, there is also evidence that FGF23 is upregulated by low iron levels [20]. FGF23 is also part of a negative feedback loop that decreases the amount of 1,25-$(OH)_2D$.

FGF23 tissues and organs

Although FGF23 is produced in bone by osteocytes, the two main target organs are the kidney and parathyroid gland. FGF23, along with the parathyroid hormone, act to downregulate the Npt2a and Npt2c transporters in the kidneys [17]. The kidney is also the primary site of action for FGF23 when bound to its co-receptor Klotho; FGF23 has also been recently shown to contribute to mineralization of bone in a pathway independent of Klotho and vitamin D levels [21].

Klotho

Klotho structure

αKlotho is found as both a membranous and a soluble protein. The membranous form consists of 5 exons, whereas soluble Klotho contains 3 exons in mice and 5 exons in humans [22]. The human *Klotho* is at 13.33 in the genome; murine *Klotho* is on chromosome 5. The membrane-bound form of Klotho (mKL) associates with FGF

receptors for FGF23-mediated intracellular signaling; the ‘cleaved’ portion of Klotho (‘cKL’) has been found to be a soluble co-receptor for FGF23.

Klotho pathway and regulation

Klotho decreases the amount of 1,25-$(OH)_2D$ by inhibiting 1-α-hydroxylase [23]. The gene was first identified from an advanced aging phenotype in deficient animals; the over-abundance of 1,25-$(OH)_2D$ and resulting hyperphosphatemia was thought to be the culprit of this phenotype by damaging organs [20]. Klotho also binds to FGF23, which increases the amount of phosphate excreted in the urine.

Klotho tissues and organs

The soluble form of Klotho is expressed in the pituitary gland, brain, testis, pancreas, thyroid, and kidneys [22]. Levels decrease early in the onset of chronic kidney disease, making it a potential biomarker for kidney function [24]. In addition, high levels of cKL in chronic hemodialysis patients has recently been associated with mortality risk [25]. Klotho is highly expressed in the kidney, where the interaction with FGF23 occurs to regulate phosphate homeostasis by inhibiting Npt2a and Npt2c in the renal proximal tubules; the net effect is a decrease in blood phosphate concentrations (Figure 2).

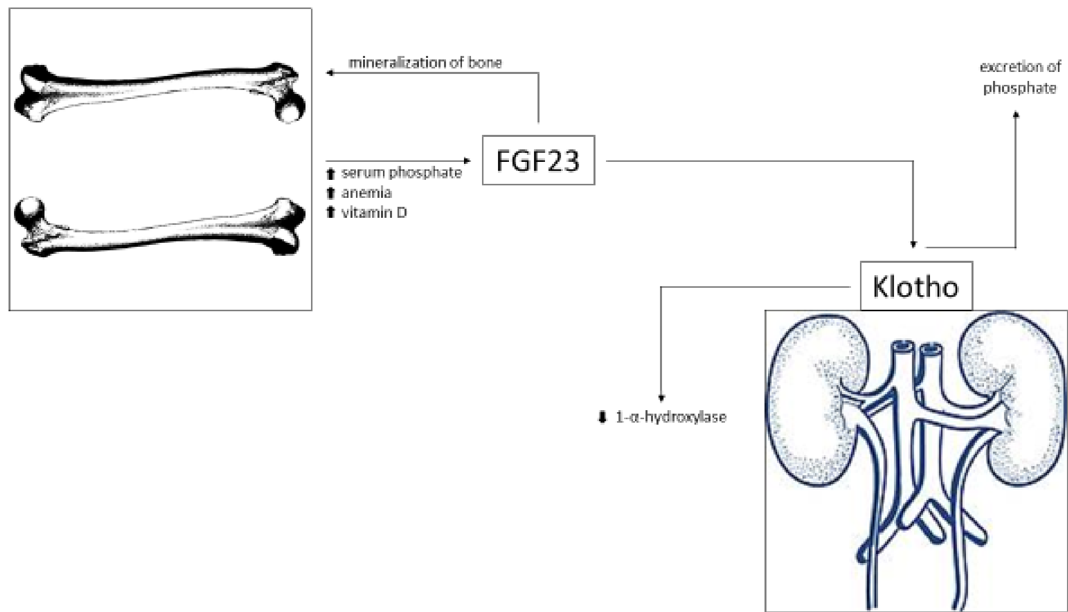

Figure 2 FGF23 and cKL pathways
Osteocytes produce FGF23, which is upregulated by increased serum phosphate, iron-induced anemia, and higher levels of vitamin D in the serum. FGF23 contributes to the mineralization of bone; in concert with Klotho in the kidneys, FGF23 increases excretion of phosphate in the urine. Klotho and FGF23 activity decrease the amount of active vitamin D by inhibiting the action of 1-α-hydroxylase.

cKL and FGF23 interactions

An interesting patient presented with hypophosphatemic rickets and elevated FGF23 levels [26]. The patient also had elevated serum PTH. When nutrition was ruled out as an underlying cause of rickets, sequencing was performed. Genes for *FGF23*, *PHEX*, and *DMP1* showed no mutations; if the cause of hypophosphatemia and hyperparathyroidism was due to a previously described disorder of phosphate handling, a mutation in one of these genes would be expected. Further testing was done by cytogenetics, and a balanced translocation was detected between chromosomes 9 and 13. The breakpoint, t(9,13)(q21.12;q13.1), occurred in the *Klotho* gene. This patient represented a novel case where a *Klotho* gene rearrangement led to Klotho over-expression and high serum levels of the cKL form as the cause of hypophosphatemia.

In response to this case study, recent research utilizing a mouse model sought to further characterize the relationship between FGF23 and Klotho [27]. cKL was overexpressed in mice via adeno-associated virus (AAV) delivery to study the effect on FGF23. Animals had significant increases in levels of bone *FGF23* mRNA along with symptoms characteristic of FGF23-dependent hypophosphatemic disorders.

Chronic kidney disease-mineral bone disorder

Mechanism

Diabetes and high blood pressure are significant risk factors for developing chronic kidney disease (CKD), which is characterized by a gradual loss of kidney function. As the kidneys lose their ability to excrete phosphate, FGF23 levels increase in an attempt to compensate. FGF23 can be utilized as a marker for the progression of CKD by following plasma concentrations of both c-terminal and intact FGF23 protein [28]. FGF23 binds to Klotho in the kidneys, where the complex increases phosphate excretion; an overabundance contributes to hypophosphatemia in a healthy individual. Hyperphosphatemia from the organ's reduced ability to excrete phosphate, along with left ventricular hypertrophy from elevated FGF23 levels, are major concerns in CKD patients. Dialysis can help a patient's overall health by filtering blood through an artificial kidney, but the specific problem of hyperphosphatemia is not alleviated by this therapy [29].

