HHS Public Access

Author manuscript

J Cell Physiol. Author manuscript; available in PMC 2023 January 01.

Published in final edited form as:

J Cell Physiol. 2022 January ; 237(1): 720–729. doi:10.1002/jcp.30541.

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

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Abstract

Klotho is an aging-suppressor gene. The purpose of this study was to investigate whether Klotho deficiency affects arterial structure. We found that *Klotho*-deficient (kl/kl) mice developed severe arterial calcification and elastin fragmentation. *Klotho*-deficient mice demonstrated higher levels of bone morphogenetic proteins (BMP2, BMP4) and runt-related transcription factor 2 (RUNX2) in aortas, indicating that *Klotho* deficiency upreglates expression of BMP2 and RUNX2 (a key transcription factor in osteoblasts). To exclude the potential involvement of hyperphosphatemia in arterial calcification, *Klotho*-deficient mice were given low phosphate diet (0.2%). Low phosphate diet normalized blood phosphate levels and abolished calcification in the lungs and kidneys, but it did not prevent calcification in the aortas in *Klotho*-deficient mice. Thus, Klotho deficiency *per se* might play a causal role in the pathogenesis of arterial calcification which is independent of hyperphosphatemia. In cultured mouse aortic smooth muscle cells (ASMCs), Klotho-deficient serum induced transition of ASMCs to osteoblasts. Klotho-deficient serum promoted BMP2/vitamin D3-induced protein expression of PIT2 and RUNX2, phosphorylation of SMAD1/5/8 and SMAD2/3, and extracellular matrix calcification. Interestingly, treatments with recombinant Klotho protein abolished BMP2/vitamin D3-induced osteoblastic transition and morphogenesis and calcification. Therefore, Klotho is a critical regulator in the maintenance of normal arterial homeostasis. Klotho deficiency-induced arterial calcification is an active process which involves osteoblastic transition of SMCs and activation of the BMP2-RUNX2 signaling.

Keywords

arterial calcification; osteoblast; BMP2; RUNX2; aortic smooth muscle cells

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AUTHOR CONTRIBUTIONS

Yi Lin, designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript.

Zhongjie Sun. contributed to conceptual design and experimental design, manuscript editing, and funding management.

Disclosure: No

Conflict of interest: No

Introduction

Arterial calcification is largely seen in individuals older than 60 years and in patients with diabetes, atherosclerosis, hypertension, and chronic kidney disease (CKD) (Zhu, Mackenzie, Farquharson, & Macrae, 2012). The frequency of arterial calcification is linked to mortality and morbidity of cardiovascular diseases (Zhu et al., 2012). Arterial calcification is characterized by the deposition of calcium minerals in arteries, likely resulting in arterial stiffening. Arterial calcification is an important risk factor for systolic hypertension, ischemic heart disease and stroke. However, the pathogenesis of arterial calcification is largely unknown.

Klotho has been considered as anti-aging gene, which encodes a single-pass transmembrane protein of 1014 amino acids (130 kDa) (Kuro-o et al., 1997; Shiraki-Iida et al., 1998). *Klotho* gene is primarily expressed in kidneys and brain choroid plexus (Kuro-o et al., 1997). Notably, short-form Klotho protein with an apparent molecular weight of 65 kDa was found in the blood as a result of alternative RNA splicing or proteolytic cleavage (C. D. Chen, Podvin, Gillespie, Leeman, & Abraham, 2007; J. Chen, Fan, Wang, & Sun, 2018; K. Chen et al., 2020; Huang, 2010; Shiraki-Iida et al., 1998). *Klotho* deficiency reduced lifespan (mouse dies at 8 to 10 weeks of age) whereas overexpression of *Klotho* extended lifespan by 20-30% (Kuro-o et al., 1997). Although Klotho is detected in the blood (Hu, Kuro-o, & Moe; Huang; Xu & Sun, 2015), the physiological function of the circulating Klotho in vessels is poorly understood.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) family that regulate bone formation (Lowery & de Caestecker, 2010). BMP2 and BMP4 have been reported to have osteogenic actions as mediators of arterial calcification (Hruska, Mathew, & Saab, 2005; Lowery & de Caestecker, 2010). BMP2 activates type I and type II serine/threonine kinase receptors which subsequently regulates intracellular Smad transcription factors. Activation of Smad signaling promotes osteoblastic transition through increasing transcription factors such as RUNX2, osterix (Osx), distal-less homeobox 5 (Dlx5), and Msh homeobox 2 (Msx2). RUN2 has been considered as an essential regulator of arterial calcification.

The purpose of this study was to investigate a hypothesis that Klotho deficiency leads to arterial calcification *via* promoting osteoblastic transition of arterial smooth muscle cells and upregulating BMP2 signaling.

Materials and methods

A detailed method section is available in the Online Supplemental Methods and Data.

Animal studies.

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). 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.

Measurements of pulse wave velocity (PWV).

PWV was measured as we described recently (K. Chen, Zhou, & Sun, 2015; Lin, Chen, & Sun, 2016).

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).

Immunohistochemistry (IHC).

IHC was performed as described previously (Lin & Sun, 2015a, 2015b). A detailed procedure is available in the Online Supplemental Methods and Data.

Cell cultures.

For details, refer to Online Supplemental Methods and Data. Klotho-deficient serum was generated as we described recently (Fan & Sun, 2016).

Western blotting.

