Klotho Deficiency Causes Vascular Calcification in Chronic Kidney Disease

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ABSTRACT

Soft-tissue calcification is a prominent feature in both chronic kidney disease (CKD) and experimental Klotho deficiency, but whether Klotho deficiency is responsible for the calcification in CKD is unknown. Here, wild-type mice with CKD had very low renal, plasma, and urinary levels of Klotho. In humans, we observed a graded reduction in urinary Klotho starting at an early stage of CKD and progressing with loss of renal function. Despite induction of CKD, transgenic mice that overexpressed Klotho had preserved levels of Klotho, enhanced phosphaturia, better renal function, and much less calcification compared with wild-type mice with CKD. Conversely, Klotho-haploinsufficient mice with CKD had undetectable levels of Klotho, worse renal function, and severe calcification. The beneficial effect of Klotho on vascular calcification was a result of more than its effect on renal function and phosphatemia, suggesting a direct effect of Klotho on the vasculature. In vitro, Klotho suppressed Na\textsuperscript{+}/H\textsuperscript{+}-dependent uptake of phosphate and mineralization induced by high phosphate and preserved differentiation in vascular smooth muscle cells. In summary, Klotho is an early biomarker for CKD, and Klotho deficiency contributes to soft-tissue calcification in CKD. Klotho ameliorates vascular calcification by enhancing phosphaturia, preserving glomerular filtration, and directly inhibiting phosphate uptake by vascular smooth muscle. Replacement of Klotho may have therapeutic potential for CKD.


The high cardiovascular mortality in patients with chronic kidney disease (CKD) is closely associated with vascular calcification (VC).\textsuperscript{1,2} Risk factors for VC include hypertension, hyperlipidemia, diabetes, plasma phosphate, homocysteine, and osteoprotegerin.\textsuperscript{3,4} Defects in endogenous anti-calcification factors such as matrix Gla protein, osteoprotegerin, carbonic anhydrase isoenzyme II, fibrillin-1, fetuin-A, fibroblast growth factor 23, and Klotho may play an important role in this dire complication of CKD.\textsuperscript{5–10} High serum phosphate is associated with significantly increased risk for death.\textsuperscript{11} Treatment with phosphorus binders improves survival of hemodialysis patients compared with no treatment with matched baseline serum phosphate levels.\textsuperscript{12} Early diagnosis and treatment is important to retard the progression of CKD. Most biomarkers in current clinical use are not early or sensitive enough.\textsuperscript{13–16} The need to find a sensitive and early biomarker is of paramount importance for early diagnosis and intervention. Various strategies have been devised to slow progression of renal disease\textsuperscript{12,17,18} with varying effectiveness.\textsuperscript{19} We are in

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dire need of additional new agents in preventing the progress
of CKD and in ameliorating VC.

*Klotho* was originally identified as an aging suppressor. Its gene product is a single-pass transmembrane protein that functions as a coreceptor for fibroblast growth factor (FGF) 23. Klotho is expressed widely, but its level is highest in the kidney. Klotho is also secreted into the cerebrospinal fluid, blood, and urine by ectodomain shedding mediated by membrane-anchored proteases. Secreted Klotho functions in an endocrine fashion as an enzyme or possibly a hormone. Klotho deficiency in rodents leads to a syndrome of premature aging where ectopic soft tissue calcification is a notable feature. Overexpression of Klotho rescues the Klotho-deficient phenotype including ectopic calcification, suggesting that Klotho may be an inhibitor of ectopic calcification.

Because of the features common to both human CKD and murine experimental Klotho deficiency (*Kl/H11002/H11002*), we postulate that Klotho deficiency may be responsible for the VC in CKD. The literature offers suggestive but limited evidence for a pathogenicrole of Klotho in CKD. Renal Klotho mRNA is lower in a five-sixths nephrectomy model of CKD and in human nephrectomy samples from end-stage sclerotic kidneys. A modest amelioration of proteinuria and renal function was observed when Klotho was overexpressed genetically in a chronic glomerulonephritis model or via viral delivery in a chronic angiotension II and a spontaneous hypertension model.

We will test three hypotheses: (1) CKD is a state of Klotho deficiency; (2) low Klotho is an early marker of CKD; and (3) Klotho deficiency contributes to VC and Klotho replacement ameliorates CKD via multiple mechanisms.

### RESULTS

**CKD Is a State of Klotho Deficiency**

The pattern of calcification of soft tissue in CKD is indistinguishable from that seen in Klotho deficiency in rodents. We found similar increases in tissue calcium content in *Kl/H11002/H11002* and CKD animals (Figure 1, A and B). We next asked whether CKD is a state of endocrine Klotho deficiency. End-stage CKD patients and animals have reduced Klotho in kidneys, but there is no data on blood or urine Klotho in CKD. Klotho was undetectable in homozygous Klotho deficiency (*Kl/H11002/H11002*) and was notably decreased in kidney and barely detectable in the blood and urine of CKD mice (Figure 1C), indicating that CKD is a state of “pan deficiency” of Klotho. Because of the lack of a reliable assay for human plasma Klotho at the time of the study, we measured urinary Klotho in CKD patients...

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**Figure 1.** Klotho levels are reduced in CKD mice and CKD patients, and soft tissue calcification is observed in CKD mice. (A) Ectopic calcification in soft tissues by Von Kossa staining and calcification in aortas and kidneys of *Kl/H11002/H11002* mice and WT CKD mice (arrows). (B) Calcium content assayed by OCPC in soft tissues (aortas and the kidneys) of *Kl/H11002/H11002* mice versus WT littermates and also of WT CKD mice versus WT Sham mice. The data are presented as the means ± SEM (*n* = 4). *P* < 0.05, **P** < 0.01 versus WT or Sham mice by unpaired t test. (C) Representative blots of Klotho protein in plasma (*n* = 3), urine (*n* = 4), and kidney (*n* = 5) of *Kl/H11002/H11002* mice or WT CKD mice. Immunoprecipitation of Klotho in 100 µl of mouse serum was followed by immunoblot. IgG heavy chain was used as the loading control. Urine Klotho was examined by directly immunoblotting approximately 40 µl of mouse urine with an identical amount of creatinine. Klotho protein in the kidney was analyzed by immunoblotting 30 µg of the total kidney lysate and qualitatively examined by immunohistochemistry. (D) Urinary Klotho protein in humans with normal kidney function and various CKD stages. The upper panel is a representative immunoblot with serial dilutions of known concentration of rMKl and concentrated human urine samples of identical amount of creatinine in same gel. The lower panel is a summary of urinary Klotho protein concentration (depicted in open bars) and of Klotho normalized by creatinine (depicted in solid bars) of normal subjects and CKD patients.
as a surrogate. Humans (Table 1) with various stages of CKD (National Kidney Foundation classification)\textsuperscript{14} have lower levels of Klotho in the urine (Figure 1D) extremely early in human CKD stage 1 (Figure 1D and Supplemental Figure 1), and the magnitude of decrease correlates with the severity of decline in estimated GFR (eGFR) in humans.

