Online Supplemental Methods and Data

Klotho Deficiency-induced Arterial Calcification Involves Osteoblastic Transition of VSMCs and Activation of the BMP Signaling

Running Title: Lin Klotho deficiency and arterial calcification

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Animal studies. Briefly, heterozygous *Klotho* mutant (*kl/*+) mice with more than 9 generations in 129/Sv background were kindly provided by Dr. Kuro-o (Kuro-o et al., 1997). *kl/*+ mice were bred with *kl/*+ mice to generate homozygous *Klotho* mutant (*kl/kl*) and wild type mice. Wild type littermate mice were used as controls. Briefly, all mice were housed in cages at room temperatures (25±1°C) and were provided with tap water ad libitum throughout the experiment. For dietary phosphate restriction, mice were fed with a low phosphate (Pi) diet containing 0.2% (wt/wt) inorganic phosphate (TD-09073, Harlan Teklad, Madison, WI) from weaning at 3 weeks of age (Xie et al., 2012). Normal diet contains 0.35% inorganic phosphate (PicoLab, Rodent Diet 20, Cat# 5053). This study was carried out according to the guidelines of the National Institutes of Health on the Care and Use of Laboratory Animals. This project was approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center.

Measurements of pulse wave velocity (PWV). PWV was measured as we described recently (Chen, Zhou, & Sun, 2015; Lin, Chen, & Sun, 2016). Doppler Signal Processing Workstation (DSPW, Indus Instruments, Houston, TX, USA) was used to measure PWV (arterial blood flow). Briefly, animals were anesthetized with Ketamine/Xylazine (90/10 mg/Kg, IP). To record the flow of aortic arch and the flow of abdominal artery, a 10-MHz Doppler probe was used. The distance between the aortic arch and the abdominal artery was measured and the time for pulse wave was calculated using the manufactory software DSPW.

Tissue collections. Animals were euthanized using an overdose of Ketamine/Xylazine (200/20 mg/Kg, IP). Blood was collected in heparin, and plasma was separated and stored at -80°C. Following blood collections, animals were perfused with heparinized saline. The ascending aorta was placed in 4% PBS-buffered paraformaldehyde (PFA) for 24 hours and then embedded in paraffin. The rest of aorta was stored in -80°C.

Plasma phosphate and calcium measurements. Plasma samples were sent to the Yale University Mouse Metabolic Phenotyping Centers for measuring plasma inorganic phosphorous and calcium as described recently (Lin et al., 2016).

Morphological Analysis. A series of cross sections of aorta (5 μm) were cut. Cross sections of aorta were stained with H&E staining assay and NovaUltra^{TM} Alizarin Red Stain Kit (Cat: IW-3001, IHCWorld, Woodstock, MD), respectively. Images of aorta from 3-5 consecutive cross sections for each animal were collected at equal exposure conditions under Nikon Eclipse Ti microscopy (object lens at 10x and 40x). The fraction area for calcified components (red color) in aortic media was obtained using NIS-Elements BR 3.0 (Nikon).

Immunohistochemistry (IHC). IHC was performed as described previously (Lin & Sun, 2015a, 2015b). A series of 5-μm-thick sections of paraffin-embedded mouse aorta were cut at a 100-μm interval on three levels. For immunohistochemical analysis, consecutive aortic cross sections were deparaffinized, rinsed in xylene, and rehydrated. After heat-induced antigen retrieval in a microwave, the cross sections were blocked first with 3% hydrogen peroxide. The sections were incubated with antibodies against MGP (10734-1-AP, ProteinTech), ALP (ab84401, Abcam), Runx2 (ab76950, Abcam), collagen I (ab765p, Millipore), smooth muscle α-actin (ab5694, Abcam), or Elastin (sc-58756, Santa Cruz) overnight at 4°C and then with appropriate secondary antibodies conjugated with HRP at room temperature for 60 minutes. Stable diaminobenzidine (DAB, Invitrogen) was used as a substrate for peroxidase. Hematoxylin was used as counterstaining. The aorta in the cross-sections for each mouse was located under a microscopy (Nikon *Eclipse* Ti). Images of consecutive cross sections for each animal were collected at equal exposure conditions and at the same magnification (40X objective lens). The staining for MGP, ALP, RUNX2, collagen I, SM α-actin, or elastin was quantified using Image J (NIH freeware) as mean gray value/pixel. Briefly, the selection line was drawn along the medium of aorta after the original RGB image was converted to a gray scale image.

