A Comparative Transcriptome Analysis Identifying FGF23 Regulated Genes in the Kidney of a Mouse CKD Model

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Abstract
Elevations of circulating Fibroblast growth factor 23 (FGF23) are associated with adverse cardiovascular outcomes and progression of renal failure in chronic kidney disease (CKD). Efforts to identify gene products whose transcription is directly regulated by FGF23 stimulation of fibroblast growth factor receptors (FGFR)/α-Klotho complexes in the kidney is confounded by both systemic alterations in calcium, phosphorus and vitamin D metabolism and intrinsic alterations caused by the underlying renal pathology in CKD. To identify FGF23 responsive genes in the kidney that might explain the association between FGF23 and adverse outcomes in CKD, we performed comparative genome wide analysis of gene expression profiles in the kidney of the Collagen 4 alpha 3 null mice (Col4a3−/−) model of progressive kidney disease with kidney expression profiles of Hypophosphatemic (Hyp) and FGF23 transgenic mouse models of elevated FGF23. The different complement of potentially confounding factors in these models allowed us to identify genes that are directly targeted by FGF23. This analysis found that α-Klotho, an anti-aging hormone and FGF23 co-receptor, was decreased by FGF23. We also identified additional FGF23-responsive transcripts and activation of networks associated with renal damage and chronic inflammation, including lipocalin 2 (Lcn2), transforming growth factor beta (TGF-β) and tumor necrosis factor-alpha (TNF-α) signaling pathways. Finally, we found that FGF23 suppresses angiotensin-converting enzyme 2 (ACE2) expression in the kidney, thereby providing a pathway for FGF23 regulation of the renin-angiotensin system. These gene products provide a possible mechanistic links between elevated FGF23 and pathways responsible for renal failure progression and cardiovascular diseases.

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Introduction
FGF23 is a bone-derived hormone that regulates phosphate and vitamin D metabolism through FGFR/α-Klotho co-receptors [1] that are expressed in a limited number of tissues, including the kidney [2]. In the kidney, FGF23 suppresses sodium-phosphate co-transporter function leading to phosphaturia and reduces 1,25(OH)2D synthesis in the proximal tubule [3,4]. Physiologically, FGF23 is part of a bone-kidney feedback loop [4,5], where circulating 1,25(OH)2D stimulates FGF23 production in bone and FGF23 suppresses 1,25(OH)2D production in the kidney [5]. FGF23 expression is also regulated by local bone-derived factors that may link bone mineralization with renal phosphate handling [6,7,8].

FGF23 plays a pathological role in hereditary hypophosphatemic disorders [8] and tumor induced osteomalacia [9]. Elevations of circulating FGF23 also occur early in the course of chronic kidney disease (CKD), where it stimulates phosphaturia to maintain phosphate balance and contributes to the development of secondary hyperparathyroidism through suppression of 1,25(OH)2D levels [10,11,12]. FGF23 is also markedly elevated in patients with end stage renal disease (ESRD) [13,14,15].

Elevated FGF23 levels are associated with left-ventricular hypertrophy and hypertension in patients with X-linked hypophosphatemia (XLH) [16]. FGF23 is also an independent risk factor for left ventricular hypertrophy [17] and cardiovascular disease [18] in the general population. In chronic kidney disease, FGF23 is one of the strongest predictors of mortality [19,20], and adverse cardiovascular outcomes [21,22]. In addition, elevated circulating FGF23 concentrations are independently associated with more rapid progression of kidney disease [23] and renal allograft loss [24].

There are many gaps in our knowledge of the molecular mechanisms whereby FGF23 regulates kidney function and leads to adverse outcomes in CKD. It is uncertain which tubular segment and FGFR receptors mediate the effects of FGF23 on the kidney [25]. In addition, knowledge of the full complement of renal gene products regulated by FGF23 in the kidney that might mediate progressive renal damage or kidney processes affecting cardiovascular disease is largely unexplored. Without this infor-
FGF-23 Responsive Genes in CKD

The top 25 upregulated genes (Table 2) showed evidence of matrix protein replacement with increased collagen synthesis (Col1a1 and Col3a1) and cellular infiltration (Cxcl1, Ly6s, Ccl5, Lyz2, Ly6, C3, VCAM1 and Ear2), consistent with the histological presence of chronic kidney disease in the mice. In total, more than 30 transcripts of protein belonging to the collagen family were increased in the kidneys of Col4a3−/− mice, as well as proteins from the TNFα superfamily (30 transcripts) and TGFβ superfamily (11 transcripts). Furthermore, TIMP1 was increased along with a substantial dysregulation in proteases in the kidney of Col4a3−/− mice, with a total of 24 metalloendopeptidases being overexpressed.

The top 25 down-regulated genes are shown in Table 3. Of note, we found evidence for reductions in DNAse1 and epidermal growth factor (EGF). In addition, we observed reductions in COP9 [33], which regulate ubiquitin-mediated proteolysis of cullin that is cause of pseudohypoaldosteronism type 2 and involved in distal tubular regulation of blood pressure and potassium homeostasis [34]. We also observed reduction in Cyp2c44, which important in producing compensatory renal artery vasodilation in response to salt-loading through the regulation of prostaglandin metabolism [35]. We also observed reduction in Slc6a19, which is a major luminal sodium-dependent neutral amino acid transporter in the proximal tubule [36] and parvalbumin, which is involved in distal convoluted sodium transport [37]. Hgdl1c, which belongs to hypoxia inducible genes that may play a role in protecting the kidney from hypoxic injury during progressive CKD [38], was also reduced in Col4a3−/− kidneys. Corin, a protease that activates atrial natriuretic peptide, was also reduced in the kidneys of Col4a3−/− mice [39].

A total of twelve up-regulated and twelve downregulated genes were randomly chosen from the renal COL4A3−/− transcriptome to be confirmed by RT-PCR as shown in Table 4. We also confirmed that the proteins encoded by the mRNAs of the most downregulated and upregulated genes, DNAse1 and Lcn2 respectively, were also altered, as shown in Figure 3.

FGF23-related Gene Transcripts in the Kidney

to establish that the Col4a3−/− microarray data set contained genes involved in FGF23 regulation of mineral metabolism, we initially focused on alterations in Cyp24a1, Cyp27b1, Npt2a, Npt2c and Klotho expression. We found that Col4a3−/− mice displayed an increase in the renal Cyp24a1 transcripts, (2.6 and 5.1 fold by microarray and RT-PCR, respectively) as well as marked increase in Cyp24a1 protein level (Figure 4). However, we failed to detect any significant changes in Cyp27b1 expression. Additionally, Npt2c (~2.0 and ~2.3 fold by microarray and RT-PCR), but not Npt2a, was down-regulated in the kidney of Col4a3−/−. Most importantly, α-Klotho, the FGF23 co-receptor, was down-regulated (~2 and ~2.2 fold by microarray and RT-PCR), and α-Klotho protein levels in the kidney were reduced by immunohistochemical staining (Figure 4).

