Supplemental Materials for

Klotho and Phosphate are Modulators of Pathologic Uremic Cardiac Remodeling

Ming Chang Hu[1,3,*], Mingjun Shi[1], Han Jun Cho[1], Beverley Adams-Huet[1,2,3], Jean Paek[1], Kathy Hill[1], John Shelton[5], Ansel P. Amaral[8,9], Christian Faul[8,9], Masatomo Taniguchi[1,2], Myles Wolf[8,#], Markus Brand[10], Masaya Takahashi[7], Makoto Kuro-o[1,5], Joseph A. Hill[3,4], and Orson W. Moe[1,3,6,*]

[1]Charles and Jane Pak Center for Mineral Metabolism and Clinical Research, [2]Departments of Clinical Sciences, [3]Internal Medicine, [4]Molecular Biology, [5]Pathology, and [6]Physiology, and [7]Advanced Imaging Research Center, University of Texas Southwestern Medical Center, Dallas, TX;

[8]Division of Nephrology and Hypertension, Department of Medicine, and [9]Department of Cell Biology and Anatomy, University of Miami Miller School of Medicine, Miami, FL;
[10]Department of Internal Medicine D, University of Münster, Münster, Germany

[#]Current address: Center for Translational Metabolism and Health, Feinberg School of Medicine, Northwestern University

*To whom correspondence should be addressed:

Ming Chang Hu, MD; PhD: Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390-8885, Phone: 214-648-9797, Fax: 214-648-2526, Email: ming-chang.hu@utsouthwestern.edu

or

Orson W. Moe; MD: Departments of Internal Medicine and Physiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390-8885, Phone: 214-648-7995, Fax: 214-645-9416, Email: orson.moe@utsouthwestern.edu

Running title: Klotho, Pi and cardiac remodeling

Supplementary Methods:

Animal experiments: Several rodent (mice and rats) models were used in this study strictly 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 (IACUC) from University of Texas Southwestern Medical Center, and the University of Münster, Germany.

Genetic Klotho-deficiency was generated by hypomorphic alleles from transgenic disruption of the *Klotho* locus and silencing of Klotho expression.[1] Transgenic mice overexpressing mouse Klotho (*Tg-Kl*) was described previously.[2, 3] All mice were *129 S1/SVlmJ* (*129 SV*) background. The homozygous (*kl*/*kl*), heterozygous (*kl*/+), and wild-type (*WT*) mice, and *Tg-Kl* mice were genotype-confirmed by PCR of genomic DNA.[1, 2]

Two CKD rodent models were used: (1) uninephrectomy plus contralateral ischemia-reperfusion injury (30 min) in *WT 129 SV* mice (12 weeks old).[2] Sham animals underwent laparotomy and manual manipulation of the kidneys. After recovery, mice were housed in cages and fed normal rodent chow (Teklad 2001, Harlan, Madison, WI 53744) phosphate for 4 weeks with free access to tap water followed by 2.0% phosphate diet (Teklad 08020, Harlan, Madison, WI 53744) for 16 weeks. (2) 5/6th nephrectomy model of CKD was induced in Sprague-Dawley rats using established methods.[4, 5]

Blood pressure: Blood pressure was measured by a computerized tail-cuff system (BP-2000, Visitech Systems, Apex, NC) in conscious animals (4 animals per restrainer). Animals 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 every day for 5 days. Each time, 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.

Cardiac magnetic resonance imaging (MRI): Cardiac function of mice were evaluated by cardiac MRI using a 7 T small animal MR scanner (Varian, Inc, Palo Alto, CA) with a 38 mm birdcage RF coil as previous described.[6] Anesthesia was by inhalational 1.5 – 2% isoflurane mixed in with medical-grade air via nose-cone. All MRI acquisitions were gated using both cardiac and respiratory triggering. Two-dimensional (2D) gradient echo images on three orthogonal planes (transverse, coronal and sagittal) were acquired to determine the orientation (long-axis) of the heart in each mouse. Axial images perpendicular to the long axis of the heart was chosen for Cine-imaging. Cine images at 12 phases per cardiac cycle was obtained with an echo time of 2.75 ms, repetition time = EKG R-R interval /12, flip angle of 45°, and 6 number of excitation. Each scan consisted of six to nine contiguous slices from apex to LV outflow with 1 mm thickness, a matrix size of 128 × 128, and a field of view of 30 x 30 mm.

