MATERIALS AND METHODS

BMP Signaling is Required for Aortic Valve Calcification
(Gomez-Stallons et al.)

Mice

Animals were maintained in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. All animal procedures were approved by the Cincinnati Children's Medical Center Animal Care and Use Committee and performed in accordance with institutional guidelines. Both male and female mice were used for all studies, with no differences observed between sexes.

Klotho-deficient (*Klotho*$^{-/-}$) mice (B6;129S5-Kl^{tm1Lex}/Mmucd) were generated by Lexicon Genetics, and mice heterozygous for the *Klotho* mutation were obtained from the Mutant Mouse Regional Resource Centers (MMRRC) (Strain ID: 011732-UCD)[1]. *Klotho* mice were genotyped according to MMRRC's instructions[2, 3]. *Bmpr1a*^{flox} mice (Bmpr1atm2.1Bhr/Mmnc 30469-UNC) were obtained from the Mutant Mouse Regional Resource Center, a NIH funded strain repository, donated to the MMRRC by Yuji Mishina, Ph.D., NIH, National Institute of Environmental Health Science. *Bmpr1a*^{flox} mice were genotyped according to MMRRC's instructions[4]. *Periostin-Cre (PostnCre)* mice[5] were kindly provided to us by Simon Conway (Indiana University). ROSA membrane Tomato/membrane EGFP (*ROSA26*^{mTmG}) reporter mice (*Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP*)Luo/J) were obtained from the Jackson Laboratory (Stock ID: 007576) and genotyped according to Jackson Laboratory protocols[6]. *Bmpr1a*^{flox} alleles were used for targeted deletion of BMP receptor 1a (Alk3) in AoV interstitial cells after breeding with *PostnCre* mice[7]. Subsequently, *Klotho*$^{-/-}$;*PostnCre*;*Bmpr1a*$^{flox/flox}$ mice were generated to determine the requirements for BMP signaling in the development of CAVD. Cohorts include littermates from a *Klotho*$^{+/-}$*;PostnCre;Bmpr1a*$^{flox/+}$ males bred with a *Klotho*$^{+/-}$*; Bmpr1a*$^{flox/+}$ females in a mixed strain background. Mice were sacrificed between 7-9 weeks of age when calcification is evident in *Klotho*$^{-/-}$ AoV.

For lineage tracing studies, *Wnt1Cre* mice were obtained from Jackson Labs (strain Tg(Wnt1-cre)11Rth Tg(Wnt1-GAL4)11Rth/J, stock number 003829)[8]. *Tie2Cre* mice were also obtained from Jackson Labs (strain B6.Cg-Tg(Tek-cre)12Flv/J, stock number 004128)[9]. *Klotho*$^{-/-}$;*Wnt1Cre*;*ROSA26*^{mTmG}, *Klotho*$^{-/-}$;*Tie2Cre*;*ROSA26*^{mTmG}, and corresponding *Klotho*$^{+/+}$ littermate controls, were sacrificed at 6 weeks of age.

Porcine AoV Interstitial Cell (VIC) Cultures

Male and female porcine hearts were obtained from a local abattoir, and AoVs were harvested, following published protocols[10, 11]. For each replicate experiment, AoVs from 3 adult pig hearts were collected and aortic VICs were isolated for cell culture experiments, as previously described[12]. Experiments were performed in triplicate and repeated 5 times with independent samples. After VIC isolation, cells were grown at 37°C with 5% CO_2. For passages (P)0 and P1, cells were cultured in M199 media with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. All experimental replicates were performed at passage 2 (P2), where cells were plated at 100,000 cells/cm^2 on uncoated 6-well tissue culture polystyrene plates in M199 media with 2% FBS and 1% penicillin/streptomycin.