Western blot was performed as described in our previous studies (K. Chen et al., 2015; Crosswhite, Chen, & Sun, 2014; Zhou, Chen, Lei, & Sun, 2015; Zhou, Chen, Wang, et al., 2015). A detailed procedure is available in the Online Supplemental Methods and Data.

RNA Isolation and RT-PCR.

The RT-PCR procedure was described previously (Lin & Sun, 2012). A detailed procedure is available in the Online Supplemental Methods and Data.

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.

Results

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Klotho deficiency caused severe arterial calcification and increased the protein levels of BMP2, BMP4, and RUNX2 in aortas

Klotho deficient mice (*kl/kl*) lived for only 8 to 9 weeks. There was a loss of aortic tissue in 8 week-old *kl/kl* mice as revealed by H&E staining (Fig. 1A). Alizarin red staining showed severe arterial calcification in *Klotho* deficient mice (Fig 1A&B), suggesting that *Klotho* deficiency caused severe arterial calcification in mice. To investigate the potential mechanism of arterial calcification in *Klotho* deficient animals, we measured several

key proteins in the regulation of osteogenesis using western blot. *Klotho* deficient mice at the earlier ages (4-6 weeks) had significantly higher levels in BMP2, BMP4, and RUNX2 in aortas (Fig. 1C-D), suggesting that Klotho deficiency upregulates these proteins. Upregulation of these factors may contribute to the development of klotho deficiency-induced vascular calcification. However, the levels of BMP2 and BMP4 in aortas of *Klotho* deficient mice decreased to the normal levels at ages of 8 weeks (Fig. 1C-D). Thus, this result suggests that BMP2 and BMP4 may not be involved in the maintenance of vascular calcification. The level of RUNX2 in aortas from *Klotho* deficient mice remained elevated at the age of 8 weeks. IHC analysis confirmed upregulation of matrix gla protein (MGP), alkaline phosphatase (ALP), and RUNX2 in *Klotho*-deficient mice (Fig. S1A-D). These data suggest that Klotho deficiency promotes osteogenesis in aortas.

Low phosphate diet did not prevent arterial stiffening in Klotho-deficient mice

It has been reported that *Klotho* deficient mice developed hyperphosphatemia (Kuro-o et al., 1997). Hyperphosphatemia has been considered as an important regulator of arterial calcification in patients with chronic kidney disease (Kendrick & Chonchol, 2011). To exclude the possible involvement of hyperphosphatemia in arterial calcification, we fed *Klotho*-deficient mice with low phosphate diet (0.2%) immediately after weaning at the age of 3 weeks. Low phosphate diet prevented premature death in *Klotho*-deficient mice and largely improved the health condition as evidenced by increased body weights (Fig. 2A&B). Pulse wave velocity (PWV), an index of arterial stiffening, was increased significantly in *Klotho*-deficient mice on normal diet starting from 6 weeks of age (Fig. 2C). These data indicate that *Klotho*-deficient mice developed arterial stiffening at their early age. Low phosphate diet did not prevent arterial stiffening in *Klotho*-deficient mice (Fig. 2C).

We found that *Klotho*-deficient mice displayed significantly higher levels of inorganic phosphate (hyperphosphatemia) and calcium levels (hypercalcemia) (Fig. 2D&E). Low phosphate diet abolished hyperphosphatemia and attenuated plasma calcium levels in *Klotho*-deficient mice (8 weeks) (Fig. 2D&E). These results indicated that Klotho deficiency-induced arterial stiffening is independent of hyperphosphatemia.

Low phosphate diet abolished lung and kidney calcification but did not prevent aortic calcification in *Klotho*-deficient mice

To determine organ specificity of calcification, we performed Alizarin red staining in aortas, lungs, and kidneys. *Klotho* deficient mice at the age of 3 weeks old did not develop obvious calcification in aortas (Fig. 3A), lungs (Fig. 3B), and kidneys (Fig. S2A). At 8 weeks old, *Klotho*-deficient mice fed with normal diet (ND) showed severe arterial calcification in aortas (Fig. 3C), lungs (Fig. 3D), and kidneys (Fig. S2B). With low phosphate diet, *Klotho*-deficient mice still developed aortic calcification albeit at a less severe degree (Fig. 3C), suggesting that Klotho deficiency-induced aortic calcification is largely independent of hyperphosphatemia. By contrast, low phosphate diet completely abolished calcification in the lung (Fig. 3D) and the kidney (Fig. S2B), suggesting that the development of calcification in lungs and kidneys is a passive calcium deposition process due to hyperphosphatemia. However, Klotho deficiency might directly promote aortic calcification in the normal blood levels of phosphate.

In addition, *Klotho*-deficient mice with normal diet displayed diminished elastin, smooth muscle α-actin, and collagen I in aortas (Fig. S2C-D), indicating arterial remodeling.

Serum levels of Klotho were dropped dramatically in *kl/kl* mice (Fig. S2E), confirming Klotho deficiency.

Klotho deficiency upregulated protein levels of BMP2, BMP4, RUNX2, and BMP signaling in aortas

To further study the potential molecular basis of aortic calcification in *Klotho* deficient animals fed on low phosphate diet, several key proteins in the regulation of osteoblastic transition were measured using western blot. Interestingly, at the age of 8 weeks, *Klotho* deficient mice fed on low phosphate diet displayed higher levels of BMP2, BMP4, and RUNX2 in aortas, compared to those of wt mice fed on low phosphate diet (Fig. 4A&B). The levels of pSMAD(1/5/8) and pSMAD(2/3) were also increased in aortas of *Klotho* deficient mice compared to those of *wt* mice (Fig. 4A&B). These results suggest that arterial calcification observed in Klotho deficient mice might be associated with the increases in arterial BMPs and/or BMP signaling.