Incidence and impact

CKD is a very common disease, affecting 1 in 8 people [30]. While rates of increase in the disease have tapered the last decade [31], CKD remains prevalent enough to increase health care costs and morbidity rates. Hyperphosphatemia due to reduced kidney function is not the only complication from chronic kidney disease. Associated deficiencies in bone and mineral metabolism have led to classification of CKD-mineral

and bone disorder (CKD-MBD) for most patients. In addition to reduced kidney function, vascular calcifications and bone abnormalities may occur in CKD-MBD patients due to abnormal serum levels of 1,25-$(OH)_2D$ or phosphorus [32]. There are several other serious health consequences that can arise from CKD-MBD, including acute kidney injury, cardiovascular disease, and mortality [31].

Hypothesis

The interaction between FGF23 and Klotho plays an important role in CKD-MBD and other disorders of phosphate regulation. A clinical case study involving a patient with hypophosphatemic rickets utilized cytogenetics when sequences of *FGF23*, *PHEX*, and *DMP1* were consistent with wild-type sequences. A balanced translocation that led to high serum *Klotho* was detected: t(9,13)(q21.12;q13.1) [26]. In addition to hypophosphatemia, serum levels of FGF23 were elevated in the patient. This case highlights the complex interactions between FGF23 and cKL. Therefore, a reliable bone cell model for these genes and their products would be beneficial for the study of phosphate handling disorders. This project was intended to test the hypothesis that there is a suitable cell line for the study of FGF23 and Klotho interactions.

Materials and Methods

WT and KL-KO mice

Animal studies were performed according to the Institutional Animal Care and Use Committee (IACUC) for Indiana University, and comply with the NIH guidelines for the use of animals. Adeno-associated virus expressing cKL (AAV-cKL, AAV-LacZ or vehicle phosphate-buffered saline, PBS) was delivered to 4 week old WT or αKL-null mice by retro-orbital injection at $1x10^{11}$ genomic copies/mouse. All mice were euthanized by CO2 inhalation followed by cervical dislocation.

WT and KL-KO AAV

The cDNA encoding residues 35-983 of the extracellular domain of mouse cKL with a CD33 N-terminal signal sequence, or β-galactosidase gene cassette (LacZ) as a control, was packaged into a recombinant hybrid adeno-associated viral vector 2/8 (AAV; RegenX Biosciences).

ROS (rat osteoblast) and MC3T3 (mouse osteoblast) cell lines

Cells were cultured in MEMα (Sygma) supplemented with 10% fetal bovine serum, 1% sodium pyruvate (Gibco by Life Technologies), 1% L-glutamine 100x solution (Hyclone), and 1% Penicillin-Streptomycin solution (Hyclone). Cells were incubated with 5% CO_2 at 37˚ C and medium was replaced after 2-3 days.

Immunoblotting

Cells were seeded at $1.0x10^5$ cells per well in 12-well plates. Each well was treated for 10 minutes with 30 nM cKL, 115 nM FGF23, or a combination of 30nM cKL and 115nM FGF23 and washed once with PBS as previously described [33]. Control wells were treated for ten minutes with 1 µl of either FGF2 or FGF8 (100 ng/mL) and washed with PBS. Each well was immediately harvested with 250 µl of 1x cell lysis buffer (Cell Signalling) with 0.1% 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, Santa Cruz). Lysates were collected in 1.7 ml microcentrifuge tubes and stored at -20˚ C.

Lysates thawed on ice were boiled for five minutes and centrifuged at maximum speed for one minute. Protein concentrations were obtained by Coomassie Plus (Bradford) Assay kit (ThermoScientific). Fresh Laemmli Sample Buffer (BioRad) with 5% β-mercaptoethanol (Fisher Scientific) was added to samples in an equal volume. Samples were then electrophoresed on an Any KD Mini-Protean TGX gel (BioRad); 30 µg of lysate was loaded in each well. TransBlot Turbo transfer system (BioRad) was used to transfer gel material to a 0.2um PVDF membrane for 3 minutes.

Membranes were blocked in a solution of 5% dry milk powder (Scientific Inc.) in 1xTBS (Tris-buffered saline, BioRad) for one hour at room temperature. Primary antibody was added according to Table 1 and incubated overnight at 4˚ C. Membranes were given four, ten-minute washes with 1xTBS and 0.05% Tween 20 (USB Corporation). Secondary

antibody was added to membranes for one hour at room temperature according to Table 1; membranes were then washed three times for ten minutes per wash with 1xTBS and 0.05% Tween 20. Enhanced chemoluminescence (ECL, GE Healthcare) was used to detect horseradish peroxidase (HRP) activity and membranes were imaged with the Amersham Imager 600 by GE.

Table 1 Antibodies for western blot

	Antibody	Vendor	Dilution
Primary (rabbit)	Phospho-ERK	Cell Signaling	1:1000
	Total ERK	Promega	1:1000
	Phospho-AKT	Cell Signaling	1:2000
Secondary	Goat Anti-Rabbit IgG-HRP Conjugated	Bio-rad	1:2000

RNA isolation and qPCR

Cells in 12-well plates, $1.0x10^5$ cells per well, were treated overnight with equal amounts of the indicated factors. Wells were washed once with PBS and harvested with 300 µl of RLT lysis buffer from the RNeasy Mini kit (Qiagen, Inc). Lysates were stored at -20˚ C if not used immediately. Either fresh lysates or those thawed on ice were then processed for RNA extraction following the RNeasy Mini kit protocol. Femur/bone RNA from mice was harvested using Trizol reagent (Life Technologies) as previously described[33]

Extracted RNA samples were diluted with RNAse-free water for a concentration of 10 ng/µl. Each well of a MicroAmp Optical 96-well reaction plate (Applied Biosystems) contained 12.5 µl TaqMan RT-PCR mix (2x), 0.625 µl TaqMan RT Enzyme Mix (40x), 0.375 µl RNase free water, 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, and 0.5 µl 5 µM probe. Primers are described in Table 2. The quantitative PCR program on the StepOnePlus Real-Time PCR System (Thermo Fisher) was 30 min 48˚ C, 10 min 95˚ C, then 40 cycles of 15 sec 95˚ C and 1 min 60˚ C. Samples were analyzed in duplicate using the comparative C_T ($\Delta\Delta C_T$) experiment workflow. The fold change of gene expression was tested by the $2^{-\Delta\Delta CT}$ method. Comparisons of control RNA expression and test sample RNA expression were calculated by Student's t-test with a threshold of $p<0.05$.

