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 mM + 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.
### SUPPLEMENTARY TABLES:

#### S. Table 1  Biochemical and physiological parameters in CKD mice

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<th>Tg-KI group</th>
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<td></td>
<td>Wt Sham</td>
<td>CKD</td>
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<tr>
<td>Serum Pi (mg/dl)</td>
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<td>Serum Ca (mg/dl)</td>
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<td>U(_{prot,V})</td>
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<td>Hct</td>
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<td>S Bp (mmHg)</td>
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FE\(_{phos}\): fractional excretion of phosphate; Cl\(_{cr}\): creatinine clearance; U\(_{prot\,V}\): urinary protein excretion; Hct: hematocrit; S Bp: 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\(^{+/+}\) group; † P<0.05, †† P<0.01 Sham K\(^{+/+}\) mice; # P<0.05, ## P<0.01 vs CKD K\(^{+/+}\) mice; $ P<0.05, $$$ P<0.01 vs Sham WT mice of Tg-KI group; ¶ P<0.05, §§ P<0.01 vs Sham Tg-KI mice; £ P<0.05; ££ P<0.01 vs CKD Tg-KI mice by one-way ANOVA followed by Student-Newman-Keuls test.
### S. Table 2  Primers used for qPCR

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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 (rMK1) 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 (Alpc0, 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:**

$\text{Na}^+$-dependent and $\text{Na}^+$-independent Pi uptake were performed following published protocol with modification. Briefly, cells were rinsed with $\text{Na}^+$-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. 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. 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 antisemur of human Klotho, 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).

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 cryosection (4 μm) were cut, and subjected to immunofluorescence staining as described 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

**KI+/− group**

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**Tg-KI group**

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B

**WT**

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**Tg-KI**

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

**A**

Plot showing the effect of Pi (mM) and KL (nM) on the uptake of \( {^{45}}\text{Ca} \) (nmol/mg/min). The x-axis represents the incubation time in minutes, ranging from 0 to 60.

**B**

Line graph depicting the time course of \( {^{45}}\text{Ca} \) uptake (nmol/mg/min) with \( {^{45}}\text{Ca} \) incubation time (minute) on the x-axis, ranging from 0 to 60.
S. Figure 6

A

$^{32}$Pi uptake (pmol/mg protein/min) vs. Pi concentration (mmol/l)

B

$^{32}$Pi uptake (pmol/mg protein/min) vs. Pi concentration (μmol/l)