Epicardial and endocardial borders were manually traced for calculation of left ventricular end systolic and end diastolic volume (LVESV, LVEDV) using NIH Image J software. Total LV volumes were calculated as the sum of all slice volumes. Stroke volume was calculated by the equation, LVEDV-LVESV. Cardiac output was calculated by the equation, stroke volume x heart rate. The left ventricular ejection fraction (LVEF) was calculated by the equation, (LVEDV-LVESV)/LVEDV ×100%. Wall thickness of left ventricle was measured at mid-ventricular short axis images (just below papillary muscle) with Image J program. Investigators performing MRI acquisition and analysis were blinded to the assignment of mice group.

Recombinant mouse Klotho: Soluble Klotho protein containing the ectodomain of mouse Klotho (amino acid number 31-982) with V5 and 6xHis tags at the C-terminus was generated using Drosophila

Expression system (Invitrogen, Carlsbad, CA), and purified from conditional medium by affinity column chromatography using anti-V5 antibody (Sigma-Aldrich) as previously described.[3]

Primary culture of neonatal rat ventricular myocytes (NRVM) and cardiac fibroblast (NRCF)

NRVM and NRCF were isolated from hearts of neonatal SD rats based on published methods.[7, 8] Briefly, hearts were excised from anesthetized neonatal 1 to 2-day-old Sprague–Dawley rats (Harlan Sprague–Dawley), minced, and digested with pancreatin 0.1%. Cells were platted on plates for 2 hr, and the medium that contained the cardiomyocyte fraction of the digested tissue was collected followed by protocol of NRVM culture, and attached cells that contained NRCF were continuously cultured followed by protocol of NRCF.

Cells were left undisturbed in plating medium at 37°C for 72 hours and then cultured in maintenance medium (DMEM with 20% Media 199, 1% insulin-transferrin-sodium selenite solution [ITS; Sigma-Aldrich] and 1% P/S) in the presence of 100 μM 5-bromo-2'-deoxyuridine (BrdU; Sigam-Aldrich) to eliminate proliferating non-myocytes resulting in a relatively pure population of isolated cardiac myocytes.[7] Cardiac myocytes were identified by immunocytochemical staining for sarcomeric α-actinin (data not shown). After 4 days, isolated cardiac myocytes were cultured in BrdU-containing maintenance medium in the presence of TGF-β1 (1 ng/ml), Ang II (1 μmol/l) or high phosphate media (3 mmol/l) with or without Klotho (0.4 nmol/l) for 30 minutes or 24 hours.

NRCF was attached and proliferated on dishes which produced virtually pure fibroblast cultures after the first passage, which was confirmed by repeated differential plating and microscopic evaluation. Cells were detached with 0.05% trypsin for passaging, and culture studies were performed at passages 2 to 4. Cells were grown in high glucose (4.5 gm/l) DMEM containing 10% heat-inactivated FBS and

antibiotics (penicillin and streptomycin)[8] and identified by immunocytochemical staining for vimentin (data not shown). NRCF were treated with same agents as mentioned for NRVM.

Cells were platted on glass and plastic surfaces, which were coated with laminin (10 μg/ml in PBS) (Invitrogen, Carlsbad, CA) at room temperature for 1 hour prior to plating. For immunofluorescence analysis, 1 x 10^6 cells were seeded per well on glass coverslips in 6-well plates. For protein and RNA isolation, 2 x 10^6 cells were seeded in 6 cm-culture dishes.

