Reduced Sox9 Function Promotes Heart Valve Calcification Phenotypes In Vivo

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Rationale: Calcification of heart valve structures is the most common form of valvular disease and is characterized by the appearance of bone-like phenotypes within affected structures. Despite the clinical significance, little is known about the mechanisms that underlie this multifactorial disease. Treatment options for valve calcification are limited, and no known therapies prevent disease progression.

Objective: This study aims to determine the role of Sox9 in maintaining connective tissue homeostasis in mature heart valves using in vivo and in vitro approaches.

Methods and Results: Using histological and molecular analyses, we report that, from 3 months of age, Sox9+/−;Col2a1-cre mice develop calcific lesions in heart valve leaflets associated with increased expression of bone-related genes and activation of inflammation and matrix remodeling processes. Consistently, ectopic calcification is observed following direct knockdown of Sox9 in heart valves in vitro. Furthermore, we show that retinoic acid treatment in mature heart valves is sufficient to promote calcific processes in vitro, which can be attenuated by Sox9 overexpression.

Conclusions: This study provides insight into the molecular mechanisms of heart valve calcification and identifies reduced Sox9 function as a potential genetic basis for calcific valvular disease. (Circ Res. 2010;106:712-719.)

Key Words: heart valves ■ calcification ■ Sox9 ■ extracellular matrix ■ mouse model

Calcification of heart valve structures affects more than 27% of the US population over 65 years of age and is the major contributor of heart valve malfunction. Despite the clinical significance, little is known about the mechanisms that underlie this multifactorial disease. Treatment options for valve calcification are limited, and no known therapies prevent disease progression.

Normal heart valve function requires organization of differentiated cell types and specialized extracellular matrix (ECM) within the valve leaflet is arranged according to blood flow. This defined tissue architecture provides the mechanical resilience and compressibility required to open and close the valve orifices effectively during the cardiac cycle. In diseased heart valves, loss of ECM organization is associated with changes in mechanical properties, ultimately leading to dysfunction. One of the most striking alterations in valve ECM homeostasis is ectopic bone-like matrix mineralization observed in calcific valve disease.

At the functional level, this histopathologic alteration results in stiffened leaflets, narrowing of the valve opening, and impaired blood flow. The mechanisms that promote the onset and progression of heart valve calcification are not clear, but recent reports suggest a complex process involving molecular and cellular phenotypes shared with bone formation and chronic inflammation. The contribution of these phenotypes in the onset and progression of calcific valvular disease is not known; however, studies have identified clinical risk factors including older age and hypercholesterolemia.

Recent reports have also shown that regulatory mechanisms common to osteogenesis play a major role in valvular calcification. Expression of bone-associated genes, including Runx2, a transcription factor required for the osteoblast gene program, and downstream target genes, including Osteopontin and Osteonectin, is increased in calcified lesions of human heart valves. The mechanisms initiating ectopic osteogenic processes in heart valves are not known, but recent reports have implicated a genetic basis and therefore developmental origins.

The diversified cell types and ECM that form the mature valve architecture are derived during embryogenesis from undifferentiated precursor cells of endocardial cushions. Following endothelial to mesenchymal transformation and cushion formation, valve precursor cells differentiate and secrete specialized ECM. The molecular mechanisms that regulate this process are not well defined but are likely essential for normal development.
structure and function of mature heart valves. Identification of such signaling pathways during development may improve understanding of adult valve pathologies associated with altered connective tissue composition and organization. Recent studies have identified parallel regulatory mechanisms between heart valve development and formation of other connective tissue systems including cartilage and tendon. Although aberrations in these signaling pathways are known to affect skeletogenesis, pathological effects on heart valve structure and function are not well defined.

First identified for its essential role in chondrocyte differentiation, the transcription factor Sox9 is known to play parallel roles in promoting expression of cartilaginous matrix proteins in developing heart valve structures. In addition to cartilage defects, mice with reduced Sox9 function develop premature skeletal ossification, suggesting an opposing role in bone formation. The mechanisms of Sox9 deficiency during skeletogenesis are not clear, although Sox9 function is sufficient to inhibit RUNX2-mediated activation of osteogenic target genes. Upstream regulators of Sox9 in cartilage and bone formation include retinoic acid (RA). RA treatment of chondrogenic proteins, and increased calcium deposition is observed on developing heart valve structures. In addition to cartilage tiation, the transcription factor Sox9 is known to play parallel regulatory mechanisms between heart valve development and formation of other connective tissue systems including cartilage and tendon. Although aberrations in these signaling pathways are known to affect skeletogenesis, pathological effects on heart valve structure and function are not well defined.