Table 2 Real-time PCR primers

Gene	Forward primer	Reverse primer	Probe	Vendor
Mouse FGF23	5’CCTTCTCCCAGTT CCTGGC 3’	5’GGGCGAACA GTGTAGAAATG C 3’	5’ FAM- CGCAGGAACGAGGTCC CGCTG-TAMRA 3’	IDT
ABI	5’ AGCCGAGCGAACA ACCCTAT 3’	5’ CGCCTTCTCATT ATTCAGAG 3’	5’ FAM- AGCACCTGACCACAGA GTCCTTTTCTGACA- TAMRA 3’	IDT
Mouse C-fos	PURCHASED FROM ABI	PROPIETARY SEQUENCE	CAT #: Mm00487425_m1	ABI
Rat EGR1	5’ AGCGCTTTCAATCC TCAAGC 3’	5’ CAGAAAAGGAC TCTGTGGTCAG G 3’	5’ FAM- AGCCGAGCGAACAACC CTACGAG TAMRA3’	IDT

Results

FGF23 and cKL measurements obtained from animal studies

The *in vivo* relationship between FGF23 and cKL was first established in wild-type animals. cKL was administered to experimental animals by AAV and control animals received phosphate buffered saline (PBS). Fold changes in *FGF23* mRNA increased significantly ($p<0.05$) in animals given cKL versus controls (Figure 3). Control mice given PBS ($n=4$) averaged 1.4 copies of FGF23 and mice administered cKL ($n=5$) averaged 354.4 FGF23 copies.

The relationship between FGF23 and cKL was further tested in mice with Klotho knocked out. Klotho knock-out mice (KL KO) were compared to wild type mice as well as to KL KO mice administered with cKL (Figure 4). Bone mRNA was isolated and analyzed for *FGF23* mRNA; FGF23 is primarily produced in bone, so induction of transcription would be evident from bone samples. Knock out animals showed a significant increase in *FGF23* mRNA in the bone compared to wild type animals. When cKL was given to KL KO animals, there was additional increase in *FGF23* mRNA levels versus PBS-treated KL KO controls. *Egr1* mRNA was also tested from the bone of wild type, KL KO, and KL KO given cKL mice (Figure 5). Egr1 has been identified as a factor that responds to FGF23-cKL activity [34, 35]. Although there was a trend, *Egr1* mRNA levels in either KL KO mice or KL KO mice given cKL did not differ significantly ($p<0.07$) from wild type mice.

Potentially, the long-term nature (4 weeks) of this study may have resulted in compensatory control and normalization of Egr1.

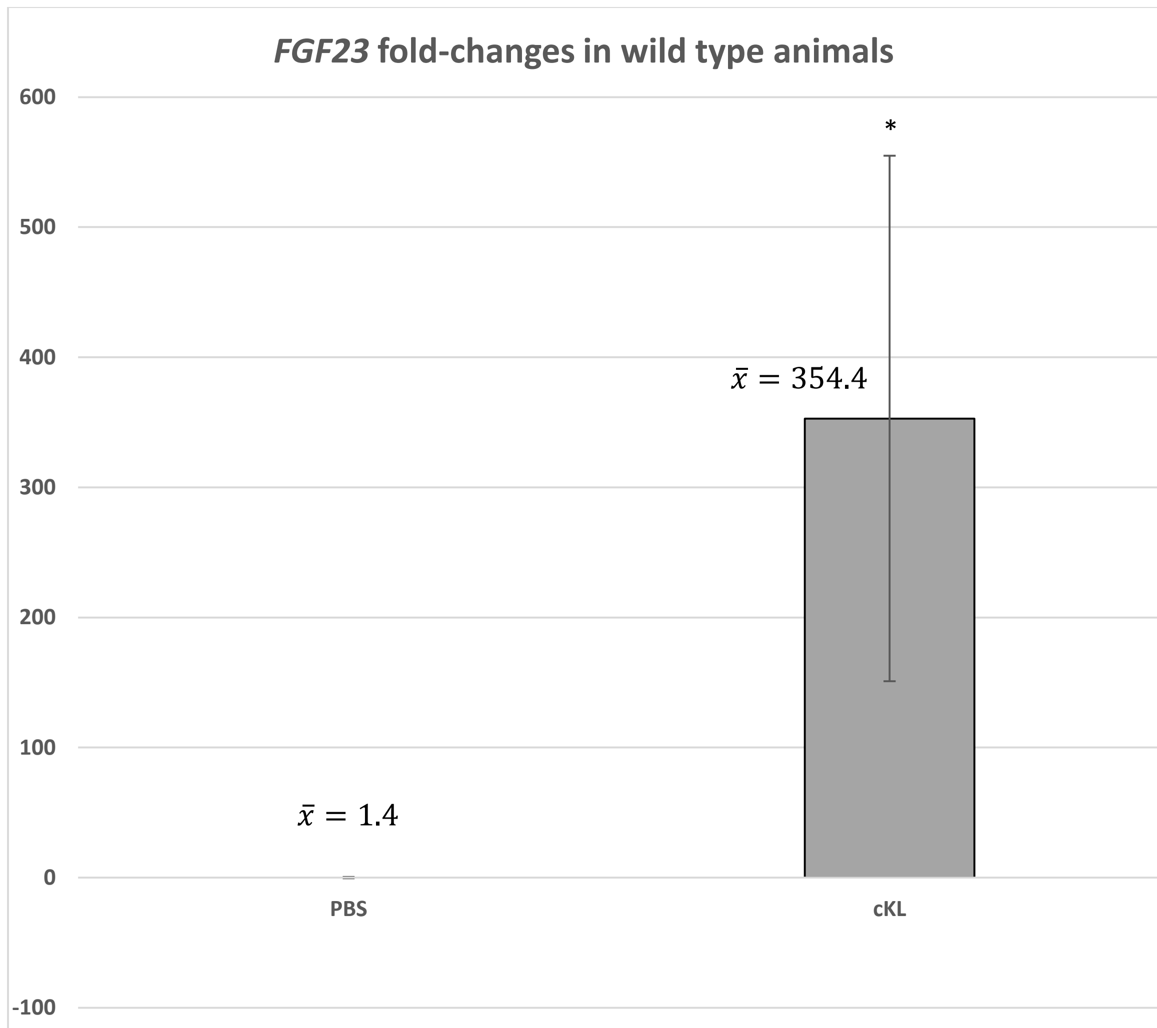

Figure 3 *FGF23* fold-changes by qPCR in wild type animals
Wild type animals were treated with either PBS or cKL and *FGF23* mRNA from bone samples was analyzed. Animals treated with cKL showed significantly higher *FGF23* mRNA levels (354.4 copies) than control animals (1.4 copies), $p<0.05$ (*). Error bars indicate SD.

