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Endothelial nitric oxide signaling regulates Notch1 in aortic valve disease

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Abstract

The mature aortic valve is composed of a structured trilaminar extracellular matrix that is interspersed with aortic valve interstitial cells (AVICs) and covered by endothelium. Dysfunction of the valvular endothelium initiates calcification of neighboring AVICs leading to calcific aortic valve disease (CAVD). The molecular mechanism by which endothelial cells communicate with AVICs and cause disease is not well understood. Using a co-culture assay, we show that endothelial cells secrete a signal to inhibit calcification of AVICs. Gain or loss of nitric oxide (NO) prevents or accelerates calcification of AVICs, respectively, suggesting that the endothelial cell-derived signal is NO. Overexpression of Notch1, which is genetically linked to human CAVD, retards the calcification of AVICs that occurs with NO inhibition. In AVICs, NO regulates the expression of Hey1, a downstream target of Notch1, and alters nuclear localization of Notch1 intracellular domain. Finally, Notch1 and NOS3 (endothelial NO synthase) display an in vivo genetic interaction critical for proper valve morphogenesis and the development of aortic valve disease. Our data suggests that endothelial cell-derived NO is a regulator of Notch1 signaling in AVICs in the development of the aortic valve and adult aortic valve disease.

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5. Conflict of interest

None.

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1. Introduction

Valvular heart disease is responsible for over 20,000 deaths each year in the United States alone and the aortic valve is commonly affected [1]. Calcific aortic stenosis affects an estimated 2–3% of the population over 75 years of age and results in progressive aortic valve stenosis, which will ultimately require valve replacement [2, 3]. Along with clinical risk factors such as hypertension, hypercholesterolemia, and diabetes, the presence of bicuspid aortic valve (BAV), a congenital malformation, increases the risk of calcific aortic valve disease (CAVD) [4]. BAV has a prevalence of 1–2% in the population and is the second most common cause of aortic stenosis [5]. The molecular mechanisms that underlie CAVD are not well understood.

Calcific aortic valve disease is manifested by the identification of calcific nodules on the arterial aspect of the aortic valve, which do not allow the valve to function properly. In addition to calcification, diseased valves display a loss of organization of the normal trilaminar valve structure [6]. The normal valve ECM is composed of collagen, elastin, and glycosaminoglycans and is interspersed with valvular interstitial cells (VICs), which are proposed to mediate the process of calcification [7]. The valve ECM and VICs are surrounded by an overlying cell layer of valve endothelium. Injury, by abnormal hemodynamics or systemic disease states, to valve endothelial cells has been postulated to be the initiating factor for the development of CAVD [8, 9].

Several studies in multiple species have demonstrated a role for endothelial nitric oxide (NO) in CAVD but the mechanism by which it affects underlying VICs is unknown [10–12]. NO is generated by three synthases, endothelial nitric oxide synthase (eNOS/NOS3), inducible NOS (iNOS/NOS2) and neuronal NOS (nNOS/NOS1). Targeted deletion of *NOS3* in mice has not been reported to result in CAVD, but interestingly is associated with BAV. The molecular mechanisms underlying this link are not known [13].

The Notch signaling pathway has been linked to aortic valve disease in humans [14]. The Notch family contains four transmembrane receptors, Notch 1–4, and functions in a broad spectrum of cell fate decisions, developmental processes and vascular disease states [15–17]. Activation of Notch receptors by the Jagged and Delta-like ligands results in a series of proteolytic cleavages that generate the Notch intracellular domain (NICD), which translocates to the nucleus and functions as a transcriptional co-activator and initiates the expression of target genes, including the *HEY* (Hes-related with YPRW motif) family of transcriptional repressors [15]. Using a traditional genetic linkage approach, heterozygous loss of function mutations in *NOTCH1* were found to be associated with autosomal-dominant BAV and CAVD [14]. In addition, *Notch1* haploinsufficient mice have been shown to develop aortic valve calcification [18, 19]. Using an established aortic valve interstitial cell (AVIC) culture system that spontaneously calcifies *in vitro*, it has been shown that inhibition of Notch signaling in AVICs accelerates the calcification process, potentially by regulating Bmp2 signaling and Sox9 [18–20]. However, the mechanisms by which Notch1 signaling is regulated in the process of CAVD has not been investigated.