Comparative Analysis of FGF23 Excess Models

to determine additional FGF23-responsive genes in the kidney of Col4a3−/− mice, we compared microarray analysis of kidneys isolated from 12 week-old WT and Col4a3−/− mice with the renal transcriptome in Hyp mice, which have hypophosphatemia and elevated FGF23 caused by inactivating mutations of Phex in osteoblasts [40], and FGF23 transgenic mice [31]. We hypothesized that shared genes in these three data sets would be enriched with FGF23-responsive transcripts.

From 13694 transcripts present in all three datasets, 31 were found to be significantly altered in the kidney of two or more
mutant mice models compared to their respective WT control mice (Figure 5). We have identified 19 of these genes that were consistently (downregulated or upregulated compared to their respective controls in all three datasets) altered in Col4a3−/−, Hyp and FGF23-transgenic mice (Table 5) by subsequently testing by PCR other populations of the same mice. Eleven gene transcripts were increased (Table 5), including lipocalin 2 (Lcn2), which was the most up-regulated transcript common to Col4a3−/− and FGF23tg databases (but not the Hyp data set). In addition, inflammatory markers, including VCAM1, which is expressed in proximal tubule cells in response to inflammatory renal diseases [41], complement factor I, a serine protease that regulates the complement cascade, and galectin-3-binding protein (LGALS3BP), were increased in all data sets. Several genes related to cell signaling were also increased, including tumor-associated calcium signal transducer 2 (Tacstd2), Receptor activity modifying protein 2 (Ramp2), guanylate binding protein 2, immediate early response 3, (ler3), phospholipase A2 (Pla2g7), phospholipid scramblase 1 (Plsc1). Lipoprotein-associated phospholipase A2 (Pla2g7), an enzyme mostly synthesized by plaque inflammatory cells (macrophages, T cells, mast cells) that hydrolyzes oxidized phospholipids in LDL was also upregulated.

With regards to down-regulated genes, 8 were reduced in all three data sets. Most interestingly, in addition to reductions in α-Klotho described above, we also found that DNase1, a secreted nuclease that eliminates DNA from necrotic cells, was dramatically

Table 1. Serum biochemistry of WT and Col4a3−/− mice.

|               | WT       | Col4a3−/− |
|---------------|----------|-----------|
| BUN (mg/dL)   | 20.29±0.67| 59.01±8.59*|
| Creatinine (mg/dL) | 0.45±0.02 | 0.82±0.15* |
| FGF23 (pg/mL) | 137.34±9.54| 1248.29±188.50* |
| PTH (pg/mL)   | 32.28±3.81 | 1772.18±452.94* |
| FEPI (%)      | 4.66±1.62 | 14.82±2.65* |
| PO4²⁻ (mg/dL) | 6.58±0.43 | 9.39±0.50* |
| Ca²⁺ (mg/dL)  | 8.88±0.33 | 9.38±0.29 |
| ALP (IU/L)    | 67.16±7.52| 87.79±10.56 |

Values are expressed as mean ± SEM from at least 13 mice per group. Comparisons were performed using one-way ANOVA and post-hoc Fisher test. BUN: Blood Urea Nitrogen; FEPI: Fractional Excretion of Phosphorus; PO4²⁻: phosphorus; Ca²⁺: total calcium; ALP: Alkaline Phosphatase. (*) P<0.05 vs. WT. doi:10.1371/journal.pone.0044161.t001

Figure 1. (A) Gross appearance and (B) body weight of 12 week-old wild-type (WT), and Col4a3−/− mice. (C) Kidney morphology showing reduced perfusion and (D) H&E renal histology showing glomerulosclerosis in the Col4a3−/− animals. Values are expressed as mean±SEM, P<0.05 vs: (*) WT, n=13 mice/group.

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Figure 2. (A) Cluster analysis of microarray performed on Kidneys from 12 week-old wild-type (WT), and Col4a3\(^{-/-}\) mice. Gene expression is represented on the heat map from the less expressed (blue) to the more expressed (red). (B) Graphic representation of transcripts expressed at least five fold in Col4a3\(^{-/-}\) as compared to WT.

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Table 2. Expression fold change of the top 25 kidney genes up-regulated in Col4a3\(^{-/-}\) mice.

| Gene Name                                      | Symbol  | Fold Change |
|------------------------------------------------|---------|-------------|
| lipocalin 2.                                   | Lcn2    | 53.8        |
| tissue inhibitor of metalloproteinase 1        | Timp1   | 23.7        |
| serine (or cysteine) peptidase inhibitor, clade A, member 3N. | Serpina3n | 20.3         |
| amiloride binding protein 1                    | Abp1    | 16.7        |
| chemokine (C-X-C motif) ligand 1.               | Cxcl1   | 14.4        |
| lysozyme (Lyzs)                                | Lyzs    | 14.4        |
| chemokine (C-C motif) ligand 5.                 | Ccl5    | 14.0        |
| collagen, type I, alpha 1.                     | Col1a1  | 13.5        |
| eosinophil-associated, ribonuclease A family, member 2. | Ear2     | 12.4        |
| collagen, type III, alpha 1.                   | Col3a1  | 12.4        |
| lysozyme 2                                     | Lyz2    | 12.4        |
| lysozyme.                                     | Lyz     | 12.3        |
| complement                                    | C3      | 12.3        |
| vascular cell adhesion molecule 1.             | Vcam1   | 11.5        |
| serine (or cysteine) peptidase inhibitor, clade A, member 10 | Serpina10 | 9.3          |
| chemokine (C-C motif) ligand 9                 | Ccl9    | 9.3         |
| immunoglobulin lambda variable 1               | Igl-V1  | 9.0         |
| B-cell leukemia/lymphoma 2 related protein A1d | Bcl2a1d | 8.4         |
| CD44 antigen                                   | Cd44    | 7.9         |
| eosinophil-associated, ribonuclease A family, member 3 | Ear3     | 7.8         |
| matrix metalloproteinase 2.                    | Mmp2    | 7.7         |
| CD14 antigen                                   | Cdi4    | 7.6         |
| ubiquitin D                                    | Ubd     | 7.5         |
| histone cluster 1, H2an                        | Hist1h2an | 7.5        |
| serine (or cysteine) peptidase inhibitor, clade A, member 3G | Serpina3g | 7.3          |

Values were obtained after clustering analysis on microarray performed in kidney of WT and Col4a3\(^{-/-}\) mice (cluster is represented in Figure 2). n = 4 samples/group. Values are expressed as fold change compared to the WT control value. Genes were selected based on a P value threshold of 0.05 and a minimum fold-change absolute value of 2.