Static heart morphology and morphometry: Heart sections were stained with Trichrome for assessment of cardiac fibrosis and imaged with a 5x objective. Images were then scanned with a Nikon SupraMax scanner and analyzed using ImagePro 1.4. To determine the scar ratio, the parameters of the program were set to recognize collagen deposition by detecting pixels in the blue range (450-475nm) versus cardiac musculature with pixels in the red range (620-750nm). The area of the cardiac musculature was determined by surrounding the outer perimeter of the cardiac tissue and then subtracting the areas occupied by the left and right ventricular cavities. Once the area of the cardiac tissue was determined, the program then scanned the pixels in this selected area to determine collagen deposition (blue range pixels) versus cardiac musculature (red range pixels). In this manner, the scar ratio is given as the area of collagen deposition divided by the area of cardiac musculature.

To measure surface area of cardiomyocytes in the hearts, paraffin-embedded sections were labeled with WGA conjugated to Alexa Fluor 555 (Invitrogen, Carlsbad, CA) following our previous method.[4] Immunofluorescence images were taken on a Leica TCS-SP5 confocal microscope with a ×63 oil objective. Leica AF6000 fluorescence software was used to quantify cross-sectional cell surface area of 25 cells per field in 4 fields along the mid-chamber free wall based on WGA-positive staining.

Plasma and urine biochemistry: plasma and urine creatinine concentrations were measured using a P/ACE MDQ Capillary Electrophoresis System and photodiode detector (Beckman-Coulter, Fullerton, CA) at 214 nm.[9] Other biochemic parameters in plasma and urine were determined with methods presented in our previous literate.[10]

Measurement of plasma full-length FGF23, PTH, and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$): Intact PTH was quantified by ELISA (Alpco, Salem, NH); 1,25-$(OH)_2VD_3$ by EIA using (Immunodiagnostic Systems, Scottsdale, AZ); and Intact FGF23 by ELISA kit (Kainos, Japan) following manufactures' instructions.

Immunohistochemistry in the heart and the kidney: Four μm sections of paraffin embedded kidney and heart tissues were stained with Hematoxylin and Eosin (H&E), and observed and photographed by a pathologist blinded to the experimental conditions using an Axioplan 2 Imaging System (Carl Zeiss Microimaging, Inc. Thornwood, NY). For immunofluorescence study, monoclonal rat antibody (KM2076) against human Klotho (1:250)[11] was used for staining and followed by secondary antibodies conjugated to fluorescin isothiocyanate (detailed methods in supplementary material). 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.

Quantifying Klotho in the kidney, urine and blood of rodents: Immunoblots for Klotho in the kidney, urine, and blood of rodents were performed as described.[2] Thirty μg protein of kidney lysate was solubilized in Laemmli sample buffer; ~ 40 μl of fresh urine were immediately mixed in Laemmli sample buffer after collection. Urine samples with identical amount of creatinine were subjected to SDS-PAGE. One hundred μl of animal heparinized plasma were subjected to immunoprecipitation with rabbit anti-serum of human Klotho, and eluted from immune complex, subjected to SDS-PAGE, transferred to

PVDF membrane, and subjected to immunoblot analysis with anti-Klotho antibody (KM2076) (0.5 μg/ml) and monoclonal mouse antibody for β−actin (1/5000 dilution, Sigma, St. Louis, MO). Signal was visualized using the ECL kit (Perkin-Elmer LAS, Inc., Boston, MA).

Immunoblot and quantitative real time PCR: Cell lysate from cardiac myocytes and fibroblasts and lysate from heart and kidney were prepared as described.[2, 4] 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 (KM2076, 0.5 μg/ml)[11, 12] monoclonal mouse antibody for β-actin (1/5000 dilution). Primary antibodies were incubated overnight at 4°C. After extensively washing, membranes were incubated with secondary antibodies conjugated with horseradish peroxidase (Amersham Life Sciences, Piscataway, NJ). Specific signal was visualized using the ECL kit (Amersham Life Sciences, Piscataway, NJ).

