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BMP Signaling is Required for Aortic Valve Calcification

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Abstract

Objective—Calcific Aortic Valve Disease (CAVD) is the most prevalent type of heart valve disease, affecting ~2% of the US population. CAVD is characterized by the presence of calcific nodules resulting in aortic valve (AoV) stenosis; however, the underlying mechanisms driving disease remain unknown. Studies of human diseased AoV provide initial evidence that BMP signaling, essential for normal bone formation, is activated during CAVD. Mice deficient in *Klotho*, an FGF23 transmembrane co-receptor, exhibit premature aging and develop AoV calcific nodules as occurs in human CAVD. The role of BMP signaling in the development of CAVD was examined in porcine aortic valve interstitial cells (VICs) and *Klotho*$^{-/-}$ mice.

Approach & Results—We show that activation of BMP signaling, as indicated by pSmad1/5/8 expression, precedes and later localizes with AoV calcification in *Klotho*$^{-/-}$ mice. In addition, cellular and ECM changes resembling features of normal bone formation are accompanied by increased osteochondrogenic gene induction in calcified *Klotho*$^{-/-}$ AoV. Likewise, osteogenic media (OM) treatment of porcine VICs results in BMP pathway activation, increased osteochondrogenic gene induction and formation of calcific nodules *in vitro*. We demonstrate that genetic inactivation of the *Bmpr1a* receptor in *Klotho*$^{-/-}$ aortic VICs, as well as BMP pathway inhibition of OM-treated aortic VICs *in vitro*, results in the inhibition of AoV calcification.

Discussion—BMP signaling and osteochondrogenic gene induction are active in calcified *Klotho*$^{-/-}$ AoV *in vivo* and calcified porcine aortic VICs *in vitro*. Importantly, BMP signaling is required for the development of AoV calcification *in vitro* and *in vivo*.

Keywords

Aortic Valve Calcification; BMP; Klotho

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DISCLOSURES

None.

INTRODUCTION

Calcific Aortic Valve Disease (CAVD) is a progressive disease, initially presenting with aortic valve (AoV) sclerosis, often leading to AoV stenosis and insufficiency later in life. The risk for developing CAVD increases with age, and CAVD is becoming more prevalent with increased average lifespan[1]. AoV stenosis affects 2.8% of individuals over the age of 75, and AoV regurgitation presents in ~2% of the elderly[2]. To date, the only clinical therapy available to patients is AoV replacement surgery, which has significant limitations of coagulation and degeneration, and is often contraindicated in the elderly[2–4]. Thus, understanding the molecular mechanisms driving CAVD pathogenesis is crucial to the development of new therapeutic approaches.

Because of its association with aging, CAVD was believed to be a passive, degenerative disease. However, recent studies have demonstrated that CAVD is an active, cellular-driven process[5]. A hallmark in the progression of CAVD is AoV stenosis caused by extensive calcific deposits[6]. In adult healthy valves, resident valve interstitial cells (VICs) remain quiescent with fibroblast-like characteristics. Interestingly, in human CAVD, markers of endochondral bone formation – including *Runx2*, *Alkaline Phosphatase*, *Osteopontin* and *Osteocalcin* – have been observed in VICs associated with areas of calcification[7, 8], supporting activation of an osteoblast-like cell phenotype in CAVD. Bone Morphogenetic Protein (BMP) signaling is essential during heart valve development and in bone formation[9, 10]. Notably, studies of human CAVD revealed increased *BMP2* and *BMP4* ligand expression in calcified AoV[11], as well as increased pSmad1/5/8, indicative of canonical BMP signaling, on the fibrosa side of AoV where most calcification occurs[8, 12]. Altogether these data demonstrate that osteochondrogenic markers and BMP signaling are active in human CAVD. However, the *in vivo* requirements of BMP signaling for osteochondrogenic gene induction and AoV calcification remain unknown.

The *Klotho*$^{-/-}$ mouse model, which exhibits premature aging associated with hyperphosphatemia, low vitamin D and increased FGF23, has been used to study age-related diseases, including chronic kidney disease and vascular calcification[13]. Similar to human CAVD, *Klotho*$^{-/-}$ mice exhibit AoV calcification at the hinge region of the fibrosa side[14]. This is in contrast to hyperlipidemic mouse models of AoV disease, in which calcification occurs throughout the aorta and valve leaflet surface, accompanied by lipid accumulation and inflammation[15, 16]. Instead, calcification occurs independent of inflammation in *Klotho*$^{-/-}$ mice, making these mice useful for examination of molecular mechanisms of AoV calcification that are distinct from hyperlipidemia.

The goal of this study is to examine the requirements for BMP signaling during AoV calcification. *Klotho*$^{-/-}$ AoV exhibit features of endochondral bone formation and localized pSmad1/5/8 activation in the hinge region preceding calcific nodule formation. The ability of a small molecule inhibitor of BMP signaling to inhibit porcine aortic VIC calcification was tested in culture. Genetic inactivation of BMP receptor *Bmpr1a* in aortic VICs of *Klotho*$^{-/-}$ mice was used to demonstrate the *in vivo* requirement for BMP signaling in the development of AoV calcification.

MATERIALS AND METHODS

Materials and Methods are available in the online-only Data Supplement.

RESULTS

Calcification of AoV in *Klotho*$^{-/-}$ mice exhibits features of endochondral bone formation

There is increasing evidence that the progression of human CAVD includes features of endochondral bone formation, including cartilage and bone-lineage gene induction[8, 11]. As demonstrated by Movat's pentachrome staining, which labels extracellular matrix (ECM) constituents of the *Klotho*$^{-/-}$ AoV, areas of cell condensation can be observed prior to calcification (Figure 1B,E,H). These condensed areas are associated with increased proteoglycans and the presence of chondrocyte-like cells as indicated by morphology (Figure 1H). Likewise, proteoglycan-rich areas are localized to calcific nodules surrounded by chondrocyte-like cells (Figure 1C,F,I). Thus, VICs localized to calcific nodules undergo cellular changes that resemble endochondral bone formation.