Here, we show that a secreted signal from endothelial cells inhibits calcification of porcine AVICs using co-culture and transwell assays. We further demonstrate that nitric oxide (NO), which is secreted by endothelial cells, prevents calcification of AVICs similar to the presence of endothelial cells, while the absence of NO increases calcification. To demonstrate that endothelial cell-derived NO is signaling to Notch1 in AVICs, we show that overexpression of a constitutively active Notch1 in AVICs prevents calcification that occurs with NO inhibition and that endothelial-derived NO signaling increases the expression of a Notch signaling target gene in AVICs. We identify a novel molecular mechanism by which endothelial cell-derived NO is communicating to neighboring cells by altering the nuclear localization of NICD in AVICs. Lastly, we show that the NO and Notch1 signaling pathways genetically interact in vivo as *NOS3;Notch1* compound mutant mice display a highly penetrant model of aortic valve disease, characterized by abnormal gene expression and valve dysfunction. These data demonstrate that NO signaling in valve endothelial cells affects Notch1 signaling pathways in AVICs and may be critical in the pathogenesis of adult aortic valve disease.

2. METHODS

2.1. Cell culture

Porcine aortic valve interstitial cells (PAVICs) were collected from juvenile pig valve cusps and isolated as previously described [20, 21]. Briefly, valve leaflets were subjected to collagenase digestion at 37° C and the endothelial cell layer gently scraped. Leaflets were cut into 1–2mm² pieces and cultured in Medium-199 (Invitrogen) supplemented with 10% FBS, L-glutamine, penicillin/streptomycin, gentamycin and amphotericin B. At confluency, cells were passaged with 0.25% trypsin-EDTA. PAVICs used for this study were between passage 3 and 7. Primary cell cultures of human umbilical vein endothelial cells (HUVECs) were cultured using EBM-2 media supplemented with the full bullet kit as directed (Lonza). HepG2 cells were obtained from ATCC and maintained in DMEM. Mouse lung endothelial cells were isolated as previously described with modifications [22]. Briefly, neonatal mouse lungs were perfused with Collagenase A (Sigma C9722) and removed after euthanization. Lungs were digested at 37° C for 30 minutes and filtered through a 70 µm cell strainer. Cell isolate was pelleted and washed before being plated on 1% gelatin-coated flasks. Cells were grown in endothelial cell-specific culture media with 0.1% heparin to inhibit growth of other cell types. All experimental studies were carried out in EBM-2 media with 0.5% FBS and 30 ng/mL VEGF-A(165) (R&D Systems). Cells were seeded on glass coverslips in 24-well plates at 40,000 cells/well, and culture media was changed every 24–48 hours. Transwell cultures were performed using 0.4µm polyester Transwell inserts (Corning) with cells being seeded at 20,000 cells/insert. Nitric oxide inhibition was performed using L-NAME (Sigma) at 2mM, DETA-NONOate (Sigma) was used as a NO donor at 20 µM, each refreshed daily. Notch inhibition was performed using DAPT (Sigma) at 10 µM. DAR-4M (Calbiochem 251765) staining was performed by washing cells with PBS and incubating for 30 minutes in 10 µM DAR-4M in phenol red-free DMEM. Cells were washed again with PBS and imaged at 580nm. Overexpression of NICD was accomplished by transfecting PAVICs using Amaxa Nucleofector system for mouse embryonic fibroblast (Lonza) with 10–100ng

constitutively active mouse NICD expression plasmid, [23] HA-tagged rat NICD plasmid (pEF1-HA-NICD1) [24] or GFP vector (pEGFP-N1; Clontech Laboratories, Cat. # 6085-1).

2.2. Calcium quantification

PAVICs were collected on coverslips by washing in cold PBS and fixing with 4% PFA for 15 minutes. Von Kossa and Alizarin red staining was performed as previously described [20, 25]. Briefly, for von Kossa staining cells were washed with distilled water and treated with 5% aqueous AgNO_3 in light at room temperature for 60 minutes. Cells were then treated with 2.5% sodium thiosulfate for 5 minutes, black representing positive staining. Alizarin red staining was performed by washing cells in distilled water and treating with 2% Alizarin red S for 5 minutes. Images were taken using a Zeiss AxioCam MRc and stereomicroscope in brightfield, quantification of positive staining was performed using ImageJ (NIH) analysis software. Calcium nodule counts were performed 5–7 days after cell seeding and were averaged as total nodule number/well.