For PCR, total RNA was extracted using RNAeasy kit (Qiagen, Germantown, MD) from mouse tissues (kidney, heart, or aorta) and cells (primary cultured rat neonatal cardiomyocytes and cardiac fibroblasts). Complimentary DNA was generated with Oligo-dT primers using SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Primers used for qPCR are shown in **Table S4** with conditions described.[2] Briefly, amplification was carried out in an ABI Prism 7000 Sequence Detector (Applied Biosystems Inc. ABI, Foster City, CA), 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). Data were expressed at amplification number of $2^{-\Delta\Delta Ct}$ by normalization of cyclophilin and comparison of controls.

Statistical analyses: Data are expressed as Means ± SD (n = 4 or more unless indicated otherwise). As appropriate, statistical analysis was performed using Student's unpaired t-test, or one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test when applicable. The association between cardiac variables (heart weight/body weight and cardiac fibrosis index) and each of plasma Klotho, FGF23, PTH, 1,25-V_2D_3, Pi, and Cr was assessed with Pearson correlation coefficients. For each plasma parameter, the partial correlation coefficients were computed to adjust for the potential confounding effects of the other five biomarkers in evaluating the association with cardiomyopathy. Analysis was performed with SAS program (v9.3) (SAS Institute, Cary, NC, USA). A value of $P \leq 0.05$ was considered statistically significant.

References:

1. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E, Iwasaki H, Iida A, Shiraki-Iida T, Nishikawa S, Nagai R & Nabeshima YI: Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature,* 390**:** 45-51, 1997.

2. Hu MC, Shi M, Zhang J, Quinones H, Griffith C, Kuro-o M & Moe OW: Klotho deficiency causes vascular calcification in chronic kidney disease. *J Am Soc Nephrol*, 22**:** 124-36, 2011.

3. Kurosu H, Yamamoto M, Clark JD, Pastor JV, Nandi A, Gurnani P, McGuinness OP, Chikuda H, Yamaguchi M, Kawaguchi H, Shimomura I, Takayama Y, Herz J, Kahn CR, Rosenblatt KP & Kuro-o M: Suppression of aging in mice by the hormone Klotho. *Science,* 309**:** 1829-33, 2005.

4. Faul C, Amaral AP, Oskouei B, Hu MC, Sloan A, Isakova T, Gutierrez OM, Aguillon-Prada R, Lincoln J, Hare JM, Mundel P, Morales A, Scialla J, Fischer M, Soliman EZ, Chen J, Go AS, Rosas SE, Nessel L, Townsend RR, Feldman HI, St John Sutton M, Ojo A, Gadegbeku C, Di Marco GS, Reuter S, Kentrup D, Tiemann K, Brand M, Hill JA, Moe OW, Kuro OM, Kusek JW, Keane MG & Wolf M: FGF23 induces left ventricular hypertrophy. *J Clin Invest,* 121**:** 4393-408, 2011.

5. Hu MC, Bankir L, Michelet S, Rousselet G & Trinh-Trang-Tan MM: Massive reduction of urea transporters in remnant kidney and brain of uremic rats. *Kidney Int,* 58**:** 1202-10, 2000.

6. Aoyagi T, Kusakari Y, Xiao CY, Inouye BT, Takahashi M, Scherrer-Crosbie M, Rosenzweig A, Hara K & Matsui T: Cardiac mTOR protects the heart against ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol,* 303**:** H75-85, 2012.

7. Yund EE, Hill JA & Keller RS: Hic-5 is required for fetal gene expression and cytoskeletal organization of neonatal cardiac myocytes. *J Mol Cell Cardiol,* 47**:** 520-7, 2009.

8. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA & Olson EN: Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci U S A,* 105**:** 13027-32, 2008.

9. Zinellu A, Caria MA, Tavera C, Sotgia S, Chessa R, Deiana L & Carru C: Plasma creatinine and creatine quantification by capillary electrophoresis diode array detector. *Anal Biochem,* 342**:** 186-93, 2005.

10. Panesso MC, Shi M, Cho HJ, Paek J, Ye J, Moe OW & Hu MC: Klotho has dual protective effects on cisplatin-induced acute kidney injury. *Kidney Int,* 85**:** 855-70, 2014.