Analysis of gene expression of 6-week-old *Klotho*$^{-/-}$ AoV demonstrates a significant increase in mRNA levels of cartilage markers *Sox9, Aggrecan, Col2a1, Col10a1* and *Mef2c* (Figure 1J), which are critical factors in chondrogenesis that precedes normal bone formation. In addition, osteogenic genes involved in bone mineralization, including *Alkaline Phosphatase* (ALP), *Runx2* and *Osteopontin* (OPN), are also significantly increased in these AoV, compared to control *Klotho*$^{+/+}$ AoV (Figure 1J). Together, these findings demonstrate that AoV calcification in the *Klotho*$^{-/-}$ mice is associated with increased cartilage and bone marker expression, supporting an endochondral bone-like process of calcification.

Calcific nodules are populated by endothelial- and neural crest-derived cells in *Klotho*$^{-/-}$ mice

Adult semilunar VICs include cells derived from endocardial cushion endothelial cells and neural crest of the developing embryo[17]. However, the contribution of each cell type to the areas of calcification and disease pathogenesis during adult CAVD remains unclear. The developmental origins of cells populating mature AoV leaflets were assessed using *Tie2Cre* labeling of endothelial-derived cells in comparison to *Wnt1Cre* lineage cells derived from embryonic neural crest. *Tie2Cre* and *Wnt1Cre* mice were crossed with *ROSA26*^{mTmG} mice in order to identify Cre-recombined cells found in the AoV. These Cre-positive cells are marked by membrane-targeted green fluorescent protein (GFP), compared to expression of membrane-targeted red fluorescence protein (RFP) in cells that do not express Cre[18]. Endothelial-derived Tie2Cre-positive cells, indicated by GFP, are distributed throughout all three leaflets in wild type AoV (Supplementary Figure I). Neural crest-derived Wnt1Cre GFP-positive cells are also present in all leaflets of wild type AoV, but are predominantly located in the hinge region (Supplementary Figure I). Thus, Tie2Cre and Wnt1Cre GFP-positive cells populate the AoV hinge region.

The embryonic origins of calcified VICs were examined in calcified *Klotho*$^{-/-}$ AoV. Both Tie2Cre and Wnt1Cre-derived cells are present in the calcified region of the AoV hinge in *Klotho*$^{-/-}$ mice (Supplementary Figure II). Approximately 60% of the Wnt1Cre-expressing

cells in the AoV hinge are localized to calcified regions in *Klotho*$^{-/-}$;*Wnt1Cre* AoV. In contrast, Tie2Cre-positive cells have a more uniform distribution in the hinge and only 20% are localized in the calcified regions. Interestingly, Tie2Cre and Wnt1Cre lineages each contribute ~25% of the total number of cells that populate the calcified areas of *Klotho*$^{-/-}$ AoV. Together these data demonstrate that both endothelial and neural crest derivatives contribute to calcified nodules in *Klotho*$^{-/-}$ AoV.

pSmad1/5/8 activation precedes and later localizes with calcific nodules in *Klotho*$^{-/-}$ AoV

The expression of pSmad1/5/8 was examined during the progression of AoV calcification in *Klotho*$^{-/-}$ mice as an indicator of BMP signaling status. pSmad1/5/8 is initially detected in the AoV hinge prior to onset of disease at 4 weeks of age (Figure 2J,N). Later, pSmad1/5/8 localizes with calcific nodules at 6 (Figure 2K,O) and 8 (Figure 2L,P) weeks in *Klotho*$^{-/-}$ AoV, compared to age-matched wild type controls where there is no positive antibody staining or calcification (Figure 2A–H). pSmad1/5/8 expression is restricted to the AoV hinge region interstitial cells, and is not detected in smooth muscle cells of the aorta that express Alpha Smooth Muscle Actin (αSMA) (Figure 2E–H and M–P). Likewise, Western blot analysis of isolated AoV at 6 weeks demonstrates increased pSmad1/5/8 protein expression in *Klotho*$^{-/-}$ mice when compared to wild type controls (Figure 2Q–R). Interestingly, there is no significant difference in the prevalence of AoV calcification between sexes. Thus BMP signaling, as indicated by pSmad1/5/8, is activated prior to and during development of AoV calcification in *Klotho*$^{-/-}$ mice.

Expression of BMP ligands and signaling intermediates was examined in *Klotho*$^{-/-}$ AoV. BMP2 protein is localized to calcific nodules of *Klotho*$^{-/-}$ AoV at 6 weeks (Figure 3C,D), but BMP2 expression was not detected in controls (Figure 3A,B). In addition, *BMP2* and *BMP4* ligand gene expression is increased in *Klotho*$^{-/-}$ AoV compared to controls at 6 weeks (Figure 3E). Gene expression of the BMP pathway downstream target *Smad6* also is significantly increased 6 weeks (Figure 3E). Thus, active BMP pathway components are localized to areas of AoV calcification in *Klotho*$^{-/-}$ mice *in vivo*.