**Klotho Levels and Progression of CKD and Vascular Calcification in CKD**

CKD and experimental Klotho deficiency both have low Klotho in blood, kidney, and urine (Figure 1C), high plasma FGF23,\textsuperscript{38} hyperphosphatemia (Table 2), and ectopic calcification (Figure 1, A and B). A critical question is whether Klotho deficiency is a mere marker or whether it contributes to the pathophysiology of CKD because the latter raises the possibility of therapeutic replacement. To this end, we examined whether Klotho levels affect CKD and its complications. Baseline Klotho was lower in KT\textsuperscript{+/−} mice compared with WT and was highest in Tg-Kl mice (Figure 2A and Supplemental Figure 2A). Klotho was decreased in all lines of mice when CKD was induced. The Klotho level in Tg-KI-CKD mice was lower than Tg-KI-Sham mice but still equivalent to that of WT-Sham mice (Figure 2A and Supplemental Figure 2A). Tg-KI mice have higher plasma Klotho levels\textsuperscript{36} and more organs expressing Klotho protein.\textsuperscript{9} In the kidneys of Tg-KI mice, almost all of the renal structures express Klotho protein (Supplemental Figure 2B).

WT-CKD mice had hypertension, anemia, increased plasma creatinine (P\textsubscript{Cr}), declined creatinine clearance (Cl\textsubscript{Cr}), increased proteinuria (Supplemental Table 1), and more severe renal histologic damage (Supplemental Figure 3). All of the changes were slightly exaggerated in KT\textsuperscript{+/−} mice but were much improved in the Tg-KI mice (Supplemental Table 1 and Supplemental Figure 3). KT\textsuperscript{+/−} mice had more severe and Tg-KI mice had milder CKD than WT mice, although all were subjected to the same insult. Hence, amelioration of CKD per se can be a potential factor for less severe soft tissue calcification when Klotho levels are maintained.

Elevation of parathyroid hormone (PTH) in WT-CKD mice was blunted by Klotho overexpression and worsened by Klotho deficiency (Figure 2B). CKD decreased plasma 1,25-(OH)\textsubscript{2}D\textsubscript{3} modestly in WT mice (Figure 2C), which is compatible with the moderate CKD (Supplemental Table 1). The increased 1,25-(OH)\textsubscript{2}D\textsubscript{3} in KT\textsuperscript{+/−} mice is compatible with Klotho being a potent suppressor of 1,25-(OH)\textsubscript{2}D\textsubscript{3} production\textsuperscript{39,40} (Figure 2C). Our in vivo data do not exclude the possibility that Klotho’s beneficial effect may be through various calcitropic hormones. The direct effect of Klotho will be examined below.

One determinant of soft tissue calcification is plasma phosphate (Pi) concentration.\textsuperscript{41–44} Both KT\textsuperscript{+/−} and WT animals with CKD had higher levels of plasma Pi and higher fractional excretion of phosphorus (FE\textsubscript{phos}) than Sham animals (Supplemental Table 1). In contrast, Tg-KI-CKD mice did not show much hyperphosphatemia; their FE\textsubscript{phos} were already high in baseline and did not increase further with CKD (Supplemental Table 1). Therefore, a second mechanism by which Klotho can lessen soft tissue calcification might be by lowering plasma phosphate levels through promotion of phosphaturia.\textsuperscript{25}

We screened for ectopic calcification in multiple organs. As expected, there was no staining in Sham animals (not shown). In CKD, there was calcification in the kidneys and aortas of both WT and KI\textsuperscript{+/−} mice (Figure 2D). In contrast, Tg-KI-CKD animals had very little or no calcification (Figure 2D). The modest and patchy calcification in the vasculature of WT CKD mice might be due to the modest renal failure and/or short duration of follow-up. The percentage of mice with detectable calcification for each CKD group was: KT\textsuperscript{+/−} mice, 69.2% (9 of 13) versus WT 57.1% (8 of 14); and Tg-KI, 25% (4 of 16) versus WT 53.3% (8 of 15). Calcium content in aortas (Figure 3A) and kidneys (Figure 3B) was higher in CKD than Sham in both the WT and KT\textsuperscript{+/−} mice. The calcium content in all organs is inversely related to Klotho levels: highest in KT\textsuperscript{+/−} mice, intermediate in WT, and lowest in Tg-KI (Figure 3, A and B).

In humans with CKD, both plasma Cr\textsuperscript{45} and Pi levels\textsuperscript{41–44} are predictors of soft tissue calcification. Soft tissue cal-

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**Table 1. Summary of ages and eGFRs of normal subjects and CKD patients**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>CKD Overall</th>
<th>CKD Stages</th>
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<tr>
<td></td>
<td>Age (years)</td>
<td></td>
<td>Stage 1</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>6/7</td>
<td>18/22</td>
<td>1/7</td>
</tr>
<tr>
<td>eGFR (ml/min per 1.73 m\textsuperscript{2})</td>
<td>105.46 ± 4.81</td>
<td>58.80 ± 6.90\textsuperscript{a}</td>
<td>116.88 ± 3.26</td>
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\(\text{eGFR is calculated with the Modification of Diet in Renal Disease equation.}\)

\(\text{aP < 0.01 versus normal subjects by one-way ANOVA followed by Student-Newman-Keul’s test.}\)