Klotho-deficient serum (FBS) increased BMP2, BMP4, and RUNX2 protein levels in primary cultures of mouse aortic smooth muscle cells (ASMCs)

To study the molecular mechanism of arterial calcification in Klotho-deficient mice, we used primary culture of ASMCs isolated from wt mouse aortas. Short-form Klotho protein was detected in mouse aortic SMCs, but *Klotho* mRNA was not detectable (Fig. 5A&B), suggesting that short-form Klotho protein might come from fetal bovine serum in culture medium. Similar results were found in a cell line of mouse aortic smooth muscle cells (MOVAS) (Data not shown).

To determine the effect of Klotho deficiency on the regulators on calcification, we generated Klotho-deficient FBS (50% reduction in Klotho) as we described recently (Fan & Sun, 2016). Klotho-deficient FBS promoted BMP2, BMP4, and RUNX2 protein expression levels in cultured primary mouse ASMCs (Fig. 5C&D). Thus, Klotho may be a negative regulator for BMP2, BMP4, and RUNX2 expression in ASMCs.

Klotho-deficient serum promoted BMP2-VitD3-induced calcification in cultured mouse aortic smooth muscle cells (MOVAS)

Since *in vitro* calcification experiment requires long-term culturing of primary ASMCs which leads to cell detachment and death, we switched to a cell line of mouse aortic smooth muscle cells (MOVAS)(Afroze et al., 2003; Mackenzie et al., 2011) for studying calcification. As shown in Fig. 6A, BMP2 induced calcification of MOVAS in the presence of vitamin D3. Interestingly, Klotho-deficient serum promoted BMP2-VitD3-induced cell calcification and calcium deposition (Fig. 6A&B). Importantly, treatments with exogenous Klotho protein completely abolished BMP2-VitD3-induced calcification (Fig. 6A&B). These data suggest that Klotho is not only necessary but also sufficient to curb BMP2-VitD3-induced extracellular matrix calcification of cultured MOVAS.

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

To further study the regulation of calcification in cultured MOVAS by Klotho, we assessed phosphorylations of SMAD and several key proteins in the regulation of calcification. BMP2 increased phosphorylations of SMAD in the presence of vitamin D3 (Fig. S3A). Importantly, Klotho-deficient serum promoted BMP2-induced phosphorylations of SMAD (Fig. S3A). Klotho deficient serum also potentiated BMP2-induced RUNX2 and PIT2 protein expressions (Fig. S3B). It is noteworthy that exogenous Klotho protein treatments completely abolished BMP2-induced phosphorylations of SMAD and protein expressions of RUNX2 and PIT2 (Fig. S3A&B). These results suggest that Klotho is both necessary and sufficient to inhibit BMP2-induced SMAD signaling and its down-stream protein expressions such as RUNX2 and PIT2 in cultured MOVAS.

Discussion

Arterial calcification is characterized by deposition of hydroxyapatite on elastic lamella of arteries. Hydroxyapatite is composed of calcium and phosphate. It was believed that aortic calcification is a passive process due to hyperphosphatemia in *Klotho*-deficient (*kl/kl*) mice(Kuro-o et al., 1997; Xu & Sun, 2015). Here, we report a surprising finding that restoring blood phosphate to normal levels by low phosphate diet did not prevent arterial calcification in *Klotho*-deficient mice (Fig. 3B). Thus, this finding provides the first evidence that Klotho deficiency might play a direct and active role in the pathogenesis of arterial calcification. Although the short-form Klotho protein (65 kDa) was detected in primary culture of mouse ASMCs, *Klotho* mRNA was not detectable (Fig. 5). Therefore, ASMCs do not express Klotho and the detected short-form Klotho protein comes from the circulation. This finding is supported by the published data that mouse arteries do not express Klotho protein (Lim et al., 2012; Lindberg et al., 2013). The short-form Klotho protein was detected in serum of *kl/kl* mouse (Fig. S2F). Therefore, these data suggest that the circulating Klotho acts directly on ASMCs as a hormone. The major source of the circulating Klotho protein is kidneys (Xu & Sun, 2015). It is interesting that a kidney-derived protein plays an important role in the maintenance of normal arterial homeostasis.

It is noticed that Klotho deficiency led to upregulation of BMP2 and BMP4 in aortas (Figs. 4A&B, S2C&D). Bone morphogenetic proteins (BMPs) are also members of the transforming growth factor-β family (Cai, Pardali, Sanchez-Duffhues, & ten Dijke). BMPs can trigger the differentiation of multipotential cells into the osteogenic lineage (Bostrom et al., 1993; Cai et al.). The BMP2 signaling pathway may promote the differentiation of myofibroblasts into the osteogenic lineage (Cheng, Shao, Charlton-Kachigian, Loewy, & Towler, 2003). Recent research showed that BMPs are involved in vascular calcification in low-density lipoprotein receptor-deficient ($LDLR^{-/-}$) mice (Derwall et al., 2012). BMP2 accelerated atherosclerotic intimal calcification in BMP2 transgenic/apoE knockout mice (Nakagawa et al., 2010). Therefore, it is expected that the increases in BMP2 and BMP4 in aortas might contribute to arterial calcification in Klotho-deficient mice.