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reduced in all three data sets. Most interestingly, angiotensin-
converting enzyme (ACE) 2, a homolog to the carboxypeptidase
ACE, was decreased in all three data sets. Finally, Them2 (thioesterase superfamily member 2) a 140-amino-acid protein of
unknown biological function was also decreased.

Finally, we performed an Ingenuity Pathway Analysis to identify
molecular interactions networks (Figure 6) related to these newly
identified transcripts. Consistent with the non-mineral metabolism
pattern of the expanded set of FGF23-regulated genes, this
analysis suggests a central role of activation of transforming growth
factor beta and tumor necrosis factor alpha (TGF-beta and TNF-
alpha), nuclear factor of kappa light polypeptide gene enhancer in
B-cells 1 (NFkB), interleukin 1, beta (IL1B), interferon, pllat-
derived growth factor (PDGF), progesterone, protein kinase C,
epsilon (PRKCE), and Chemokine (C-C motif ligand 13 (CCL13)
pathways in the common genes regulated in the three data sets,
consistent with activation of inflammatory and immunoregulatory
processes.

Independent Confirmation of Newly Identified FGF23-
responsive Genes

We have used complementary in vivo approaches to verify
FGF23 regulated genes in the kidney. First, we tested the effects of
chronic daily administration of rat recombinant rFGF23 on genes
identified from the comparative microarray analysis, plus addi-
tional genes (Cyp24a1, Cyp27b1, Npt2a and Npt2c) in FGF23−/−
and compound Col4a3−/− mice. The FGF23 null background was used to minimize the effects of endogenous

![Figure 3. (A) Western blots and corresponding (B) quantification of the most upregulated and downregulated gene product in WT
and Col4a3−/− mice.](doi:10.1371/journal.pone.0044161.g003)
FGF23 production peaks, as well as the amount of FGF23 injected to these animals. The administration of 50ng/g of rFGF23 twice daily to FGF23−/− and compound Col4a3−/− FGF23−/− mice resulted in a ~12-fold increase in Cyp24a1 and induced a decrease in Cyp27b1 expression (Table 6), consistent with known actions of FGF23 on these gene products. Additionally, we have found that chronic FGF23 administration induced elevations in 5 genes (lcn2, cfi, vcam1, gbp2 and plscr1) and decreased the expression of 4 genes (dnase1, car14 ace2 and slca2) in FGF23 genes (lcn2, cfi, vcam1, gbp2 and plscr1) and decreased the expression of 4 genes (dnase1, car14 ace2 and slca2) in FGF23−/− mice.

Secondly, we have transferred FGF23−/− mice on the Col4a3−/− background, thus identifying genes that respond to CKD progression independently of FGF23. We found that lcn2, cfi, pla2g7, and ier3 were upregulated by kidney disease progression. In addition, we also administered rFGF23 to compound FGF23−/−Col4a3−/− mice, to attempt separation of CKD effects from those mediated by FGF23, as well as possible interactions between FGF23 and CKD. The transfer of FGF23−/− on the CKD background, singled out genes that respond to FGF23 only with decline in renal function (upregulated: tacstd2, lgals3bp, ramp2; downregulated: afm, them2). Renal failure and FGF23 interacted to further increase FGF23 actions on lcn2, cfi and ier3, while CKD, although without independent regulatory actions per se, potentiated the effects of FGF23 on gbp2,,plscr1, dnase1 and slc2a2. Interestingly, Klotho is upregulated by rFGF23 in FGF23−/− mice and normalized in animals with impaired renal function.

Since the chronic adnistration of FGF23 may lead to systemic changes, we also evaluated the rapid, short-term response to rFGF23 administration. This was accomplished by examining the acute effects of rFGF23 administration in C57Bl6 mice after 1 and 12 h. We found that injection of rFGF23 resulted in a 10-fold increase in Cyp24a1 one hour after injection that persisted after 12 h. We also observed that rFGF23 induced a decrease in Cyp27b1 and Npt2c, but had no effect on Npt2a (Table 6). Lipocalin2 was confirmed to be increased in the kidney following rFGF23 administration. We also found that GBP2 Tacstd2 and Plscr1 were increased in response to acute FGF23 elevation. Furthermore, Dnase1 and Car14 were decreased by FGF23, consistent with the microarray data. However, we could not confirm FGF23 regulation of ACE2 and Them2 in these short-term FGF23 administration studies.

To investigate if FGF23 directly regulates these genes, we examined the effects of rFGF23 on distal 209 renal tubular cells in vitro. By real-time PCR, we found that 209 cells express z-klotho and FGFR4, and lesser amounts of FGFR1 or FGFR3 transcripts (data not shown). We found that 8 of 10 genes tested were directly modified by FGF23 in vitro, including FGF23 stimulation of

| Gene Name | Symbol | Fold Change |
|-----------|--------|-------------|
| deoxyribonuclease I. | Dnase1 | −8.4 |
| hemoglobin, beta adult minor chain | Hbb-b2 | −7.2 |
| cytochrome P450, family 2, subfamily d, polypeptide 12 | Cyp2d12 | −6.8 |
| COP9 (constitutive photomorphogenic) homolog, subunit B | COP9 | −4.9 |
| 4-hydroxyphenylpyruvic acid dioxygenase. | Hpd | −4.8 |
| hemoglobin, beta adult major chain | Hbb-b1 | −4.6 |
| cytochrome P450, family 2, subfamily c, polypeptide 44 | Cyp2c44 | −3.6 |
| erythropoietin delta-aminolevulinate synthase 2 | Alas2 | −3.6 |
| solute carrier family 6 (neurotransmitter transporter) | Slc6a19 | −3.5 |
| epidermal growth factor | Egf | −3.3 |
| parvalbumin | Pvalb | −3.2 |
| camello-like 1 | Cml1 | −3.2 |
| UDP glucuronosyltransferase 1 family, polypeptide A7C | Ugt1a7c | −3.0 |
| G protein-coupled receptor 112 | Gpr112 | −3.0 |
| ureidopropionase, beta | Upb1 | −2.9 |
| hypoxia inducible domain family, member 1C | Hid1c | −2.9 |
| hydroxycarboxylate oxidase (glycolate oxidase) 3. | Hao3 | −2.8 |
| bisphosphate 3′-nucleotidase 1 | Bpnt1 | −2.8 |
| ureidopropionase, beta | Upb1 | −2.8 |
| sorbitol dehydrogenase | Sord | −2.7 |
| endothelial cell-specific molecule 1 | Esm1 | −2.7 |
| glycine N-methyltransferase | Gmnt | −2.7 |
| adenylate kinase 3 alpha-like 1 | Ak3l1 | −2.7 |
| corin | Corin | −2.7 |

Values were obtained after clustering analysis on microarray performed in kidney of WT and Col4a3−/− mice (cluster is represented in Figure 2). n = 4 samples/group. Values are expressed as fold change compared to the WT control value. Genes were selected based on a P value threshold of 0.05 and a minimum fold-change absolute value of 2.