2.3. Protein Expression

Western blotting of PAVICs was accomplished by rinsing cells with cold PBS, then adding Laemmli sample buffer (Bio-Rad) to cell culture wells. Cells were removed with a plastic cell scraper and boiled for 10 minutes before being loaded onto an acrylamide gel. Proteins were blotted on PVDF membranes and blocked with 5% BSA in TBS containing 0.5% Tween-20 (Sigma). Blots were probed using antibodies against Hey1 (Millipore AB-5714, 1:1000) and Actin (Abcam Ab1801, 1:5000) and visualized using HRP-conjugated anti-rabbit secondary antibodies (Santa Cruz Biotechnology sc-2313 1:5000) and Pierce ECL Western Blotting Substrate (Thermo). For nuclear fractionation, the NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo) was utilized. Western blotting of cell fractions was performed as described above using antibodies against NICD (Abcam ab8925 1:2000), Lamin A/C (Santa Cruz Biotechnology sc-7292 1:40,000) and GAPDH (Novus Bio NB300-221, 1:1000). Densitometry analysis was performed using ImageJ software. For immunofluorescence, cells were fixed in 4% PFA for 15 minutes and washed with PBS. Blocking was performed using PBS with 0.3% Trion X-100 (Arcos Organics) and 5% goat serum (Vector Labs). Treatment with primary antibody against NICD (Abcam ab8925 1:400), HA tag (Abcam ab9134 1:500) or pSMAD-1/5/8 (Millipore AB3848) was performed using PBS with 0.3% Trion X-100 and 5% goat serum at 4°C overnight. Visualization was accomplished using Texas Red-conjugated (Vector Labs) or Alexa Fluor 488 (Invitrogen A11008) anti-rabbit antibodies at 1:200 for 60 minutes in PBS with 0.3% Triton X-100 and 5% goat serum.

2.4. Breeding and collection of mouse embryos

Research was approved by the Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children's Hospital. Genotyping of *NOS3*^{-/-} and *Notch1*^{+/-} mice was performed as previously described [26, 27]. Compound mutant mice were obtained by breeding *NOS3*^{+/-} and *NOS3*^{-/-} mice to *NOS3*^{+/-};*Notch1*^{+/-} animals.

2.5. Valve morphology and histology

For gross valve morphology, hearts of 6–8 week old mice were fixed in 10% Formalin (Fisher) at 4°C overnight. The aortic valve was dissected out and categorized in a blinded fashion. Images were taken using a Zeiss AxioCam MRc and stereomicroscope. For valve histology, *NOS3*^{-/-}; *Notch1*^{+/-}, *Notch1*^{+/-}, *NOS3*^{-/-} and wild type mice were placed on high fat diet (Teklad TD.88137) at 6–8 weeks of age and sacrificed after 12 weeks. Hearts were removed and fixed, and the aortic valves were dissected out and embedded in paraffin before sectioning. Alcian blue counterstaining was performed as previously described using 1% Alcian blue in 20% acetic acid [28]. Immunohistochemistry was performed using Immunocruz ABC Staining System with primary antibodies against Sox9 (Abcam ab26414) or alpha-smooth muscle actin (Sigma A5228).

2.6. Echocardiographic Imaging

Transthoracic echocardiographic studies were performed on *NOS3*^{-/-}; *Notch1*^{+/-}, *Notch1*^{+/-} and *NOS3*^{-/-} mice anesthetized with isoflurane (2% in 100% oxygen at a flow rate of 1.5 L/min) at 18–20 weeks of age following 12 weeks of high fat diet (*NOS3*^{-/-}; *Notch1*^{+/-} n=4, *Notch1*^{+/-} n=2, *NOS3*^{-/-} n=3, wild type n=2). Studies were carried out using a Vevo 2100 device equipped with 18–38 MHz linear-array transducer with a digital ultrasound system (Visualsonics Inc., Canada). Body temperature was monitored throughout the procedure using a rectal probe thermometer. Pulsed wave Doppler interrogation across the aortic valve was used in parasternal long axis view to obtain aortic transvalvular maximal velocity. The average of three consecutive cardiac cycles was used for each measurement. All echocardiographic imaging and measurements were performed by an experienced investigator blinded to the study design.