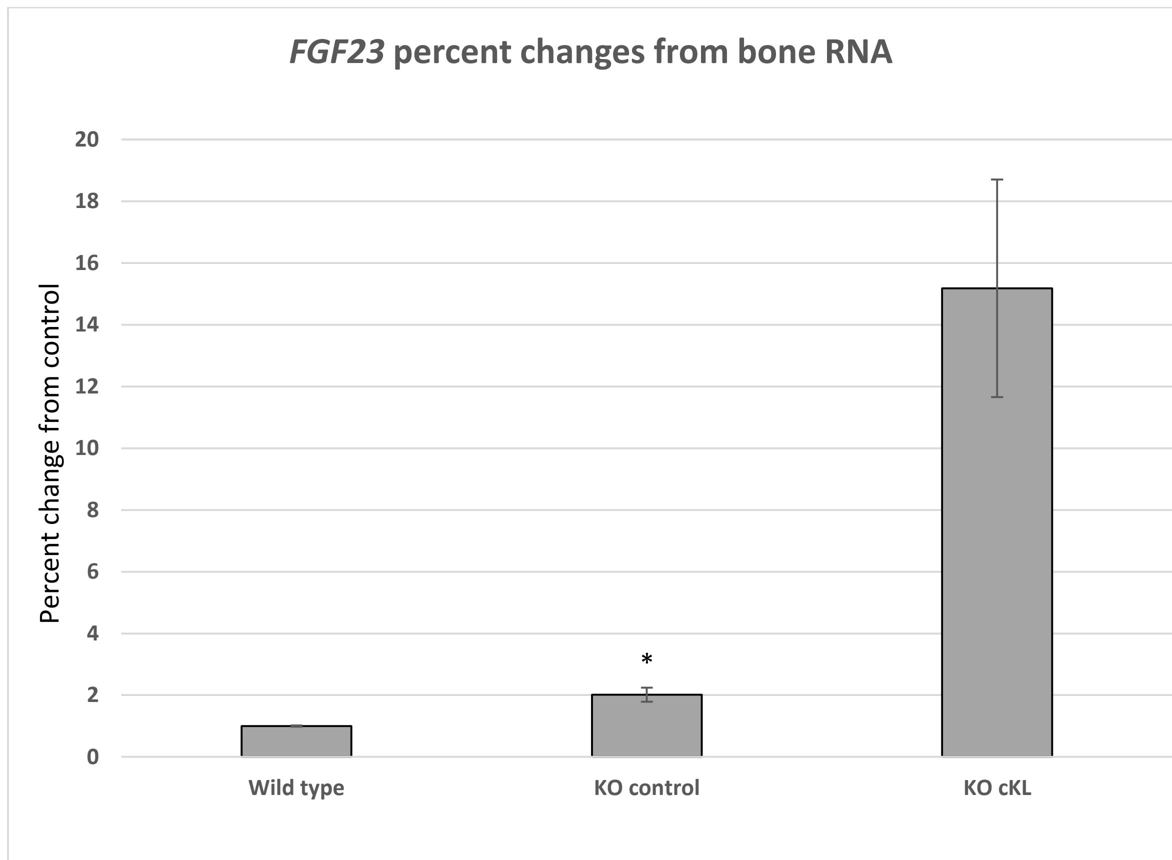

Figure 4 *FGF23* percent changes in KL KO mice
Klotho knockout mice showed a significant increase in *FGF23* mRNA compared to wild type mice, $p<0.001$ (*). *FGF23* mRNA was further increased in comparison to wild type when knock out animals were administered cKL, but the increase was not significant by Student's t-test ($p<0.07$). Error bars indicate SD.

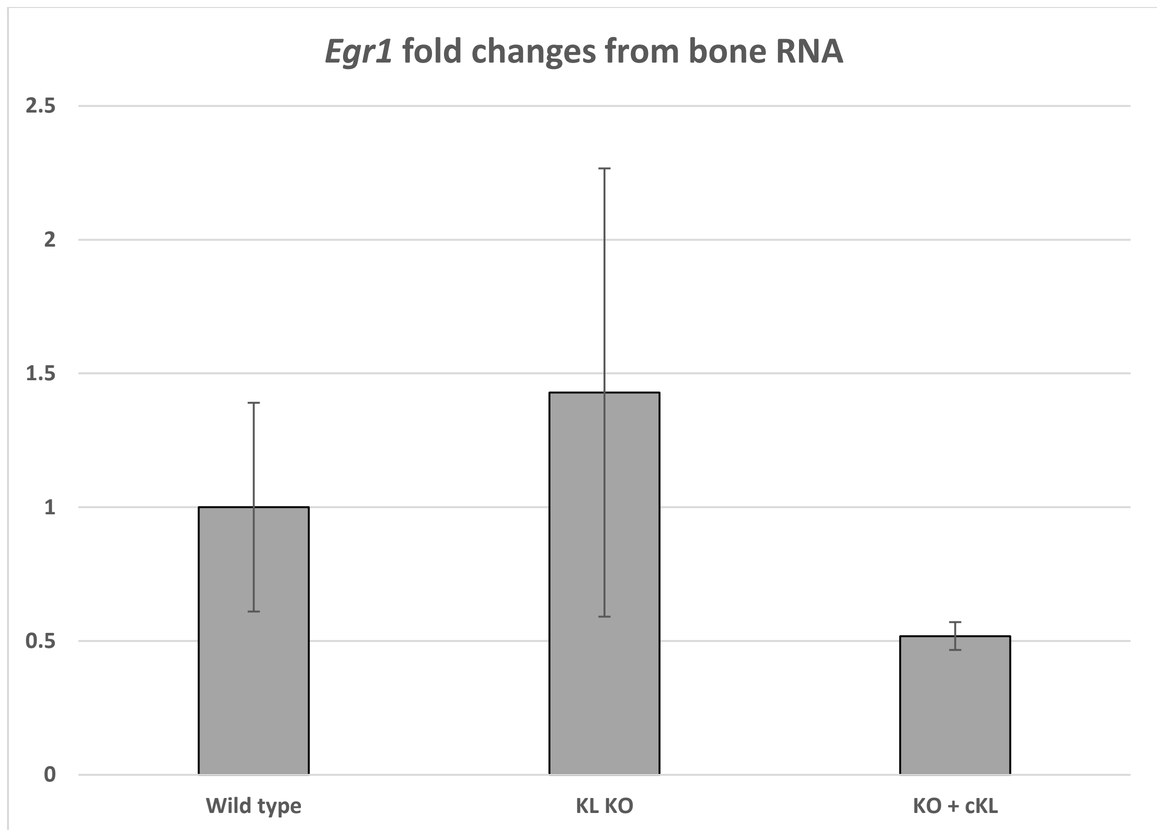

Figure 5 *EGR1* mRNA changes in KL KO mice
The amount of *EGR1* mRNA from the bones of KL KO mice did not differ significantly ($p<0.32$) from EGR1 mRNA levels in wild type mice, although there was a slight increase. KL KO mice given cKL also did not show a significant change in *EGR1* mRNA compared to wild type mice. Error bars indicate SD.

C-fos, another indicator of FGF23-cKL activity [34, 35], was compared in KL KO animals and KL KO animals administered cKL (Figure 6). The levels of *Egr1* mRNA from bone samples were similar between both groups, as were *c-fos* mRNA levels. Although *c-fos* levels were slightly higher than *Egr1* levels, there was not a significant increase ($p<0.28$).