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increments in Cfi and Ramp1 and decrements in α-Klotho, Car14, Slc2a2, ACE2, DNase 1, and Afn in distal tubule cells after treatment with FGF23 (Table 8).

Discussion

Comparative analysis of gene expression profiles of the Col4a3−/− mice, a CKD model of elevated circulating levels of FGF23, and two other models of FGF23 excess and normal renal function [31,40,42,43], along with confirmation of FGF23 regulation of these transcripts in vivo and in vitro, identified novel genes not previously recognized to be regulated by FGF23 as well as confirmed the regulation of genes known to be regulated by FGF23 by FGF23 in the kidney.

The effects of FGF23 on phosphate and vitamin D metabolism are mediated by the regulation of Npt2a, Cyp27b1 and Cyp24a1 functions in the proximal tubule [9]. With the exception of Npt2a, we have evidence in Col4a3−/− mice of alterations in Cyp24a1, Nap2a, Npt2c by FGF23, consistent with their known involvement in mediating FGF23 effects on kidney phosphate, calcium and vitamin D metabolism. The failure to observe changes in Npt2a gene transcripts likely points to the important role of post-translational regulation of brush border membrane insertion of this gene transcripts likely points to the important role of post-transcriptional regulation of Cyp27b1, FGF23, and two other models of FGF23 excess and normal renal function [31,40,42,43], along with confirmation of FGF23 regulation of these transcripts in vivo and in vitro, identified novel genes not previously recognized to be regulated by FGF23 as well as confirmed the regulation of genes known to be regulated by FGF23 in the kidney.

While serum soluble α-Klotho concentrations are inversely correlated with serum FGF23 [46] and reductions in α-Klotho mRNA levels in the kidney have been observed with CKD and other states of FGF23 excess FGF23 [47], direct regulation of α-Klotho by FGF23 has not been previously demonstrated. Rather, reductions in α-Klotho in CKD has been attributed to a primary decrement α-Klotho caused by loss of renal tubular cells in the diseased kidney, leading to secondary increments in FGF23 [48]. Both α-Klotho message and protein were decreased in kidneys of Col4a3−/− mice, and the administration of rFGF23 results in decrements in α-Klotho expression in the kidney of wild-type mice and in cultured distal tubular cells. Interestingly however, chronic administration of rFGF23 to both Fgf23−/− and Col4a3−/− mice, failed to suppress α-Klotho message levels, which may be due to offsetting effects of 1,25(OH)2D, which is known to stimulate α-Klotho gene transcription [49], in this model. Regardless, FGF23 suppression of α-Klotho might have several physiological effects, including providing a mechanism to desensitize FGF23 signaling responses through FGFR [50] as well as regulate circulating forms of α-Klotho produced by the distal tubule that potential act as a hormone and/or paracrine co-factor for several growth factor receptors [28,48] [46,51].

We identified other gene products that could potentially account for the associations between elevated circulating FGF23 concentrations renal failure progression and cardiovascular mortality that have been found in clinical association studies. At present it is not certain if these untoward effects associated with elevations in FGF23 are due to direct effects of FGF23 on FGFR/α-Klotho complexes in the kidney, off “target effects” of high levels of FGF23 to directly activate FGFRs in the absence of α-Klotho in the heart [22], or represent epiphenomena caused by effects of CKD to increase FGF23 levels. In support of on-target actions, we identified FGF23-regulated renal genes with mechanistic linkages to cardiovascular diseases. In this regard, we found that ACE2 is reduced by excess FGF23 in all three models and that FGF23 suppresses ACE2 expression in the distal tubule cultures. ACE2 is a negative regulator of the renin-angiotensin system (RAS) that has vasodilator and natriuretic effects, leading to reduced blood pressure [52]. A direct effect of FGF23 to suppress ACE2 provides an alternative explanation for the recently proposed associations between vitamin D deficiency, activation of the renin-angiotensin system and regulation of α-Klotho expression [53]. FGF23 direct suppression of ACE2 expression could lead to activation of RAS, establishing a linkage between increased FGF23 and increased mortality.

Table 4. Expression fold change of selected genes confirmed by RT-PCR in Col4a3−/− and WT mice.

| Gene Symbol | Microarray | RT-PCR | Gene Symbol | Microarray | RT-PCR |
|-------------|------------|--------|-------------|------------|--------|
| FGF23 regulated genes involved in mineral metabolism |
| Cyp24a1 | 2.6 | 5.1 | Npt2c | -2.0 | -2.3 |
| Cyp27b1 | 1.3 (NS) | 1.1 (NS) | Npt2a | -1.4(NS) | -1.6(NS) |
| Genes significantly modified in microarray dataset |
| Lcn2 | 53.8 | 313.3 | Dnase1 | -8.4 | -7.8 |
| Timp1 | 23.7 | 157.8 | Hbb-b2 | -7.2 | -7.2 |
| Vcam1 | 11.5 | 25.8 | Cyp2d12 | -6.8 | -6.8 |
| MGP | 6.1 | 9.1 | Hbb-b1 | -4.6 | -6.9 |
| Adamts2 | 4.9 | 26.7 | Cyp2c44 | -3.6 | -4.0 |
| STAT3s1 | 2.4 | 4.6 | Aqp 11 | -2.4 | -5.1 |
| Slc34a2 | 4.0 | 7.9 | Cyp 51 | -2.3 | -2.2 |
| Cfi | 3.2 | 5.8 | Car14 | -2.5 | -3.8 |
| Pla2g7 | 1.7 | 6.5 | Afn | -2.2 | -1.9 |
| Lgal33bp | 2.1 | 18.7 | Slc2a2 | -1.6 | -1.7 |

Values are expressed as fold change compared to the WT value. n = 4 samples/group. Comparisons were performed using Student T test. P<0.05 vs: WT. doi:10.1371/journal.pone.0044161.t004
We also identified renal gene products that may mediate a direct effect of FGF23 to accelerate the progression of chronic kidney disease. In addition to α-Klotho, which has been shown to modulate renal damage [54], we also observed FGF23 regulation of several other genes associated with renal injury, including, Cfi, which has been shown to contribute to inflammatory and acute renal injury [55,56], and suggesting an effect of FGF23-mediated complement activation; DNase1, which is associated with systemic lupus erythematosus (SLE) [57,58], implicating a role of FGF23 in stimulating inflammatory responses in the kidney; carbonic anhydrase 14 (car14), whose inactivation in transgenic mice leads to progressive renal injury [59,60]. Additionally, both slc2a2 and afamin were found to be downregulated in the distal tubular cell line by Fgf23. Slc2a2 (also known as the glucose transporter gene GLUT2) is a disease causing gene for Fanconi-Bickel syndrome, which has systemic as well as characteristic tubular nephropathy.