For osteogenic medium (OM) treatment, 0.2mM ascorbic acid and 10mM β-glycerophosphate were included in the culture media (2% FBS M199). For BMP pathway studies, cells were treated with 100ng/mL inhibitor LDN-193189 (Selleckchem S2618, diluted in DMSO) or 100ng/mL human BMP2 (R&D 355-BM-010), and control cells were treated with equal volumes of DMSO (0.1% DMSO)[13, 14]. Plating day was considered Day 0 and cell culture media was changed at Day 3 and Day 6. At Day 9, cells were harvested for analysis of VIC calcification, gene expression, or protein expression.

Histology & Immunohistochemistry

Murine hearts were harvested and processed for either paraffin embedding or cryo-preservation and sectioned at 7μm[12]. For paraffin embedding, hearts were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, dehydrated through a graded ethanol series, cleared in xylenes, and embedded in paraffin wax. For cryo-preservation, hearts were fixed in 4% PFA for 4 hours at room temperature, placed in 30% sucrose overnight at 4°C, and embedded in Tissue-Tek OCT compound (Sakura). Cryo-preservation was performed for mice containing the *ROSA26*^{mTmG} allele, in order to detect Cre-recombined cells by EGFP positive fluorescence, as well as non-recombined cells by RFP positive fluorescence. For analysis of calcification in porcine VICs, adherent cells were fixed in 4% PFA for 20 minutes at room temperature prior to staining.

Murine AoV tissue sections and fixed porcine VICs were stained with Alizarin Red (2% Alizarin Red S (MP Biomedicals) in water, pH 4.2) and von Kossa (Diagnostic BioSystems) for the detection of calcific nodules. Pentachrome (American MasterTech) staining of murine hearts was used to determine the extracellular matrix composition of AoVs. Images were captured using an Olympus BX51 microscope with a Nikon DS-Ri1 camera and NIS-Elements D 3.2 software. For immunohistological studies, all slides were treated with a citric acid antigen retrieval reagent (Vector Laboratories) for 5 minutes prior to antibody incubation. The primary antibodies used were: pSmads1/5/8 (Millipore AB3848, 1:200), α-Smooth Muscle Actin (Sigma A5228, 1:300), and BMP2 (Abcam ab14933, 1:100). The following secondary antibodies (Life Technologies, 1:200) were used for antibody fluorescent detection: goat anti-rabbit 488 (A11008), goat anti-rabbit 568 (A11011), goat anti-mouse 488 (A11001) and goat anti-mouse 568 (A11004). Nuclei were counterstained with To-Pro3 (Life Technologies, 1:100), or 4', 6-Diamidino-2-Phenylindole, DAPI (Life Technologies, 1:10,000). Images were captured using a Nikon A1-R confocal system with NIS-Elements D 3.2 software.

Frozen sections of whole hearts taken from *Tie2Cre;ROSA26*^{mTmG}, *Klotho*$^{-/-}$*;Tie2Cre; ROSA26*^{mTmG}, *Wnt1Cre;ROSA26*^{mTmG}, and *Klotho*$^{-/-}$*;Wnt1Cre;ROSA26*^{mTmG} mice were washed twice for 5 minutes in distilled water to remove OCT compound. Specimens were exposed to a 1% citric acid solution (H-3300, Vector Laboratories) for 45 minutes at room temperature. Following thorough washing in 1xPBS, nuclei were counterstained using DAPI (Life Technologies, 1:10,000) and images of GFP and Tomato protein fluorescence were captured using a Nikon A1-R confocal in combination with NIS-Elements D 3.2 software.

For quantitative analysis of aortic valve calcification in *Klotho;PostnCre* mice, sample sizes were n=5 for the control group and n=6 for the *Klotho*$^{-/-}$;*PostnCre*;*Bmpr1a*$^{flox/flox}$ experimental group. For von Kossa staining, 10 slides (selecting every 5th slide) from each mouse heart were analyzed, starting at the base of the aortic root through the entire aortic valve. The area of aortic valve calcification (μm^2) was quantified per slide, and the values for all 10 slides were added to obtain the total calcified area for each mouse heart.