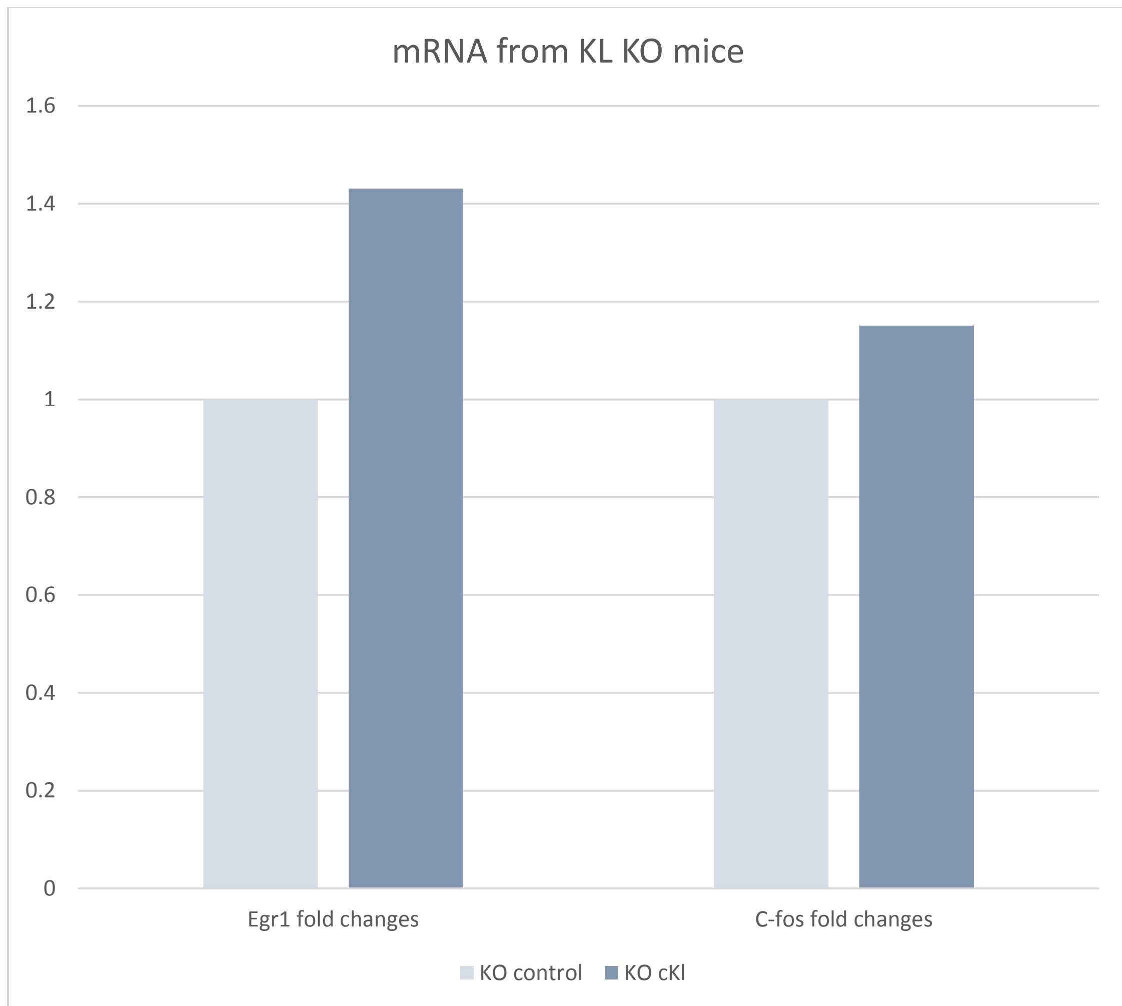

Figure 6 *EGR1* and *C-fos* mRNA from KL KO mice
mRNA isolated from bones of KL KO animals given cKL was analyzed for *Egr1* and *C-fos* mRNA levels. Despite a slight increase, there was no significant difference when compared to KL KO control animals (n=5, $p<0.28$).

Assessing FGF23 and Klotho activity in cell model studies

Two murine cell lines were examined to search for a suitable model to test FGF23-Klotho interactions and bioactivity. ROS (rat osteoblast) cells and MC3T3 (mouse osteoblast) cells were both cultured and exposed to cKL and FGF23, and the mRNA encoding the transcription factor Egr1 was used as a readout for FGF and cKL activity. FGF2 and FGF8 were added to cell cultures as positive controls for Egr1 activity. RNA extracted from lysates of cells treated with PBS, FGF2, FGF8, FGF23, Klotho, and a combination of FGF23+Klotho were tested for *Egr1* mRNA levels as an indicator of cell responsiveness. In ROS cells, a significant increase in Egr1 mRNA was seen only in cells treated with the positive control FGF2; the experimental conditions of adding FGF23, Klotho, or FGF23+Klotho did not induce activity in the cells (Figure 7). MC3T3 cells had higher *Egr1* mRNA compared to PBS negative control conditions in cells treated with the positive control FGF8 and in cells treated with the combination of FGF23 and Klotho (Figure 8). The increase in activity induced by FGF23+Klotho indicates that MC3T3 cells are responsive to a combination of FGF23 and Klotho activity. MC3T3 cells treated with Klotho alone also exhibited an increase in *Egr1* mRNA levels compared to MC3T3 cells treated with FGF23 alone.