Figure 4. Immunohistochemistry of Cyp27b1, Cyp24a1 and α-klotho in the kidneys of WT and Col4a3^{−/−} mice. doi:10.1371/journal.pone.0044161.g004
abnormalities [61]. Decrements in afamin (Afm), a vitamin E-
binding protein, related to vitamin D binding protein [62], are
observed in early acute renal allograft rejection [63].

Regarding additional genes involved in renal injury, we found
that Lcn2 (or NGAL) was markedly increased in the Col4a3−/−
and the FGF23Tg data set as well as in Fg23−/− and Col4a3−/−
Fgf23tg mice receiving rFGF23. Lcn2 mRNA is normally
expressed in the kidney [64] where it promotes epithelial
differentiation of the mesenchymal progenitors, leading to the
generation of glomeruli, proximal tubules, Henle’s loop and distal
tubules [65,66]. Lcn2 expression is markedly induced in injured
epithelial cells through NF-κB dependent pathways [67], and plays
a central role in controlling cell survival and proliferation [68-69].
Lcn2 has been shown to contribute to CKD progression both in mice and humans [70]. The magnitude of the increase in Lcn2 as a response to FGF23 was greater in animals with CKD, suggesting that other factors other than FGF23 are also stimulating transcription of this gene. Additionally, 8 other genes which are significantly expressed during the repair stage in AKI were also increased in the kidneys of Col4a3−/− mice, including C5, Vcam1, Serpin10 m C3, Lyz, Col3a1, Col1a1, and abp1 [71]. Finally, Them2, which belongs to a family of enzymes that play an important role in lipid metabolism and might contribute to tubular toxicity [72] was also increased in models of FGF23 excess.

Finally, pathway analysis identified TGF-β and TNF-α
signaling pathways as being involved in FGF23 responses in the
kidney. The TGF-β pathway are increased in most forms of CKD
in humans and experimental animals and controls including fibrogenesis, apoptosis, epithelial-to-mesenchymal transition, and
inflammation leading to glomerulosclerosis and tubulointerstitial
fibrosis [73]. Three additional genes observed to be altered in a
Tgfβ1 Tg mouse model of CKD, were also in the top modified
genes in the Col4a3−/− kidneys, including Timp1, Lcn2 and
Ccl11 [73]. TNF alpha is a central proinflammatory agonist
mediator that is generated in a wide variety of innate and adaptive
immune responses inflammatory mechanisms regulated by TNF
might contribute to renal disease progression and cardiovascular
events [74,75,76], and even in non-calcified aortas in patients with
CKD display increased TNF immunoreactivity [77]. The central
role of these pathways, together with IL1-beta, another pro-
inflammatory cytokine, demonstrate that the inflammatory state
that correlates with kidney disease may be modified by FGF23.
We also found FGF23 associated increases in VCAM1, which is
expressed in proximal tubule cells in response to inflammatory
renal diseases [41], and interferon-induced guanylate-binding
protein 2 (GBP2), which regulates cell growth and matrix
metalloproteinase expression [78].

Our analysis has several limitations. Data sets from Hyp and
FGF23Tg mice have fewer number of genes analyzed than the
Col4a3−/− (~13,000 vs. ~45,000, respectively), with the resulting
possibility that some genes may have been missed. The presence
of CKD could mask FGF23-responsive genes since both FGF
receptors and klotho expression and function are altered.
However, this appears to be an minor issue, since we confirmed
that the known FGF23 regulated genes were still altered in this
model. In addition, the microarray analysis was performed in
whole tissues, which gives a composite read out of all cell types.
We also did not define the specific tubular segments or the role of
Klotho/FGFR complexes in the FGF23-mediated changes in gene
expression in the kidney. Further studies will be needed to
determine the cell-type specific alterations in gene expression. Age
difference between the animals of all three databases may also
confound the interpretation. The functional significance of FGF23
regulation of these genes remains to be established.

Regardless, we have discovered novel and potentially important
FGF23 regulated genes involved in inflammation and progressive
renal fibrosis as well as alterations in factors with systemic effects,
such as ACE2, which might impact on cardiovascular function.
Further studies are needed to test the role of these factors in linking
FGF23 to mortality and progressive renal dysfunction.

Materials and Methods

Animals and Genotyping
All mice were maintained on a standard diet (7912, HarlanTeklad, Madison, WI, USA). Animal care and protocols were in
accordance with the guidelines established by the University of
Tennessee Institutional Animal Care and Use Committee as
detailed in the “Guide for Care and Use of Laboratory Animals,”
prepared by the Institute on Laboratory Animal Resources,
National Research Council (Department of Health & Human
Services Publication NIH 86–23, National Academy Press, 1996)
and UTHSC IACUC specifically approved this study (protocol
1884). Animals were anesthetized before serum collection and
sacrifice by ip injection of ketamin (120 mg/Kg) and xylazin
(20 mg/Kg), followed by cervical dislocation. During the entire
period of the study, activity, respiratory rate, muscle strength via
grip strength, feeding and drinking, fur loss were the major signs
Table 5. Expression fold change of 19 renal genes modified in models of excess FGF23.