Another interesting finding is that Klotho protein suppressed BMP2/Vitamin D3-induced calcification in cultured mouse aortic smooth muscle cells (MOVAS) (Fig. 6). It is well

documented that high level of phosphate or phosphate glycerol is required to generate extracellular matrix calcification in cell culture (Mackenzie et al., 2011). Our data suggest that both BMP2 and Vitamin D3 are required for the development of cell calcification in the absence of high level of phosphate. In our experimental condition, recombinant BMP2 protein itself did not directly induce calcification in cultured MOVAS. On the other hand, vitamin D3 has been shown to promote calcification in various vascular SMCs in the presence of 10 mM β-glycerophosphate (Jono, Nishizawa, Shioi, & Morii, 1998; Mantadakis et al., 2007). Indeed, high concentration of vitamin D was found both in *Klotho* deficient mice and *Klotho* knockout mice (Tsujikawa, Kurotaki, Fujimori, Fukuda, & Nabeshima, 2003; Yoshida, Fujimori, & Nabeshima, 2002). In this study, we found that Klotho deficiency promoted BMP2/vitamin D3-induced calcification in cultured MOVAS (Fig. 6). Importantly, treatment with recombinant Klotho protein completely abolished this Klotho-less serum-BMP2/vitamin D3-induced calcification. Therefore, Klotho is an important regulator in BMP2/vitamin D3-induced smooth muscle cell calcification.

Several lines of evidence showed that the BMP2–canonical Smad1/5/8 signaling pathway is linked to the bone remodeling processes including osteoblast differentiation and osteoclastogenesis and to the transition from vascular SMCs to osteoblast-like cells (Broege et al., 2013; Kee et al., 2014). Klotho deficiency upregulated phosphorylation of SMAD1/5/8 in aortas (Fig. 4). Interestingly, Klotho-deficient serum promoted BMP2-induced phosphorylations of SMAD1/5/8 and SMAD2/3 in cultured mouse ASMCs whereas Klotho protein treatments abolished BMP2-induced phosphorylation of SMAD (Fig. S3). Therefore, these data suggest that Klotho is a negative regulator of the BMP signaling. A further study is required to investigate how Klotho interacts with BMP2 and/or BMP2 receptors. Given that some effects of Klotho are mediated by FGF23 signaling (Kurosu et al., 2006; Razzaque, 2009; Urakawa et al., 2006), it would be interesting to investigate if the regulation of the BMP signaling by Klotho is FGF23-depedent.

RUNX2 (runt-related transcription factor) is a critical regulator for osteoblast differentiation and chondrocyte maturation (Komori et al., 1997; Otto et al., 1997). RUNX2 is a marker of osteoblasts and has been shown to play an important role in VSMC calcification *in vitro* and *in vivo* (Byon et al., 2008; N. X. Chen, Duan, O'Neill, & Moe, 2006; Mori, Shioi, Jono, Nishizawa, & Morii, 1999; Speer, Li, Hiremath, & Giachelli, 2010; Sun et al., 2012). Here, we showed that Klotho deficiency increased arterial RUNX2 levels associated with arterial calcification in mice (Fig. 4). Klotho-less serum directly upregulated RUNX2 levels in cultured mouse ASMCs (Fig. 5). In addition, Klotho-less serum enhanced BMP2-induced RUNX2 expression and matrix calcification in MOVAS in the presence of vitamin D3 (Fig. 6 & S3). These findings provide the first evidence that Klotho deficiency promoted osteoblastic transition. Interestingly, Klotho treatments abolished the upregulation of RUNX2 and matrix calcification in cultured MOVAS (Fig. 6 & S3). Taken together, these results suggest that RUNX2 might be a down-stream of Klotho-BMP pathway in the regulation of arterial calcification (Fig. S4). Most recent data indicated that functional cooperation between vitamin D receptor and RUNX2 is required for arterial calcification in response to vitamin D3 (Han et al., 2013).

Our results also suggest that Klotho might regulate PIT2 protein levels in matrix calcification in cultured MOVAS. PIT-1 and PIT-2 are sodium-dependent phosphate (NaPi) cotransporters, which have been identified as the predominant phosphate transporters in rat and human VSMCs (Li, Yang, & Giachelli, 2006; Villa-Bellosta, Bogaert, Levi, & Sorribas, 2007). PIT1 and PIT2 have been shown to regulate phosphate uptake and phosphate-induced matrix calcification in cultured vascular smooth muscle cells redundantly (Crouthamel et al., 2013). Currently, there is no antibody against mouse PIT1. Here, we showed that Klotho-deficient serum promoted BMP2-induced PIT2 protein levels in cultured MOVAS which was reversed by Klotho treatments (Fig. S3B). A further study is warranted to determine how Klotho regulates BMP2-induced PIT2 protein level in mouse aortic smooth muscle cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Source of Funding

This work was supported by NIH R01 HL154147, HL118558, AG062375 and AG049780 grants.

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Perspective

Low phosphate diet normalized blood phosphate levels and abolished calcification in lungs and kidneys but did not prevent the development of arterial calcification in Klotho-deficient mice. Klotho deficiency upregulated BMP2, BMP4 and RUNX2 expressions in aortas leading to aortic calcification in the absence of hyperphosphatemia. Klotho-deficient serum directly induced osteoblastic transition in cultured SMCs as evidenced by upregulation of BMP2 and RUNX2. Klotho-deficient serum promoted BMP2/VitD3-induced calcification, protein expressions of PIT2 and RUNX2, and phosphorylations of SMAD1/5/8 and SMAD2/3 in SMCs. Interestingly, treatments with Klotho protein abolished BMP2/VitD3-induced morphogenetic effects. Thus, Klotho deficiency-induced arterial calcification is an active process which requires activation of the BMP2-RUNX2 signaling pathway. Klotho may be a therapeutic target for vascular calcification.