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Previous studies have shown that heart valves from mice with targeted homozygous loss of Sox9 in Type II Collagen (Col2a1)-derived cells express diminished levels of cartilage-associated proteins, and increased calcium deposition is observed on valves from viable heterozygotes. Sox9 overexpression in murine heart valves, we used a targeted approach using the Cre/loxP system. Breedings of Col2a1-cre with Rosa26R reporter mice reveal recombination by X-gal staining in a subset of cells along the edges of the mitral (Figure 1C), aortic (Figure 1D), tricuspid, and pulmonic valve leaflets during remodeling stages at E17.5. To determine the role of Sox9 in murine heart valves, we used a targeted approach using the Cre/loxP system. Breedings of Col2a1-cre with Rosa26R reporter mice reveal recombination by X-gal staining in a subset of cells along the edges of the mitral (Figure 1C), aortic (Figure 1D), tricuspid, and pulmonic valve leaflets (data not shown) from E15.5, consistent with Sox9 expression (Figure 1A and 1B). In heart valves from 3-month-old Sox9fl/+;Col2a1-cre−/− (Sox9fl/) mice, Sox9 is expressed in cells throughout the valve leaflet (Figure 1E). However, following recombination in Sox9fl/+;Col2a1-cre mice, Sox9 expression is moderately reduced along the edges of the valve leaflets (arrows, Figure 1F) associated with Col2a1-derived cells (arrows, Figure 1C). This is in comparison to adjacent nonrecombined cells (arrowheads, Figure 1F) and cells along the edges of valves from cre-negative Sox9fl/+ littermate controls (Figure 1E). PE-CAM staining indicates the endothelial cell layer (Figure 1E and 1F). These data validate successful recombination of Sox9 in targeted cells.

**Methods**

Sox9fl/+ female mice were bred with Col2a1-cre males (The Jackson Laboratory) to generate heterozygous offspring at expected Mendelian ratios. Sox9fl/+;Col2a1-cre mice and Sox9fl/+ littermate controls were age-matched and subject to echocardiography as described. Following functional analysis, hearts were dissected and RNA extracted from atrioventricular regions containing the mitral and tricuspid valves) were whole hearts were fixed, cryoembedded, and sectioned for histological staining, in situ hybridization, and immunofluorescence as described. Real time PCR-based TaqMan Low Density Array (TLDA) cards were used to quantitatively identify changes in mRNA transcript levels of target genes as described. For in vitro studies, neonate mouse or embryonic day (E)10 chick mitral valve explants were treated with DMSO, RA 1 µmol/L, adenovirus (Ad)-green fluorescent protein (GFP), Ad-Sox9, or Ad-Cre.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Results**

**Generation of Mice With Targeted Reduction of Sox9 Function in Heart Valves**

Our previous studies have shown that Sox9 is highly expressed during early stages of endocardial cushion development. Using immunofluorescence, we also detect Sox9 expression in the mitral (Figure 1A), tricuspid, pulmonic (data not shown), and aortic valve (Figure 1B) leaflets during remodeling stages at E17.5. To determine the role of Sox9 in murine heart valves, we used a targeted approach using the Cre/loxP system. Breedings of Col2a1-cre with Rosa26R reporter mice reveal recombination by X-gal staining in a subset of cells along the edges of the mitral (Figure 1C), aortic (Figure 1D), tricuspid, and pulmonic valve leaflets (data not shown) from E15.5, consistent with Sox9 expression (Figure 1A and 1B). In heart valves from 3-month-old Sox9fl/+;Col2a1-cre−/− (Sox9fl/) mice, Sox9 is expressed in cells throughout the valve leaflet (Figure 1E). However, following recombination in Sox9fl/+;Col2a1-cre mice, Sox9 expression is moderately reduced along the edges of the valve leaflets (arrows, Figure 1F) associated with Col2a1-derived cells (arrows, Figure 1C). This is in comparison to adjacent nonrecombined cells (arrowheads, Figure 1F) and cells along the edges of valves from cre-negative Sox9fl/+ littermate controls (Figure 1E). PE-CAM staining indicates the endothelial cell layer (Figure 1E and 1F). These data validate successful recombination of Sox9 in targeted cells.