BMP pathway inhibition prevents calcific nodule formation and osteochondrogenic gene induction in aortic VICs cultured *in vitro*

As previously shown[19], porcine VICs treated with Osteogenic Media (OM) form calcific nodules, as indicated by positive Alizarin Red (Figure 4C) and von Kossa (Figure 4G) staining after 9 days in culture. In contrast, VICs maintained in control media do not form calcific nodules (Figure 4A,E). The necessity for BMP signaling in OM-induced calcific nodule formation was determined using small molecule inhibitor of BMP signaling, LDN-193189[20, 21]. LDN-193189 treatment inhibits OM-induced formation of calcific nodules (Figure 4D,H) when compared to cells treated only with OM (Figure 4C,G), as demonstrated by quantification of the number of nodules/well (Figure 4I–J). In addition, LDN-193189 treatment significantly inhibits phosphorylation of Smads1/5/8 as illustrated by Western blot analysis and quantification (Figure 5A,B). Moreover, OM-induced *BMP2,* but not *BMP4,* ligand expression is suppressed by LDN-193189 treatment (Figure 5C,D). Furthermore, addition of LDN-193189 to OM-treated cells results in decreased expression of OM-induced chondrogenic gene *Hapln1* (Figure 5E), as well as osteogenic genes

Osteopontin (Figure 5F) and *Osteocalcin* (Figure 5G). However, BMP2 treatment alone in the absence of OM is not sufficient to promote calcification of aortic VICs *in vitro* (Supplementary Fig III). Notably, treatment of porcine VICs for 9 days does not result in apoptosis (Supplementary Figure IV). Together, these data demonstrate that BMP signaling is required, but is not sufficient, for the formation of calcific nodules and osteochondrogenic gene induction in cultured aortic VICs.

Genetic inactivation of *Bmpr1a* does not affect AoV development or maturation *in vivo*

The requirement for BMP signaling in normal aortic VICs was examined *in vivo* using *PostnCre;Bmpr1a*$^{flox/flox}$ mice. *PostnCre*[22] is active in aortic VICs, but not in endothelial cells, and is expressed robustly throughout the AoV[23]. The BMP Type IA receptor ALK3 (*Bmpr1a*) is required for BMP-mediated phosphorylation of Smad1/5/8 in many tissues, including heart valve progenitors[24]. *Tie2Cre* and *Wnt1Cre* are active in a subset of aortic VICs, and deletion of *Bmpr1a* in either of these lineages causes embryonic lethality[25, 26]. Therefore, the requirements for *Bmpr1a* in *PostnCre* lineage cells, present in embryonic and adult valves after endocardial cushion formation, were examined. Although BMP signaling plays essential roles during endocardial cushion EndMT[24, 26], *PostnCre;Bmpr1a*$^{flox/flox}$ mice are viable and the hearts are grossly normal at postnatal day (P)1, similar to *Bmpr1a*$^{flox/flox}$ control mice (Supplementary Figure V). In addition, Pentachrome staining demonstrates normal heart morphology, in addition to normal heart valve stratification and ECM composition of *PostnCre;Bmpr1a*$^{flox/flox}$ aortic and mitral valves (Supplementary Figure V), when compared to control P1 hearts. *PostnCre* expression is observed in other regions outside of the AoV VICs, including fibroblasts located at the atrioventricular junction[22]. However, since *PostnCre* is activated after endocardial cushion formation[23], these data demonstrate that *PostnCre*-driven deletion of *Bmpr1a* does not affect late stage AoV development or neonatal maturation.

PostnCre;Bmpr1a$^{flox/flox}$ mice are viable and fertile as adults, with no observed embryonic or postnatal lethality. AoV analyzed at 8 weeks of age exhibit normal heart morphology, as well as normal AoV and ECM composition, as indicated by Pentachrome staining when compared to control *Bmpr1a*$^{flox/flox}$ mice (Supplementary Figure VI). Although the valves are apparently normal, *PostnCre;Bmpr1a*$^{flox/flox}$ mice exhibit a significant decrease in body weight compared to control mice (Supplementary Figure VI). In spite of this difference, *PostnCre*-driven deletion of *Bmpr1a* does not affect overall heart and valve structure and homeostasis in adult mice.

Genetic inactivation of *Bmpr1a* prevents development of AoV calcification in *Klotho*$^{-/-}$ mice with CAVD

The requirement for BMP signaling, through *Bmpr1a*, in the development of CAVD in *Klotho*$^{-/-}$ mice was examined. In contrast to *Tie2Cre* or *Wnt1Cre*-lineages, *PostnCre* derived cells are located throughout the *Klotho*$^{-/-}$ AoVs (Supplementary Figure VII) and adult mice are viable for analysis. Thus 7–9 week-old *Klotho*$^{-/-}$;*PostnCre;Bmpr1a*$^{flox/flox}$ mice were used to determine if BMP signaling is required in the development of AoV calcification. *Klotho*$^{-/-}$ mice with or without *PostnCre*-mediated *Bmpr1a* deletion are smaller by weight than normal adult mice[27] (Figure 6H). However, inactivation of *Bmpr1a*

in *Klotho*$^{-/-}$ AoV results in a significant decrease of AoV calcification, as evident by analysis of von Kossa staining, when compared to control mice that exhibit AoV calcification (Figure 6A,D). BMP pathway inactivation through loss of *Bmpr1a* also preserves normal ECM organization and cellular morphology (Figure 6B,E). Importantly, no pSmad1/5/8 immunoreactivity is observed in *Klotho*$^{-/-}$;*PostnCre; Bmpr1a*$^{flox/flox}$ AoV (Figure 6F). Total areas of calcification were measured and quantified for *Klotho*$^{-/-}$; *PostnCre;Bmpr1a*$^{flox/flox}$ and control *Klotho*$^{-/-}$ AoV von Kossa stained images. Quantitative data show a significant decrease in the total calcified area of AoV in *Klotho*$^{-/-}$;*PostnCre;Bmpr1a*$^{flox/flox}$ mice when compared to *Klotho*$^{-/-}$ mice (Figure 6G). Together these data demonstrate the necessity of BMP-pSmad1/5/8 signaling for the development of AoV calcification in *Klotho*$^{-/-}$ mice.

