Valve Endothelial Cell–Derived Tgfβ1 Signaling Promotes Nuclear Localization of Sox9 in Interstitial Cells Associated With Attenuated Calcification

Danielle J. Huk, Blair F. Austin, Tori E. Horne, Robert B. Hinton, William C. Ray, Donald D. Heistad, Joy Lincoln

Objective—Aortic valve disease, including calcification, affects >2% of the human population and is caused by complex interactions between multiple risk factors, including genetic mutations, the environment, and biomechanics. At present, there are no effective treatments other than surgery, and this is because of the limited understanding of the mechanisms that underlie the condition. Previous work has shown that valve interstitial cells within the aortic valve cusps differentiate toward an osteoblast-like cell and deposit bone-like matrix that leads to leaflet stiffening and calcific aortic valve stenosis. However, the mechanisms that promote pathological phenotypes in valve interstitial cells are unknown.

Approach and Results—Using a combination of in vitro and in vivo tools with mouse, porcine, and human tissue, we show that in valve interstitial cells, reduced Sox9 expression and nuclear localization precedes the onset of calcification. In vitro, Sox9 nuclear export and calcific nodule formation is prevented by valve endothelial cells. However, in vivo, loss of Tgfβ1 in the endothelium leads to reduced Sox9 expression and calcific aortic valve disease.

Conclusions—Together, these findings suggest that reduced nuclear localization of Sox9 in valve interstitial cells is an early indicator of calcification, and therefore, pharmacological targeting to prevent nuclear export could serve as a novel therapeutic tool in the prevention of calcification and stenosis. (Arterioscler Thromb Vasc Biol. 2016;36:328-338. DOI: 10.1161/ATVBAHA.115.306091.)

Key Words: animal model cardiovascular disease ■ aortic valve ■ endothelial cell ■ heart valve ■ signaling pathways

Calcific aortic valve disease (CAVD) is the most prevalent valvular disorder accounting for ≈55,000 hospitalizations and 15,000 deaths annually in the United States. To date, no preventative medical therapies exist, and valve replacement surgery remains the only effective treatment for this disease. Despite the high morbidity and mortality rates, the mechanisms underlying CAVD remain largely unknown.

The normal aortic valve (AoV) is composed of 3 cusps, and function is largely achieved by a highly organized connective tissue consisting of 3 layers of diversified extracellular matrix (ECM) and 2 major cell populations. The ECM provides all the necessary biomechanical properties for coaptation during the cardiac cycle and predominantly consists of collagens, proteoglycans, and elastin arranged relative to blood flow. The stratified ECM is established and maintained by valve interstitial cells (VICs) that reside within the core of mature cusps as quiescent fibroblast-like cells in the absence of disease. The valve cusps are encapsulated by a single layer of valve endothelial cells (VECs) that serve as a physical barrier between VICs and the hemodynamic environment. In addition, findings from in vitro studies suggest that VECs may influence VIC behavior and ECM production. Together, the extracellular and cellular components of the valve create an integrated and balanced connective tissue to maintain heart valve structure and function throughout life.

CAVD is a progressive disorder characterized by alterations in connective tissue homeostasis that result in valve stiffening and incomplete opening. Although the precise pathogenesis of CAVD remains unknown, mutations in NOTCH1 have been associated with AoV disease in humans, and a variety of nongenetic risk factors, including diabetes mellitus, aging, hypertension, hypercholesterolemia, and...
smoking, have been identified.6 CAVD pathogenesis is complex, and although once thought of as a degenerative disorder, it is now considered an active process whereby quiescent VICs undergo phenotypic changes and ectopically express osteogenic markers, including Runx2, Osteocalcin, and Spp1, that facilitate deposition of mineralized ECM and formation of calcific nodules.3,8,9 These cellular and extracellular changes alter valve biomechanics and lead to a less flexible and more stiffened cusp that progressively results in stenosis and impaired blood flow. Although the contribution of VICs in the formation of calcific nodules has been well studied in CAVD, little is known about how this process is initiated.

We previously showed that the transcription factor Sox9 is highly expressed in VICs and plays a causative role in the onset of AoV disease.10,11 Sox9fl/+;Col2a1-cre mice develop early onset calcification phenotypes with an associated down-regulation of healthy cartilaginous ECM proteins.10,11 These phenotypes are consistent with the diverse roles of Sox9 in positively regulating chondrogenic target genes (type II collagen, aggrecan, cartilage link protein) and repressing osteogenic markers (RUNX2, Spp1) in the developing skeletal system.5,12–15 Although our previous studies identified a causative role for reduced Sox9 function in CAVD in mice, the mechanisms of its regulation have yet to be determined. The valve endothelium has been shown to regulate VIC phenotypes in vitro,5,19–21 and in human patients, endothelial cell dysfunction accelerates the onset and progression of many cardiovascular diseases.22–25 In this current study, we examine whether the nuclear export signal of Sox9 is required for this process, pA VICs were treated with 5 ng/mL of the nuclear export signal inhibitor Leptomycin B for 7 days. As shown, the progressive loss of Sox9 nuclear localization in cultured pA VICs began as early as 24 hours after culture (Figure 1G), suggesting that this precedes calcific nodule formation detected at day 7. To determine whether the nuclear export signal of Sox9 is required for this process, pAVICs were treated with 5 ng/mL of the nuclear export signal inhibitor Leptomycin B for 7 days. As shown, treatment retained Sox9 in the nucleus (Figure IJ versus 1H) and significantly attenuated Alizarin Red staining (Figure 1I). These studies suggest that Sox9 nuclear localization is reduced in VICs before the onset of calcification in vitro, and this process is dependent on the nuclear export signal.