11. Kato Y, Arakawa E, Kinoshita S, Shirai A, Furuya A, Yamano K, Nakamura K, Iida A, Anazawa H, Koh N, Iwano A, Imura A, Fujimori T, Kuro-o M, Hanai N, Takeshige K & Nabeshima Y: Establishment of the anti-Klotho monoclonal antibodies and detection of Klotho protein in kidneys. *Biochem Biophys Res Commun,* 267**:** 597-602, 2000.

12. Hu MC, Shi M, Zhang J, Pastor J, Nakatani T, Lanske B, Razzaque MS, Rosenblatt KP, Baum MG, Kuro-o M & Moe OW: Klotho: a novel phosphaturic substance acting as an autocrine enzyme in the renal proximal tubule. *FASEB J,* 24**:** 3438-50, 2010.

Supplemental figures and legends

Supplemental Figure 1. Renal and systemic Klotho levels. (A) Representative immunoblots for Klotho protein in the kidney, plasma and urine of homozygous (*kl/kl*) and heterozygous (*kl/+*) *Klotho*-deficient mice, *Wild-type* littermates (*WT*) and transgenic *Klotho* overexpressing (*Tg-Kl*) mice at 6 and 12 weeks of age. **(B)** Summary of densitometric analysis of Klotho protein levels in the kidney, plasma and urine of *kl/kl*, *kl/+*, *WT* and *Tg-Kl* mice. Data are expressed as means ± SD of 4 animals from each group and statistical significance was assessed by one-way ANOVA followed by Student-Newman-Keuls test, and significance was accepted when *:P<0.05; **:P<0.01.

Supplemental Figure 2. Renal fibrosis in 5/6 nephrectomized rats. Rats with 5/6th Npx. Representative micrographs of left ventricular sections stained with Sirius red (**left panel**). Summary of semi-quantification of Sirius red stain positive area over whole heart section by Image J program (**right panel**). Data expressed as means ± SD of 4 animals from each group and statistical significance was assessed by Student *t* test. Significant differences were accepted when **: P <0.01 between groups.

Supplemental Figure 3. Interaction between aging, genetic Klotho deficiency, and high phosphate diet on cardiac hypertrophy and fibrosis in mice. *kl/+*, *WT* and *Tg-Kl* mice at 6 or 12 months old were fed normal rodent chow or high phosphate diet for 12 weeks. Hearts were harvested at 9 or 15 months for immunohistochemistry with WGA to visualize cardiomyocyte (representative micrographs shown in **Fig. 5D~W**). **(A)** Summary of cardiomyocytes area visualized by WGA in cross sections. (**B~C**) Hearts were harvested for immunoblot to examine Collagen I, α-actinin and β-actin in left ventricular lysates from *kl/+*, *WT* and *Tg-Kl* mice **(B)** at 9 or (**C**) 15 months old fed with normal rodent chow or high phosphate (2.0% Pi) diet. Summary was shown in the bottom panel. Data are expressed as means ± SD of 4 animals from each group and statistical significance was assessed by one-way ANOVA

followed by Student-Newman-Keuls test, and significant differences were accepted when *:$p<0.05$; **:$p<0.01$.

Supplementary movie: Cardiac magnetic resonance imaging (MRI):