DISCUSSION

Here, we demonstrate that expression of pSmad1/5/8, indicative of active BMP signaling, precedes and later localizes with AoV calcification in *Klotho*$^{-/-}$ mice with CAVD. Likewise, BMP ligands *BMP2* and *BMP4* are significantly increased in *Klotho*$^{-/-}$ AoV. Consistent with previous studies, BMP signaling is required for osteogenic gene induction and calcification in cultured porcine VICs, similar to human VICs[28–30]. pSmad1/5/8 activation and increased *BMP2* and *BMP4* expression have been reported in human CAVD[8, 11, 12]. However, *in vivo* requirements for BMP signaling in AoV calcification have not been demonstrated previously. Here, we show that BMP pathway inhibition through genetic inactivation of *Bmpr1a* in *Klotho*$^{-/-}$ aortic VICs prevents AoV calcification *in vivo.* Thus BMP signaling is required for CAVD.

BMP signaling has previously been implicated in valve development and disease. In developing valves, *Bmpr1a*-mediated BMP signaling is required for proper formation of the atrioventricular junction and endocardial cushions[24, 26]. Moreover, genetic deletion of the related receptor ALK2 (*Acvr1)* in the cushion mesenchyme leads to AoV defects, including Bicuspid Aortic Valve (BAV)[31]. In this study, we show that *PostnCre*-driven deletion of *Bmpr1a* after endocardial cushion formation does not affect valve development or adult homeostasis, but does inhibit AoV calcification in *Klotho*$^{-/-}$ mice. Interestingly, increased BMP signaling due to haploinsufficiency of the BMP inhibitor *Smad6* is sufficient to promote aortic calcification[32]. Thus BMP signaling has multiple critical roles in valve development and disease.

There is increasing evidence that AoV calcification during CAVD occurs via an endochondral bone formation-like process[11]. VICs derived from both endothelial and neural crest embryonic lineages are calcified, suggesting that the potential to calcify is associated with location within the valve, rather than a specific embryonic origin or cell lineage. Interestingly, BMP signaling via pSmad1/5/8 plays essential roles during endochondral bone formation by regulating expression of critical cartilage and bone factors[33, 34]. In accordance, osteochondrogenic gene induction, accompanied by cellular and ECM changes that resemble features of normal endochondral bone formation, occurs in calcified *Klotho*$^{-/-}$ AoV, where BMP signaling is active. Loss of BMP signaling through genetic deletion of Bmpr1a *in vivo* or inhibition of BMP receptor kinase activity by LDN-193189 in culture leads to loss of

AoV osteochondrogenic characteristics and prevents formation of calcific nodules. Together these data demonstrate that BMP signaling is required for induction of endochondral bone formation-like processes during AoV calcification.

There are a number of risk factors associated with CAVD, including valve developmental defects, notably BAV, inflammation due to increased lipid deposition, and hyperphosphatemia as a result of chronic kidney disease (CKD). Consequently, the underlying pathogenesis of CAVD may vary depending on the accompanying risk factors[6, 35–37]. Previous studies have shown that AoV calcification in *Klotho*$^{-/-}$ mice occurs independent of inflammation[14], or a myofibroblast intermediate[19]. Furthermore, *Klotho*$^{-/-}$ mice develop hyperphosphatemia secondary to CKD and prior to the onset of AoV calcification along with other age-related phenotypes[13, 27]. The Klotho protein is a cofactor for fibroblast growth factor FGF23 and *Klotho*-deficiency leads to abnormal mineral metabolism characterized by increased phosphate, calcium and vitamin D serum levels[38]. *Klotho*-deficiency also leads to increased Wnt signaling[39, 40], which together with BMP signaling, promotes endochondral bone formation and contributes to vascular calcification[41]. BMP signaling is not sufficient to induce AoV calcification, but the contributions of these additional factors in conjunction with increased BMP signaling to AoV pathogenesis remains to be elucidated.

CAVD is a progressive disease, and currently there are no pharmacologic-based therapies that can prevent or inhibit CAVD. The current standard of treatment is AoV replacement, which is associated with significant complications that increase with age[42]. This study shows that BMP pathway inhibition prevents calcific nodule formation, a hallmark of CAVD pathology, demonstrating that BMP signaling is required for the development of CAVD. Importantly, *in vitro* treatment of porcine aortic VICs with BMP inhibitor LDN-193189 leads to inhibition of OM-induced calcific nodule formation. LDN-193189 is a small molecule inhibitor that specifically targets the BMP receptor Bmpr1a and has been shown to reduce soft tissue calcification[21], as well as vascular calcification and atherosclerosis[43, 44]. However, LDN-193189 has not yet been tested in the context of CAVD. Our data demonstrates that targeting BMP signaling might serve as a new therapeutic approach in the prevention of AoV calcification during CAVD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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NONSTANDARD ABBREVIATIONS AND ACRONYMS

CAVD	Calcific Aortic Valve Disease
AoV	Aortic Valve
VIC	Valve Interstitial Cell
BMP	Bone Morphogenetic Protein
αSMA	Alpha Smooth Muscle Actin

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HIGHLIGHTS

- Increased BMP signaling, osteochondrogenic gene induction, cellular morphology and ECM changes resembling endochondral bone formation are observed in calcified *Klotho*$^{-/-}$ AoV, supporting an endochondral bone-like process of calcification via BMP signaling.
- Treatment with BMP receptor inhibitor LDN-193189 inhibits OM-induced osteochondrogenic gene induction and formation of calcific nodules *in vitro.*
- Genetic inactivation of the *Bmpr1a* receptor in *Klotho*$^{-/-}$ aortic VICs, results in the inhibition of AoV calcification. Thus BMP signaling is required for AoV calcification *in vivo*.