**Results**

**Sox9 Nuclear Localization Is Reduced in VICs and Precedes Calcification In Vitro**

Reduced Sox9 function in vivo promotes calcific AoV phenotypes, suggesting a causative role.10,11 To examine the regulatory mechanisms of Sox9 in the onset of calcification, we used an in vitro pAVIC calcification system. After 7 days of culture on glass, pAVICs formed calcific nodules as detected by Alizarin Red staining (Figure 1A–1C). This was associated with reduced Sox9 expression and nuclear localization (Figure 1D–1E) and led to decreased Col2a1 (chondrogenic) and increased Runx2 (osteogenic; Figure 1F). To determine whether these changes were associated with apoptosis, Cleaved Caspase-3 expression was examined by Western blot, but insignificant differences were observed between days 1 and 7 (Figure IIA in the online-only Data Supplement). However, it should be noted that Western blot analysis identifies Cleaved Caspase-3 expression in the entire cell population, and therefore, associations between Alizarin Red reactivity and apoptosis cannot be made at the single cell level, but should be considered for future work. The progressive loss of Sox9 nuclear localization in cultured pAVICs began as early as 24 hours after culture (Figure 1G), suggesting that this precedes calcific nodule formation detected at day 7.
during the neonatal period because of respiratory distress, and echocardiograms are not routinely performed. Therefore, causative links of SOX9 misexpression to human AoV disease has been challenging. Here, we examined correlations between Sox9 expression in valve tissue excised from humans (∼70 years) undergoing surgical AoV replacement as a result of end-stage calcification and stenosis. As shown in Figure 2B, SOX9 was significantly reduced in VICs located close to the calcific region (Figure 2B, *) compared with age-matched nondiseased controls (Figure 2A, arrows). Interestingly, of the remaining SOX9 expression in diseased valves, its localization was predominantly cytoplasmic (Figure 2B, arrowhead, inset box). By Western blot, nuclear Sox9 expression was undetectable in calcified adult valves, whereas adult controls and pediatric noncalcified diseased valves expressed an abundance (Figure 2C and 2D). Cytoplasmic SOX9 was also reduced in CAVD patients; however, this effect was not as dramatic as changes in nuclear expression. These findings suggest that reduced Sox9 expression correlates with CAVD in the human population; however, because these samples were taken from end-stage disease, we are unable to distinguish between cause and effect. *P<0.05 compared with 1 day cultures, n=3.

Endothelial Cells Maintain Sox9 Nuclear Localization in VICs and Attenuate Calcification

To examine the role of VECs in regulating Sox9 expression and calcification, pAVICs were cocultured in a transwell assay in the absence (Figure 3A and 3C) or presence (Figure 3B and 3D) of pAVECs. After 7 days, Alizarin Red detected calcification in pAVICs cultured alone (Figure 3B, arrows). Interestingly, reduced Sox9 expression was noted in 11-, but not in 3-, month-old Reversa mice (Figures IIIC and IIID in the online-only Data Supplement) before the onset of calcification detected at 22 months (Figure 2G and 2H). Together, these findings suggest that reduced Sox9 expression is associated with AoV calcification in human patients and occurs before the deposition of calcified nodules in a hypercholesterolemic mouse model.
nuclear expression in pAVIC/pAVEC cocultures (Figure 3E and 3F), which was associated with increased expression of the transcriptional target gene Col2a1 (Figure 3G). The ability of VECs to protect VICs against calcification by retaining Sox9 nuclear localization was also observed in human VIC cocultures with human umbilical vein endothelial cell (Figure IV in the online-only Data Supplement), suggesting conservation across species. As the transwell system is designed to prevent physical contact between endothelial cells and VICs, we hypothesized that the factor emanating from VECs to prevent calcification in VICs is secreted.

**Tgfβ1 Treatment Is Sufficient to Promote Sox9 Nuclear Localization and Prevent Calcification of pAVICs**

In heart valves, Sox9 expression and localization has been shown to be regulated by several signaling pathways, including BMP2, Notch1, and β-catenin. However based on their mechanisms of action and patterns of expression, we excluded these as candidates for the VEC-mediated regulation of Sox9 in VICs. Tgfβ1 and its downstream signaling mediator pSmad2 are highly enriched in VECs compared with VICs (Figure VA–VC in the online-only Data Supplement). To determine whether Tgfβ1 is sufficient to recapitulate the protective effects of VECs on VIC-mediated calcification, pAVICs were plated for 48 hours and treated with human recombinant TGFβ1 (10 ng/mL) or BSA every 48 hours for an additional 5 days. As shown in Figure 4A, TGFβ1 treatment attenuated calcification compared with BSA as indicated by Alizarin Red reactivity. In addition, TGFβ1 treatment re-established Sox9 nuclear localization in pAVICs (Figure 4B) similar to coculture with pAVECs, and this was associated with increased Col2a1 and decreased Runx2 (Figure 4C). To further investigate this, whole postnatal AoV explants from Tgfβ1−/− mice were cultured and treated with a AdV-Cre or AdV-GFP (Figure 4D and 4E). von Kossa staining revealed that AdV-Cre treatment increased calcium deposition (Figure 4D–4F) as a result of Tgfβ1 knockdown (Figure 4G) and decreased Sox9.
expression (Figure 4H and 4I). To further support a role for TGFβ1 signaling in this process, pAVICs were cocultured in a transwell assay with Tgfβ1fl/fl CD31+ murine cardiac endothelial cells and treated with AdV-Cre or AdV-GFP. After 48 hours, cocultured pAVICs treated with AdV-Cre similarly developed calcific nodules (Figure VD–VF in the online-only Data Supplement). To determine whether TGFβ1 treatment affected Sox9 function, luciferase assays were performed in pAVICs using plasmids containing the minimal promoter and Sox9-responsive intron 1 of Col2a1 (4x48bp-Col2a1) or just the minimal promoter lacking SRY (sex determining region Y)-binding sites (-89-+6bp-Col2a1). As shown, TGFβ1 treatment increases the transcriptional activity of 4x48bp-Col2a1, and this was dependent on Sox9 response elements (Figure 4J). To confirm that TGFβ1 signaling emanating from VECs was responsible for maintaining Sox9 nuclear localization and preventing VIC-mediated calcification, transwell assays were repeated in the presence of the TGFβ1-receptor inhibitor, SB431542. As shown in Figure 4K, SB431542 treatment reduced nuclear Sox9 expression (Figures VI and VJ in the online-only Data Supplement) and enhanced calcification (Figure 4L; Figures VG and VH in the online-only Data Supplement).