\(\text{bP < 0.05 versus normal subjects by one-way ANOVA followed by Student-Newman-Keul’s test.}\)

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**Table 2. Blood Pi and creatinine clearance in Klotho\textsuperscript{−/−} mice and WT CKD mice**

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<thead>
<tr>
<th></th>
<th>Klotho\textsuperscript{−/−} Model</th>
<th>CKD Model in WT Mice</th>
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<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KT\textsuperscript{+/−}</td>
</tr>
<tr>
<td>Serum Pi (mg/dl)</td>
<td>5.9 ± 0.4</td>
<td>8.1 ± 0.5\textsuperscript{a}</td>
</tr>
<tr>
<td>Cl\textsubscript{Cr} (\mu l/min/g body wt)</td>
<td>15.41 ± 1.03</td>
<td>10.32 ± 2.14\textsuperscript{b}</td>
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\(\text{The data are represented as the means ± SEM (n ≥ 4).}\)

\(\text{aP < 0.01 versus Sham by unpaired t test.}\)

\(\text{bP < 0.05 versus WT by unpaired t test.}\)
cium content is positively related to plasma Pi and Cr in all mice (Figure 3C). When we divided the animals into subgroups on the basis of their genetic Klotho status, despite the overlap, one could see that for a given plasma Pi and Cr concentration, Tg-Kl mice had the lowest soft tissue calcium content, and Kl/Kl mice had the highest, with WT in between (Figure 3C). Therefore, differences in plasma Pi or Cr are insufficient to explain the different levels of ectopic calcification in the various Klotho background. The data suggest that Klotho has a direct protective effect on soft tissue calcification above and beyond that of the renal effects of phosphaturia and preservation of glomerular filtration.

**Pi Uptake and Pi-induced Mineralization and Dedifferentiation in Cultured Cells**

Elevated plasma Pi is associated with VC in experimental animals and in CKD patients.41–44 Pi influx is believed to be mediated by NaPi-3 group of Na\(^+\)/H\(^+\)-coupled transporters (Pit-1...
In the aorta, control the balance between differentiation and dedifferentiation of Klotho had the opposite effect. Therefore, Klotho may increased both transcription-PCR; data not shown). High Pi treatment inhibited Pit1 abundance than except for Pit1 in adipocytes (Figure 6B). Both Pit1 and Pit2 (−10× lower abundance than Pit1) transcripts are present in A10 cells (NaPi-2a, NaPi-2, and NaPi-2c are not detectable by reverse transcription-PCR; data not shown). High Pi treatment increased both Pit1 and Pit2 mRNA in A10 cells, and Klotho blocked this increase (Figure 7A). A similar inhibition by Klotho was also found in osteoblast cells and adipocytes except for Pit1 in adipocytes (Figure 6C). High Pi induced upregulation of Runx2 and downregula-

Figure 3. The levels of calcium content in the kidneys and the aortas of Sham and CKD mice are correlated with genetic levels of Klotho. (A and B) Calcium content was assayed using OCPC in the aortas (A) and the kidneys (B) of Sham and CKD mice at different genetic Klotho levels: KI+/− (red) and Tg-Kl (blue) and their WT littermates (black). The data are represented as the means ± SEM (n = 7). *P < 0.05; **P < 0.01 versus Sham WT mice of KI+/− group; †P < 0.05; ††P < 0.01 Sham KI+/− mice; ‡P < 0.05; ‡‡P < 0.01 versus CKD KI+/− mice; §P < 0.05; §§P < 0.01 versus Sham WT mice of Tg-Kl group; &P < 0.05; &&P < 0.01 versus Sham Tg-Kl mice; *P < 0.05; **P < 0.01 versus CKD Tg-Kl mice by one-way ANOVA followed by Student-Newman-Keul’s test. (C) Relationship of calcium content in the aortas and the kidneys with blood Pi and blood Cr, respectively, in Sham (triangles) and in CKD (circles) mice at three different genetic Klotho levels: KI+/− (red) and Tg-Kl (blue) and their WT littermates (black). C, CKD; S, Sham.
tion of SM22 mRNA and protein in A10 cells (Figure 7), suggesting that dedifferentiation of smooth muscle occurred with high Pi. Klotho reversed these changes and blocked Pi-induced dedifferentiation of A10.

**DISCUSSION**

This is the first report that CKD is a state of Klotho deficiency in the kidney, plasma, and urine and that Klotho downregulation is not merely an early biomarker for kidney damage but also plays a pathogenic role in the progression of CKD as well as one of the principal complications of CKD, namely VC. Klotho bestows its anti-calcification effect possibly via at least three mechanisms: a phosphaturic hormone, the preservation of GFR, and a direct effect on soft tissues including the vascular smooth muscle (Figure 8). The potential utility of Klotho in clinical practice is at least two-fold. First, Klotho can serve as an early and sensitive biomarker of CKD. Second, Klotho replacement therapy may be in the horizon in slowing progression of CKD as well as preventing and reversing complications.

**CKD Is a State of Klotho Deficiency**

Our animals have disease equivalent to human CKD stage 3 to 4, which comprises up to approximately 85% of human CKD. Decreased renal Klotho expression was shown in human renal tissue from end stage kidneys and animals with five-sixths nephrectomy. Because secreted Klotho exerts multiple effects on distant sites, it is crucial to explore Klotho protein levels in blood and urine. We found commensurate Klotho deficiency in the kidney, plasma, and urine in rodent CKD and in urine in human CKD. The mechanism of how kidney disease lowers Klotho expression is unknown presently but can potentially involve ischemia, oxidative stress, angiotensin II, and inflammation. CKD lowered Klotho even in the Tg-Kl mice, despite the fact that the transgene was driven by a constitutive promoter. This can be due to the fact that
endogenous renal Klotho expression actually constitutes 50% of the renal Klotho in the transgenic animals.36 In addition, CKD may have a translational or post-translational effect on the transgenic Klotho.