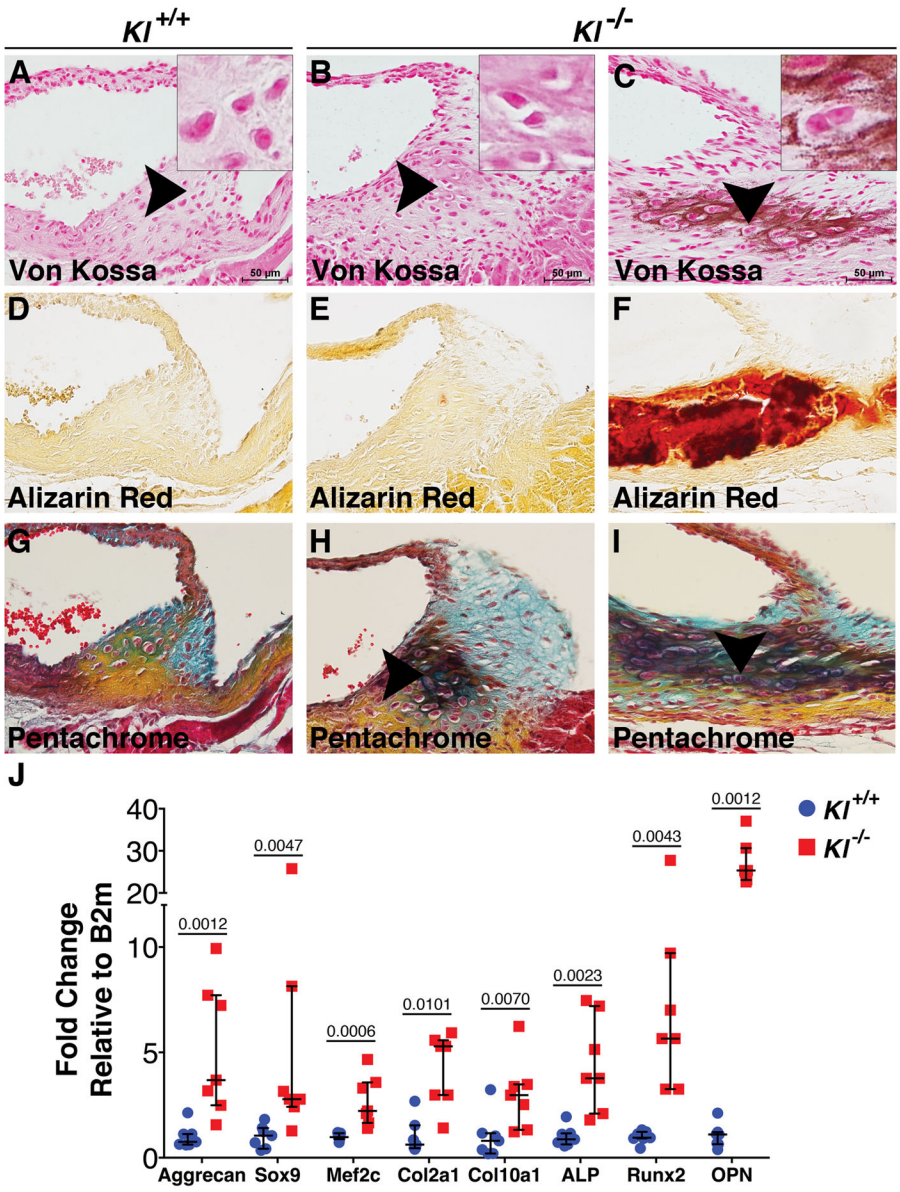

Figure 1. Calcification of *Klotho*$^{-/-}$ AoV has features of endochondral bone formation

Von Kossa staining detects the presence of calcific deposits in 6-week old *Klotho*$^{-/-}$ AoV (brown, C) compared to controls (A). Chondrocyte-like cells are present in *Klotho*$^{-/-}$ AoV hinge regions (B,C arrowheads and insets). As a secondary method of detection for calcification, Alizarin Red staining was performed (red, D–F). Movat's Pentachrome staining shows changes in the ECM in *Klotho*$^{-/-}$ AoV (H,I arrowheads). Yellow stains collagen, blue stains glycosaminoglycan, deep green denotes intense overlapping yellow and blue staining. At least 3 hearts were analyzed for each phenotype at 6 weeks of age (A–F). Osteochondrogenic gene expression was determined in *Klotho*$^{-/-}$ AoV compared to control AoV at 6 weeks (n=7/group). Gene expression was normalized to Beta-2-microglobulin (B2m) control expression. Values were normalized to wild type control averages and are

represented as fold changes (J). Error bars are shown as interquartile means and scatter plot. Statistical significance was determined by Mann-Whitney U-test ($p < 0.05$ are indicated).