Supplemental Table 1. Summary of CKD mice induced by UNX-IRI

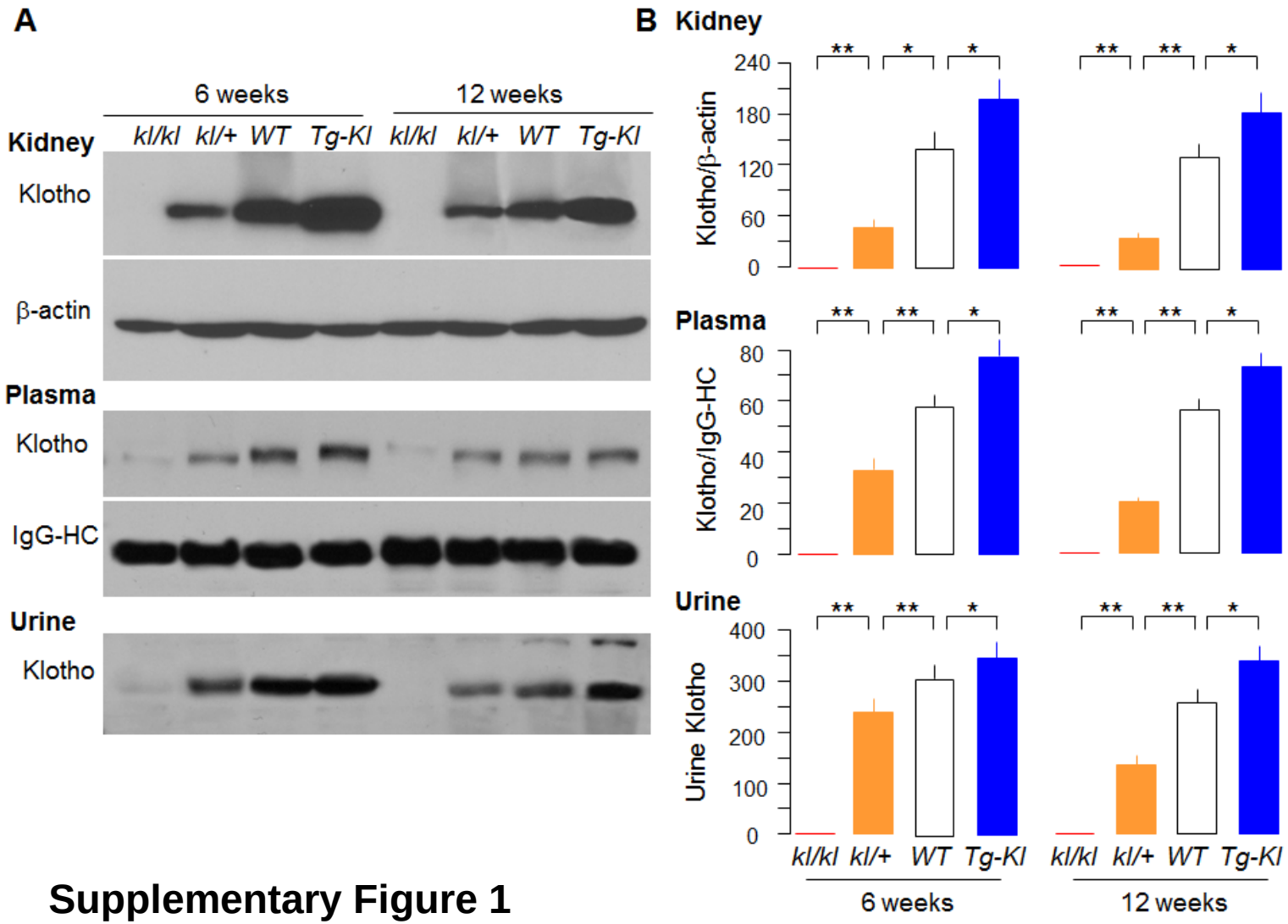
	Sham	UNX-IRI	P*
BW (gram)	27.3±5.3 (10)	26.0±7.2 (12)	=0.420
HW (mg)	138.8±11,7 (10)	143.5±16.1 (12)	=0.034
HW/BW (mg/g)	5.13±0.27 (10)	5.43±0.28 (12)	<0.01
MAP (mmHg)	110.1±7.6 (10)	117.5±8.7 (12)	<0.04
Plasma Cr (mg/dl)	0.14±0.01 (9)	0.25±0.02 (11)	<0.01
Plasma Pi (mg/dl)	7.63±0.59 (10)	8.65±0.61 (12)	<0.001
Plasma Ca (mg/dl)	7.39±0.46 (10)	7.23±0.50 (12)	=0.448
Urinary protein (mg/24 h)	1.69±0.19 (10)	4.75±0.23 (12)	<0.001
PTH (pg/ml)	229.6±15.9 (7)	275.4±18.1 (7)	<0.001
$1,25(OH)_2D_3$ (pg/ml)	210.4±48.8 (6)	42.7±9.8 (8)	<0.001
FGF23 (pg/ml)	243.6±20.1 (7)	432.1±74.4 (7)	<0.001