**Rho Kinase Is Required for TGFβ1–Mediated Sox9 Regulation in pAVICs**

In chondrocytes, Rho kinase (ROCK) functions downstream of TGFβ1, and therefore, to determine whether ROCK facilitates TGFβ1-mediated Sox9 nuclear localization, pAVICs were pretreated with the inhibitor Y27632 before TGFβ1 exposure. Although TGFβ1 treatment increased nuclear Sox9 expression in pAVICs (Figure 5A, lane 2 and Figure 5B), pretreatment with Y27632 abolished this effect (Figure VA, lane 4 and Figure VB in the online-only Data Supplement). As expected,
pSmad is increased with Tgfβ1 treatment, but the addition of the ROCK inhibitor does not significantly affect levels, suggesting that Smad and ROCK function through differential Tgfβ1-mediated signaling pathways (Figure 5C). Col2a1 expression is increased in response to TGFβ1, and this effect seems to be dependent on ROCK activity (Figure 5D). From these data, we speculated that ROCK might regulate Sox9 via phosphorylation, and published reports have shown that phosphorylation at Serine (S) 64 and 181 drive nuclear localization.36 To test this, we performed luciferase assays and showed that compared with wild-type Sox9, transactivation of Col2a1 was attenuated (∼20%) when cotransfected with an S64 and S181 mutant (Figure 5E). Interestingly, inhibition of ROCK activity alone by Y27632 was sufficient to increase Alizarin Red reactivity (Figure 4).
Red reactivity in cultured pAVICs after 48 hours (Figure 5F and 5G). These observations suggest that Sox9 phosphorylation by ROCK may play a role in VIC-mediated calcification.

Targeted Deletion of Tgfβ1 in VECs Leads to Aortic Valve Disease In Vivo

To investigate whether endothelial Tgfβ1 plays a role in AoV disease in vivo, we targeted loss of function using an Nfatc1ENCre transgenic line that recombines in VECs, but not VICs or any other endothelial cells other than the early endocardium28 (Figure VIC in the online-only Data Supplement). Using this approach, Tgfβ1 was ablated in VECs of Tgfβ1fl/fl;Nfatc1ENCre+ mice (Figure VIA and VIB in the online-only Data Supplement), and subsequently, pSmad2 expression was reduced (Figure VIIA and VIIB in the online-only Data Supplement) compared with littermate controls (Tgfβ1fl/fl;Nfatc1ENCre−; Figure VIA in the online-only Data Supplement). By postnatal stages, AoVs from Tgfβ1fl/fl;Nfatc1ENCre+ mice were thickened, and Sox9 was detected at high levels in VICs in the absence of calcification (Figure 6A, 6B, and 6G). At 3 months (Figure 6D), Sox9 expression was dramatically reduced by immunohistochemistry (Figure 6D) along with Col2a1 (Figure VIIIC and VIID in the online-only Data Supplement). By 6 months, Sox9 remained low, and positive Alizarin Red and von Kossa reactivity indicated calcific nodule formation (Figure 6E–6G; Figure VIE–VIJ in the online-only Data Supplement) in Tgfβ1fl/fl;Nfatc1ENCre+ mice. Similar to observations in vitro (Figure 1A and 1B; Figure IIIA in the online-only Data Supplement), these phenotypes were not associated with increased cell apoptosis as determined by undetectable Cleaved Caspase-3 expression (Figure IIIB–IIID in the online-only Data Supplement). As Sox9 is a potent positive regulator of collagen, trichrome staining was performed at 6 months (Figure VII and VIIJ in the online-only Data Supplement); however, no significant differences were noted relative to increased thickness, although Col2a1 was reduced at 3 months (Figure VIIIC and VIID in the online-only Data Supplement). The phenotypic changes in cusp thickness and calcification at 6 months of age were associated with subtle, but significant increased AoV peak velocity (Figure 6H) and blood flow regurgitation (Figure VIK and VI in the online-only Data Supplement) as determined by echocardiography. Together, these data imply that loss of Tgfβ1 in VECs leads to reduced Sox9 expression in VICs at 3 months, which precedes calcification onset and associated dysfunction detected at 6 months in vivo.

Discussion

Calcific aortic stenosis is the most predominant form of valve pathology affecting >25% of adults over the age of 65.1 At present, there are no effective treatments other than intervensional surgery, and pharmacological mechanistic-based
therapies can only be developed if the regulatory processes that initiate CAVD onset and progression are identified. In this current study, we expand on our previous work demonstrating a causative role for the transcription factor Sox9 in CAVD\textsuperscript{10,11} and show that Tgf\textsuperscript{β} \textsubscript{1} signaling from VECs is essential for promoting Sox9 nuclear localization in VICs to prevent calcification. In two CAVD mouse models (\textit{Tgf}\textsuperscript{β}\textsubscript{1}fl/fl; \textit{Nfatc1ENCre}+) and a calcification in vitro assay, reduced Sox9 expression in VICs preceded calcific nodule formation, supporting a role during early stages of disease onset. This study directly shows that VEC dysfunction at the level of regulatory pathways is sufficient to promote CAVD and, in addition, highlight the potential of targeting Sox9 nuclear localization as a novel therapeutic strategy.