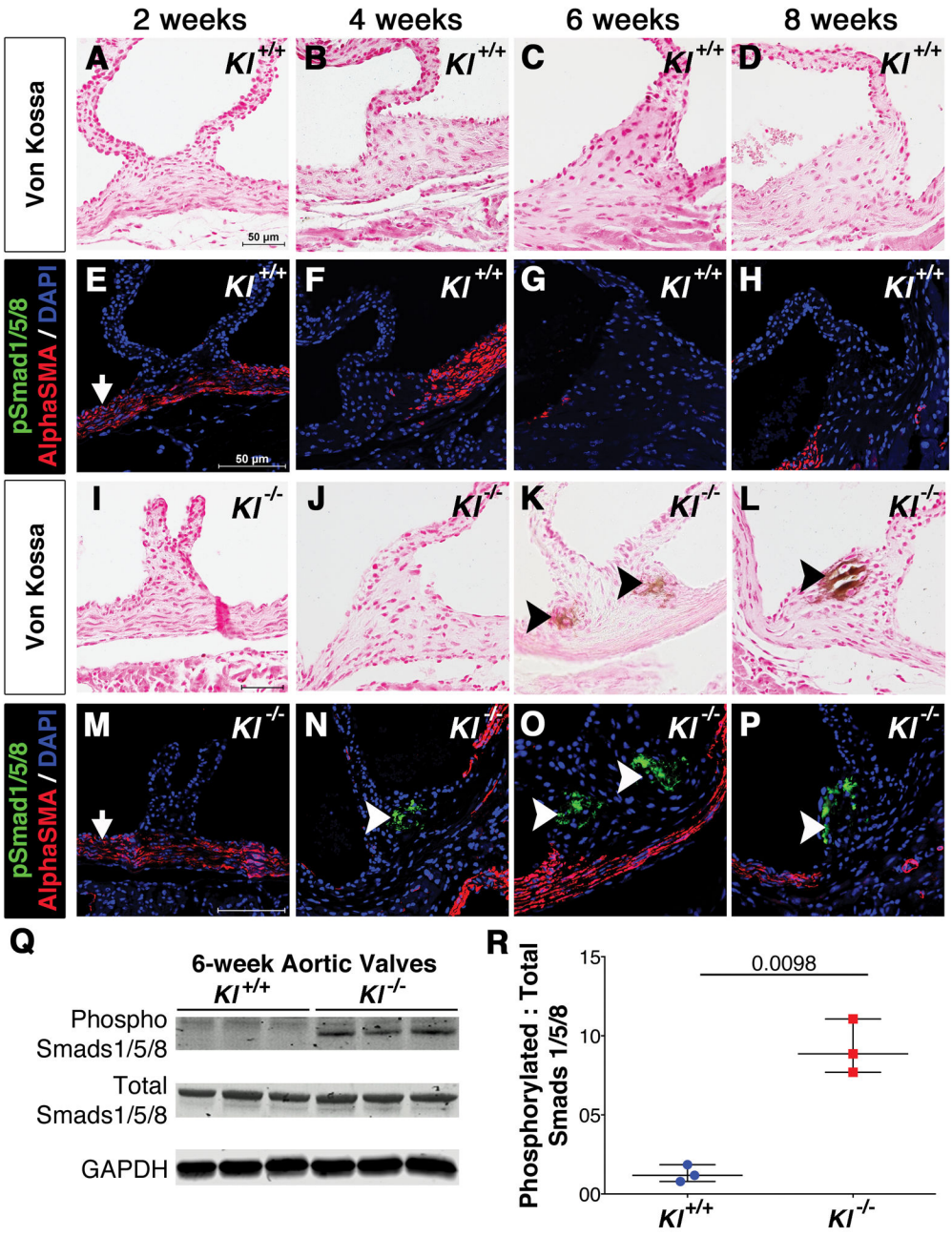

Figure 2. pSmad1/5/8 activation precedes and later localizes with calcific nodules in *Klotho*$^{-/-}$ AoV

AoV calcification in *Klotho*$^{-/-}$ mice (I–L) compared to WT littermate controls (A–D) was examined at 2, 4, 6 and 8 weeks of age. Evidence of calcification is first observed at 6 weeks and persists through 8 weeks of age, as detected by von Kossa staining (K–L, arrowheads). pSmad1/5/8 (green) activation precedes calcification at 4 weeks (N, white arrowheads), and then localizes with calcific nodules at 6–8 weeks (O–P) in *Klotho*$^{-/-}$ AoV. Smooth muscle cells of the aorta are detected by AlphaSMA staining (red) in Klotho$^{-/-}$ and control AoV (E–H, M–P). At least 3 hearts were analyzed for each phenotype and time-point. Western blot analysis of phosphoSmad1/5/8, total Smad1/5/8 and GAPDH protein levels in *Klotho*$^{-/-}$

AoV compared to WT littermate control (Q). Quantification of phosphoSmad1/5/8 levels normalized to total Smads and GAPDH (R); n=3 was used for each *Klotho*$^{-/-}$ and control group in which each n is equal to 10–12 AoV. Error bars are shown as mean ±SEM and scatter plot. Statistical significance was determined by unpaired t-test with Welch's correction ($p < 0.05$).

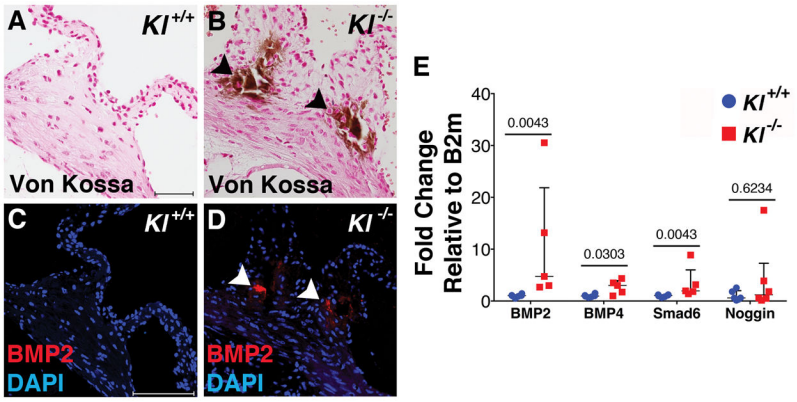

Figure 3. BMP pathway components are active in calcified *Klotho*$^{-/-}$ AoV

BMP2 ligand is expressed in calcified AoV of *Klotho*$^{-/-}$ mice (B,D) but not control AoV (A,C) at 6 weeks of age, as determined by immunofluorescence (red). BMP signaling network gene expression was evaluated by qRT-PCR of RNA isolated from *Klotho*$^{-/-}$ AoV compared to WT controls at 6 weeks of age (E). There is a significant increase in *BMP2* and *BMP4* ligand mRNA levels, as well as *Smad6* (n=5/group). Gene expression was normalized to B2m control expression. Values were normalized to wild type control averages and are represented as fold changes. Error bars are shown as interquartile means and scatter plot. Statistical significance was determined by Mann-Whitney U-test ($p < 0.05$).