RNA Isolation and Quantitative PCR

RNA was isolated from valve leaflets including the hinge region after micro-dissection from murine AoV roots, exclusive of aortae, of *Klotho*$^{-/-}$ and *Klotho*$^{+/+}$ controls (6 weeks of age) and used for transcript analysis. A total of 9 leaflets from 3 AoV roots were pooled for each sample (n=1). 5-7 independent samples (n=5-7) were used for each wild type control and *Klotho*$^{-/-}$ group at 6 weeks. mRNA was isolated using Trizol reagent (Invitrogen) following manufacturer's protocols. cDNA was synthesized with 400ng of RNA from each sample using Superscript First-Strand Synthesis System (Life Technologies) following manufacturer's instructions. For porcine VIC studies, RNA was isolated using Trizol and 800ng of RNA was used from each sample for cDNA synthesis, following protocols described for murine tissue.

For murine qPCR studies, Taqman Fast Universal mix (Life Technologies) was used for gene expression assays. Primer IDs for each gene analyzed are shown in *Supplementary Table 1*. *Beta-2-microglobulin* (*B2m*), found on the surface of all nucleated cells, was used as a normalization control to compare cDNA expression in Taqman assays[15, 16]. For porcine qPCR studies, Power SYBR green mix (Life Technologies) was used for gene expression analysis. Primer sequences were designed using NCBI primer BLAST for the following *Sus Scrofa* mRNA sequences provided by NCBI: *BMP2* (NM_001195399.1), *BMP4* (NM_001101031.2), and *Smad6* (XM_003480446.2). These sequences and additional primer sets used are listed in *Supplementary Table 2*. Experiments were completed using the StepOnePlus real time PCR system (Applied Biosystems) with StepOne Software version 2.2.2. For Power SYBR green reactions, gene expression was analyzed using the standard curve method; while the $\Delta\Delta$Ct method was used to calculate gene expression fold-changes analyzed with Taqman probes[17, 18].

Protein Isolation and Analysis

Total protein was isolated from micro-dissected AoV leaflets of 6-week-old *Klotho*$^{-/-}$ and wild type control mice. Leaflets from a total of 10-12 AoV roots were pooled for each sample (n=1). 3 replicate samples (n=3 containing 30-36 valves) were used for each wild type and *Klotho*$^{-/-}$ sample group. Isolated AoV samples were lysed using a mixture of Cell Lysis Reagent (Sigma), Protease Inhibitor (Thermo) and Phosphatase Inhibitor (Thermo). For cell culture studies, total protein was isolated from each culture well (n=1) of control and OM-treated aortic VICs, in the presence or absence of LDN-193189. For each experiment, 3 replicate wells were used for each treatment group, and 5 independent replicate experiments were performed. For apoptosis analysis, WT SV40 MEFS treated with 200nM Staurosporine for 8 hours[19] were used as positive controls for detection of cleaved Caspase-3 expression. After protein isolation, 30ng was used per sample for each Western blot lane[20]. For murine studies, primary antibodies used were: pSmad1/5/8 (Millipore AB3848), total Smad1/5/8 (1:500, Santa Cruz, SC-6031-R), and GAPDH (Cell Signaling 5174). For porcine studies, antibodies used were: pSmad1/5/9 (Cell Signaling 13820), Smad1 (Cell Signaling 6944), GAPDH (Cell Signaling 5174), Caspase-3 (Cell Signaling 9662), cleaved Caspase-3 (Cell Signaling 9664), and beta-Actin (Sigma A5316). For both murine and porcine studies, secondary antibodies used for fluorescent protein detection were: IRDye 800CW and IRDye 680RD (LI-COR). Blots were imaged using an Odyssey scanner with ImageStudio version 3.1.4 software (LI-COR).