There is strong evidence to show that the process of calcification is mediated by VICs as a result of abnormal activation, apoptosis, ECM remodeling, and calcium deposition.\textsuperscript{37} However, based on findings from other cardiovascular diseases, it has been speculated that dysfunction of the valve endothelium could also play a role. Here, we show that VECs prevent calcific nodule formation by VICs (Figure 3; Figure IV in the online-only Data Supplement), consistent with other reports demonstrating a protective role for the valve endothelium against disease processes.\textsuperscript{5,19,21,38} In vivo, VECs are in direct contact with the hemodynamic environment and, therefore, exposed to shear stress and circulating signaling molecules, cytokines, and risk factors, including cholesterol, lipids, and inflammatory cells. As the VECs encapsulate the valve

\textbf{Figure 6.} Targeted deletion of \textit{Tgf}\textsuperscript{β}\textsubscript{1} in murine valve endothelial cells (VECs) leads to decreased Sox9 expression, calcific nodule formation, and aortic valve (AoV) dysfunction in vivo. \textit{A–F}, Immunohistochemistry to show Sox9 expression (green, arrows) in AoV from control (\textit{Tgf}\textsuperscript{β}\textsubscript{1}fl/fl; \textit{Nfatc1ENCre}+) and \textit{Tgf}\textsuperscript{β}\textsubscript{1}fl/fl; \textit{Nfatc1ENCre}+ (\textit{B, D, F}) mice at postnatal (\textit{A and B}) and 3 (\textit{C and D}) and 6 months (\textit{E and F}) of age. Representation images shown based on \textit{n}=3. \textit{G}, Alizarin Red reactivity in \textit{Tgf}\textsuperscript{β}\textsubscript{1}fl/fl; \textit{Nfatc1ENCre}+ mice at each time point relative to age-matched controls, \textit{n}=3. \textit{H}, Echocardiography to determine AoV peak velocity in \textit{Tgf}\textsuperscript{β}\textsubscript{1}fl/fl; \textit{Nfatc1ENCre}+ mice at 6 months of age compared with controls, \textit{n}=6. *\textit{P}<0.05 compared with controls.
cusp, VICs do not experience the same exposure, yet mediate pathological processes in response to abnormal mechanical stress or risk factor exposure.\(^{9,20,39}\) Therefore, VECs likely serve as sensors and molecularly relay external information to underlying VICs within the leaflets during both pathological and physiological conditions, and if damaged, lost, or injured, these protective mechanisms are likely lost, and the VICs lose their molecular communications and become directly exposed to the external environment.

In this current study, we identify Tgfβ1 signaling as a critical VEC-mediated growth factor that positively regulates Sox9 expression and nuclear localization in VICs (Figure 4) via ROCK (Figure 5) to prevent calcification. Our findings also suggest that in addition to being anti-osteal, this pathway also promotes chondrogenic-like phenotypes (Figures 1F, 3G, and 4C) in pAVICs, consistent with our previous work\(^{42}\) and suggesting pivotal roles in heart valve homeostasis. Tgfβ1 is predominantly localized to VECs (Figure VIA in the online-Only Data Supplement),\(^{35}\) whereas Tgfβ2 and Tgfβ3 seem more widespread throughout the VIC population. These ligands, along with their receptors, play multiple roles in valve development,\(^{40,41}\) and inhibition of Tgfβ signaling in mouse models of myxomatous degeneration alleviates valvular phenotypes, suggesting Tgfβ-dependency in disease states.\(^{42–49}\) We recognize that findings from our study are dissimilar to previous reports, showing that Tgfβ1 inhibition are pro-osteogenic in cultured VICs.\(^{47,49–51}\) The reasons for such disparities in response to increased Tgfβ1 signaling or Y27632 treatment in vitro are unclear, but could be dependent on the sensitivity of culture conditions, including VIC passage number,\(^{52}\) species,\(^{53}\) cell contacts,\(^{54}\) substrate,\(^{55}\) endothelial cell contamination,\(^{19}\) Tgfβ1 dosage, and endogenous Sox9 levels. In vivo, the pro-osteogenic dependency of Tgfβ1 in heart valves has not been reported, and this study is the first to suggest that regulated levels of Tgfβ1 secretion by VECs are required to maintain valve homeostasis and prevent calcification, whereas loss of function in VECs could be pathogenic. In vivo, the environment is different from in vitro conditions, and as Tgfβ and ROCK signaling are responsive to biomechanical cues, the hemodynamic environment or valve compliance could also influence their mechanisms of action on downstream targets and the pathological process.

Although this study has shown that increased Tgfβ1 signaling in VECs prevents calcification mediated by VICs, systemic therapeutic targeting of Tgfβ1 could be problematic based on its wide-spread function in many systems and possible dosage dependency in the valves as discussed earlier. Therefore, nuclear retention of Sox9 in VICs would be an attractive alternative in the prevention of calcification. CRM1-dependent nuclear export signal is required for nuclear export of Sox9 during the process of calcification as Leptomycin B treatment attenuated calcification in vitro (Figure 1H–1K). In addition, our findings suggest that promoting phosphorylation or preventing phosphatase activity at S64 and S181 may be an additional mechanism to maintain Sox9 in the nuclei of VICs and prevent disease onset and progression (Figure 5E). However, therapeutic targeting of Sox9 localization needs to be tightly regulated because we show that this transcription factor plays pivotal roles in osteogenic and chondrogenic programs. Although reduced nuclear localization increases Runx2 (osteogenic), this is at the expense of Col2a1 (chondrogenic; Figure 1F). Similarly, nuclear retention of Sox9 in pAVICs by the presence of endothelial cells (Figure 3G) or Tgfβ1 (Figure 4C) attenuates calcification (Runx2), but significantly increases Col2a1. Therefore, homeostatic mechanisms need to be considered.