2.7. Statistics

Statistical analysis were performed using Student's t-test or one-way ANOVA with a P value <0.05 being considered significant.

3. RESULTS

3.1. Secreted endothelial cell signal inhibits calcification of AVICs

We and others have investigated signaling pathways in AVICs which accelerate calcification, but the mechanisms by which endothelial cells communicate to AVICs has not been investigated.[2, 20] Using a physical co-culture assay, we asked if endothelial cells are signaling to porcine AVICs in the onset and progression of calcification. Co-culturing porcine AVICs with human umbilical vein endothelial cells (HUVECs) resulted in decreased formation of calcific nodules when compared to porcine AVICs alone (Fig. 1A,B,G). The decreased calcification observed with HUVEC co-culturing was quantified by staining with Alizarin red, which marks calcium, and von Kossa, a marker of mineralization (Fig. 1C–H). This effect was specific to HUVECs as co-culturing with HepG2 cells, a human liver carcinoma cell line, did not significantly decrease calcification (Supp. Fig. 1). In order to determine if the anti-calcific effect of endothelial cells was due to direct physical contact, we performed transwell studies. In the transwell system, HUVECs maintained the ability to reduce the calcification of porcine AVICs as evidenced by a decreased formation

of calcific nodules, von Kossa, Alizarin red and phospho-Smad 1/5/8 staining (Fig. 1I–P). These studies indicated that a secreted factor from endothelial cells attenuates the calcification of porcine AVICs.

3.2. Nitric oxide signaling affects AVIC calcification

Endothelial NO signaling has been linked to CAVD, and we asked if NO signaling affected calcification in our porcine AVIC culture system. Consistent with previous work, the addition of a non-specific NO donor (DETA NONOate) prevented the spontaneous calcification of porcine AVICs (Supp. Fig. 2 B,D,E), whereas the addition of L-NAME, an inhibitor of nitric oxide synthase (NOS), resulted in increased levels of calcification (Supp. Fig. 2C–E). [10, 11] To determine if endothelial cell-derived NO, specifically, could be involved, we co-cultured porcine AVICs in a transwell system with primary mouse lung endothelial cells (MLECs) derived from either wild type or *NOS3*^{−/−} mice. While wild type MLECs were able to attenuate calcification similarly to the effect seen with HUVECs, *NOS3*^{−/−} MLECs were unable prevent calcification of porcine AVICs as measured by calcific nodules and calcium staining (Fig. 2). This effect was accompanied by decreased NO levels (Supp. Fig. 3) and decreased Hey1 levels in cell co-cultured with *NOS3*^{−/−} MLECs (Supp. Fig. 4A). These data suggested that endothelial-derived NO is mediating the process of calcification in porcine AVICs.

3.3. Endothelial nitric oxide signaling is a regulator of Notch1 signaling in AVICs

We previously published that inhibition of Notch signaling in AVICs accelerates the development of calcification and inducible NO has been shown to regulate Notch signaling in cholangiocytes [20, 29]. We hypothesized that the effects of endothelial-derived NO in AVICs are mediated by Notch1 signaling. Therefore, we asked if overexpression of the constitutively active form of Notch1 (NICD) prevents the AVIC calcification observed with NOS inhibition. Porcine AVICs transfected with NICD displayed decreased calcification as compared to those transfected with GFP control plasmid, while as expected, treatment with L-NAME resulted in an increased amount of calcification (Fig. 3A–C). Overexpression of NICD in the setting of L-NAME significantly decreased nodule formation and calcification of AVICs to levels similar to NICD alone and GFP control (Fig. 3D–F). Consistent with a model of Notch1 signaling regulation by endothelial cell-derived NO, increased expression of the Notch1 downstream target, Hey1, was noted in porcine AVICs when cultured with HUVECs or treated with NO donor (Fig. 3G–H). Increased Hey1 expression was also observed in cells overexpressing NICD (Supp. Fig. 4B). Moreover, we were not able to rescue the increased calcification that occurs with Notch inhibition with the addition of NO donor (Supp. Fig. 5).