**Increased Calcium Deposition in Heart Valve Leaflets From Adult Sox9fl/+;Col2a1-cre Mice**

As determined by von Kossa reactivity, calcium deposits are observed in aortic (Figure 2A and 2B), mitral (Figure 2C and 2D), and tricuspid (data not shown) valve leaflets of Sox9fl/+;Col2a1-cre mice from 3 months of age (Figure 2B and 2D) but not in leaflets from control Sox9fl/+ mice (Figure 2A and 2C). In all cases, von Kossa staining reveals calcium deposition on the leaflet surface adjacent to blood flow (red arrows). Lack of von Kossa reactivity in neighboring tissue sections from Sox9fl/+;Col2a1-cre mice treated with counterstain only (inset, Figure 2D), or decalcified with EDTA (data not shown), eliminates misidentification of pigmented melanocytes present in the valves.
Heart Valves From Sox9<sup>fl/fl</sup>;Col2a1-cre Mice Show Increased Osteogenic Signaling and Inflammatory and ECM Remodeling Processes at Twelve Months of Age

Recent studies have described human calcific valvular disease as an active process associated with activation of regulatory pathways common to bone development and increased inflammatory processes and ECM remodeling. To identify similar processes in Sox9<sup>fl/fl</sup>;Col2a1-cre mice, high-throughput quantitative real-time PCR was performed using custom TLDA cards designed to identify changes in genes characteristic of advanced human heart valve calcification.

In heart valves from 12-month-old Sox9<sup>fl/fl</sup>;Col2a1-cre mice, transcript levels of several bone-related genes are significantly increased over controls. Similar to previous observations in human calcific valvular disease, these include increased expression of Runx2 (2-fold), Osteonectin (1.8-fold), Osteopontin (8.7-fold), Osteoprotegerin (3.9-fold), and Smpd3 (1.9-fold) compared to Sox9<sup>fl/fl</sup> littermate controls (Figure 3A). Online Table II shows results for all the genes examined in Sox9<sup>fl/fl</sup>;Col2a1-cre mice and controls at 3, 6, and 12 months of age. In situ hybridization confirms increased osteonectin transcript in 12-month-old Sox9<sup>fl/fl</sup>;Col2a1-cre valve leaflets over Sox9<sup>fl/fl</sup> controls (Figure 3B and 3C).
Consistent with previous observations in calcified human valves, inflammatory responses appear active in heart valves from 12-month-old Sox9\textsuperscript{fl/fl}/H11001;Col2a1-cre mice compared to controls. This is indicated by increased expression of vascular cell adhesion molecule-1 (Vcam-1) (1.9-fold), a cell adhesion molecule involved in proinflammatory signal transduction; colony-stimulating factor 1 receptor (Csf1r) (1.6-fold), indicative of macrophage infiltration; and Toll-like receptor 2 (Tlr2) (2.1-fold), important in cytokine release in immune response. Spatially, increases in Vcam-1 expression by immunostaining were observed throughout the valve leaflet (data not shown). Furthermore, increases in inflammation-related transcripts observed in Sox9\textsuperscript{fl/fl}/H11001;Col2a1-cre mice are significantly higher at 12 months compared to 3 months of age.

Figure 3. Transcript levels of bone-related genes are increased in 12-month-old Sox9\textsuperscript{fl/fl}/H11001;Col2a1-cre mice. A, Normalized fold changes in transcripts levels of osteogenic-signaling genes Runx2, Osteonectin (ON), Osteopontin (OP), Osteoprotegerin (OPG), and Smad3 in Sox9\textsuperscript{fl/fl}/H11001;Col2a1-cre vs Sox9\textsuperscript{fl/fl}/H11001 control mice at 3, 6, and 12 months, as determined by TLDA. *P<0.05. Osteonectin, undetected by in situ hybridization in control mitral valve leaflets (B), is detected in a 12-month Sox9\textsuperscript{fl/fl}/H11001;Col2a1-cre mouse mitral valve leaflet (arrows, C). Low-magnification images (B and C) are available in Online Figure II.

Figure 4. Inflammation and ECM remodeling–related genes are increased in 12-month-old Sox9\textsuperscript{fl/fl}/H11001;Col2a1-cre mice. A, Inflammation–associated genes Vcam-1, Csf1r, and Tlr2 are increased in Sox9\textsuperscript{fl/fl}/H11001;Col2a1-cre mice relative to controls at 3, 6, and 12 months (TLDA analysis). B, Increases in ECM remodeling and fibrosis-related genes Col1a1, Col1a2, Timp1, and Tenascin-C (Ten-C) are also observed. *P<0.05 relative to age-matched controls; †P<0.05 relative to 3 month Sox9\textsuperscript{fl/fl}/H11001;Col2a1-cre mice. Elastin (green) is detected on the atrial surface of the mitral valve in control Sox9\textsuperscript{fl/fl}/H11001 mice (arrows, C). Elastin fibers are fragmented and disorganized in Sox9\textsuperscript{fl/fl}/H11001;Col2a1-cre mitral valve leaflets (arrows, D).