Pathogenic Role of Klotho in Progression of CKD and Its Complications

The data clearly show that Klotho is more than a mere marker for CKD. Klotho overexpression lessens progression of CKD, improves Pi metabolism, and protects the vasculature from calcification. Previous studies showed that overexpressing Klotho by viral-based gene transfer34 or genetic manipulation33 attenuated progressive renal injury, but the Klotho status and systemic complications of CKD were not studied. Because Klotho is present in multiple body fluids,25,27,36,57 the restoration of Klotho clearly exerts multiple systemic effects in addition to renoprotection.

Disturbed mineral metabolism is implicated in hyperparathyroidism, osteodystrophy, and vascular calcification in CKD.38–61 Pi overload accelerates calcification in CKD, and control of Pi reduces calcification in CKD.62–64 Klotho exerts its phosphaturic effects by inhibiting renal NaPi-2a and NaPi-2c in the renal proximal tubule.25,38 Maintenance of high Klotho in CKD preserves phosphaturia and lessens phosphate retention. Hyperphosphatemia is also an important contributor to VC observed in Klotho-deficient mice.65

Tg-Kl animals have better renal function when subjected to the same renal insult. The mechanisms whereby Klotho protects kidney from injury are unknown but potentially include anti-oxidation, anti-apoptosis,55–56 and anti-senescence.33 In addition to preservation of phosphaturia and GFR, Klotho has a direct effect on the vasculature. Pathologic calcium phosphate deposition in the blood vessels and heart is found in aging, diabetes,67,68 hyperlipidemia,69 and CKD.3–4,7 NaPi-3 proteins (Pit1 and Pit2) are broadly expressed and believed to play housekeeping as well as pathologic roles in different cells.70–73 Extracellular Pi stimulates calcification in VSMC through inorganic Pi influx by NaPi-373–75 and cell-

Figure 5. Klotho regulates Pi-induced mineralization and Pi uptake but not calcium uptake in cultured rat VMSC (A10). (A) A10 cells in a six-well plate were incubated in medium containing 1.0 or 2.0 Pi mM with or without 0.4 nM Klotho for 10 days to examine the Klotho effect on Pi modulated calcium content in A10 cells measured by OCPC assay. The data are presented as the means ± SEM (n = 8). *P < 0.05; **P < 0.01 versus Pi 1.0 mM + Kl 0 nM; †P < 0.05; ††P < 0.01 versus Pi 2.0 mM + Kl 0 nM; ‡P < 0.05; ‡‡P < 0.01 versus Pi 1.0 mM + Kl 0.4 nM by one-way ANOVA followed by Student-Newman-Keul’s test. (B) Effect of Pi on calcium content in A10 cells: dose dependence. The half-maximal effect was achieved at 0.72 mM Pi in the absence of Klotho and at 1.15 mM in 0.4 nM Klotho. (C) Effect of Klotho effect on calcium content in A10 cells: dose dependence. (D) A10 cells were incubated in medium containing 1.0 or 2.0 Pi mM with or without 0.4 nM Klotho for 3 days. Na+-dependent and Na+-independent isotopic Pi uptake was determined. The data are presented as the means ± SEM (n = 6). *P < 0.05; **P < 0.01 versus Pi 1.0 mM + Kl 0 nM; †P < 0.05; ††P < 0.01 versus Pi 2.0 mM + Kl 0 nM; ‡P < 0.05; ‡‡P < 0.01 versus Pi 1.0 mM + Kl 0.4 nM by one-way ANOVA followed by Student-Newman-Keul’s test. (E) Effect of Pi on Na+-dependent uptake on A10 cells: dose dependence. Vmax = 35.2 pmol/mg/min and Km = 12.2 mM Pi in the absence of Klotho, and Vmax = 31.3 pmol/mg/min and Km = 17.6 mM with 0.4 nM Klotho. (F) Effect of Klotho effect on Pi uptake in A10 cells: dose dependence. A10 cells were incubated in medium containing 1.0 or 2.0 Pi mM with or without 0.4 nM Klotho for 3 days.
surface Pit2 reorganization. High ambient Pi accelerates mineralization and stimulates surrogate markers of osteogenesis in VSMC, spawning the hypothesis that high Pi stimulates “ossification” of VSMC. Soluble Klotho not only suppresses baseline NaPi-3 activity but also abrogates high Pi-induced upregulation of NaPi-3 mRNA and activity and suppresses calcification and maintains differentiation of VSMC. Pit1 might also act through mechanisms independent of Pi influx, and suppression of Pit1 may affect cell proliferation.

One minor caveat of the A10 in vitro model is that we do not know whether cultured rat cells mimic all features of human VSMC in vivo.

FGF23 signal transduction generally requires transmembrane Klotho as a coreceptor. CKD subjects have high levels of full length FGF23 and upregulation of this signal pathway. We did not measure FGF23 levels in our animals. It is possible that part of the beneficial effects of Klotho on soft tissue in CKD result from improvement of FGF23 signal transduction.

**Potential Clinical Utility**

These preclinical studies lay the foundation for two major potential applications. Extensive effort has been devoted to search for early biomarker for kidney diseases focusing mostly on acute kidney disease and less on CKD. Proteins such as adiponectin, γ-glutamyltransferase, cystatin C, N-acetyl-β-D-glucosaminidase, fatty acid-binding protein 1, and endothelin-1 were proposed as biomarkers. We documented that patients with early stage CKD (stage 1 and 2) already have significantly lower urinary Klotho, and urinary Klotho is progressively lowered with declining eGFR. Urinary Klotho protein might be an ideal early biomarker for CKD. One important goal of CKD treatment is to prevent or postpone the progression to end stage. Klotho supplementation might be a good strategy not only to preserve remnant kidney function but also to minimize complications of CKD through multiple mechanisms.

**CONCISE METHODS**

**Human Study**

A total of 53 human subjects were included in this study: 13 normal volunteers and 40 CKD patients (Table 1) at different stages according to the National Kidney Foundation. This study was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center at Dallas. All of the human subjects were given and accepted an informed consent form. None of the human subjects were receiving treatment with Pi binder, calcium, or active vitamin D or
analogs or renal replacement therapy when their urine samples were collected. For measurement of urinary Klotho protein, 4 ml of fresh urine was concentrated to 0.2 ml through Amicon Ultra-4 filters with 100-kD cutoff (Millipore, Billerica, Massachusetts). The concentrated urines (with identical urine creatinine) along with recombinant murine Klotho (rMKI) protein of known concentration were subjected to immunoblot. Specific signals on the basis of bands on films were obtained with free Image J program (National Institutes of Health), and the Klotho protein concentrations in urine samples were quantified using the rMKI as a standard curve.