Interestingly, although our data shows that calcification is associated with reduced Sox9 nuclear localization, we do not observe significant increases in cytoplasmic localization. This could be because of rapid degradation of cytoplasmic or unphosphorylated Sox9, and this may explain why an overall reduction in Sox9 expression and not nuclear localization is observed in Tgfβ1\(^{10,9}\)Nfatc1ENCre\(^{−}\) mice; however, further work is required to test this. The relevance of nuclear Sox9 to prevent calcific nodule formation by VICs is intriguing, and we and others have shown that as a transcription factor, Sox9 binds and positively regulates cartilaginous matrix genes highly expressed in the valves and represses osteogenic gene programs associated with valve calcification, including Spp1 and Runx2.\(^{9,12–18}\) Therefore, suggesting that in the nuclei of VICs, Sox9 plays pivotal transcriptional roles in promoting healthy (cartilaginous) phenotypes and preventing calcification, which are dysregulated in valve disease (Figure VIII in the online-only Data Supplement). Interestingly, we observe Sox9 expression in VECs of 3-month-old wild-type animals (Figure 6C), and although this cell type does not typically undergo osteoblast-like changes, it is considered that Sox9 may play an additional role related to maintaining endothelial integrity, which if disrupted may have secondary effects on calcification.

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**Disclosures**

None.

**References**


Heart valve disease is prevalent, yet the mechanisms underlying onset and progression are poorly understood. At present, there are no pharmacological therapies available to treat valve pathologies, and surgical repair or replacement remains the only effective treatment. We previously showed that reduced Sox9 function in mice promotes calcific aortic valve disease; however, to date, the mechanisms of this process remain elusive. Here, we demonstrate that reduced Sox9 expression and nuclear localization in valve interstitial cells precedes the onset of calcification. In vitro, Sox9 nuclear export and calcific nodule formation is prevented by valve endothelial cells, and in vivo, loss of endothelial-derived Tgfβ1 signaling leads to reduced Sox9 expression and calcific aortic valve disease. Together, this work identifies a novel signaling pathway between valve endothelial cells and valve interstitial cells that is critical in the prevention of aortic valve disease.
Interstitial Cells Associated With Attenuated Calcification

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Supplemental Figure I. Negative controls for pAVIC assays. (A, B) DIC images of pAVICs following 1 (A) and 7 (B) days of culture of glass. Arrows denote calcific nodules. (C-F) No primary antibody reactions in pAVICs treated for 1 (C) and 7 (D) days, or treated with BSA (E) or TGFβ1 (F).
Supplemental Figure II. Apoptosis is not significantly altered in heart valve calcification assays. (A) Western blot analysis to show Cleaved Caspase-3 expression in pAVICS cultured for 1, or 7 days. Note that calcification occurs by day 7. (B-E) Immunohistochemistry to detect Cleaved Caspase-3 expression in aortic valve (AoV) leaflets from control (Tgfβ1<sup>−/−;Nfatc1ENCre<sup>−</sup>) (B, D) and Tgfβ1<sup>−/−;Nfatc1ENCre<sup>+</sup> mice. Arrows indicate autofluorescent red blood cells.
**Supplemental Figure III.** Reduced Sox9 expression precedes calcification in hypercholesterolemic mice. (A, B) Alizarin Red staining revealed undetectable calcification in 3 (A) and 11 (B) month old hypercholesterolemic Reversa mice. (C, D) Immunohistochemistry to indicate Sox9 expression in VICs of 3 (C) and 11 (D) month old hypercholesterolemic mice. Note reduced expression in the absence of calcification at 11 months. Representation images shown from n=3.
Supplemental Figure IV. HUVECs attenuate human VIC-mediated calcification in vitro. (A, B) Alizarin Red reactivity to detect calcific nodule formation and (C, D) Sox9 immunostaining to determine localization in pAVICs co-cultured with pAVICs (A, C) or HUVECs (B, D). (E, F) Alizarin Red staining in human VICs (hVICs) co-cultured with hVICs (E) or HUVECs (F). (G) Quantitation of Sox9 nuclear localization from C, D. (H) Quantitation of Alizarin Red reactivity from E, F. * p ≤ 0.05 compared to VIC/VIC controls, based on n=4.
**Supplemental Figure V.** Loss of Tgfβ1 in vitro leads to reduced Sox9 expression in VICS and calcific nodule formation. (A) ELISA analysis of secreted Tgfβ1 in pAVICs and pAPECs, n=4. (B) Western blot to show pSmad2 and tSmad2 expression in pAVICs co-cultured alone, or with pAPECs, based on n=3. (C) Quantitation of B. (D, E) Alizarin Red staining (arrows) in pAVICs cultured in transwell assays with CD31+ murine cardiac endothelial cells (mCECs) isolated from Tgfβ1−/− mice and treated with AdV-GFP (D) or AdV-Cre (E). Alizarin Red reactivity is quantitated in (F), based on n=3. (G, H) Alizarin Red reactivity to detect calcification on pAVICs co-cultured with pAPECs and treated with BSA (G) or the Tgfβ inhibitor, SB31542 (H). (I, J) Sox9 immunoreactivity in pAVICs cultured as in G, H.
Supplemental Figure VI. Histological and functional analysis of Tgβ1<sup>fl/fl</sup>;Nfatc1ENCre mice. (A, B) Immunohistochemistry to show Tgfβ1 expression in VECs of control (Tgβ1<sup>fl/fl</sup>;Nfatc1ENCre) (A) and Tgβ1<sup>fl/fl</sup>;Nfatc1ENCre<sup>+</sup> (B) mice. (C) Rosa-Tomato;Nfatc1ENCre<sup>+</sup> mice to show Cre recombination in aortic VECs. (D) Quantitation of AoV area (2D) in control and Tgβ1<sup>fl/fl</sup>;Nfatc1ENCre<sup>+</sup> mice at 6 months of age. von Kossa (E, F), Alizarin Red (G, H) and Trichrome (I, J) staining of AoVs from 6 month old Tgβ1<sup>fl/fl</sup>;Nfatc1ENCre<sup>+</sup> (F, H, J) and control (E, G, I) mice. Arrows indicate calcific nodule formation. (K, L) Color Doppler imaging to show AoV regurgitation (L, red) in 6 month old Tgβ1<sup>fl/fl</sup>;Nfatc1ENCre<sup>+</sup> mice relative to controls (n=6). AoV, Aortic valve. *p<0.05 compared to controls. Representative images are shown from n=3.
**Supplemental Figure VII.** Chondrogenic and osteogenic proteins are altered in *Tgfβ1^{fl/fl};Nfatc1ENCre^+* mice. Immunohistochemistry to detect changes in pSmad (A, B), Col2a1 (C, D) and Runx2 (E, F) expression in control (*Tgfβ1^{fl/fl};Nfatc1ENCre^-*) (A, C, E) and *Tgfβ1^{fl/fl};Nfatc1ENCre^+* (B, D, E) mice at 3 months of age. Arrows indicate expression, arrowheads highlight auto fluorescent staining from red blood cells.
**Supplemental Figure VIII.** Model representation of the progression of calcific aortic valve disease. Based on findings from this study we hypothesize that in normal AoVs, Tgfβ1 (red) is expressed in the endothelium to promote nuclear localization of Aox9 expression (blue) in VICs to prevent calcification. This mechanism is potentially regulated by ROCK signaling to promote phosphorylation and nuclear localization of Sox9. As a result, nuclear Sox9 regulates transcription of chondrogenic-like (activate) and osteogenic-like (repress) gene programs to maintain healthy valves. In early stage CAVD prior to the onset of calcific nodule formation, Tgfβ1 expression is reduced in the damaged endothelium (brown), and therefore Sox9 nuclear localization is no longer maintained (diffuse blue). By end stage CAVD, these molecular changes promote VIC-mediated calcific nodule formation (surface of cusp) and stenosis.
**Cell culture systems**