Cell cultures. Klotho-deficient serum was generated as we described recently (Fan & Sun, 2016). Klotho-deficient FBS was generated with a Pierce® Direct IP kit (Cat: 26148, Thermo Scientific).Mouse aortic smooth muscle cells were isolated with collagenase Type II digestion as described before (Ray, Leach, Herbert, & Benson, 2001). Cells from passage 3 to 4 were used as the primary culture of mouse ASMCs. A cell line of mouse aortic smooth muscle cells, MOVAS was purchased from ATCC (cat#: CRL-2797). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, SCRR-30-2020, ATCC), 100 μg/ml of streptomycin and 100 U/ml of penicillin (Sigma) at 37°C, 5% CO2. Culture medium was changed every 2 or 3 days. Recombinant human BMP2 (355-BM-010, R&D System) and recombinant human Klotho (5334-KL-025, R&D System) were used in some cell experiments. 1α,25-Dihydroxyvitamin D3 (D1530) was purchased from Sigma (St. Louis, MO).

Western blotting. Briefly, frozen aorta or cells were lysed in Ripa buffer containing protease inhibitor cocktail (Sigma), 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 2 mM sodium vanadate, 1 mM EDTA, and 1 mM EGTA. The protein concentration was measured using the Pierce BCA assay (Thermo Scientific). The lysates (30 μg protein/well) under reduced conditions were directly subjected to SDS-PAGE (4-15% Tris-HCL precast gel, Bio-Rad) followed by western blotting with antibodies against BMP2 (sc-6895, Santa Cruz), BMP4 sc-6896, Santa Cruz), Klotho (AF1819, R&D Systems), Runx2 (ab76950, Abcam), pSmad1/5/8 (9511, Cell Signaling) , pSmad2/3 (8828, Cell Signaling), and Pit2 (sc-377326, Santa Cruz). The blot was then rinsed and reprobed with antibodies against α-tubulin (2135, Cell Signaling), β-actin (Abcam), or total Smad (sc-7153, Santa Cruz) for the loading control. mouse serum albumin and IgG was removed with a Proteome Purify 2 Mouse Serum Protein Immunodepletion Resin kit (MIDR002-020, R&D Systems) before the circulating KL in the serum was measured using WB.

RNA Isolation and RT-PCR. The RT-PCR procedure was described previously (Lin & Sun, 2012). Total RNA was purified from mouse kidneys, primary cultures of mouse

aortic SMCs, and MOVAS using TRIzol® Reagent, followed by Qiagen RNeasy® Mini Kit. RNA (500 ng) was reverse-transcribed using SuperScript^{TM} III Reverse Transcriptase with OligodT20 in the presence of 10ul dNTP for 1h at 50ºC. The resulting cDNAs were used as templates for PCR with oligonucleotides primers to amplify *Klotho* mRNA and *β-actin* mRNA. The two specific primer pairs for mouse *Klotho* mRNA were used as described previously (Lin & Sun, 2012). The primers for the β-actin gene were used as the internal control (Lin & Sun, 2012). PCR reactions were conducted as described previously (Lin & Sun, 2012). The PCR products were separated on 1.5% agarose gels and stained with ethidium bromide and the bands were visualized using a ChemiDoc System BioRad Imager.

Alizarin red staining for cell matrix calcification. A modified Alizarin red staining was used to detect cell matrix calcification (Hsu, Artigues, & Villar, 2008). Briefly, cells grown on 24-well plates were washed with phosphate buffered saline. Cells were fixed with 10% formaldehyde at room temperature for 15 minutes. The fixative was removed with an excess of distilled water. Cells were stained with Alizarin Red Stain Solution (1%) (RICCA Chemical, Cat # 500-16) for 20 minutes. The cell layer containing calcium deposits was stained bright red by the Alizarin Red Solution after washing with water. Images of cell calcification were collected at equal exposure conditions under Nikon Eclipse Ti microscopy (object lens at 10x and 40x).

Calcium deposition. A modified assay for calcium deposition was used (Zavaczki et al., 2011). Briefly, cells grown on 24-well plates were washed with PBS (phosphate buffered saline) and decalcified with 0.6 mol/l HCl for 24 h. Calcium content of the supernatants was determined by the QuantiChrome Calcium Assay Kit (Gentaur, Hayward, CA). After decalcification, cells were solubilized with a solution of 0.1 mol/l NaOH and 0.1% sodium dodecyl sulfate, and protein content of the samples were measured with the BCA protein assay kit (Thermo Scientific, Rockford, IL). Calcium content of the cells was normalized to protein content and expressed as mg/mg protein.