To determine how NO is regulating Notch1 signaling in porcine AVICs, we examined the expression and localization of Notch1 in porcine AVICs that were exposed to gain and loss of NO. Nuclear fractionation of porcine AVICs treated with NO donor and L-NAME was performed. NICD protein levels were increased in the nuclear fraction of AVICs treated with NO donor while L-NAME decreased nuclear expression of NICD as determined by immunoblotting (Fig. 3I–L). This change in nuclear localization of NICD was also noted by immunofluorescence in primary porcine AVICs with gain and loss of NO (Supp. Fig. 6). A

similar effect on nuclear localization was noted with NO donor and L-NAME when a transfected HA-tagged NICD was utilized (Supp. Fig. 7). Examination of whole cell lysate did not appear to suggest that changes in NO affected the total levels of NICD (Fig. 3I and J). These studies demonstrate a molecular pathway by which endothelial cell-derived NOS modulates Notch1 signaling in AVICs by regulating nuclear localization of the active form of Notch1.

3.4. Genetic Interaction of NOS3 and Notch1

To determine if Notch1 and endothelial cell-derived NO signaling pathways exhibited an in vivo genetic interaction in the process of aortic valve disease, we generated compound mutant mice that were null for endothelial nitric oxide synthase (*NOS3*^{-/-}) and heterozygous for Notch1 (*NOS3*^{-/-}; *Notch1*^{+/-}). Mice which are homozygous for a null mutation in endothelial NOS (*NOS3*^{-/-}) are viable and display a partially penetrant phenotype of BAV [13]. *Notch1*-null mice are embryonic lethal secondary to vascular defects and *Notch1* heterozygote mice display mild levels of aortic valve calcification [18, 19, 30]. We bred *NOS3*^{+/-}; *Notch1*^{+/-} mice with *NOS3*^{+/-} or *NOS3*^{-/-} mice and found that *NOS3*^{-/-}; *Notch1*^{+/-} mice suffered an approximate 50% embryonic or early neonatal lethality by post-natal day 10 secondary to a spectrum of cardiac malformations (manuscript in preparation). We examined the aortic valves of surviving 8–16 week old *NOS3*^{-/-}; *Notch1*^{+/-} animals. Careful inspection of the aortic valve demonstrated severely malformed aortic valves in 10 out of 11 *NOS3*^{-/-}; *Notch1*^{+/-} mice examined (Fig. 4A,H,I). Mice null for only *NOS3* displayed an incidence of BAV in 27% (8/30), consistent with previous reports (Fig 4. A,F,G) [13]. The *Notch1*^{+/-} mice had a low incidence of BAV (8%=1/12) while the aortic valve was normal in wild type mice (Fig. 4A–E). We next asked if these pathways were important for aortic valve disease in the setting of a three leaflet aortic valve and placed *NOS3*^{-/-}; *Notch1*^{+/-}, *Notch1*^{+/-} and *NOS3*^{-/-} mutant mice on a Western diet for twelve weeks. Echocardiographic examination of these adult mice demonstrated aortic stenosis in 3 out of 3 *NOS3*^{-/-}; *Notch1*^{+/-} mice while *NOS3*^{-/-}, *Notch1*^{+/-} and wild type mice had no evidence of aortic stenosis (Fig. 5A–D). Four out of 4 *NOS3*^{-/-}; *Notch1*^{+/-} mice had aortic insufficiency while this was not observed in *NOS3*^{-/-}, *Notch1*^{+/-} and wild type animals. Gross and histologic examination of these valves demonstrated thickened aortic cusps in *NOS3*^{-/-}; *Notch1*^{+/-} mice when compared to *NOS3*^{-/-}, *Notch1*^{+/-} and wild type mice (Fig. 5E–H). In addition, the *NOS3*^{-/-}; *Notch1*^{+/-} mice developed aortic valve disease as indicated by increased Alcian blue staining and alpha-smooth muscle actin (SMA) expression (Fig. 5I–P). While no change in the expression of Hey1 was noted in *NOS3*^{-/-}; *Notch1*^{+/-} valves, the expression of Sox9, a downstream target of Notch1 linked to CAVD, was decreased (Fig. 5Q–T; data not shown) [28]. These in vivo studies demonstrated a genetic interaction between endothelial NOS and Notch1 that is critical for the development of aortic valve disease.