Animal Models

All of the work on mice was conducted following the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health and was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

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Figure 7. Klotho suppresses Pit1/2 expression and Pi-induced dedifferentiation in cultured rat VMSC (A10). (A) A10 cells were incubated in medium containing 1.0 or 2.0 Pi mM with or without 0.4 nM Klotho for 3 days, and mRNA levels of Pit1, Pit2, Runx2, and SM22 were assayed by qPCR. The data are presented as the means ± SEM (n = 6). (B) The representative immunohistochemistry for Runx2 and smooth muscle actin (SMA) was shown from three independent experiments in A10 cells. (C) A representative immunoblot for Runx2, SMA, and β-actin is displayed from four independent experiments of A10 cells. (D) A summary of densitometric quantification of all samples is shown. The data are presented as the means ± SEM (n = 4). *P < 0.05; **P < 0.01 versus Pi 1.0 mM + KI 0 nM; #P < 0.05; ##P < 0.01 versus Pi 2.0 mM + KI 0 nM; £P < 0.05; ££P < 0.01 versus Pi 1.0 mM + KI 0.4 nM by one-way ANOVA followed by Student-Newman-Keul’s test for (A) and (D).

Figure 8. Proposed model of potential effects of Klotho on vascular calcification. Klotho can protect the vasculature against calcification in CKD probably by three actions: (1) slowing progression of CKD; (2) maintenance of normophosphatemia through induction of phosphaturia; and (3) direct inhibition of Pi influx into VSMC, which in turn suppresses the dedifferentiation of VSMC.
The mice used for preparation of CKD model were: (1) one line of transgenic mice with overexpression of Klotho, EFmKL46 (Tg-Kl), whose genetic background is a mixture of C57BL/6 and 129 and (2) heterozygous for Klotho-deficient mice (Kl+/−), whose genetic background for is C57BL/6 and C3H/1. WT littermates were generated during cross-breeding for Tg-Kl and Kl+/− mice. Kl+/− mice and WT littermates used in this study ranged from 6 to 8 weeks; Kl+/−, Tg-Kl, and WT mice were approximately 12 weeks in age. CKD model was generated using uninephrectomy plus ischemia-reperfusion injury in contralateral kidney. Sham mice underwent laparotomy and manual manipulation of the kidneys. After recovery, the mice were housed in normal cages and fed with 1.0% phosphorus diet for 4 weeks with free access to tap water followed by 2.0% phosphorus diet for 8 weeks. For the metabolic study, the mice were transferred to individual metabolic cages. After acclimatization, 24-hour urine was collected, blood was drawn, and tissues were harvested. Plasma and urine chemistry were analyzed by Vitros Chemistry Analyzer (Ortho-Clinical Diagnosis, Rochester, New York) by the Animal Core Facility in University of Texas Southwestern Medical Center. BP was measured by a computerized tail-cuff system (BP-2000; Visitech Systems, Apex, North Carolina) in conscious animals throughout our study. Plasma intact PTH and 1,25-(OH)2 vitamin D3 were measured using Apex, North Carolina) in conscious animals throughout our study. Plasma intact PTH and 1,25-(OH)2 vitamin D3 were measured using ELISA kits from Alpco (Salem, New Hampshire) and Immunodiagnostic Systems (Scottsdale, Arizona), respectively.

**Von Kossa Staining and Calcium Content**
The calcium content in soft tissues was measured using the o-cresolphthalein complexone (OCPC) method (Sigma, St. Louis, Missouri). Quantitation (μg/mg protein) was normalized to protein concentration determined by Bradford protein assay. Kidney, heart, and aorta were stained with Von Kossa and counterstained with nuclear Fast Red for evaluation of calcium precipitation. Positive signal for calcium precipitation would be seen in black or brown-black.

**Cell Culture**
Rat vascular muscle cell (A10), mouse osteoblasts (MC-3T3-E1), mouse adipocytes (3T3-L1), and canine kidney cell (MDCK) were cultured and maintained in condition as described previously. The cells were treated with Pi and/or soluble Klotho protein (amino acid number 31 to 982) as previous described. At given time points, the cells were harvested for Von Kossa staining, OCPC assay, RNA extraction, and for 32P-phosphate and 45Ca uptake (detailed protocol in Supplemental Methods).

**Kidney Histology and Immunohistochemistry**
Four-μm sections of frozen kidney tissues were stained with hematoxylin and eosin and observed and photographed by a renal pathologist (JZ) blinded to the experimental conditions using an Axioplan 2 Imaging System (Carl Zeiss, Thornwood, New York). For immunofluorescence study, a monoclonal rat antibody (KM2076) against human Klotho (1:250) was used for staining and followed by secondary antibodies conjugated to fluorescin isothiocyanate (detailed methods in the supplemental materials). Rhodamine-phalloidin (1:50) (Molecular Probes, Eugene, Oregon) for staining β-actin filaments was applied for double staining. The sections were visualized with a Zeiss LSM-510 laser scanning microscope.

**Quantifying Klotho in the Kidney, Urine, and Blood of Mice**
Kidney total lysates were prepared as described. Thirty μg of protein of kidney lysate was solubilized in Laemmli sample buffer; approximately 40 μl of fresh urine were immediately mixed in Laemmli sample buffer after collection. Urine samples with identical amounts of urine creatinine were subjected to SDS-PAGE. One hundred μl of mouse serum were subjected to immunoprecipitation with 4 μl of rabbit anti-serum of human Klotho, followed by immunoblot analysis with anti-Klotho antibody (KM2076) (1:2500), goat anti-rabbit antibody conjugated with horseradish peroxidase (1:5000 dilution) for IgG heavy chain, and monoclonal mouse antibody for β-actin (1/5000 dilution; Sigma). Specific signal was visualized using the ECL kit (PerkinElmer LAS, Inc., Boston, Massachusetts).