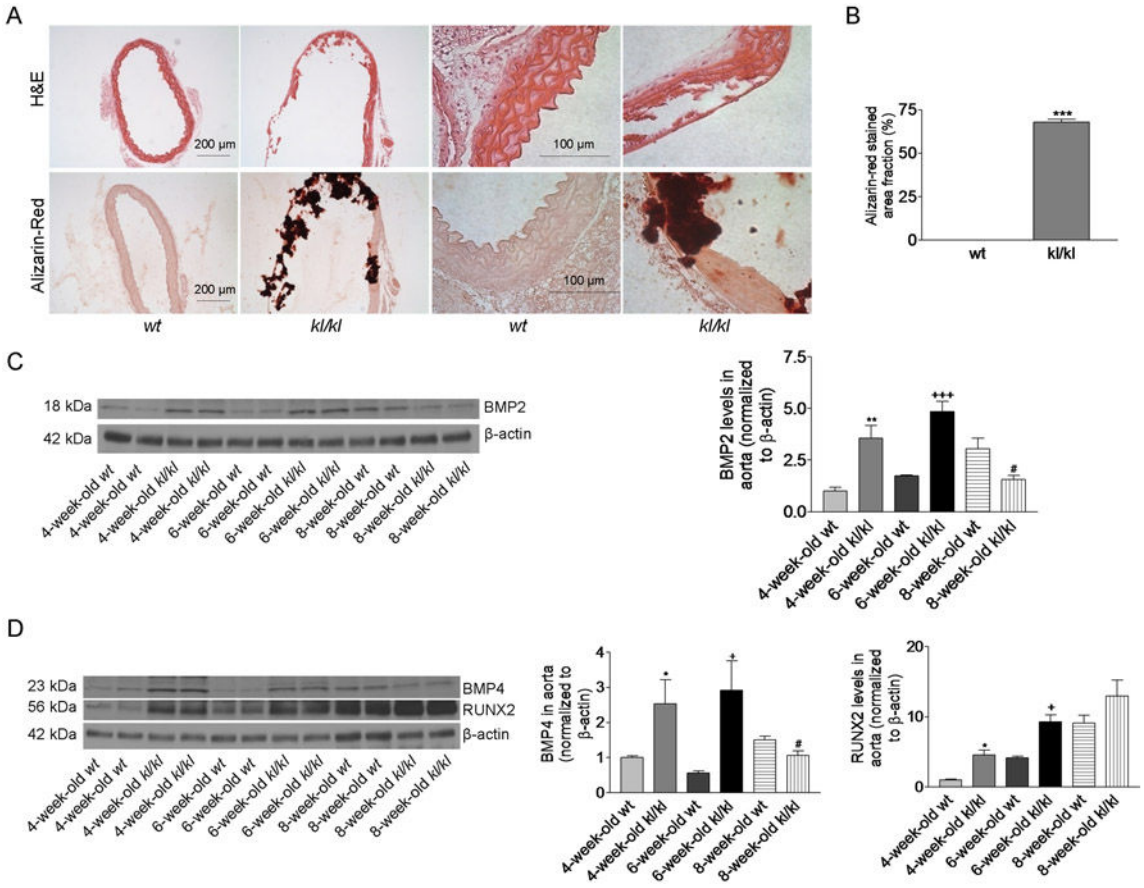

Figure 1. *Klotho* deficiency caused severe arterial calcification and increased the protein levels of BMP2, BMP4, and RUNX2 in aortas.

(**A**), H&E and Alizarin-Red staining of thoracic aorta tissue sections (original magnifications: ×200 and x100) from 8-week-old *wt* and *kl/kl* deficient mice. (**B**), The area fraction of Alizarin-Red positive-staining in aorta section (n=5). ***p<0.001, compared with *wt* mice. (**C**), Western blot analysis of BMP2 in aortas. (**D**), Western blot analysis of BMP4 and RUNX2 in aortas. n=5; *p<0.05, **p<0.01 vs 4-week-old *wt*; +p<0.05, +++p<0.001 vs 6-week-old *wt*; #p<0.05 vs 8-week-old *wt*. Data=means±SEM.