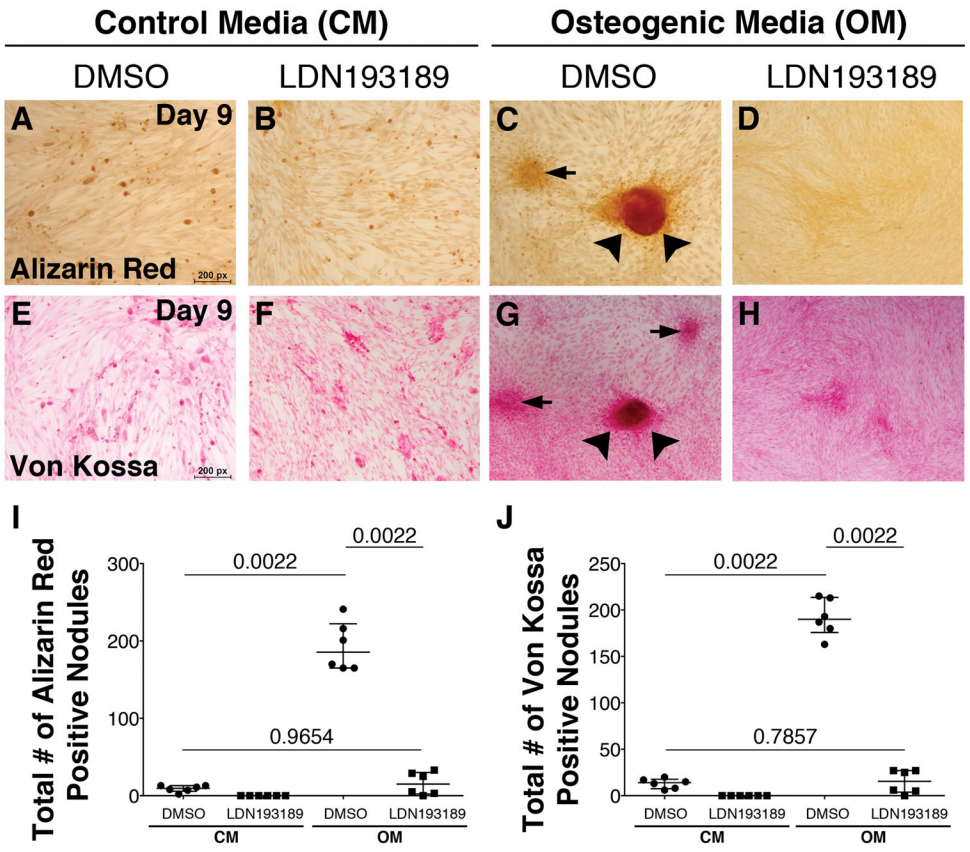

Figure 4. LDN-193189 treatment prevents formation of calcific nodules in porcine aortic VICs *in vitro*

Porcine aortic VICs were cultured in Osteogenic Media (OM) or Control Media (CM) for 9 days, ± BMP inhibitor LDN-193189. OM-induced calcific nodules can be observed by Alizarin Red (red, C) and von Kossa (brown, G) staining, and formation of these nodules is inhibited by LDN-193189 (D,H). The total number of calcific nodules for each treatment group, as detected by Alizarin Red (I) and von Kossa (J) staining, was quantified. Each dot is representative of the total number of calcific nodules quantified per well for each treatment group. Error bars are shown as interquartile means and scatter plot. Statistical significance was determined by Mann-Whitney U-test ($p < 0.05$) for comparisons indicated by lines.

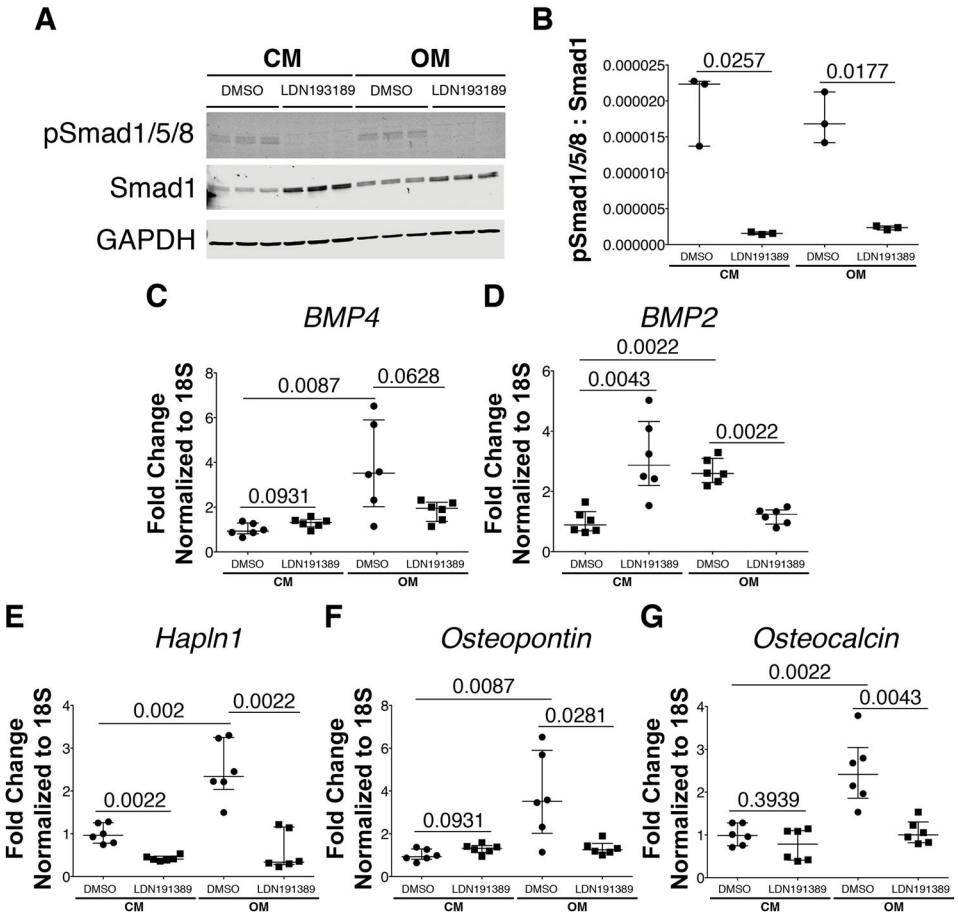

Figure 5. LDN-193189 treatment inhibits OM-induced osteochondrogenic gene induction in porcine aortic VICs *in vitro*