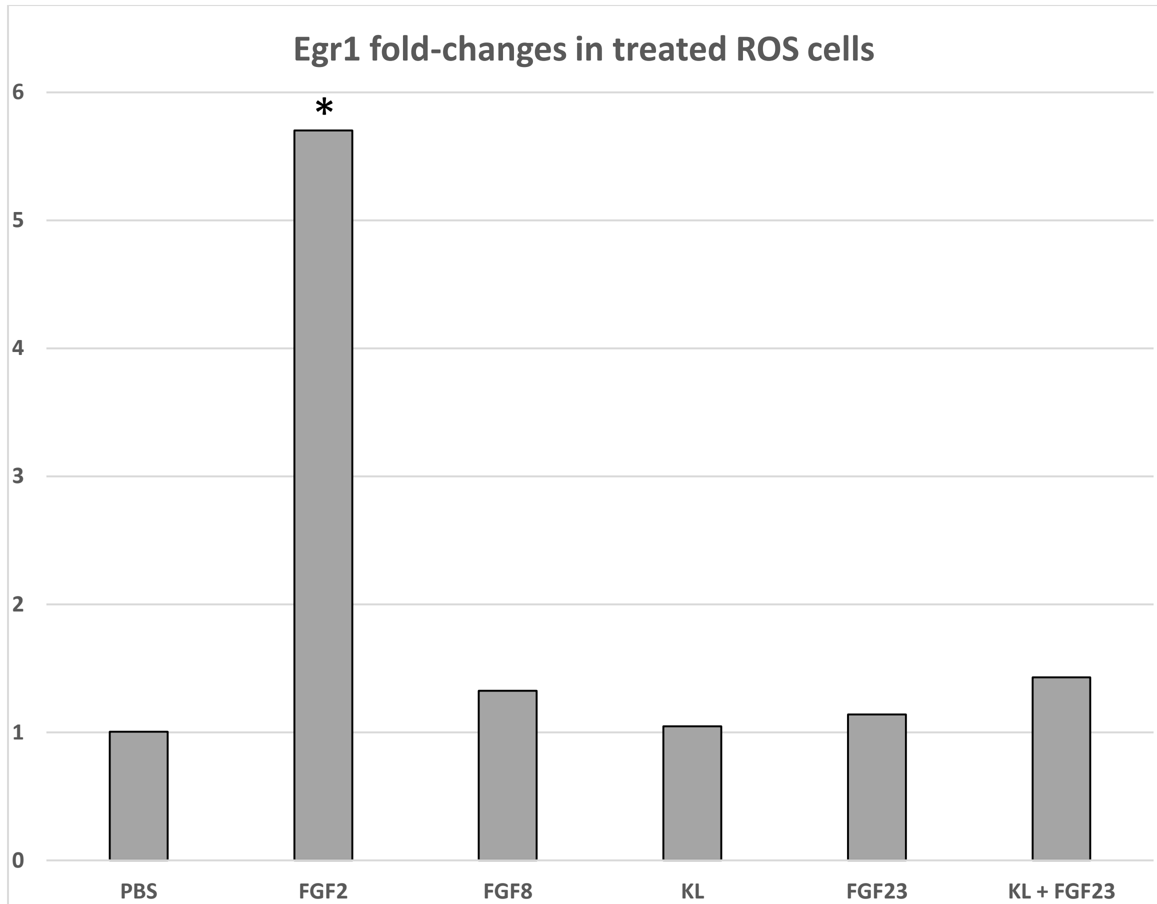

Figure 7 ***Egr1*** **mRNA fold-changes in ROS cells** Cells in culture were treated with either PBS, FGF2, FGF8, Klotho, FGF23, or Klotho+FGF23. Resulting lysates were tested for *Egr*1 mRNA by qPCR**.** ROS cells treated with FGF2 showed a significant increase in *Egr1* mRNA, $p<0.05$ (*). Only a modest increase in *Egr1* mRNA was seen in cells treated with both cKL and FGF23. Experiment was repeated once.

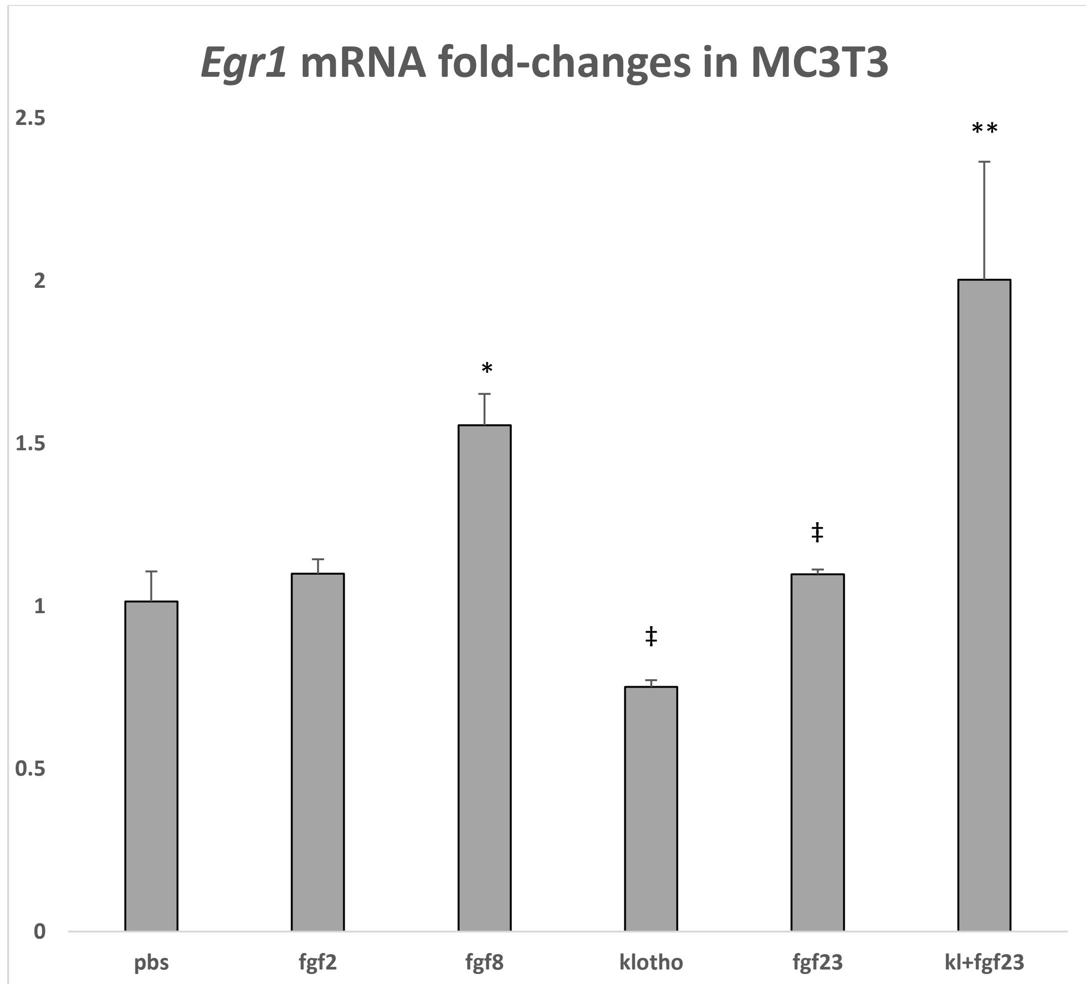

Figure 8 *Egr1* mRNA fold-changes in MC3T3 cells MC3T3 cells treated with cKL or FGF23 alone showed no effects, however a combination of cKL+FGF23 increased expression of *Egr1* mRNA compared to PBS (untreated), $p<0.05$ (**). The positive control FGF8 increased *Egr1* mRNA $p<0.005$ (*). There is also a significant increase of *Egr1* mRNA in FGF23-treated cells compared to those treated with cKL, $p<0.0001$ (‡). Error bars indicate SD. PBS (n=5); FGF2 (n=3); FGF8 (n=4); Klotho (n=3); FGF23 (n=4); KL+FGF23 (n=5).