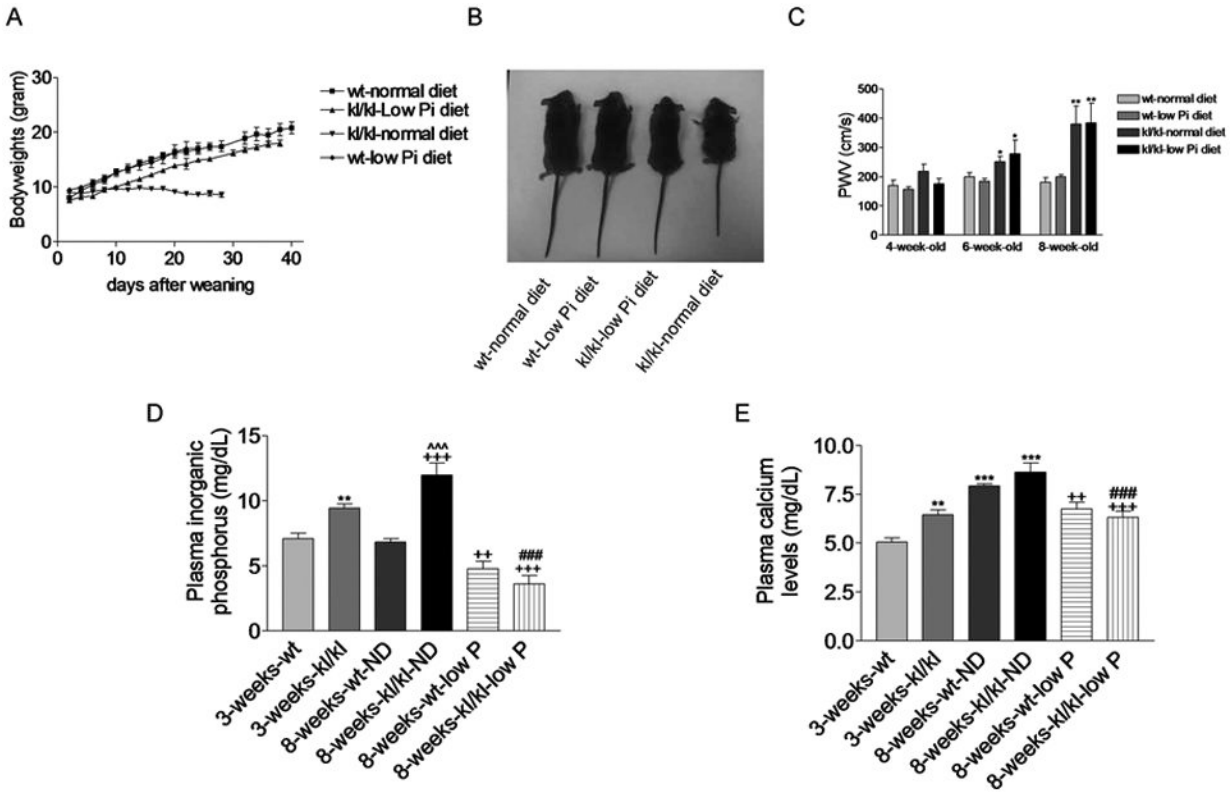

Figure 2. Low phosphate did not prevent arterial stiffening in *Klotho* deficient mice.

(**A**), Body weights. (**B**), Images of animals at 8 weeks old. (**C**), PWV in mice at different ages. n=5; * p<0.05, ** p<0.01 vs wt-normal diet. (**D**). Blood phosphorus levels. (**E**), Blood calcium levels. n=6; * *p<0.01, ***p<0.001 vs 3-weeks-wt; $^{++}$p<0.01, $^{+++}$p<0.001 vs 8-weeks-wt-ND; ^^^p<0.001 vs 3-weeks-kl/kl; ###p<0.001 vs 8-weeks-kl/kl-ND. ND, normal diet. Data=means±SEM.

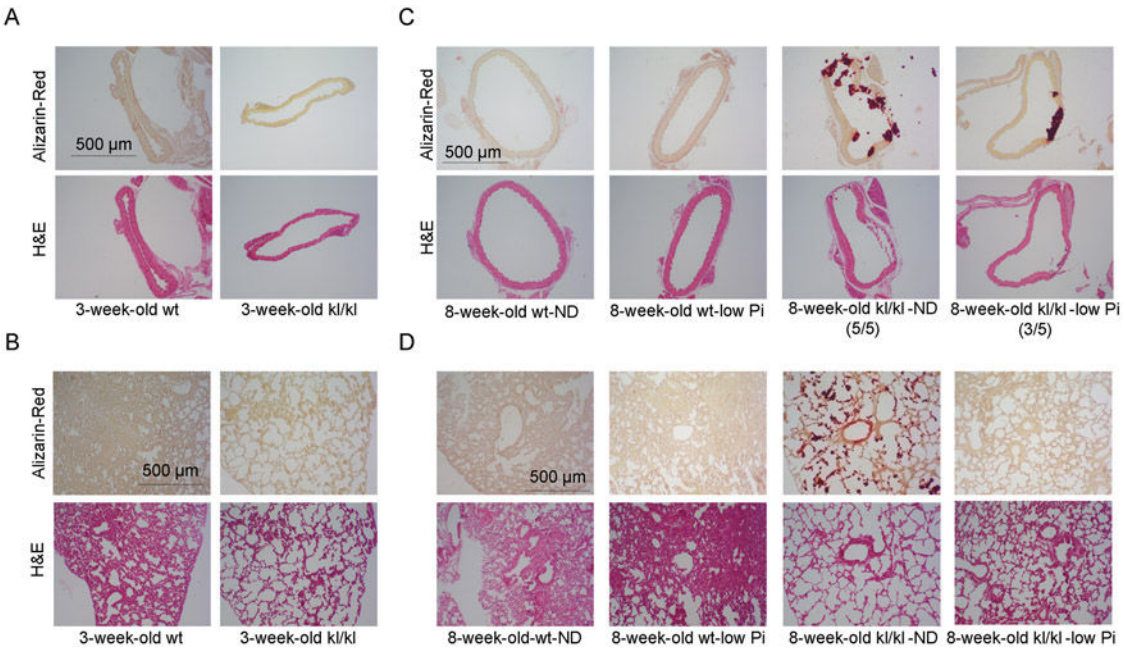

Figure 3. Low phosphate diet abolished lung calcification but did not prevent aortic calcification in *Klotho*-deficient mice.