Western blot analysis shows pSmad1/5/8 relative to total Smad 1 and GAPDH control proteins in porcine aortic VICs cultured in Osteogenic Media (OM) or Control Media (CM), ± BMP inhibitor LDN-193189 (A,B). Gene expression profiles of BMP ligands *BMP4* (C) and *BMP2* (D), cartilage marker *Hapln1* (E) and bone markers *Osteopontin* (F) and *Osteocalcin* (G), evaluated by qRT-PCR of RNA isolated from porcine aortic VICs cultured in OM and CM for 9 days, ± BMP LDN-193189. Displayed values represent 2 independent experiments performed in triplicate (C–G). Values are representative of 5 different independent experiments. Gene expression was normalized to 18S control expression. Values were normalized to control DMSO averages and are represented as fold changes. Error bars are shown as interquartile means in scatter plots. Statistical significance was determined by Mann-Whitney U-test ($p < 0.05$).

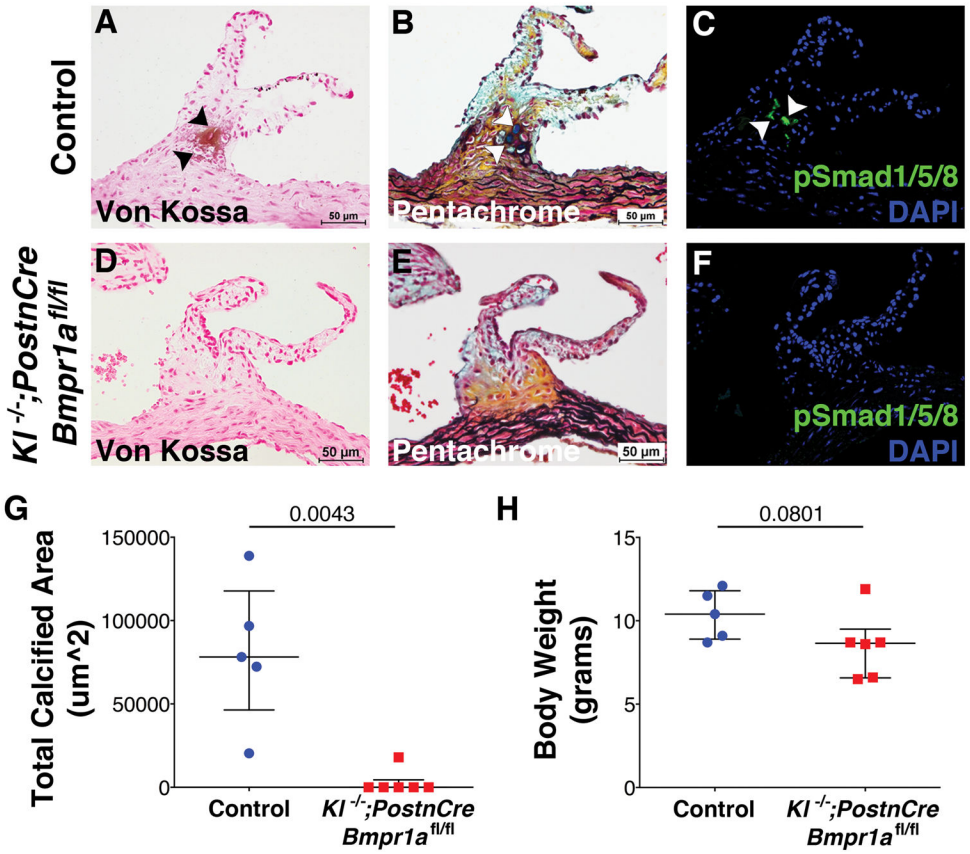

Figure 6. Genetic inactivation of the BMP receptor Bmpr1a prevents AoV calcification in *Klotho*$^{-/-}$ mice

Von Kossa staining was used for the detection of AoV calcification in *Klotho*$^{-/-}$;*PostnCre;Bmpr1a*$^{flox/flox}$ compared to control mice at 7–9 weeks of age (A,D, black arrowheads). Control mice include both *Klotho*$^{-/-}$;*PostnCre;Bmpr1a*$^{+/+}$ and *Klotho*$^{-/-}$;*Bmpr1a*$^{+/+}$, since there is no difference in the amount of AoV calcification seen in both phenotypes. Pentachrome staining illustrates ECM and cellular composition (B,E). IHC for pSmad1/5/8 (green) was used for detection of active BMP signaling (C,F). Total calcified area of the AoV was quantified from von Kossa stained histological sections of *Klotho*$^{-/-}$;*PostnCre;Bmpr1a*$^{flox/flox}$ (n=6) mice compared to control mice (n=5) (G). A significant decrease in the total calcified area in *Klotho*$^{-/-}$;*PostnCre;Bmpr1a*$^{flox/flox}$ AoV is observed when compared to calcified areas in control AoV (C, p= 0.0043). Total body weight is not significantly different between groups (H). Error bars are shown as interquartile means in scatter plots. Statistical significance was determined by Mann-Whitney U-test ($p< 0.05$).