Figure 5. Sox9 knockdown increases calcification phenotypes in mouse valve explants. A and B, von Kossa reactivity in Sox9\textsuperscript{fl/fl} neonatal mouse valve explants infected with GFP-adenovirus (Ad-GFP) (A) or Cre-adenovirus (Ad-Cre) (B). C, Quantification of von Kossa reactivity in Ad-GFP– and Ad-Cre–infected explants as a percentage of total area defined by Alcian blue. D, Sox9 transcript levels are significantly decreased following infection with Ad-Cre, whereas Runx2 and osteopontin (OP) are increased relative to GFP-treated controls. *P>0.05.
Valvular disease is frequently associated with ECM disorganization and excess collagen deposition, leading to fibrosis. In Sox9fl/fl/H11001;Col2a1-cre mice, transcript levels of genes associated with tissue fibrosis are increased, including Col1a1 (1.9-fold), Col1a2 (1.7-fold), and Tenascin-C (ten-C) (4.3-fold) (Figure 4B). In addition, the matrix metalloproteinase inhibitor Timp1 is increased 4-fold, indicative of valve leaflet remodeling. Disorganized elastic fibers have previously been reported with calcified heart valves. In valves from Sox9fl/fl/H11001;Col2a1-cre mice, changes in elastin transcript levels are not observed; however, mature elastic fibers appear fragmented and ectopically distributed throughout the valve leaflet in Sox9fl/fl/H11001;Col2a1-cre mice (Figure 4C), compared to parallel bundles localized along the atrial surface in controls (Figure 4D). Collectively, these findings indicate that increased calcium deposition observed in Sox9fl/fl/H11001;Col2a1-cre mice is associated with activation of osteogenic, inflammation, and ECM remodeling programs.

Knockdown of Sox9 in Mature Heart Valves In Vitro Promotes Calcific Phenotypes
To support in vivo findings and determine a direct role for Sox9 in promoting heart valve calcification, atrioventricular valve explants from neonate Sox9fl/fl mice were infected with adenovirus targeting Cre recombinase (Ad-Cre) (Figure 5C) or Ad-GFP (Figure 5B) as a negative control. In association with a >2-fold knockdown of Sox9 expression, explants infected with Ad-Cre display 28% von Kossa reactivity (Figure 5B and 5C), compared with negligible levels observed in Ad-GFP controls (<5%) (Figure 5A and 5C), or explants from Sox9fl/+ mice infected with Ad-Cre (data not shown). This increase in calcium deposition is associated with a significant increase in Osteopontin and a trend toward increased Runx2 expression. These data support a direct role for reduced Sox9 function in promoting calcific heart valve phenotypes.

RA Treatment Reduces Sox9 Expression and Promotes Osteogenic-Like Processes in Mature Chick Heart Valves
RA has previously been identified as an upstream regulator of Sox9 during cartilage and bone development. To determine similar roles in mature heart valves intact avian mitral valve explants were subject to RA treatment in vitro. At E10, avian heart valves express high levels of Sox9 and exhibit highly organized ECM patterning indicative of maturation. Following 1 μmol/L RA treatment for 48 hours, von Kossa staining reveals significantly increased calcium deposition (21.7%) compared to DMSO controls (2.9%) (Figure 6A, 6B, and 6G). Furthermore, RA treatment is associated with significant decreases in Sox9 transcript levels (5.3-fold) (Figure 6H).

To determine the role of reduced Sox9 in RA-induced valve calcification, avian explants treated with DMSO or 1 μmol/L RA were infected with adenovirus-expressing full-length mouse Sox9 (Ad-Sox9) or GFP (Ad-GFP). Consistent with Figure 6A, negligible von Kossa reactivity is observed in DMSO-treated
expansions infected with Ad-GFP (Figure 6C) or Ad-Sox9 (Figure 6E). In DMSO-treated explants, endogenous chicken Sox9 expression does not change, whereas mouse Sox9 expression is significantly increased in Ad-Sox9–infected explants (Figure 6H), confirming targeted overexpression. As expected, RA treatment in Ad-GFP–infected explants significantly increases von Kossa reactivity (>30%) (Figure 6D) and decreases Sox9 expression (Figure 6H) compared to DMSO controls infected with Ad-GFP (Figure 6C and 6H). Notably, RA treatment in Ad-Sox9–infected explants (Figure 6F) does not increase von Kossa reactivity to levels observed in RA-treated Ad-GFP–infected (Figure 6D) explants. These findings suggest that increased Sox9 expression prevents RA-induced heart valve calcification in vitro, supporting a direct and causative role for reduced Sox9 function in promoting calcific valve phenotypes.