Statistical Analysis. Data were analyzed using a one-way ANOVA. The Newman-Keuls procedure was used to assess differences between means. To compare data between two groups, student t test was used. Data were expressed as mean ± SEM. A probability value with $p<0.05$ were considered significant.

References

Chen, K., Zhou, X., & Sun, Z. (2015). Haplodeficiency of Klotho Gene Causes Arterial Stiffening via Upregulation of Scleraxis Expression and Induction of Autophagy. *Hypertension, 66*(5), 1006-1013. doi:HYPERTENSIONAHA.115.06033 [pii]
10.1161/HYPERTENSIONAHA.115.06033 [doi]

Fan, J., & Sun, Z. (2016). The Anti-aging Gene Klotho Regulates Proliferation and Differentiation of Adipose-derived Stem Cells. *Stem Cells*. doi:10.1002/stem.2305 [doi]

Hsu, H. H., Artigues, A., & Villar, M. T. (2008). Induction of calcification by serum depletion in cell culture: a model for focal calcification in aortas related to atherosclerosis. *Lipids Health Dis, 7*, 2.

Kuro-o, M., Matsumura, Y., Aizawa, H., Kawaguchi, H., Suga, T., Utsugi, T., . . . Nabeshima, Y. I. (1997). Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature, 390*(6655), 45-51. doi:10.1038/36285 [doi]

Lin, Y., Chen, J., & Sun, Z. (2016). Antiaging Gene Klotho Deficiency Promoted High-Fat Diet-Induced Arterial Stiffening via Inactivation of AMP-Activated Protein Kinase. *Hypertension, 67*(3), 564-573. doi:10.1161/hypertensionaha.115.06825

Lin, Y., & Sun, Z. (2012). Antiaging gene Klotho enhances glucose-induced insulin secretion by up-regulating plasma membrane levels of TRPV2 in MIN6 beta-cells. *Endocrinology, 153*(7), 3029-3039. doi:10.1210/en.2012-1091

Lin, Y., & Sun, Z. (2015a). Antiaging Gene Klotho Attenuates Pancreatic beta-Cell Apoptosis in Type 1 Diabetes. *Diabetes, 64*(12), 4298-4311. doi:db15-0066 [pii]
10.2337/db15-0066 [doi]

Lin, Y., & Sun, Z. (2015b). In Vivo Pancreatic beta-Cell-Specific Expression of Antiaging Gene Klotho: A Novel Approach for Preserving beta-Cells in Type 2 Diabetes. *Diabetes, 64*(4), 1444-1458. doi:db14-0632 [pii]
10.2337/db14-0632 [doi]

Ray, J. L., Leach, R., Herbert, J. M., & Benson, M. (2001). Isolation of vascular smooth muscle cells from a single murine aorta. *Methods Cell Sci, 23*(4), 185-188.

Xie, J., Cha, S. K., An, S. W., Kuro, O. M., Birnbaumer, L., & Huang, C. L. (2012). Cardioprotection by Klotho through downregulation of TRPC6 channels in the mouse heart. *Nat Commun, 3*, 1238. doi:ncomms2240 [pii]
10.1038/ncomms2240 [doi]

Zavaczki, E., Jeney, V., Agarwal, A., Zarjou, A., Oros, M., Katko, M., . . . Balla, J. (2011). Hydrogen sulfide inhibits the calcification and osteoblastic differentiation of vascular smooth muscle cells. *Kidney International, 80*(7), 731-739. doi:S0085-2538(15)55120-X [pii]
10.1038/ki.2011.212 [doi]