ROS cells were also probed by western blot for phospho-Erk (p-Erk) and total Erk protein levels (Figure 9). Lysates from cells treated with FGF2 or FGF8 showed strong signal compared to untreated cells for p-Erk along with lysates treated with a combination of FGF23 and Klotho. Increased signal on the blot corresponds to increased expression of the protein that is being probed, so this result shows the combination of FGF23 and Klotho increase the amount of Erk that is phosphorylated in ROS cells. The phosphorylated Erk indicates that the treatment induced the MAPK pathway. The profile for total Erk was the opposite: stronger signals were seen for lysates of cells treated with PBS, FGF23 alone, and Klotho alone. MC3T3 cells were also immunoblotted blotted for p-Erk and total Erk (Figure 10). The same signal strengths were seen as in the ROS cells; FGF2, FGF8, and the combination of FGF23 and Klotho increased protein expression of p-Erk. MC3T3 cells also produced more total Erk when treated with PBS, FGF23 alone, and Klotho alone.

In summary, the results show that cKL and FGF23 delivered together have bioactivity in bone. The studies with WT and KL KO mice confirm that administering cKL induced FGF23 expression *in vivo*. Furthermore, as shown in the MC3T3 and ROS cell lines, a combination of cKL and FGF23 were required to increase cell signaling of the pERK activity. Importantly, these studies show that both cell lines respond to cKL and FGF23 administration, and thus provide new cell models for mechanistic testing as well as targeting bone cKL-FGF23 bioactivity.

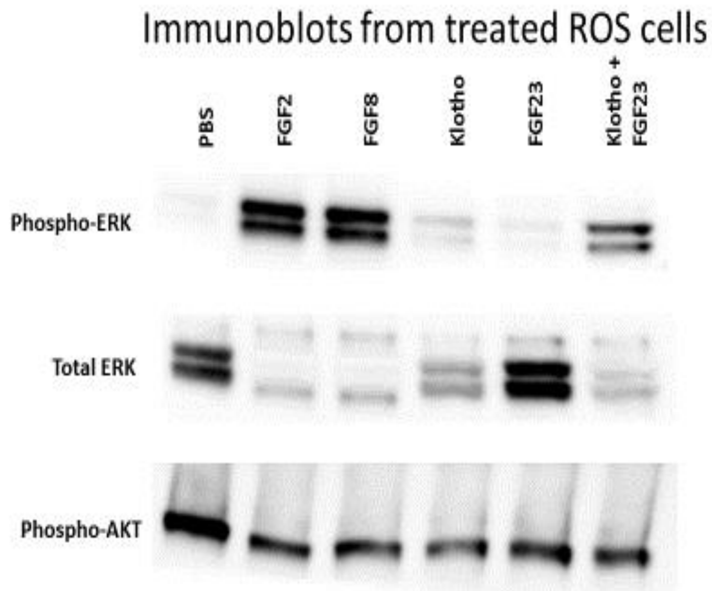

Figure 9 Western blot of treated ROS cells
Lysates from treated ROS cells were probed for phospho-ERK, total ERK, and phospho-AKT (loading control). Positive controls FGF2 and FGF8 showed strong signal for phospho-ERK, as well as lysates from cells treated with Klotho and FGF23 in combination. Total ERK showed strong signal for PBS, Klotho alone, and FGF23 alone treated lysates. Repeated once; values quantitated by ImageJ.

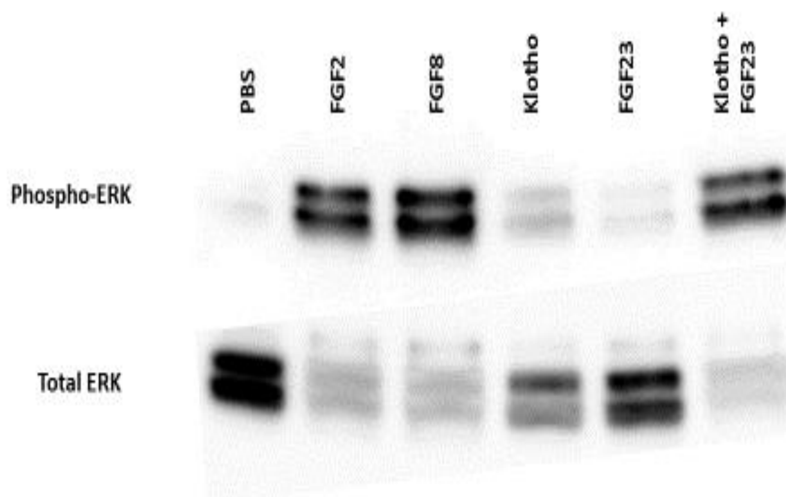

Figure 10 Western blot of treated MC3T3 cells
Lysates from treated ROS cells were probed for phospho-ERK and total ERK. Positive controls FGF2 and FGF8 showed strong signal for phospho-ERK, as well as lysates from cells treated with Klotho and FGF23 in combination. Total ERK showed strong signal for PBS, Klotho alone, and FGF23 alone treated lysates. Repeated once; values obtained by ImageJ analysis.

Discussion

Rationale

A major consideration for the management of CKD-MBD is lowering phosphate levels of patients. A better understanding of the interactions between FGF23 and Klotho with respect to the impact on phosphate metabolism will help guide research for therapeutics. Klotho, in particular, is not yet well understood. Although it has been shown in animal models that the protein has atheroprotective effects, high levels of Klotho have been associated with higher mortality rates in CKD patients [24]. This risk of severe complications makes cKL problematic as a treatment for hyperphosphatemia in CKD and CKD-MBD patients, although treating patients with cKL is an appealing option for reducing serum phosphate levels. A reliable cell model for Klotho studies would be useful in determining more detailed pathways involving FGF23 and Klotho, and also potential therapeutics targeting these or other proteins in the relevant pathways. The aim of this thesis was to study potential cell models that could serve the purpose of FGF23 and Klotho testing.

Klotho upregulates *FGF23 in vivo*

The *in vivo* data assembled here reinforces the relationship between FGF23 and Klotho. qPCR data showed induction of *FGF23* mRNA expression in wild type mice when treated with cKL via adenoviral delivery. This result indicates that *FGF23* mRNA is directly

upregulated by increased cKL levels. An increase in *FGF23* mRNA was also seen in Klotho knock out mice that were administered cKL. This result also shows the positive effect of cKL on the amount of *FGF23* mRNA. In both of these mice models, cKL clearly upregulated *FGF23*. This relationship agrees with results found in a previous study that utilized AAV-delivered cKL in a mouse model [27]. The levels of *FGF23* were also increased according to the amount of cKL delivered.