*Porcine and human valve cell assays.*

Porcine aortic valve (AoV) interstitial (pAVIC) and endothelial cells (pAVEC) were isolated from AoV cusps of juvenile staged pigs as described,\(^1\) and plated at 4x10\(^4\) cells/cm\(^2\) in growth media containing DMEM, 10% L-glutamine, 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (pen/strep). Human mitral valve interstitial cells (hVICs) were a kind gift from Dr. Alain Colige (University of Liege).\(^2\)

*Isolation of murine cardiac endothelial cells.*

Murine cardiac endothelial cells (mCECs) were isolated from post natal day 6-8 Tgfβ1\(^{fl/fl}\) mice using a magnetic bead isolation technique (Invitrogen).\(^3\) Sheep anti-rat IgG DynaBeads (Invitrogen) were coated with either CD31 or ICAM-2 (BD Biosciences) according to the manufacturer’s instructions (Invitrogen). Whole hearts were dissected and cells were dissociated by incubation in 1mg/ml collagenase/dispase solution (Roche) for 45 minutes at 37°C. The resulting cell suspension was incubated with CD31-conjugated DynaBeads (BD biosciences) and bound cells were isolated using the magnetic bead separation system (Invitrogen). The cell pellet consisting of antibody-conjugated cells was resuspended in endothelial cell media (EBM-2 containing 0.5% FBS, 1% pen/strep, and 0.75% Fibroblast Growth Factor) and plated onto a gelatin-coated T-75 flask. Confluent endothelial cells were further purified using ICAM-2-conjugated Dynabeads (BD Biosciences) and maintained on gelatin-coated flasks for subsequent experiments.

*AoV explant assays.*

AoV explants were isolated from post natal Tgfβ1\(^{fl/fl}\) mice and cultured on membrane filters as previously described.\(^4\)

*Transwell and calcification assays.*

For calcification assays, 4x10\(^4\) pAVICs were seeded on glass coverslips in 24-well cell culture plates and allowed to reach confluency. Cultures were then incubated for 1-7 days, changing media every 48 hours to allow for calcification (n=4). For transwell-culture systems endothelial cells (HUVECs, pAVECs, or mCECs) were seeded into transwell inserts (Millipore) at a density of 2x10\(^4\) and cultured overnight to allow for cell attachment. Transwell inserts were then placed into wells containing pAVICs or hVICs, and VIC media was replaced with mCEC media (see above) and cultured for 5-7 days with a media change every 48 hours with or without TGFβ1 (10ng/ml) or SB431542 (100nM) (n=4). Following culture, cells were fixed in 4% paraformaldehyde (PFA)/PBS and subject to immunohistochemistry or quantitative PCR (qPCR) (see below).

*Treatment of primary valve cells*

For treatments, pAVICs were grown to confluency and fresh media was supplemented with the following. TGFβ1 (10ng/ml), Y27632 (10µmol/L), or BSA control (0.0002% final concentration) for 48 hours (n=4). For cultures treated with both TGFβ1 and Y27632, cells were pre-treated with Y27632 30 minutes prior to the addition of Tgfβ1. 5ng/ml Leptomycin B or ethanol vehicle (0.1% final concentration) for 6 hours, then replaced with VIC media for an additional 7 days (n=3). 100nM SB431542 was added to mCEC media and supplemented every other day for 7 days (n=3). Following treatments, cells were subject to RNA or protein isolation, or fixed for immunostaining (see below). AoV explants from Tgfβ1\(^{fl/fl}\) post natal pups were infected immediately with 1×10\(^{10}\) PFU AdV-Cre or control AdV-GFP in serum free medium for 6 hours. Following infection, explants were incubated in VIC media (see above) for an additional 48 hours. Following infection
and incubation, treated explants were removed from the membrane filter and processed for histological, Western blot or RNA analysis (see below).