**Discussion**

Identification of signaling pathways that mediate disease onset or progression is critical for the development of new treatments for calcific valvular disease. Here, we present findings from a mouse model with targeted loss of Sox9 function during valve development that displays increased susceptibility to calcific valve phenotypes in adulthood. This is marked by the appearance of calcific lesions and increased expression of osteogenic genes including Runx2 in valve leaflets from adult Sox9fl/+; Col2a1-cre mice. Furthermore, these calcification processes are associated with activation of genes characteristic of ECM remodeling and inflammation. Despite these pathological phenotypes, valve function in adult Sox9fl/+; Col2a1-cre mice is comparable to Sox9fl/++; litterate controls (Online Table I). The ability of Sox9 deficiency to directly promote valvular calcification is supported by increased von Kossa reactivity following Sox9 knockdown in murine valve explants in vitro. Furthermore, similar calcification is observed following RA treatment in vitro, and overexpression of Sox9 is sufficient to attenuate RA-induced calcification. Collectively, these findings suggest that Sox9 plays important roles in maintaining connective tissue homeostasis in mature heart valve structures and provide insights into a genetic basis for calcific disease.

**Sox9 Is Required to Maintain Heart Valve Connective Tissue Homeostasis**

In mature heart valves, ECM composition and organization are essential for maintaining valve structure and function.\(^3\),\(^4\),\(^36\) Previous studies have shown that mature valve leaflets are rich in proteoglycans and express markers characteristic of cartilage tissue.\(^4\) Furthermore, our group has shown that cartilage-associated gene expression in developing heart valves requires Sox9, similar to findings in chondrogenic systems.\(^20\),\(^22\),\(^54\),\(^55\) These findings highlight an important role for Sox9 in establishing the desired connective tissue composition in normal heart valves. In this present study, we show that reduced Sox9 function leads to ectopic formation of calcified matrix and therefore highlight an additional role for Sox9 in maintaining connective tissue homeostasis of mature valves. However, the mechanisms of Sox9 function in these processes are not clear, but our findings suggest that Sox9 plays pivotal roles in promoting cartilage-like phenotypes and preventing osteogenic processes in normal heart valve structures. Therefore, dysregulation of Sox9 function likely has profound effects in promoting proteoglycan or osteogenic-related valvular disease associated with “floppy” or “stiffened” valve function, respectively.

**Sox9 Function and Calcific Valvular Disease**

Sox9fl/+; Col2a1-cre mice develop calcific lesions on the surface of heart valve leaflets adjacent to blood flow, therefore highlighting similarities between our mutant mouse model and human calcified valvular disease.\(^9\) However, it is appreciated that the gross changes in ECM organization observed in human calcified valves cannot be thoroughly analyzed in this model because of size limitations of rodent heart valves.\(^3\) At the molecular level, we again observe similarities with clinical pathology, including increased expression of bone-associated genes including Runx2 and downstream osteogenic target genes.\(^7\),\(^50\) The underlying etiology that promotes pathological bone signaling in heart valves is not understood, but recent studies have identified NOTCH1 mutations in patients with bicuspid aortic valve and aortic valve calcification.\(^14\) Interestingly, both NOTCH1 and Sox9 have been shown to repress RUNX2 activity.\(^14\),\(^25\) Therefore, one might predict that the osteogenic phenotypes observed in Sox9fl/+; Col2a1-cre mice are attributable to lost repression of Runx2. However, Notch1 expression was not significantly different in heart valves from Sox9fl/+; Col2a1-cre mice, and therefore interactions between Sox9 and Notch signaling on Runx2 activity cannot be discerned.

Unlike calcified human valves, where pathological calcification is associated with functional defects including stenosis, heart valve function in Sox9fl/+; Col2a1-cre mice is normal, and using conventional echocardiography, valve leaflet fusion was not observed. This suggests that the calcific lesion size (<4%) within the valve leaflet is not sufficient to affect mechanical properties of the valve at 12 months of age. Studies in mouse models susceptible to valve calcification have effectively aggraded phenotypes with additional exposure to known clinical risk factors including hypercholesterolemia and renal dysfunction, highlighting the multifactorial nature of this disease and therefore exposing Sox9fl/+; Col2a1-cre mice to such factors would be a plausible approach for future studies.\(^53\),\(^56\),\(^57\)