Alizarin-Red and H&E staining of aorta from 3-week-old *kl/kl* deficient mice (**A**) and 8-week-old *kl/kl* deficient mice (**C**). Alizarin-Red and H&E staining of lung from 3-week-old *kl/kl* deficient mice (**B**) and 8-week-old *kl/kl deficient* mice (**D**).

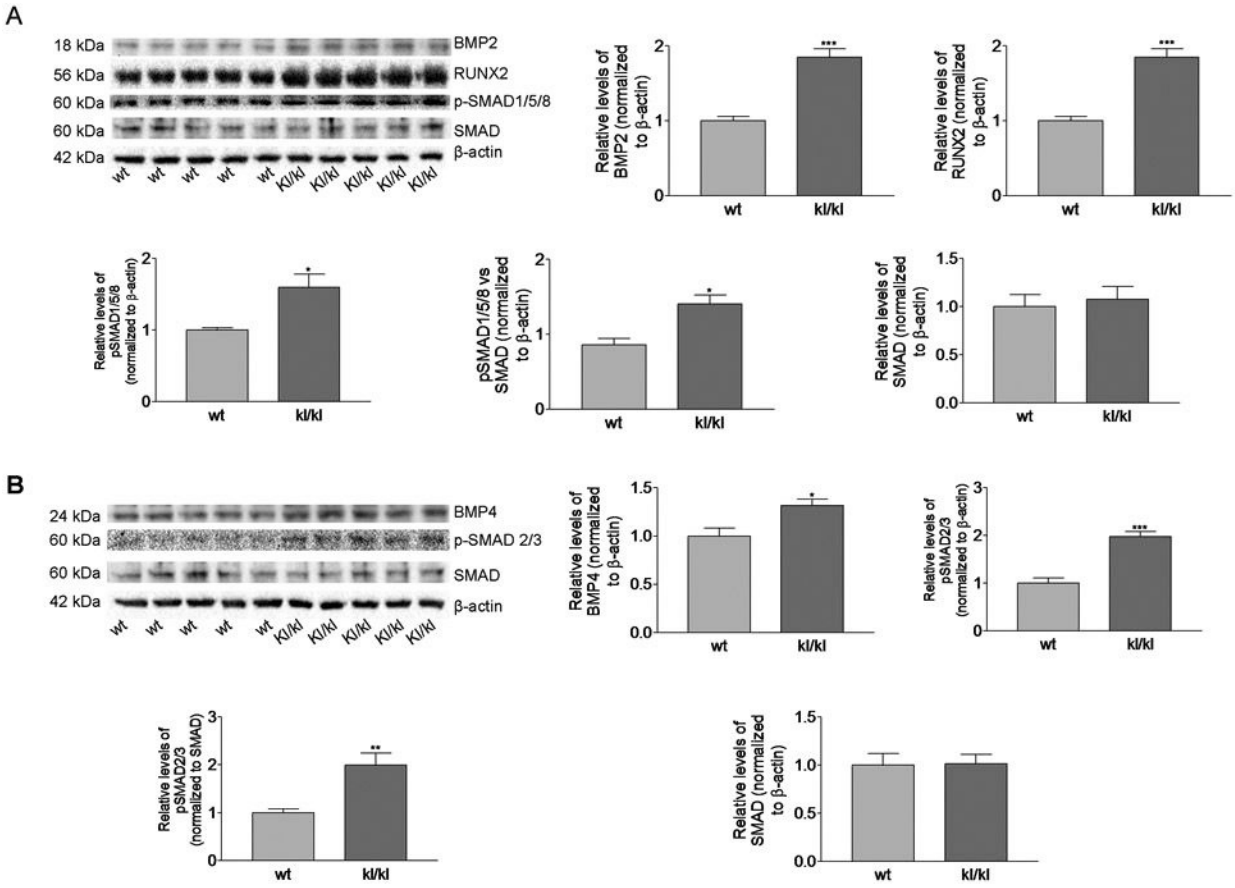

Figure 4. Klotho deficiency upregulated protein levels of BMP2, BMP4, RUNX2, and BMP signaling in aortas.

(**A**), Western blot analysis of BMP2, RUNX2, p-SMAD(1/5/8), and total SMAD in aorta lysates from mice. (**B**), Western blot analysis of BMP4, pSMAD(2/3), and total SMAD in aortic lysates. n=5; *p<0.05, **p<0.01, ***p<0.001 vs 8-week-old *wt* mice fed on low phosphate diet. Data=means±SEM.