| Gene Name                  | Symbol | Col4a3−/− | Hyp | FGF23tg |
|----------------------------|--------|-----------|-----|---------|
| **Increased**              |        |           |     |         |
| Lipocalin 2                | Lcn2   | 53.8      | NM  | 2.0     |
| Vascular cell adhesion molecule 1 | Vcam1 | 11.5      | 1.4 | 1.4     |
| Complement component factor i | Cfi    | 3.2       | 3.2 | 1.6     |
| Tumor-associated calcium signal transducer 2 | Tacstd2 | 2.5 | 1.3 | 1.4 |
| Lectin, galactoside-binding, soluble, 3 binding protein | Lgals3bp | 2.1 | 1.3 | 1.3 |
| Phospholipase A2, group VII | Pla2g7 | 1.7       | 1.9 | 2.0     |
| Immediate early response 3 | Ier3   | 1.6       | 1.8 | 2.2     |
| Transporter 1, ATP-binding cassette, sub-family B | Tap1 | 1.6 | 1.5 | 1.2 |
| Receptor (calcitonin) activity modifying protein 2 | Ramp2 | 1.5 | 1.5 | 1.6 |
| Phospholipid scramblase 1 | Plscr1 | 1.4       | 1.4 | 1.3     |
| Guanylate binding protein 2 | Gbp2   | 1.3       | 1.6 | 1.3     |
| **Decreased**              |        |           |     |         |
| Deoxyribonuclease 1        | Dnase1 | −8.4      | −4.3| −5.0    |
| Carbonic anhydrase 14      | Car14  | −2.5      | −1.6| −1.7    |
| Afamin                     | Afm    | −2.2      | −1.6| −1.5    |
| Klotho                     | Kl     | −2.0      | −1.4| −1.6    |
| Abhydrolase domain containing 14A | Abhd14a | −1.9 | −1.5 | −1.3 |
| Angiotensin I converting enzyme 2 | Ace2 | −1.8 | −2.3 | −1.9 |
| Solute carrier family 2, member 2 | Slc2a2 | −1.6 | −1.4 | −1.3 |
| Thioesterase superfamily member 2 | Them2 | −1.6 | −1.4 | −1.2 |

Values are expressed as mean±SEM and as a relative percentage of the respective WT control value. Comparisons were performed using Student T test. P<0.05 vs: WT. NM, gene not present in the dataset.

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Figure 6. Ingenuity Pathway analysis (IPA) performed on 31 listed genes. The network is built according the identified interconnected pathways involving the highest majority of genes. Genes represented in pink color belong to the cluster. Genes represented in bold font are central regulators of the identified pathways that do not belong to the cluster. Genes represented in white color are other intermediary regulators that do not belong to the cluster.

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Table 6. Expression fold change of renal genes modified after injection during 8 weeks of rFGF23.

| Gene Name                   | Symbol  | Fgf23+/− | Col4a3+/− | Col4a3+/− |
|-----------------------------|---------|----------|-----------|-----------|
| **FGF23 regulated genes involved in mineral metabolism** |         |          |           |           |
| cytochrome P450, family 24, subfamily A, polypeptide 1 | Cyp24a1 | +12.6    | +1.5      | +1.5      |
| cytochrome P450, family 27, subfamily B, polypeptide 1 | Cyp27b1 | −7.7     | +1.3      | −3.9      |
| solute carrier family 34 (sodium phosphate), member 3 | Npt2c   | −1.2 (NS)| −1.8      | −2.6      |
| solute carrier family 34 (sodium phosphate), member 1 | Npt2a   | −1.2 (NS)| 1.0 (NS)  | −1.2 (NS) |
| **Increased in microarray comparative analysis** |         |          |           |           |
| lipocalin-2                  | Lcn2    | +1.9     | +2.6      | +6.3      |
| complement component factor i | Cfi     | +2.3     | +1.3      | +2.9      |
| vascular cell adhesion molecule 1 | Vcam1  | +2.9     | −1.1 (NS)| +4.5      |
| guanylate binding protein 2  | Gbp2    | +2.1     | +1.2 (NS)| +3.4      |
| tumor-associated calcium signal transducer 2 | Tacstd2 | −2.4     | −1.2 (NS)| +1.6      |
| lectin, galactoside-binding, soluble, 3 binding protein | Lgals3bp| −3.4     | −1.5      | +1.8      |
| phospholipase A2, group VII  | Pla2g7  | −1.1 (NS)| +1.4      | −1.2 (NS)|
| immediate early response 3   | Ire3    | −1.2 (NS)| +2.0      | +2.5      |
| transporter 1, ATP-binding cassette, sub-family B | Tap1    | −1.2 (NS)| −1.2 (NS)| +1.1 (NS)|
| receptor (calcitonin) activity modifying protein 2 | Ramp2   | −1.7     | +1.1 (NS)| +1.5      |
| phospholipid scramblase 1    | Plscr1  | +1.5     | −1.2 (NS)| +2.1      |
| **Decreased in microarray comparative analysis** |         |          |           |           |
| deoxyribonuclease 1          | Dnase1  | −2.4     | +1.5      | −8.1      |
| carbonic anhydrase 14        | Car14   | −1.4     | +1.3      | −1.9      |
| Aflamin                      | Aflm    | +1.0 (NS)| +1.0 (NS)| −2.5      |
| αKlotho                      | αKl     | +1.9     | +1.5      | +1.0 (NS)|
| angiotensin I converting enzyme 2 | Ace2  | −16.7    | (−1.3) NS| −15.6     |
| solute carrier family 2, member 2 | Slc2a2 | −2.2     | −1.1 (NS)| −4.5      |
| thioesterase superfamily member 2 | Them2  | −1.1 (NS)| −1.2 (NS)| −1.9      |

Values are expressed as mean±SEM and as a relative fold change of the non injected Fgf23+/− mice control value. Single (Fgf23+/−) and compound (Col4a3+/− Fgf23+/−) mice were injected (+ rFGF23) or not twice a day with 50 ng/g of recombinant rat FGF23 during 8 weeks. Comparisons were performed using Student T test. P<0.05 vs. Ctr. N=3/group. NS, gene is present but is not significantly different.

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and symptoms that have been monitored three times a week by the investigator team and daily by the Comparative Medicine employees. If any signs of discomfort or infection were observed, the animal was euthanized by CO2 inhalation followed by cervical dislocation and excluded from the study.

Heterozygous Col4a3+/− mice were initially obtained from Jackson Laboratories (Westgrove, PA, USA). To obtain the compound Col4a3+/− Fgf23 mice we first crossed heterozygous Col4a3+/− females to Fgf23 mice +/− males to obtain Col4a3+/−/Fgf23+/− mice and then crossed Col4a3+/−/Fgf23+/− males to Col4a3+/−/Fgf23+/−/Fgf23−/−.

Tail or ear biopsies were collected to genotype the mice. REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA) was used for DNA extraction and PCR amplification. Mice were genotyped for col4a3 mutation and PCR was repeated in all mice after sacrifice to exclude artifacts and ensure the correct genotype [30,79].

Administration of Rat Recombinant FGF23

Rat recombinant FGF23 (rFGF23) was administered intraperitoneally (ip) to WT, Fgf23+/− and Col4a3+/−/Fgf23−/− mice. To test the chronic effects of FGF23, Fgf23−/− and compound Col4a3+/− Fgf23−/− mice were administered twice daily (every 12 hours) with 50 ng/g rFGF23 during eight weeks. Kidneys were collected 12 hours after the last rFGF23 administration. This procedure partially corrected the circulating FGF23 levels in serum samples collected 6 and 12 hours after the last injection (69±22 and 47±16 in FGF23−/− mice and 65±14 and 41±12 in Col4a3−/− Fgf23−/−). To test the acute effects of excess FGF23, C57Bl6 mice were given a single injection of 50ng/g rFGF23 and the kidneys were collected 1 and 12 hours after the injection. Experimental animals were compared to animals of the same genotype receiving 0.9% NaCl vehicle.