Online Supplemental Data

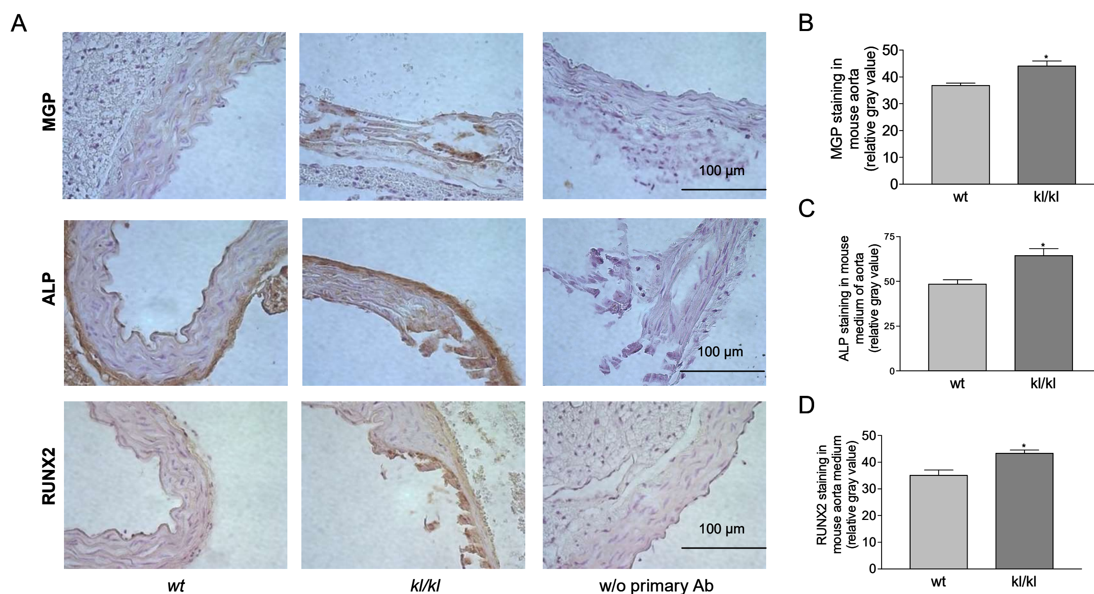

Figure S1. Analysis of bonemorphogenetic factors in aortas. Immunohistochemistrical analysis of matrix gla protein (MGP), alkaline phosphatase (ALP), and RUNX2 (brown staining) of aorta tissue sections from 8-week-old *wt* and *kl/kl* mice fed on normal diet (**A**). Quantifications of MGP staining (**B**), ALP staining (**C**), and RUNX2 staining (**D**) of aorta sections using the NIH Image J software. n=4, *p<0.05 compared with the *wt* mice.

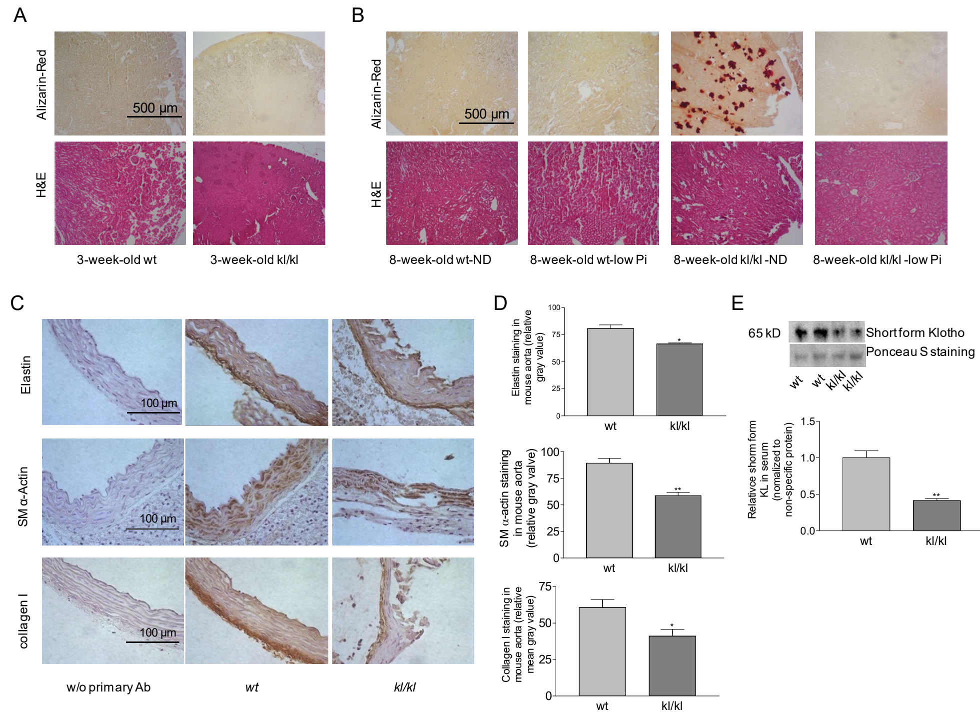

Figure S2. **Analysis of calcification in kidneys and expression of elastin, SM α-actin, and collagen I in aortas.** Alizarin-Red and H&E staining of kidney from 3-week-old *kl/kl* mice (**A**) and 8-week-old *kl/kl* mice (**B**). Elastin, SM α-actin, and collagen I staining in aortic sections (**C**) and quantifications of Elastin, SM α-actin, and Collagen I staining of aortic sections using the NIH Image J software (**D**). Short form Klotho protein in mouse serum **(E)** was detected using Western blot following removal of mouse serum albumin and IgG. n= 4, *p<0.05, **p<0.01, compared with *wt* mice.