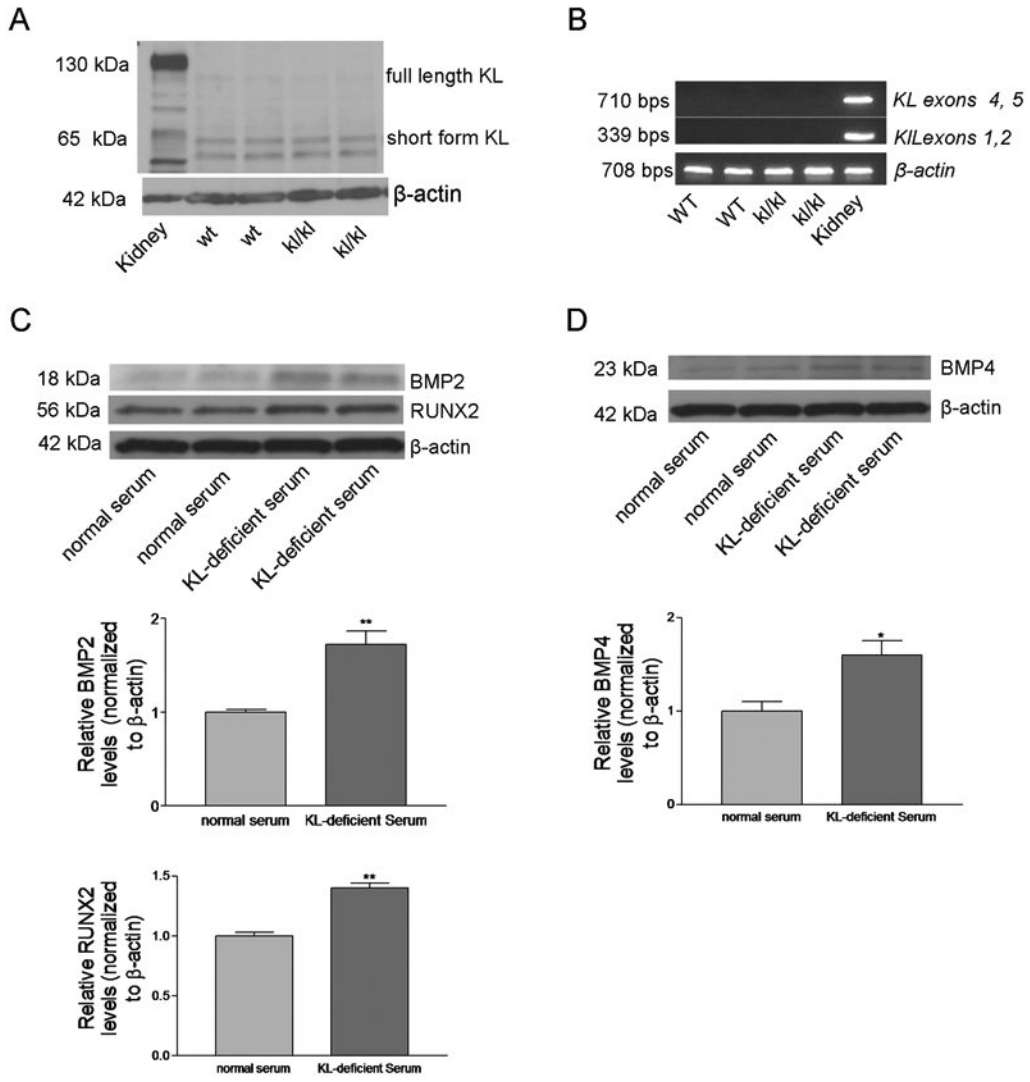

Figure 5. Klotho-deficient serum (FBS) increased BMP2, BMP4, and RUNX2 protein levels in primary cultures of mouse aortic smooth muscle cells (SMCs).

Aortic smooth muscle cells (SMCs) were isolated from 6-week-old *kl/kl* and *wt* mice. (**A**), Short form Klotho protein in primary mouse aortic SMCs was assessed by Western blot. (**B**), *Klotho* mRNA in primary mouse ASMCs were detected using RT-PCR. **(C),** BMP2 and RUNX2 protein expression were measured in cells incubated with Klotho-deficient serum (10%) or normal serum (10%) for 5 days. (**D**), BMP4 protein was measured after incubation with Klotho-deficient serum (10%) or normal serum (10%) for 5 days. n=5; *p<0.05, **p<0.01 vs normal serum. Data=means±SEM.

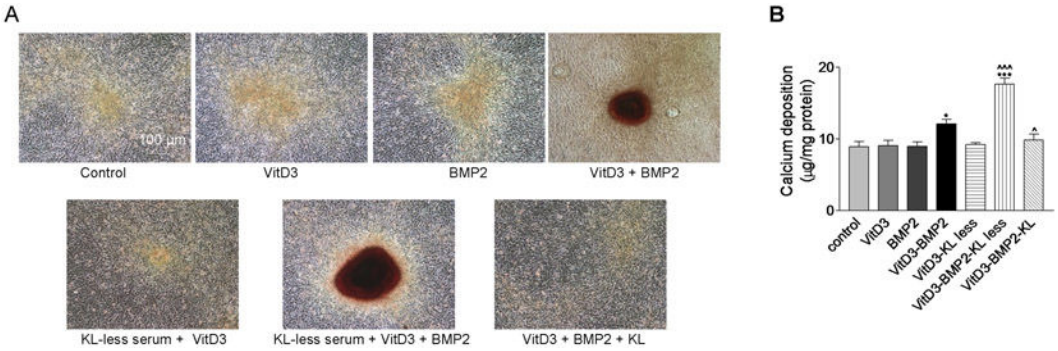

Figure 6. Klotho-deficient serum promoted BMP2-VitD3-induced calcification in cultured mouse aortic smooth muscle cells (MOVAS).

Confluent cells were incubated with 2.5% normal FBS or 2.5% KL-deficient FBS in the presence or absence of 1 nM of vitamin D3 or 200 ng/mL of BMP2 for two weeks. Culture medium was changed every 3 days. Representative photographs of Alizarin Red staining at the magnification of 100 (**A**). Quantification of calcium contents in extracellular matrix (**B**). n=4 independent experiments. *p<0.05, ***p<0.001 vs the control; ^p<0.05, ^^^p<0.001 vs VitD3-BMP2. Data=means±SEM.