**The RA-Sox9 Pathway Is a Potential Therapeutic Target for Treatment or Prevention of Calcific Valvular Disease**

Previous approaches in the treatment of calcific valvular disease have focused on treating underlying risk factors. However, clinical trials using lipid-lowering therapies have been inconclusive and patient outcomes are not improved.\(^58\)–\(^60\) Therefore, alternative strategies are needed to improve disease prognosis and insights will likely be gained by targeting signaling pathways active during the onset and progression of calcific disease. Our findings suggest that RA signaling regulates Sox9 function to promote osteogenic processes in mature heart valves, highlighting RA-Sox9 signaling as a potential target for alternative therapeutic approaches. Manipulation of the RA signaling pathway has already proven pharmacologically effective in the treatment of several bone-associated pathologies, including the use of small-molecule RA inhibitors such as antioestrogenic agents.\(^61\)–\(^63\) Therefore, future work investigating the benefits of
retinoid pathway antagonism in the prevention or treatment of calcific valvular disease is warranted. Collectively, our studies identify a previously unappreciated role for Sox9 function in maintaining connective tissue homeostasis in mature heart valve structures. In addition, there is evidence to suggest that reduced Sox9 function during embryonic development later leads to calcific valvular disease manifested in the adult.

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Disclosures

None.

References

Novelty and Significance

What Is Known?
- Heart valve calcification is the most frequently acquired valvular disease, but the etiology is not clear.
- Sox9 is expressed in developing and mature heart valves and is required for expression of cartilage-related proteins.
- Mice deficient for Sox9 fail to form skeletal cartilage and develop bone prematurely.

What New Information Does This Article Contribute?
- Reduced Sox9 function promotes calcific valve phenotypes in vitro and in vivo.
- Heart valve calcification in mice with reduced Sox9 function is associated with increased osteogenic signaling and activation of inflammatory and extracellular matrix processes.
- Retinoic acid treatment promotes calcific valve phenotypes that can be rescued by Sox9 overexpression.

Calcification of heart valve structures is the most common form of valvular disease and most often results in surgical replacement. Despite the significance, the mechanisms that promote disease onset and progression are largely unknown. In this present study, we have identified that reduced Sox9 function in a subset of type II collagen-derived valve cells during embryonic development promotes calcific valve phenotypes in vivo. This pathological state is associated with increased signaling of genes active during bone development and activation of inflammatory and matrix remodeling processes in calcified valves from Sox9 mutant mice. Furthermore, we have identified retinoic acid as an upstream repressor of Sox9 function in promoting calcification in vitro. This study generated a mouse model of human pathology and identified a novel genetic-based mechanism for calcific valve disease. Findings from this study provide insights into the molecular mechanisms that promote the onset of heart valve calcification that will undoubtedly contribute to the development of alternative therapeutic strategies.
Detailed Methods

Generation of Mice
Sox9\textsuperscript{flox/flox} female mice\textsuperscript{1} were bred with Col2a1-cre males (Jackson Laboratories)\textsuperscript{2} to generate heterozygous offspring (Sox9\textsuperscript{flox/+};Col2a1-cre) and Cre negative Sox9\textsuperscript{flox/+} littermate controls at expected Mendelian ratios. To determine recombination, Col2a1-cre mice were crossed with Rosa26R-lacz (Rosa26R) reporter mice and sacrificed at postnatal day 1. Genotyping was performed by RT-PCR as previously described for adult genomic DNA.\textsuperscript{1,3}

Tissue Preparation
Sox9\textsuperscript{flox/+};Col2a1-cre mice and Sox9\textsuperscript{flox/+} littermate controls were sacrificed at 3, 6, and 12 months of age. Hearts were removed and fixed in 4% paraformaldehyde overnight at 4°C. Tissue was processed through 10, 20 and 30% sucrose solutions before embedding in OCT freezing compound (Tissue-Tek), and sectioning 10µm-thick. Frozen sections were stored at -20°C and allowed to dry at 42°C for 20 minutes and rinsed in 1X PBS prior to immunohistochemical, in situ hybridization or histological staining.\textsuperscript{3} Hearts from E18.5 Col2a1-cre;Rosa26R mice were fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin and sectioned for X-Gal staining as described.\textsuperscript{3,4}