**PCR**
For real time PCR, total RNA was extracted from mouse tissues (kidney and aorta) and cell lines (rat vascular smooth muscle cells, A10; mouse osteoblast-like cells, MC-3T3-E1; mouse adipocytes, 3T3-L1). Complimentary DNA was generated. Primers for quantitative PCR (qPCR) are shown in Supplemental Table 4 with conditions described previously. The detailed methods are described in the supplemental materials.

**Statistical Analyses**
The data are expressed as the means ± SEM (n = 8 or more unless indicated otherwise). The detailed analyses were described in the supplemental materials.

**ACKNOWLEDGMENTS**
This work was supported primarily by the Simmons Family Foundation. The authors also received supported from the National Institutes of Health (AG-19712, AG-25326, DK-48482, and DK-54392), the George M. O’Brien Kidney Research Core Center/University of Texas Southwestern Medical Center at Dallas (NIH P30DK-07938), the American Heart Association (0865235F), Eisai Research Fund, Ellison Medical Foundation, Ted Nash Long Life Foundation, and a Grant from the Charles and Jane Pak Center of Mineral Metabolism and Clinical Research. Parts of this work was published in abstract form in the Journal of the American Society of Nephrology (18: 7A–8A, 2007). The authors are grateful to Mr. Lei Wang for breeding and maintaining Kl+/− and Tg-Kl mice and their wild-type littermates, to Ms. Olga Sineshchekova for Klotho protein preparation, and to Ms. Rebecca Arichta for assistance in measurement of PTH and 1,25-(OH)2 vitamin D3.

**DISCLOSURES**
None.
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BASIC RESEARCH


Supplemental information for this article is available online at http://www.jasn.org/.

Supplementary materials for:

Klotho Deficiency and Vascular Calcification in Chronic Kidney Disease

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Running title: Klotho in chronic kidney disease

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SUPPLEMENTARY INFORMATION

SUPPLEMENTAL FIGURES

Supplemental Figure 1: Relationship of urinary Klotho concentration. Individual data points used for bar graph in Figure 2. (A) Urinary Klotho and (B) urinary Klotho/Cr ratio with eGFR of normal (red circles) and of CKD patients (black circles).

Supplemental Figure 2: Klotho protein expression in the kidneys of mice. (A) CKD and Sham mice of different Klotho levels. Frozen kidney sections were stained with monoclonal rat antibody for Klotho followed by ant-rat IgG conjugated to FITC. (B) Colocalization of Klotho protein (green) and NaPi-2a protein (red) in the kidneys of Tg-Kl and WT mice.

Supplemental Figure 3: Kidney histology in Sham and CKD mice. Kidneys were harvested and frozen in O.C.T compound (Tissue TeK) using liquid N2. Cryosections (4 μm) were subjected for Hematoxylin-Eosin (HE) staining. (A) the sham and CKD mice of Kl+/− and WT littermates; (B) the sham and CKD mice of Tg-Kl and WT littermates.

Supplementary Figure 4: Effect of soluble Klotho protein on mineralization induced by high Pi culture media in several cell lines: Rat vascular muscle cell (A10) (A), canine kidney cell (MDCK) (B), mouse osteoblasts (MC-3T3-E1) (C), and mouse adipocytes (3T3-L1) (D) were incubated with 1.0 or 2.0 mM Pi culture media with Klotho or vehicle for 10 days. The extent of mineralization in cells was estimated by Von Kossa staining. Pixels per plate were taken with ImageJ software (NIH), quantified and shown in bottom panel in each cell line. Data is presented as means ± SEM; n = 4. *: P<0.05, **: P<0.01 vs Pi 1.0 mM + Kl 0 nM; #: P<0.05; ##: P<0.01 vs Pi 2.0 mM + Kl 0 nM; £: P<0.05; ££: P<0.01 vs Pi 1.0 μM + Kl 0.4 nM by one-way ANOVA followed by Student-Newman-Keuls test.
**Supplementary Figure 5: Effect of Klotho effect on Ca uptake in A10 cells:** A10 cells were incubated in medium containing 1.0 or 2.0 Pi mM with or without 0.4 nM Klotho for 3 days, and isotopic Ca uptake (A) were determined. Means ± SEM; n = 4. There are no significant statistical differences between groups by one-way ANOVA followed by Student-Newman-Keuls test. Substrate kinetics of Ca uptake by rat VSMC cell line (A10) (B).

**Supplementary Figure 6: Substrate kinetics of Pi uptake by rat VSMC cell line (A10):** (A) Pi uptake by A10 cells at different Pi concentrations with Na⁺ (close circle) or without Na⁺ (open circle) media containing 5.0 μM ³²P-K₂PO₄. (B) Na⁺-dependent (close circle) and Na⁺-independent (open circle) Pi transport by A10 cells in lower Pi concentration.
## S. Table 1  Biochemical and physiological parameters in CKD mice

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<tr>
<td></td>
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<td>Serum Pi (mg/dl)</td>
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<tr>
<td>Serum Ca (mg/dl)</td>
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<td>FE\textsubscript{phos}</td>
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<td>Serum Cr (mg/dl)</td>
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<td>±0.02 ±0.02</td>
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<tr>
<td>Cl\textsubscript{cr} (µl/min/BWg)</td>
<td>13.03 7.87</td>
<td>10.95 8.36</td>
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<td>±1.90 ±1.06</td>
<td>±1.89 ±0.93</td>
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<tr>
<td>U\textsubscript{protV} (µg/min/BWg)</td>
<td>0.052 0.134</td>
<td>0.043 0.147</td>
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<td>±0.004 ±0.005</td>
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<td>±3.5 ±1.2</td>
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<td>SBp (mmHg)</td>
<td>111.3 122.4</td>
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<td>±1.9 ±2.8</td>
<td>±5.0 ±5.8</td>
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FE\textsubscript{phos}: fractional excretion of phosphate; Cl\textsubscript{cr}: creatinine clearance; U\textsubscript{protV}: urinary protein excretion; Hct: hematocrit; SBp: systolic blood pressure. Data is represented as Mean±s.e.m.; n ≥ 7. * P<0.05, ** P<0.01 vs Sham WT mice of K\textsuperscript{IvC} group; ¥ P<0.05, ¥¥ P<0.01 Sham K\textsuperscript{IvC} mice; # P<0.05, ## P<0.01 vs CKD K\textsuperscript{IvC} mice; ¥¥# P<0.05, ¥¥## P<0.01 vs Sham WT mice of Tg-K\textsuperscript{I} group; § P<0.05, §§ P<0.01 vs Sham Tg-K\textsuperscript{I} mice; £ P<0.05, ££ P<0.01 vs CKD Tg-K\textsuperscript{I} mice by one-way ANOVA followed by Student-Newman-Keuls test.
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<td>Pit1</td>
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<td>Cyclophilin</td>
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DETAILED METHODS