4. DISCUSSION

Endothelial cell injury has been proposed to be the initiating factor for CAVD, and in this study we have defined a molecular pathway linking endothelial cells to molecular changes in AVICs that lead to the development of calcification and aortic valve malformations. We

show that endothelial cell secreted NO affects the calcification process using a well-established porcine AVIC culture system. Our data uncover a novel mechanism by which NO modulates Notch1 signaling in neighboring AVICs by altering the nuclear localization of NICD. In addition, we describe a previously unrecognized in vivo genetic interaction between *NOS3* and *Notch1*, demonstrating compound mutant mice (*NOS3*^{-/-};*Notch1*^{+/-}) develop aortic valve disease. The findings demonstrate a novel molecular mechanism for aortic valve disease that links the endothelium to the underlying interstitial cells.

CAVD has similar risk factors to coronary atherosclerosis and many of these are associated with endothelial cell dysfunction [31]. Consistent with this, evidence of oxidative stress as measured by superoxide was demonstrated in a hypercholesterolemic mouse model of CAVD [32]. To our knowledge, this study provides the first molecular evidence linking endothelial cell dysfunction and its resultant abnormal signaling to alter gene expression in underlying AVICs. We demonstrate that NO is a molecular signal that mediates this crosstalk between the endothelium and interstitial layers of the aortic valve. Other signaling pathways also implicated in valve calcification include Wnt/Lrp5 and Bmp2 however further investigation is required to determine if endothelial NO intersects with these other molecular pathways in AVICs [33–35].

In the course of this work, we have uncovered a novel mechanism by which NO signaling modulates gene expression in neighboring cells. We found that endothelial cell-produced NO signaling effects the expression of Hey1, a downstream mediator of Notch1 signaling. We show that this effect is associated with altered nuclear localization of NICD. A similar finding has been reported with inducible NO in cholangiocytes, where expression of inducible NOS induces nuclear translocation of NICD suggesting that this may be a more general mechanism by which NO regulates gene expression [29]. It is known that NO regulates downstream signaling pathways by two potential mechanisms, either by the canonical pathway involving cGMP/PKG or by S-nitrosylation. A recent publication demonstrates that NO regulates the nuclear localization of the transcription factor Nrf2 by S-nitrosylation of Keap1, which normally functions to sequester Nrf2 in the cytoplasm [36]. Additional research is required to determine if NO utilizes a similar strategy to modify NICD localization in the aortic valve. Interestingly, the interaction between these two signaling pathways may be more complex as Notch has been shown to directly regulate the expression of soluble guanyl cyclases, which act as NO receptors, suggesting both pathways may have the ability to regulate each other [37]. Our observations are similar to the report of *NOS3* functioning as a genetic modifier of *Tbx5* in atrial septal formation [38]. Furthermore, while our data connect NO to Notch signaling, it does not preclude the possibility of alternative Notch1 activation or attenuation in parallel to our system.

The first evidence linking *NOTCH1* mutations to aortic valve disease was described in families with autosomal dominant form of BAV and CAVD [14]. Interestingly, a BAV was not found in all family members with CAVD as some family members had CAVD in the setting of a three leaflet aortic valve. This initial observation suggested that Notch1 may function in both adult aortic valve disease and morphogenesis [39]. While multiple studies have provided evidence for a role of Notch1 signaling in adult aortic valve disease, here we have demonstrated a molecular link to *NOS3* in the overlying endothelium. This observation

is also supported by the finding that patients having a BAV display decreased levels of NOS3 in the aortic endothelium [40]. Furthermore, our data suggest that alteration of Notch signaling in the AVICs, not the endothelial cells, leads to calcification. Additional investigation is required to determine if the Notch signaling pathway is perturbed in other models of aortic valve disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We examine the role of endothelial cell signaling in aortic valve disease
- Endothelial cells are able to prevent calcification of porcine aortic valve interstitial cells
- Nitric oxide modulates Notch1 activity in porcine AVICs to affect calcification
- NOS3 and Notch1 genetically interact *in vivo* as compound mutant mice display aortic valve disease

Figure 1.