Taqman Low Density Array (TLDA) and Real-Time PCR
RNA was isolated from atrioventricular regions from Sox9\textsuperscript{flox/+};Col2a1-cre and Sox9\textsuperscript{flox/+} littermate controls at 3, 6, and 12 months of age. 400ng of RNA was subject to cDNA synthesis according to the manufacturer’s instructions (Applied Biosystems) and TaqMan Low Density Array (TLDA) or real-time PCR was performed. TLDA cards consist of 384 wells pre-loaded with selected 4 x 96 Taqman oligos including 2 endogenous controls, for high-throughput, low reaction volume, real-time PCR. For each TLDA card, 10µl of each cDNA sample was diluted in 90µl of double distilled water and mixed with 100µl of Taqman master mix (#4304437, Applied Biosystems). Standard recommended PCR protocols were performed (50°C for 2 minutes, 94.5°C for 10 minutes, 97°C for 30 seconds, 59.7°C for 1 minute, with steps 3 and 4 repeated for 40 cycles) using the Applied Biosystems 7900 HT Fast Real-Time PCR System. ΔCT (cycle count to threshold) values were determined by normalization to 18s using the RQ SDS manager software (Applied Biosystems). For real-time PCR, individual TaqMan inventoried detection assays (Applied Biosystems) were used against chicken Sox9, mouse Sox9, mouse Runx2 and mouse Osteopontin according to manufacturer’s instructions and normalized to 18s expression levels. Sample wells with a CT of 40 were excluded from analysis as outliers when more than two standard deviations above the mean of replicates. Statistically significant differences in transcript level were determined using Student’s t-test or one-way ANOVA with p<0.05 considered significant.

Histology and Immunostaining
Tissue sections were prepared as described above and blocked in 10% heat inactivated goat serum, 0.1% Tween-20 in 1xPBS for 1 hour at room temperature. For elastin staining, tissue sections were subject to antigen retrieval by boiling in unmasking solution (Vector Labs) for 10 minutes. Primary antibody incubations (in 1:1 block solution:PBS) were carried out overnight at 4°C at the following dilutions: PECAM (sc1505, Santa Cruz) (1:50), Sox9\textsuperscript{5} (1:1000) and Elastin (sc-17581, Santa Cruz) (1:100). Following primary antibody incubation, sections were washed in 1x PBS and incubated with respective anti-rabbit, anti-goat or anti-mouse Alexa 488 or 566 secondary antibodies at 1:400 dilution for 1 hour at room temperature, washed, and incubated with DAPI (blue) for identification of nuclei before mounting in Vectashield (Vector Laboratories). Alternatively for Vcam-1 detection, colorimetric ABC Staining System (Santa Cruz) was used according to manufacturer’s instructions.

For von Kossa staining, tissue sections were prepared as described above, rinsed in deionized water, and incubated in 5% silver nitrate solution for 1 hour under direct light (Schott Modulamp), in a reflective chamber. Slides were washed in water, differentiated in 5% sodium thiosulfate pentahydrate for five minutes, rinsed and counterstained for 20 minutes in 1% Alcian blue in 20% acetic acid for visualization of proteoglycan-
rich normal valve tissue. To prevent misidentification of valvular melanocytes as calcium deposits (black, silver-stained areas), neighboring sections were either treated with 0.38M EDTA overnight to dissolve calcium deposits or processed as described above, omitting the silver nitrate step. Quantification of von Kossa reactive area was performed using Image Pro Plus software and calculated as a percentage of von Kossa positive area (black) over total valve section area (blue).

For In situ hybridization analysis, the antisense Osteonectin probe was obtained from Dr. Katherine Yutzey. Procedures were performed on 10µM-thick frozen sections as previously described.

Echocardiogram
Transthoracic echocardiography was performed on 3, 6, and 12 month old Sox9$^{flox/+}$;Col2a1-cre mice and Sox9$^{fl/+}$ littermate controls using the VisualSonics 770 system (Toronto, Canada) as described. Mice were anesthetized with 1% isoflurane 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. For each parameter measurement, the average reading was recorded from at least three distinct frames from each of 4-8 animals and the standard deviation calculated. Statistical significance was determined using Student’s t-test ($P<0.05$).

Chick Valve Explant Culture
Mitral valves were dissected from 10-day chick embryos and placed on 10mm-wide 0.1µm pore filters (VCWP, Millipore) with culture media (1% chicken embryo extract (Accurate Chemical, USA), 1% Penicillin/Streptomycin, 1 × M199 (Invitrogen), 10% FBS (Invitrogen)). Mitral valve explants from 5-6 embryos were attached to each filter. 1µmol/L retinoic acid in DMSO or DMSO alone (0.001% final concentration) was added after plating and cultures were incubated for 48 hours. Explants were collected in Trizol for RNA isolation or mounted intact to microscope slides and fixed in 4% PFA/PBS for von Kossa staining as described above.