Human study:

Thirteen normal volunteers and 39 chronic kidney disease (CKD) patients were recruited with approval by the Institutional Review Board of UT Southwestern Medical Center at Dallas. CKD patients with varying severity and etiologies were consecutively recruited from Renal Clinic at Parkland Memorial Hospital at the University of Texas Southwestern Medical Center. According to the NKF classification of CKD, our study cohort contained 7-9 patients in each CKD stage. All patients provided written informed consent. Random spot urine samples were collected during routine clinic visit. For measurement of urinary Klotho protein, 4 ml fresh urine was centrifuged at 1500 g at 4°C to remove urinary sediment followed by concentrated to 0.2 ml through Amicon Ultra-4 filters with cutoff 100 kDa (Millipore, Billerica, MA) at 5000 g at 4°C for 20 minutes. The concentrated urine samples were immediately mixed with Laemmli sample buffer and stored at -80°C. The concentrated urines with identical urine creatinine content were subject to immunoblot on the same SDS gels along with recombinant mouse Klotho (rMKl) protein of known concentration as standards.

Rodents:

All rodent work was conducted following the Guide for the Care and Use of Laboratory Animals by The National Institutes of Health (NIH); and was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center at Dallas. Mice used for preparation of CKD model were: (1) transgenic murine line with over-expression of Klotho, EFmKL46 (Tg-Kl); and (2) heterozygous for Klotho deficient mice (Kl+/−). The reason that we used Kl+/− mice instead of Kl−/− mice was that Kl−/− mice are very fragile and do not tolerate surgery. WT littermates were generated during cross breeding for Tg-Kl and Kl+/− mice, and were used as controls of Tg-Kl and Kl+/− respectively. The age of Kl+/− and WT mice used in this study ranges from 6 - 8 weeks; Kl+/−, Tg-Kl and WT mice about 12 weeks.
Model of CKD:

Chronic kidney disease was generated using nephrectomy plus ischemia-reperfusion injury (IRI). Under general anesthesia, left kidney was first decapsulated to preserve the adrenal gland followed by removal of left kidney. Then right renal artery was isolated and cross-clamped for 30 minutes to induce ischemia followed by reperfusion by removing clamps. Kidneys were visually inspected to assure ischemia and reperfusion periods. Sham mice underwent laparotomy when both kidneys were decapsulated and renal arteries of both sides were dissected, but renal clamps were not applied. After recovery, mice were monitored for post-operative complications. Mice were fed with 1.0% phosphorus diet for 4 weeks with free access to tap water followed by 2.0% phosphorus diet for 8 weeks. For metabolic study, mice were transferred to individual metabolic cages. After adaptation for 2 days, 24-hour urine was collected; blood was drawn; and tissues were harvested. Plasma and urine chemistry of animals were analyzed using Vitros Chemistry Analyzer (Ortho-Clinical Diagnosis, Rochester, NY) by Animal Core Facility in UT Southwestern Medical Center.

Immunoreactive PTH assay and serum 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃):

Murine intact PTH was quantified by ELISA (Alpco, Salem, NH) (sensitivity 3.0 pg/ml; within- and between-run coefficients of variation are 3.9% and 8.9%, respectively). Murine 1,25-(OH)₂D₃ was determined by EIA using (Immunodiagnostic Systems, Scottsdale, AZ) (sensitivity 2.5 pg/ml; within- and between run coefficients of variation in our laboratory are <10% and <15%, respectively).

Blood pressure measurement:

Blood pressure was measured by a computerized tail-cuff system (BP-2000, Visitech Systems, Apex, NC) in conscious animals throughout our study following
manufacture instruction. On each occasion, four animals were placed in the restrainers. Mice were trained for 3 consecutive days in the prewarmed (98 ± 0.5°F) tail-cuff device to familiarize them with the procedure, followed by measurements of systolic and diastolic blood pressure everyday for 5 days. During each procedure, the 10 trial cycles were repeated followed by 10 recorded cycles for one test. At least 4 successful procedures per day were carried out and the results were averaged for each individual animal for 5 consecutive days. The mean values of all analyses were used for comparisons.

Von Kossa staining and calcium concentration measurement:

Kidney, heart, aorta, and stomach were stained for calcium precipitation with Von Kossa. Tissue sections were incubated with 1% silver nitrate solution under ultraviolet light for 30 minutes followed by incubation with 5% sodium thiosulfate for 5 minutes to remove the un-reacted silver. Sections were counterstained with nuclear fast red, photographed blindly by a renal pathologist (JZ) using Axioplan 2 Imaging (Carl Zeiss MicroImaging, Inc. Thornwood, NY). The calcium concentration in tissues was measured using o-cresolphthalein complexone method (Sigma, St. Louis, MO). The calcium content (μg/mg protein) was quantified by normalization of protein concentration, as determined by Bradford protein assay.

Kidney histology:

Four μm sections of frozen kidney tissues were made and stained with Hematoxylin plus Eosin (HE), then and observed and photographed blindly by independent investigators using a microscopy of Axioplan 2 Imaging (Carl Zeiss MicroImaging, Inc. Thornwood, NY).