Endothelial cells inhibit calcification of porcine aortic valve interstitial cells (AVICs) via a secreted signal. Porcine AVICs spontaneously calcify in culture (A,C,E,G,H), however co-culturing porcine AVICs with HUVECs reduced calcification as measured by the lack of nodule formation (B,G) and significant decrease in Alizarin Red (D,H) and Von Kossa (F) staining. Inhibitory effects on calcification by HUVECs were demonstrated using a transwell co-culture system (I–P). The addition of HUVECs decreased nodule formation as well as Alizarin Red (J,O,P), von Kossa (K,L) and phospho-Smad1/5/8 (M,N) as compared to porcine AVICs alone (I,K,M,O,P), suggesting the effect is caused by a secreted signal and is contact independent. Alizarin Red, reddish brown; Von Kossa, brown; phospho-Smad, bright green; DAPI, blue. PAVIC, porcine AVIC; HUVEC, human umbilical vein endothelial cell; * p value < 0.05; scale bar 200 μ m.

Figure 2.

Nitric oxide signaling affects calcification of porcine aortic valve interstitial cells (AVICs). Porcine AVICs cultured in transwell with wild type mouse lung endothelial cells (MLEC) displayed decreased calcification as determined by nodule counts (D), phospho-Smad1/5/8 (A–C, green) and Alizarin red (E–H, red) staining as compared to porcine AVICs alone (A,E). Transwell culture with *NOS3*^{-/-} MLECS did not attenuate calcification of AVICs (B,F,D,H). Alizarin Red, reddish brown; phospho-Smad, bright green; DAPI, blue. PAVIC, porcine AVIC; EC, endothelial cell; * p value < 0.05; scale bar 200 μ m.

Figure 3.

Nitric oxide regulates Notch1 signaling in porcine AVICs. Porcine AVICs transfected with NICD alone demonstrate an inhibition of calcification as shown by a decrease in nodule formation (C,E) and Alizarin red staining (C,F) compared to control GFP-transfected porcine AVICs (A). L-NAME treatment (2 mM) of GFP-transfected porcine AVICs results in increased calcification (B), that is inhibited by the transfection of NICD (D) as shown by a decrease in nodule formation (E) and Alizarin red staining (F). (A–D) are stained with Alizarin Red. (G,H) Co-culturing of porcine AVICs with HUVECs or adding NO donor (20 μ M DETA-NONOate) results in increased Hey1 protein expression by immunoblot and densitometry. Actin expression is shown as loading control. (I–L) Cell fractionation experiments of treated PAVICs demonstrate increased nuclear expression of NICD with NO donor (I,J) and decreased nuclear expression of NICD with L-NAME (K,L) by immunoblotting and densitometry analysis. Lamin A/C and GAPDH are shown as loading controls. The graphs in J and L are representative of 3 independent experiments in which NICD expression was normalized to Lamin A/C. PAVIC, porcine AVIC; HUVEC, human umbilical vein endothelial cell; * p value <0.05; scale bar 200 μ m.

Figure 4. Abnormal aortic valve morphology in *NOS3*^{-/-};*Notch1*^{+/-} animals. (A) Table summarizing aortic valve morphology in different genotypes. (B–I) Representative images of aortic valves of 8–16 week old wild type (B,C), *Notch1*^{+/-} (D,E), *NOS3*^{-/-} (F,G) and *NOS3*^{-/-};*Notch1*^{+/-} (H,I) animals. AoV, aortic valve; BAV, bicuspid aortic valve; scale bar 200 μm.

Figure 5.

Aortic valve disease in adult *NOS3*^{-/-};*Notch1*^{+/-} mice. Echocardiographic evaluation displayed aortic valve stenosis in *NOS3*^{-/-};*Notch1*^{+/-} animals (D) compared to normal flow in wild type, *NOS3*^{-/-} and *Notch1*^{+/-} mice (A,B,C). Histological examination demonstrated thickened valves of compound mutants (H) compared to wild type, *NOS3*^{-/-} and *Notch1*^{+/-} mice (E, F, G). *NOS3*^{-/-};*Notch1*^{+/-} animals display increased Alcian blue staining (I–L), SMA expression (M–P) and decreased Sox9 expression (Q–T) compared to *NOS3*^{-/-} and *Notch1*^{+/-} mice. (wild type, n=2; *NOS3*^{-/-}, n=3; *Notch1*^{+/-}, n=2; *NOS3*^{-/-};*Notch1*^{+/-}, n=4) WT, wild type; scale bar 200 μm.