**Generation of mice**

Tgfβ1<sup>fl/fl</sup> female mice (Jackson Laboratories, stock #010721, C57BL/6J) were bred with Nfatc1ENCre<sup>5</sup> males (backcrossed to C57BL/6J) to generate heterozygous offspring (Tgfβ1<sup>fl/+</sup>;Nfatc1ENCre). Heterozygous males were then bred with Tgfβ1<sup>fl/fl</sup> females to create homozygotes (Tgfβ1<sup>fl/fl</sup>;Nfatc1ENCre<sup>−</sup>) and Cre negative (Tgfβ1<sup>fl/fl</sup>;Nfatc1ENCre<sup>+</sup>) littermate controls. Tgfβ1<sup>fl/fl</sup>;Nfatc1ENCre<sup>+</sup> and control mice were then harvested at post natal (PND3-5), 3 and 6 months of age and analyzed (see below). To determine recombination, Nfatc1ENCre mice were crossed with Rosa26R-Tomato reporter mice (Jackson Labs, stock #007676, C57BL/6J) and sacrificed at post natal day 1. Genotyping was performed as previously described for adult genomic DNA.<sup>5, 6</sup> Reversa hypercholesterolemic and normocholesterolemic control mice were obtained from Dr. Donald Heistad.<sup>7</sup> Normocholesterolemic animals were generated following deletion of microsomal triglyceride transfer protein (Mttp) using the interferon-inducible Mx1-Cre transgene and receiving 4 injections of polyinosinic-polycytidylic acid (225 µg IP) at 2-day intervals and maintained on a chow diet for 6 or 12 months. While hypercholesterolemic animals maintained Mttp function and were fed Western diet for 6 months and then given 4 injections of polyinosinic-polycytidylic acid (225 µg IP), switched to a chow diet, and followed up for an additional 6 months. Hearts from these mice were harvested at 3, 11, and 22 months of age for immunohistological analysis (see below).

**Human AoV specimens**

Human diseased aortic valve specimens (n=3) were obtained from patients undergoing valve replacement surgery. Patients with a history of infective endocarditis, rheumatic heart disease, or a genetic syndrome were excluded. Control AoVs (n=3) were obtained from age-matched individuals at the time of autopsy who died of non-cardiac causes. AoV tissues were fixed in 10% formalin, dehydrated through a graded ethanol series, washed in xylenes, and embedded in paraffin wax. Studies were approved by the Institutional Review Boards at Cincinnati Children’s Hospital Medical Center and the University of Cincinnati (RBH).

**Histology**

**Animal models:** Hearts were dissected from Tgfβ1<sup>fl/fl</sup>;Nfatc1ENCre<sup>+</sup> mice and controls (Tgfβ1<sup>fl/fl</sup>;Nfatc1ENCre<sup>−</sup>) at PND3-5, 3 and 6 months of age, and hypercholesterolemic and normocholesterolemic Reversa mice at 3, 11 and 22 months. Whole hearts were fixed in 4% PFA/PBS overnight and processed for paraffin embedding and cut at 6µm-thick as previously described.<sup>8</sup> Tissue sections containing AoVs were then subject to Pentachrome, Trichrome or von Kossa staining as described and counterstained for 20 minutes in 1% Alcian Blue.<sup>4, 8</sup> Valve thickness was determined using Image Pro Plus software by tracing the perimeter of the AoV cusps identified by Alcian Blue staining, and measuring the internal area (pixels) for n=4 mice per group (n=4 per genotype) for Tgfβ1<sup>fl/fl</sup>;Nfatc1ENCre<sup>+</sup> mice compared to Tgfβ1<sup>fl/fl</sup>;Nfatc1ENCre<sup>−</sup> controls. For Alizarin Red staining, tissue sections were stained with freshly prepared 2% Alizarin Red S (pH 4.1-4.3, Sigma) for 10 minutes. Quantification of von Kossa and Alizarin Red reactivity were performed using Image Pro Plus software following auto-contrast of colored images in Photoshop. Quantitation of von Kossa and Alizarin Red reactivity was calculated as a percentage of positive staining (black) over the total valve area as identified by Alcian Blue and statistical significance was determined by Student’s t-test in
In vitro assays: Following treatment, post natal AoV explants were mounted onto glass slides, fixed in 4% PFA and subject to von Kossa staining and Alcian Blue counterstaining as described above. Quantitation of von Kossa reactivity was calculated as a percentage of positive staining (black) over the total valve area and statistical significance was determined by Student’s t-test in AdV-Cre treated explants compared to AdV-GFP controls. For cultured pAVICs, Alizarin Red reactivity was calculated as an average of positive (red) area (in pixels) in 10 fields per sample. Statistical significance in treated cells was determined using the Student’s t-test as compared to respective vehicle treated controls, or pAVICs cultured alone for transwell assays.

Immunohistochemistry
Animal models: For antibody staining, fixed tissue sections of AoV from human subjects, Tgfβ1fl/fl;Nfatc1ENcre mice, Reversa hypercholesterolemic, or respective control mice were subjected to antigen retrieval by boiling for 10 minutes in unmasking solution (Vector Laboratories) prior to overnight incubation at 4°C with primary antibodies against Sox9 (Millipore, 1:500), Cleaved Caspase 3 (Cell Signaling 1:200) Tgfβ1 (Abcam 1:200), Col2a1 (Abcam, 1:100), Runx2 (Santa Cruz, 1:100) or pSmad2 (Cell Signaling, 1:100). For human tissue, colormetric detection of Sox9 was performed using diaminobenzidine according to the manufacturer’s instructions (ABC staining system, Santa Cruz Biotechnology) and slides were counterstained with hematoxylin for 5 minutes. For tissue sections and cultured cells, immunofluorescent staining against Sox9 and Tgfβ1 was performed using a donkey-anti-rabbit-488 Alexa-Fluor secondary antibody (Invitrogen, 1:400) and visualized using an Olympus BX51 microscope.