Klotho and FGF23 interact *in vitro*

One function of Klotho is the regulation of FGFRs. When Klotho forms heteromeric complexes with FGFRs in the kidney, FGF23 is able to bind very strongly to this receptor complex [20]. ROS (rat osteoblast) and MC3T3 (mouse osteoblast) cell lines were tested with FGF23 and cKL to find a model that potentially reflected the relationship between FGF23 and cKL that is seen in mouse models. Cell models may be more useful for studies that require large screens. Egr1 is known to be activated by the FGF23-cKL complex [34]; this mRNA was measured in cells treated overnight with a combination of FGF23 and cKL. Cell were incubated for the optimal time to allow transcription of *Egr1* to occur. The results were inconclusive for the ROS cells with regard to Egr1 as an early indicator of the action of FGF23 combined with Klotho. MC3T3 cells, however, exhibited the expected FGF23-cKL activity response as evidenced by increased *Egr1* mRNA levels. These results indicate that of the two cell lines tested, MC3T3 may be a better choice for the study of FGF23-cKL *in vitro*.

In addition to qPCR studies to measure mRNA levels, immunoblotting for protein expression levels was also performed on cKL- and FGF23-treated ROS and MC3T3 cells. Total Erk and phosphorylated Erk, a member of the MAPK pathway, were probed to detect FGF23-cKL activity [36]. Erk activity, probed for as phosphorylated Erk, was detected in both of the controls, FGF2 and FGF8, and also in cells treated with a combination of FGF23 and Klotho [33]. Although the ROS cells showed a response in phosphorylated Erk protein, there was not an increase in *Egr1* mRNA when treated with a combination of FGF23 and Klotho. On the other hand, protein and mRNA data from MC3T3 cells indicate they are promising as a means of further studying FGF23-cKL interactions; both mRNA and protein assays show that MC3T3 cells respond to FGF23-cKL.

Future research

Future directions for research include testing agonist/antagonist models to aid in phosphate homeostasis for CKD patients. Results from this study indicate that MC3T3 cells are more appropriate for the study of FGF23-cKL interaction based on the protein and mRNA studies. Therapies that target either Klotho or FGFRs could be used to regulate the action of FGF23 in the kidneys by affecting the binding of the complex. These therapies could increase the affinity for FGF23, which could decrease phosphate reabsorption and promote the phosphaturic action of FGF23. While FGFRs were not tested here, the MC3T3 cell line may be useful in terms of FGF23 and Klotho interactions

with FGFRs. In addition, based on the findings presented in this thesis, the MC3T3 line could be used to test small molecules capable of interacting with FGF23, Klotho, FGFRs, or the FGF23-cKL complex.

In addition to Klotho and associated FGFRs, FGF23 itself can be studied as a potential therapeutic target. Indeed, the anti-FGF23 antibody therapeutic 'KRN23' is undergoing clinical trials [37]. Certainly, targeting FGF23 in late stage disease could potentially ameliorate the 'off target' cardiovascular manifestations of markedly elevated FGF23 [38]. Additionally, promoter studies using the FGF23 promoter could provide the molecular mechanisms whereby Klotho regulates FGF23 transcription. Indeed, previous promoter studies have identified key elements for the 1,25D regulation of FGF23 [18, 39]. If the exact portion of the promoter responsive to Klotho is ascertained, the direct effect of Klotho on FGF23 could potentially be controlled. For example, experiments could include isolating the promoter and performing pull-down assays with Klotho. MC3T3 cells have been shown to be responsive to FGF23-cKL activity and would be an ideal model for testing various promoters *in vitro* after initial protein studies were complete.

Conclusions

The mice data presented herein agreed with previous studies [27] characterizing the relationship between FGF23 and Klotho. Sustained delivery of cKL to mice has been

shown to increase FGF23 mRNA [27], and the same results were duplicated when mRNA from bone was probed for FGF23. ROS cells and MC3T3 bone cells were compared for their FGF23-cKL activity. Although both cell lines reflected FGF23-cKL protein activity as seen on western blots, only MC3T3 cells showed an increase in *Egr1* mRNA by qPCR analysis as an early indicator of FGF23-cKL activity. In conclusion, the MC3T3 cell line is a promising tool for the study of FGF23-cKL interactions, which could lead to future targeting of this pathway to control FGF23 production in both rare and complex diseases.

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Anna Bonfitto

Education

2016	Master of Science in Medical and Molecular Genetics IUPUI, Indianapolis, IN
2006	Bachelor of Science in Biology; minor in history Indiana University, Bloomington, IN
2002	Bachelor of General Studies; mathematics and science concentration Indiana University, Bloomington, IN

Experience

1/13-present **Laboratory Analyst, Vertellus.** Perform gas chromatography, titrations, and high performance liquid chromatography. Standardize equipment and maintain assigned GCs. Analyze chromatograms and report information through laboratory information management system. Run and analyze samples on gas chromatography-mass spectrometer. Prepare and analyze waste water samples for environmental compliance. Stock laboratory benches and fume hoods, clean glassware, and contribute to general laboratory housekeeping. Complete safety audits quarterly.

6/11 to 6/12 **Laboratory Technician, Heritage Crystal-Clean.** Performed used oil extractions and other sample preparations of asphalt, vacuum gas oil, and distillate fuel. Communicated with unit operators regarding samples and results. Operated and troubleshot Karl Fisher, inductively coupled plasma, gas chromatography, viscometer (manual and automatic), cold crank tester, and flash point testers. Completed manual titrations, pH testing, data entry, ordering of supplies, and all relevant paperwork. Ordered and unpacked supply orders and kept laboratory properly cleaned and stocked.

2/09 to 7/09 **Laboratory Technician, Lively Lab, Indiana University.** Assisted in research concerning interactions between populations of

pathogenic bacteria *Xenorhabdus bovienii*. Prepared media and reagents, ordered supplies, performed and scored inhibition assays, prepared polymerase chain reactions, and sequenced DNA samples. Coordinated research plans with supervising post-doctoral fellow and attended weekly lab meetings.

1/07 to 8/07 **Research Associate, Ybe Lab, Indiana University.** Performed protein purification from large *E. coli* growths, assembly assays, western blotting, pull-downs, and circular dichroism experiments on clathrin and Huntingtin-interacting protein. Created primers for point mutations and verified by DNA sequencing. Kept an excellent laboratory notebook and presented results at lab meetings. Helped train undergraduate hourly employees and volunteers. Organized disposal of hazardous wastes. Prepared SDS-PAGE gels and purified proteins for labmates.

11/03 to 7/04 **Scientist, KP Pharmaceuticals.** Worked with investigation of new drugs at a contract research and development pharmaceutical company. Performed physical and chemical assays, including pH testing, hardness testing, and solubility. Wrote and revised standard operating procedures, set up new equipment, and performed annual internal quality control audit. Kept daily records of controlled environment storage areas. Participated in manufacture of tablets and parenterals, including clean room processes.

Awards

2014-2015 **AAUW Career Development Grant:** Competitive scholarship provided for education expenses

Volunteer Work

2013 **Indianapolis Animal Care and Control**: walked and bathed dogs, spent time socializing cats

2013-2014 **Girls, Inc**: taught sessions on body image and bullying, participated in panel discussion on career paths