VIC Calcific Nodule Quantification

After fixation in 4% PFA, cultured porcine VICs were washed in 1X PBS, followed by distilled water, and stained with Alizarin Red (2% Alizarin Red S in water, pH 4.2; Sigma-Aldrich) or von Kossa (Diagnostic BioSystems) for the detection of calcific nodules, as previously described[12, 21]. Using a Nikon SMZ1500 camera with NIS-Elements D 3.2 software, 14 images were taken per culture well and the total number of calcified nodules (positive for Alizarin Red and von Kossa) per well was counted from the images. The average number of nodules/well was obtained from biological triplicates for each treatment group and repeated for each of the 3 replicate experiments. For quantification purposes, only nodules that were fully attached to the plate surface were counted. For Alizarin Red staining, a red nodule depicted calcified nodules. For von Kossa staining, only nodules that were brown in color were counted as positive calcified nodules.

Valve Cell Lineage Analysis *in vivo*

Klotho$^{+/+}$*;Tie2Cre;ROSA26*^{mTmG} and *Klotho*$^{+/+}$*;Wnt1Cre;ROSA26*^{mTmG} animals were harvested at 6 weeks of age. Hearts were washed in ice-cold 1xPBS and aortic roots were micro-dissected away from the rest of the heart. Subsequently, the aortic roots were bisected along the proximal-distal axis, between the right coronary and left coronary AoV cusps, and

fixed in 4% PFA overnight at 4°C. Following fixation, aortic roots were washed twice in ice-cold 1xPBS for 10 minutes total. After washing, the whole aortic roots were mounted on specimen slides in 50% glycerol/50% 1xPBS. Specimens were analyzed using a Nikon A1-R confocal system with NIS-Elements D 3.2 software by capturing Z-stack images throughout the entire volume of the aortic root.

For quantification of the contribution of *Tie2Cre* and/or *Wnt1Cre* derivatives to the regions of AoV calcification in *Klotho*$^{-/-}$ mice, at least 3 cryo-sections from calcified regions of aortic valve hinge regions in at least 3 biological specimens were used. Quantification was based on the following number of specimens: *Klotho*$^{-/-}$*;Tie2Cre;ROSA26*^{mTmG} (n=3) and *Klotho*$^{-/-}$*; Wnt1Cre;ROSA26*^{mTmG} (n=5). To define the region of calcification, cryo-sections were stained with Alizarin Red as described above and brightfield images were obtained using an Olympus BX51 light microscope with NIS Elements BR 3.2 software. Brightfield images of sister-sections stained for calcification were overlaid onto the confocal images using Photoshop CS4 software and the calcified area was outlined.

Using the mTmG (RFP/GFP) lineage trace reporter, independent calculations were performed for *Tie2Cre*-expressing cells and *Wnt1Cre*-expressing cells, in order to highlight the likelihood of each lineage cell type (EGFP$^+$ cells) to localize to calcified areas in comparison to the whole hinge region of the aortic valves. The total number of EGFP$^+$ cells localized to the calcified areas for each lineage was calculated by counting EGFP$^+$ cells localized to calcified areas, compared to the total number of EGFP$^+$ cells localized to the hinge region of the AoV (representing 100% of the cells). In addition, the total percent of Tie2Cre and Wnt1Cre-positive cells contributing to calcific nodules was calculated by counting the total number of EGFP$^+$ cells found in the calcified areas, compared to the total number of nuclei (DAPI$^+$) found in the calcified region (representing 100% of the cells). For this, the total number of DAPI-stained nuclei localized to each calcific nodule was quantified for each section. Using Alizarin Red staining, the calcified area of the aortic valves was identified.

Statistical Analysis

The sample population for *in vivo* and *in vitro* RT-PCR analysis do not follow a Gaussian distribution, thus nonparametric Mann-Whitney U-tests were used. For RNA expression analysis, a non-parametric, two-tailed Mann-Whitney U- test was performed to determine statistical significance of differences observed between experimental groups. For Western analysis, an unpaired t-test with Welch's correction was used to determine statistical significance between experimental groups. For lineage tracing analysis, a non-parametric, two-tailed Mann-Whitney U-test test was performed to calculate statistical significance of observed differences between experimental groups. For all statistical tests, a p-value of 0.05 or less was considered statistically significant. All statistical tests were performed using GraphPad Prism 6.0 software.

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