Serum Biochemistry

Serum samples were collected by intracardiac exsanguination. Serum calcium was measured using a Calcium CPC Liquicolor Kit (Stanbio Laboratories, Boerne, TX, USA) and serum phosphorus was measured using the phosphomolybdate-ascorbic acid method, as previously described [80]. Serum parathyroid hormone (PTH) levels were measured using the Mouse Intact PTH ELISA Kit (Immutopics, Carlsbad, CA, USA). Serum 1,25(OH)2D and 25OH2D levels were measured using the vitamin D EIA Kits (Immundiagnostic Systems, Fountain Hills, AZ, USA).
USA). Serum FGF23 levels were measured using the FGF23 ELISA kit (Kainos Laboratories, Tokyo, Japan).

RT-PCR and Microarray

RT-PCR and microarray analysis were performed on kidneys from 12 week-old mice. Total RNAs were isolated using TRI-reagent (Molecular Research Center, Cincinnati, OH, USA) according to previously published method [81]. First-strand cDNA was synthesized from the kidney RNAs using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). The 20 μL reverse transcriptase reaction was based on 1 μg total RNA. The iCycler iQ Real-Time PCR Detection System and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) were used for real-time quantitative PCR analysis. The expression was normalized by glyceraldehyde-3-phosphate dehydrogenase (Gapdh) in the same sample and expressed as 100% of the control (WT). Sequences of primers used for real-time quantitative RT-PCR are listed in Table 9. The expression of 45,000 genes was tested on the kidney samples using the Illumina.SingleColor.MouseWG-6_V2_0_R1_11278593_A chip (Illumina, San Diego, CA, USA) at the DNA Discovery Core of University of Tennessee Health Science Center on 4 male mice per group. The resulting data were compared with previously published data reflecting the renal transcriptome in Hyp [40] and FGF23 transgenic mice [31].

### Table 7. Expression fold change of renal genes modified 1 and 12 h after injection or rFGF23.

| Gene Name | Symbol | 1 h | 12 h |
|-----------|--------|-----|------|
| FGF23 regulated genes involved in mineral metabolism | | | |
| cytochrome P450, family 24, subfamily A, polypeptide 1 | Cyp24a1 | 10.8 | 3.5 |
| cytochrome P450, family 27, subfamily B, polypeptide 1 | Cyp27b1 | −3 | NS |
| solute carrier family 34 (sodium phosphate), member 3 | Npt2c | −1.3 | −1.5 |
| solute carrier family 34 (sodium phosphate), member 1 | Npt2a | NS | NS |
| Increased in microarray comparative analysis | | | |
| Lipocalin-2 | Lcn2 | 3.3 | NS |
| complement component factor i | Cfi | 2.2 | 3.1 |
| vascular cell adhesion molecule 1 | Vcam1 | NS | NS |
| guanylate binding protein 2 | Gbp2 | 2.2 | NS |
| tumor-associated calcium signal transducer 2 | Tacstd2 | 1.5 | 2.3 |
| lectin, galactoside-binding, soluble, 3 binding protein | Lgals3bp | NS | 2.3 |
| phospholipase A2, group VII | Pla2g7 | 1.4 | −1.5 |
| intermediate early response 3 | Ire3 | NS | NS |
| transporter 1, ATP-binding cassette, sub-family B | Tap1 | NS | NS |
| receptor (calcitonin) activity modifying protein 2 | Ramp2 | NS | NS |
| phospholipid scramblase 1 | Plscr1 | NS | 2 |
| Decreased in microarray comparative analysis | | | |
| deoxyribonuclease I | Dnase1 | NS | −2.0 |
| carbonic anhydrase 14 | Car14 | NS | −2.2 |
| Afamin | Afm | NS | NS |
| αKlotho | α-Kl | −1.5 | 1.7 |
| angiotensin I converting enzyme 2 | Ace2 | NS | NS |
| solute carrier family 2, member 2 | Slc2a2 | 1.3 | 1.5 |
| thioesterase superfamily member 2 | Them2 | NS | NS |

Values are expressed as mean±SEM and as a relative fold change of the non injected control WT mice (Ctr)value. Comparisons were performed using Student T test. P<0.05 vs. Ctr. N=4/group. NS, gene is present but is not significantly different.

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### Table 8. Expression fold change of selected genes confirmed by RT-PCR in a distal cell culture model, after 12 h of rFGF23 treatment.

| Gene Symbol | Distal (209) Cells |
|-------------|-------------------|
| Vcam1 | NS |
| Cfi | 2.0 |
| Pla2g7 | NS |
| Ramp2 | 1.3 |
| αKl | −1.8 |
| Car14 | −3.7 |
| Slc2a2 | −2.2 |
| Ace2 | −1.7 |
| Dnase1 | −1.4 |
| Afm | −1.4 |

Distal (209) tubular cell lines were cultured during 1 week and treated with 2 μg of rFGF23 per well. Values are expressed as mean±SEM and as a relative fold change of the untreated control. Comparisons were performed using Student T test. N=4; P<0.05 vs: untreated control.
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Western Blotting and Immunohistochemistry

These techniques were performed as described previously [8,82,83]. Briefly, total proteins from kidneys were extracted in 1ml lysis buffer of T-PER Tissue protein extraction reagent (Pierce, IL, USA), supplemented with protease inhibitors (Roche Applied Science, IN, USA). Protein lysates (25 μg/sample) were reduced and extracted in LDS Sample buffer (Invitrogen, CA, USA) heated for 10 min at 70°C, migrated on NuPAGE Novex 10% Bis-Tris Gels (Invitrogen, CA, USA), and then analyzed by Western blotting using the ECL Advance WB Detection Kit (GE Healthcare, UK). The immunoreactive bands were visualized using enhanced chemiluminescence detection reagents (GE Healthcare, UK) on a Fluor-S Multi Imager (BioRad, CA, USA). Band intensities were determined by densitometry using ImageJ (NIH, USA).