Data are expressed as the means ± SD, and n is the sample size. *Student *t* test was used for statistical analysis between Sham and UNX-IRI groups. BW: body weight; Ca: total calcium; CKD: chronic kidney disease; Cr: creatinine; FGF23: fibroblast growth factor-23; HW: heart weight; MAP: mean arterial pressure; UNX-IRI: unilateral nephrectomy plus contralateral ischemia reperfusion injury followed by high phosphate diet for 12 weeks; Pi: inorganic phosphate; PTH: parathyroid hormone; $1,25(OH)_2D_3$: 1,25-dihydroxyl-vitamin D_3; *WT*: wild type.

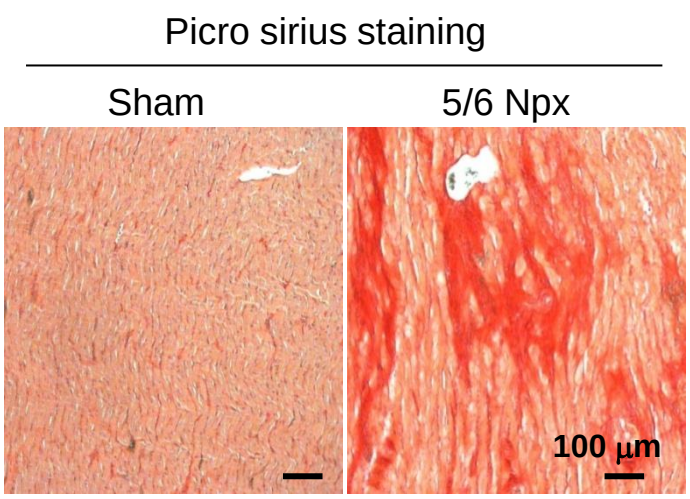
Supplemental Table 2. Primers used for qPCR

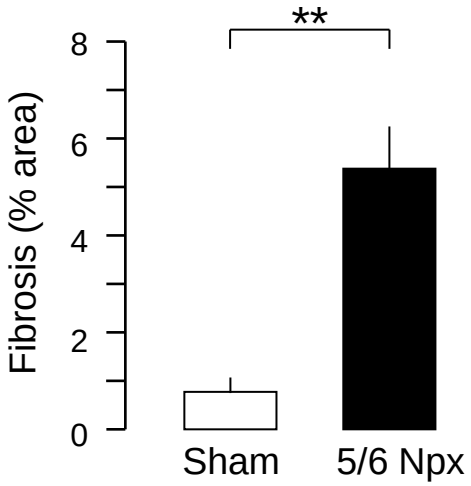
	Forward primer	Reverse primer
α-MHC	GAG ATT TCT CCA ACC CAG	TCT GAC TTT CGG AGG TAC T
β-MHC	CTA CAG GCC TGG GCT TAC CT	TCT CCT TCT CAG ACT TCC GC
ANP	CAT CAC CCT GGG CTT CTT CCT	TGG GCT CCA ATC CTG TCA ATC
BNP	CAC CGC TGG GAG GTC ACT	GTG AGG CCT TGG TCC TTC AA
CTGF	CTG GAA GAC ACA TTT GGC CC	CAG AAG GTA TTG TCA TTG GT
α-SMA	GAG AAG CCC AGC CAG TCG	CTC TTG CTC TGG GCT TCA
cyclophilin	TGC TCT TTT CGC CGC TTG CT	TCT GCT GTC TTT GGA ACT TTG TCT G

α-MHC: α-myosin heavy chain; *ANP*: atrial natriuretic peptide; *β-MHC*: β-myosin heavy chain; *BNP*: brain (B-type) natriuretic peptide; *CTGF*: connective tissue growth factor; *α-SMA*: α-smooth muscle actin