Real time PCR:

Total RNA was extracted using RNeasy kit (Qiagen) from mouse tissues (kidney, heart, or aorta) and cell lines (rat vascular smooth muscle cells, A10; mouse
osteoblast-like cells, MC-3T3-E1; mouse adipocytes, 3T3-L1). Complimentary DNA was generated with Oligo-DT primers using SuperScript III First Strand Synthesis System (Invitrogen) according to manufacturer’s protocol. Primers used for qPCR were shown in S. Table 3 with conditions described in literature. Briefly, amplification was carried out in an ABI Prism 7000 Sequence Detector (Applied BioSystems), with one cycle of 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The reaction was performed in triplicate for each sample. PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide (data not shown), and also were amplified using Big Dye Terminator 3.1 chemistry (Applied Biosystems Inc. ABI), and analyzed on ABI capillary instruments by DNA Sequencing Core Facility in UT Southwestern Medical Center at Dallas.

Recombinant mouse Klotho:

Soluble Klotho protein containing the entire extracellular domain of mouse Klotho (amino acid number 31-982) with V5 and 6x His tags at the C-terminus was generated using Drosophila Expression system (Invitrogen), and purified from conditional medium by affinity column chromatography using anti-V5 antibody (sigma-Aldrich) as previous described.

Cell culture:

Rat vascular muscle cell lines (A10), mouse osteoblasts (MC3T3-E1), and mouse adipocytes were cultured and maintained in condition as described previously. Cells were grown in 6-well plates and treated with Pi and/or Klotho. Fresh culture media were added every other day. Ten days after treatment, cells were harvested for Von Kossa staining and OCPC assay. Cells on 24-well plates were for an uptake of $^{32}$P-phosphate and $^{45}$Ca-CaCl$_2$.

Pi uptake:

Na$^+$-dependent and Na$^+$-independent Pi uptake were performed following published protocol with modification. Briefly, cells were rinsed with Na$^+$-free
solution, then incubated with uptake solution containing 0.05 mM KH$_2$PO$_4$ (5 μCi/ml, Perkin Elmer, Boston, MA) for Na$^+$-coupled Pi uptake if uptake solution contains 137 mM NaCl; and for Na$^+$-independent Pi uptake if NaCl was replaced with 137 mM Choline Cl. The Pi uptake (pmol/mg protein*min) in a given time was quantified by normalization to protein concentration determined by Bradford protein assay. Each transport reaction was performed in triplicate.

Calcium uptake:

Calcium uptake were performed following published protocol with modifications.$^{12}$ Briefly, cells were treated 1.0 or 2.0 mmol/l Pi culture medium for 48 hours. Klotho or vehicle was added at specified times and concentrations prior to addition of $^{45}$Ca into culture medium. $^{45}$Calcium chloride (5 μCi/ml, Perkin Elmer, Boston, MA) was added for 20 minutes, medium was removed, and cells were rinsed with pre-cold PBS followed by PBS-3 mM LaCl$_3$ and 2 mM EGTA for 3 times. Cells were lysed in 0.5N NaOH. Radioactivity of cell lysate (nmol/mg protein/min) was measured by scintillation and protein concentration determined by Bradford protein assay. Each uptake of sample was performed in triplicate.

Immunoblot:

Cell lysate and kidney total lysate were prepared as described.$^{13-14}$ Thirty μg protein was solubilized in Laemmli sample buffer, electrically fractionated on SDS-PAGE, transferred to PVDF membrane, and subjected to immunoblot using specific antibodies: monoclonal rat antibody for Klotho (1:1000 dilution), polyclonal rabbit antibody for Runx2 (1/200 dilution, Santa Cruz biotechnology), monoclonal mouse antibody for smooth muscle actin (SMA) (1/3000 dilution, Sigma-Aldrich), monoclonal mouse antibody for β-actin (1/5000 dilution, Sigma-Aldrich). Primary antibodies were incubated overnight at 4°C. After extensively washing, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (Amersham Life Sciences). Specific signal was visualized using the ECL kit (Amersham Life Sciences).
Measurement of Klotho protein in rodent blood and urine:

Forty μl of fresh urine were collected and immediately mixed in Laemmli sample buffer after collection. Four ml of human fresh urine were collected and immediately concentrated to 200 μl through Amicon Ultra-4 filters (Millipore, Billerica, MA) followed by mixing with 1X Laemmli sample buffer. One hundred μl of mouse serum were subjected to immunoprecipitation with 4 μl of rabbit anti-serum of human Klotho, 15 and immune complex was eluted from protein G beads with 2.5X Laemmli sample buffer. Protein samples in Laemmli sample buffer were electrically fractionated by SDS-PAGE, transferred to PVDF membrane, and subjected to immunoblot analysis with rat anti-human Klotho monoclonal antibody (KM2076). 15

Kidney immunohistochemistry:

In some experiments, for clear visualization of signal in the proximal tubules, kidney were fixed in situ with perfusion of 2.5% paraformaldehyde via distal aorta of renal arteries before the kidneys were moved, while in most experiments, kidneys without in situ fixation were harvested and directly frozen in O.C.T compound (Tissue TeK) using liquid N2. Kidney cropysection (4 μm) were cut, and subjected to immunofluorescence staining as described 14 or kept in -20°C until use. Monoclonal rat antibody for Klotho (1:250) followed by secondary antibodies (ant-rabbit, or rat or mouse IgG conjugated to fluorescin isothiocyanate. Finally rhodamine-phalloidin (1:50) (Molecular Probes, Eugene, OR) for staining β-actin filaments was applied for double staining. Sections were visualized with a Zeiss LSM-510 laser scanning microscope.

Statistical analyses:

Data are expressed as the means ± SEM (n = 8 or more unless indicated otherwise). As appropriate, statistical analysis was performed using Student’s unpaired or paired t-test, or analysis of variance (ANOVA) followed by Student-
Newman-Keuls test when applicable. A value of $P \leq 0.05$ was considered statistically significant. Unless stated otherwise, representative figures reflect the results in a minimum of 4 independent experiments. Linear regression was used to examine the association between calcium content in kidneys or in aortas and blood Pi and creatinine.
REFERENCES:


S. Figure 1
A

$Kl^{+/−}$ group

$Kl^{+/−}$

WT

Sham CKD

Sham CKD

$Tg-Kl$ group

WT

Tg-Kl

Sham CKD

Sham CKD

B

WT

Tg-Kl

100 μM

S. Figure 2
A

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B

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S. Figure 3
S. Figure 4
S. Figure 5