For immunohistochemistry, left kidneys were dehydrated in absolute ethanol and embedded in paraffin. 5 μm thick sections were cut on a rotary microtome. Sections were dried overnight on pre-charged pre-cleaned slides (VWR Scientific, PA, USA), deparaffinized and rehydrated. Nonspecific sites were blocked with 1X animal free blocker (Vector Laboratories Inc., CA, USA) and then sections were incubated with specific primary antibodies for 1 hour. An Immunohistological Vectastain ABC kit (Vector Laboratories Inc., CA, USA) was subsequently used for detection of the target protein and slides counterstained with DAPI, dehydrated and mounted with entellan. The following primary antibodies have been used: goat-raised anti-human Klotho (sc-22218), goat-raised anti-human Cyp27b1 (sc-49642), goat-raised anti-human Cyp24a1 (sc-32165), goat-raised anti-mouse lipocalin2 (sc-18698), goat-raised anti-mouse DNase1 (sc-19269) from Santa Cruz Biotechnology (Santa Cruz, CA).

### Table 9. Sequences of primers used for RT-PCR.

| Target Gene | Forward Primer | Reverse Primer |
|-------------|----------------|----------------|
| Ace2        | CTTCTTCTCAGTGGCACCACCCA | CCGTGGGCAAGATCCTCAT |
| Adama2s     | CTGAGGCAGGGCGCGCTT | CCGGCTGAGCGCTGTGCAC |
| Atp8        | AGT GAC GAG TTC GCC TGC GT | CTG GCA CTG GCT TG GTC GGT |
| Car14       | TGG GAT CCT GCC TGC AGA TGG G | TGG CCA ATG GTC CTG ACC GTG |
| Cfi         | AGA CTG GGC CCC GCA CTC CT | CAC ACA CTG GGC TGC CAG CC |
| Cyp 51      | CCC TCA GAC GGT GGC AGG GT | GTC CAA GGG CTC TGC CCA GG |
| Cyp24a1     | GTT CTG CTC ACG GTA GGC | CCA GTC TTC GCA GTT GTC C |
| Cyp27b1     | ACA CTG CTC ACG AGT TAC G | TTA GCA ATC CGC AAG CAC |
| Cyp2c44     | CCC AAG GGC ACC GCT GT T | AGC TCA ATG CCG GGC AAA C |
| Cyp2d12     | AGC CCA GAT CCC AAG GGC AGT | GGT GAC TGG GCA GGG TGC CA |
| Dnase1      | TGC CTG AGC AGC CCT GA | TGA GCC CCC GAG TGC CTA |
| Gbp2        | ACA CTG CCT GTG AGA GAG GAC AGA | CTG TGC GGT AGA GGC CCA CGA |
| Hbb-b1      | GCT TCT GT TCT GTG TGT ACT TGC | GAC AAC CAG CAC CCT GCC CA |
| Hbb-b2      | AGG CCC TGG GCA GGT TGA | GCC ATG GGC CTT CAC GTT GGG |
| Ier3        | GCC GGC AGC TAC CAA CCG AG | GAC CGG GGC GGC AGT AAT GG |
| Lcn2        | TGG CAG GCA ATG CGG TAC AG | CCG TGG TGG CCA CTT GCA CA |
| Lgal3b      | AAG TGG TGG GCA GCA GCC GCC TC | GTC CGA ACA GCT CCT GGG GC |
| Mgp         | GCAGGCGGAGGCGAATAA | AGGAAGGAGTGGGGCAGCGAG |
| sKlotho     | AGG CAG ATG ACC TAC CAA CCG | GCA TGA TCT GAT ATT AGC |
| Npt2a       | ATG CTG GCT TCT TCC TAC | CCA CAA TGT TCA TGC CTT CT |
| Npt2c       | CGT GGG GAC TGT TAT CAA TG | TAC TGG GCA TGC ACG TTT CT |
| Pla2g7      | TGC TGC CTC CCA TGG TGC CA | AGC CGG CAG CAG ACA TCA CC |
| Plscr1      | GAG TCC CTC CTG CCA GGG AAA GC | CCC CGG TGG ACA ATG CAG TGG A |
| Ramp2       | GAC AGC GTT GTG CCT CTC TCC | GTC GCA CCA GGG AGT ATG |
| Slc2a2      | CCA GTC GTG CAG TGG GGC GA | CCC AGG GCA CCC CTG AGT |
| Slc34a2     | AAA TGC CCA GCC CAA CCC CG | GTC CGG CCA CTT TGC CTC CA |
| STAT3s1     | CCCCCACGGCCACCCAGTTA | TGTCGAGCTGTGGTTGCA |
| Tacstd2     | GGC ATG GGC ACC CG GCT TTT TG | GAC CCC GGC TGG GCC ATT TG |
| Tap1        | GCC CTG GAC GCC TTA TCG GCG | ATG AGA CAA GTG GCC TGG TGC TG |
| Them2       | TTT CCT GCC AGC AGC AG | GGA GCA GGC GAG ACA AGT |
| Timp1       | CAC GGG CCT AAG GGA CG | TTC GTG GCA GGC AAG CAA AGT |
| Vcam1       | GTT CAA CGT TTC CCC CAA GGA | GCC ATC CTG CAG CTG TGC CT |

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Cell Culture

Immortalized renal tubular cells were kindly donated by Peter Friedman [84]. Cells were plated in standard 25 cm² flasks and allowed to grow until confluence. Cells were removed with 0.25% trypsin solution containing 0.02% EDTA (Sigma- Aldrich, St. Louis, MO, USA) and plated on 6-well plates for 7 days, then treated with 2 µg/well of rFGF23 or vehicle for 24 hours.

Statistics

Differences among the two groups were tested by Student T test using the Statistica software (Statssoft, Tulsa, OK, USA). The differences were considered statistically significant at p<0.05.

Microarray analysis and filtering was performed as previously described [88]. Briefly, microarray data were analyzed using GeneSpring GX7.3 software (Agilent Technologies, Santa Clara, CA, USA). The Robust Multichip Averaging probe summarization algorithm was used to perform background correction, normalization, and probe summarization. Data were normalized per chip and per gene to the median. Genes were filtered to include only those that were expressed in at least one of the eight samples. The statistical analysis was performed using a one-way ANOVA followed by Benjamini-Hochberg multiple test correction assuming variances were equals to minimize the false positive discovery. P value was set at 0.05. Cluster analysis using a gene tree classification, Pearson correlation and average linkage was then performed to identify groups of genes for which the patterns of expression were similar. Pathway analysis was performed using the Ingenuity program (Ingenuity Systems, Redwood City, CA, USA) to match the identified genes of interest to already known broader networks of genes contained in the literature database.

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Author Contributions

Conceived and designed the experiments: VD VJ. Performed the experiments: BD VJ. Analyzed the data: VD AM BD. Contributed reagents/materials/analysis tools: AM HL JH YJ WG. Wrote the paper: VD VJ.

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