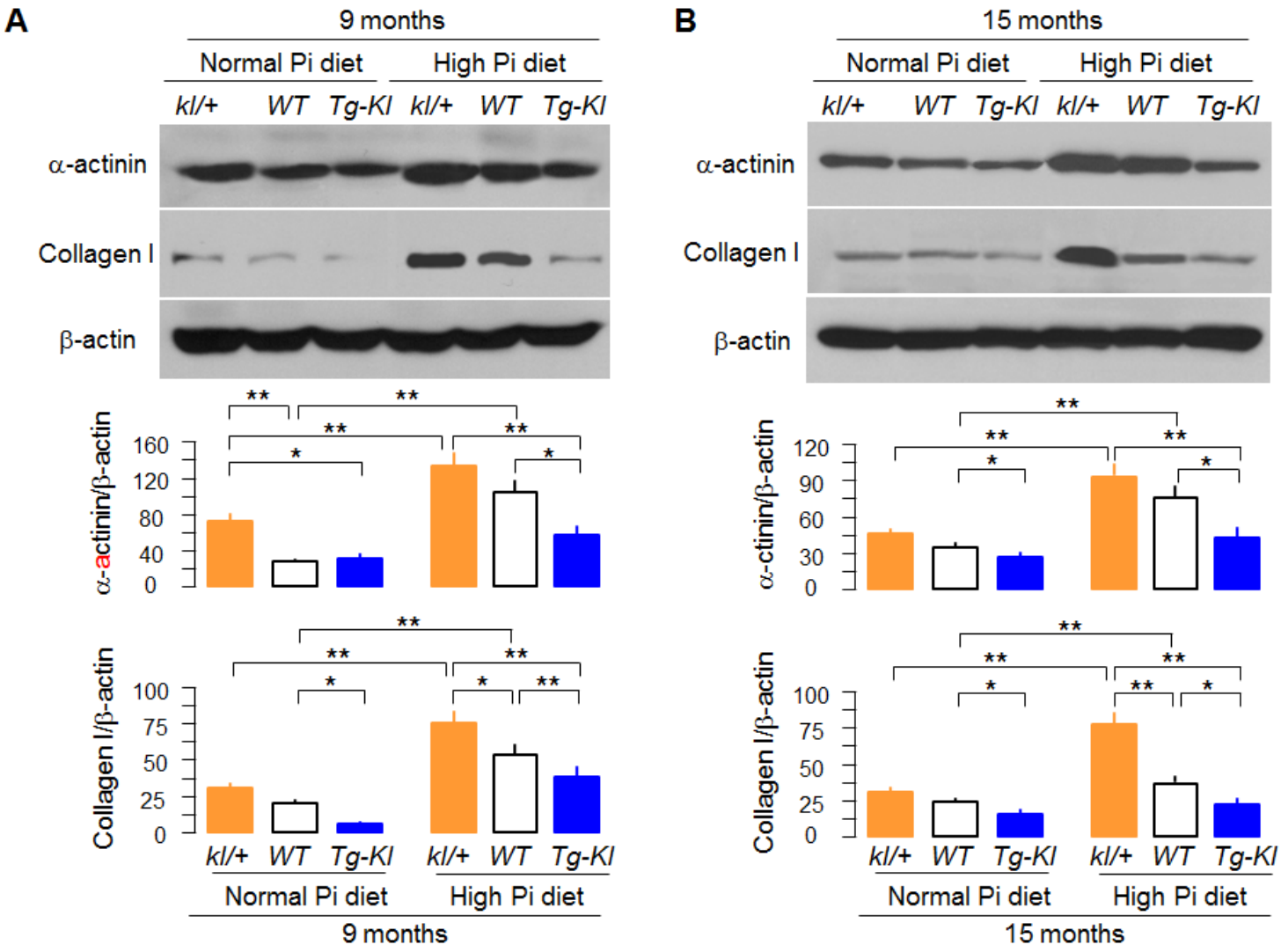


Supplementary Figure 1





Supplementary Figure 2.



Supplementary Figure 3.