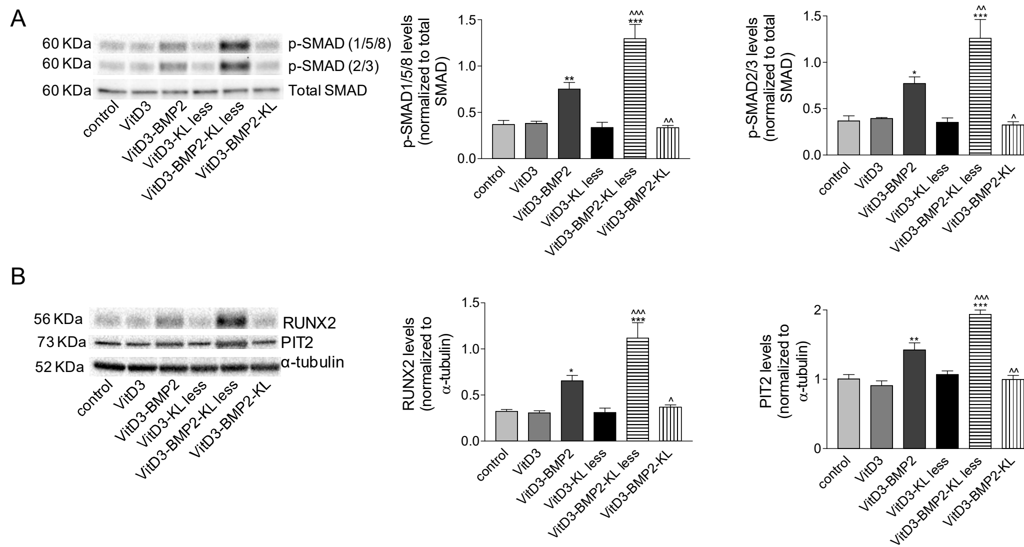

Figure S3. **Klotho-deficient serum promoted BMP2-induced phosphorylations of SMAD1/5/8 and SMAD 2/3 and increased BMP2-induced RUNX2 and PIT2 expression in cultured MOVAS.** Confluent MOVAS cells were incubated with 2.5% normal FBS or 2.5% klotho-deficient FBS in the presence or absence of 1 nM of vitamin D3 or 200 ng/mL of BMP2 for two weeks. (**A**), Western blot analysis of p-SMAD1/5/8 and p-SMAD2/3 levels. (**B**), Western blot analysis of pSMAD1/5/8, RUNX2 and PIT2 protein levels. n=5; *p<0.05, **p<0.01, ***p<0.001 vs control*;* ^p<0.05, ^^p<0.01, ^^^p<0.001 vs VitD3-BMP2. Data=means±SEM.

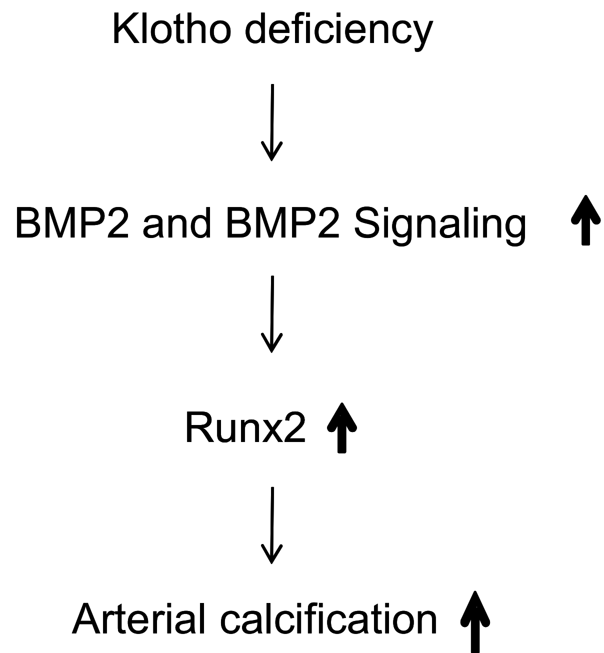

Figure S4. The working hypothesis of arterial calcification. Klotho (kl/kl) deficiency in blood results in an increase in BMP2 and activation of BMP2 signaling in artery. Activation of BMP2 signaling promotes RUNX2, leading to arterial calcification.