In vitro assays: For cultured cells, differential Interference Contrast (DIC, Nomarski) images were taken after 1 and 7 days of culture using an Olympus BX51 microscope equipped with a DIC prism and analyzer using polarized light. In addition, cells were incubated overnight with anti-Sox9 diluted in block (as above) or no primary antibody (block alone) at 4°C, followed by a 1 hour incubation with donkey-anti-rabbit-488 Alexa-Fluor secondary antibody (Invitrogen, 1:400). Reactivity was visualized and captured using an Olympus BX51 microscope and Cellsense software set at the same exposure as primary antibody treated samples. Quantitation of Sox9 nuclear localization in vitro was performed blinded and calculated as the number of cells exhibiting nuclear Sox9 staining over the total number of cells as detected by DAPI nuclear stain, in a total of 10 fields per sample (n=3). Staining was considered nuclear if the intensity of nuclear staining (co-localized with nuclear DAPI) was greater than the intensity of cytoplasmic staining. Statistical significance was determined against respective controls using the Student’s t-test.

Western Blotting and ELISA
For nuclear and cytoplasmic protein extracts, cells were collected in NE-PER extraction reagent (Pierce) supplemented with complete EDTA free protease inhibitor cocktail, and lysed according to the manufacturer’s protocol. Total protein extracts were obtained by lysing cells in RIPA buffer supplemented with complete EDTA free protease inhibitor cocktail. Protein extracts were collected and 5-10µg of total, nuclear, or cytoplasmic protein was run on a 12% SDS PAGE gel and transferred to nitrocellulose membranes using an iBlot dry blot system (Invitrogen). Membranes were blocked in 2% BSA for 1 hour and probed with antibodies against Sox9 (1:2000, Millipore), pSmad2 (1:1000, Cell Signaling), tSmad2 (1:1000, Cell Signaling) Actin (1:5000, Cell Signaling) TBP (1:1000,
Abcam) Cleaved Caspase-3 (1:1000, Cell Signaling) or Histone H3 (1:1000, Cell Signaling) followed by incubation with anti-rabbit-horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000, Cell Signaling). Membranes were then washed three times in 1× TBST for 10 minutes. Western blots were developed using Super Signal West Femto Substrate (Pierce) and BioMax MR film (Eastman Kodak). Densitometry was performed using ImageJ software and calculated as a ratio of Sox9 band density over the loading control in n=3 samples. Statistical significance in treated samples was determined using the Student’s t-test comparing loading control normalized band density values.

Secreted Tgfβ1 levels were determined using the Quantikine ELISA kit (R&D Systems). pAVICs or pAVECS were serum starved for 6 hours prior to collection of cell culture supernatant. Samples were activated and assayed according to the manufacturers protocols. Optical densities of samples were determined using a microplate reader set to 450nm, and Tgfβ1 concentrations were calculated using a standard curve (n=5). Statistical significance was determined using the Students t-test comparing absolute secreted levels in pAVECS versus pAVICs.

**Polymerase Chain Reaction**
Changes in gene expression were determined by quantitative real-time PCR using a Step One Plus Real Time PCR system (Applied Biosystems) according to the manufacturer’s instructions using TaqMan assays (Applied Biosystems) for Tgfβ1, Sox9, Col2a1 and Runx2; normalized to 18s. RNA was extracted from pAVICs, pAVECs, and HUVECs using standard Trizol protocols (Invitrogen) and cDNA and PCR reactions were performed as previously described. Significant differences in gene expression were reported as a fold change compared to control (n=3-6).

**Transfections and Luciferase Assays**
The pGL3-Col2a1 constructs were obtained from Dr. Michael Underhill. The 4x48bp-Col2a1 construct contains the minimal promoter of murine Col2a1 in addition to the Sox9-responsive first intron, while the 89-6bp-Col2a1 lacks the first intron. For luciferase assays, pAVICs were plated at 4x10⁴/cell (24-well plate) 16-20 hours prior to transfection with Lipofectamine and Plus Reagent (Invitrogen) according the manufacturer's instruction. 500ng of 4x48bp-Col2a1 or 89-6bp-Col2a1 were transfected into each well along with 50ng pGL4 (Renilla luciferase, Promega). For Tgfβ1-treated assays, TGFβ1 (10ng/ml) or BSA was added to the pAVIC media following transfection. Alternatively, pAVICs were co-transfected with 500ng WT-Sox9 or pmutant-Sox9 obtained from Dr. Martin Cheung. All transfections were performed in 0.5mL OptiMem for 4 hours before the addition of 0.5mL normal growth media. Cell lysates were collected 48 hours following transfection according to the manufacturer’s instructions for dual luciferase assays (Promega). Data for TGFβ1-treated assays are represented as a fold change in luciferase activity of TGFβ1 treatment compared to BSA-treated controls (n=6). For the p-mutant co-transfections, data are reported as an average percent of luciferase activity of the pGL3-4x48Col2a1 co-transfected with WT-Sox9 (set at 100%) and normalized to pGL4 Renilla signal (n=3). Statistical analysis was performed using the Student’s t-test.

**Echocardiography**
Transthoracic echocardiography was performed on Tgfβ1fl/fl:Nfatc1ENCre+ and Tgfβ1fl/fl:Nfatc1ENCre− mice (n=6) at 3 and 6 months of age using a VisualSonics 2100 system (Toronto, Canada). Mice were anesthetized with 1% isofluorane inhalation and
placed on a heated platform. Two-dimensional imaging was recorded with a 40-hertz transducer to capture long- and short-axis projections with guided M-Mode, B-Mode and PW Doppler recorded. The average reading for each parameter measured was recorded from at least 10 frames from each animal and the standard deviation calculated. Statistical significance was determined using Student’s t-test (P<0.05) comparing absolute peak velocities of $Tgf\beta_1^{fl/fl};Nfatc1 ENCre^+$ against $Tgf\beta_1^{fl/fl};Nfatc1 ENCre^-$ littermate controls.