Adenoviral-Sox9 (Ad-Sox9) and adenoviral-GFP (Ad-GFP) were produced according to manufacturer’s instructions (AdEasy XL, Stratagene) using the full-length mouse Sox9 cDNA (for Ad-Sox9) or no insert (Ad-GFP) in pShuttle-IRESHrGFP-1, purified, and tittered with AdEasy Viral Titer Kit (Stratgene). In Sox9 over-expression experiments Ad-Sox9 or Ad-GFP was applied at 1x10⁶ PFU per filter in 50ul serum free culture media for 4 hours, followed by overnight incubation in 1mL serum free culture media prior to RA or DMSO application. Explants were further incubated in 2% serum media for 48 hours and collected for histology and RNA isolation as described above. Statistical significance was determined using Student’s t-test on at least 3 independent experiments.

Mouse Valve Explant Culture
Mitral and tricuspid valve structures were dissected from 1-3 day postnatal homozygous Sox9$^{fl/fl}$. To prevent de-differentiation, valves were cultured as explants on pore filters as described above for chick valve culture. Adenovirus targeting Cre Recombinase (Ad-Cre) (Vector Labs) or GFP (Ad-GFP) was added at 1x10⁶ PFU/ per filter in DMEM supplemented with 4mM L-glutamine and 1% Penicillin/Streptomycin. In parallel control experiments, Ad-Cre and Ad-GFP were also added to valve explants from C57/Bl6 wild-type mice). After overnight incubation explants were supplemented with 2% FBS and maintained for an additional 48 hours prior to collection for histology and RNA isolation as described above for chick valve explants. For real-time PCR, statistical significance was determined based on fold changes in Ad-Cre infected Sox9$^{fl/fl}$ explants relative to Ad-GFP infected littermate controls (n=3). Similar results were observed in Ad-Cre infected wild-type (C57/Bl6) explants (n=2, data not shown).
Online Supplement References


Online Figure I. Lower magnification images corresponding to Figure 2. Calcium deposition was examined by von Kossa reactivity in aortic (Ao) (A, B) and mitral (mv) (C, D) valve leaflets from 12 month old Sox9^{fl/+};Col2a1-cre (B, D) and Sox9^{fl/+} littermate control mice (A, C). Note increased reactivity in Sox9^{fl/+};Col2a1-cre mice. Red arrows indicate direction of blood flow; black boxes indicate areas depicted at higher magnification in Figure 2.
Online Figure II. Lower magnification images corresponding to Figure 3. In situ hybridization to determine Osteonectin (ON) expression in the septal mitral valve leaflet from 12 month old control (Sox9fl/+ (A) and Sox9fl+/;Col2a1-cre (B) mice. Black boxes indicate areas depicted at higher magnification in Figure 3.
**Table I**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Control</th>
<th>Col2a1cre;Sox9flox/+</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS:d</td>
<td>0.96 ±0.15</td>
<td>0.86 ±0.21</td>
<td>0.307</td>
</tr>
<tr>
<td>LVID:d</td>
<td>4.163 ±0.75</td>
<td>3.75 ±0.47</td>
<td>0.221</td>
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<tr>
<td>LVPW:d</td>
<td>0.94 ±0.14</td>
<td>0.81 ±0.09</td>
<td>0.045</td>
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<tr>
<td>LVID:s</td>
<td>2.45 ±1.09</td>
<td>2.73 ±0.68</td>
<td>0.549</td>
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<tr>
<td>LVPW:s</td>
<td>1.33 ±0.16</td>
<td>1.18 ±0.15</td>
<td>0.109</td>
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<tr>
<td>LV Vol;d</td>
<td>80.38±33.12</td>
<td>70.55 ±30.85</td>
<td>0.554</td>
</tr>
<tr>
<td>LV Vol;s</td>
<td>29.55±20.30</td>
<td>30.77 ±19.50</td>
<td>0.906</td>
</tr>
<tr>
<td>%EF</td>
<td>66.32±10.71</td>
<td>54.19 ±23.37</td>
<td>0.186</td>
</tr>
<tr>
<td>%FS</td>
<td>36.88±7.528</td>
<td>30.03±11.02</td>
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<tr>
<td>IVRT</td>
<td>18.05±4.45</td>
<td>18.75 ±2.51</td>
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<tr>
<td>MV E</td>
<td>883.85±195.81</td>
<td>800.51 ±140.01</td>
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<tr>
<td>MV A</td>
<td>478.49±237.032</td>
<td>467.36 ±206.84</td>
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<tr>
<td>MV E/A</td>
<td>1.90 ±0.12</td>
<td>1.73 ±0.20</td>
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<tr>
<td>AV Peak Vel</td>
<td>835.43 ±48.38</td>
<td>896.00 ±91.51</td>
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<tr>
<td>AV Peak Grad</td>
<td>2.86 ±0.34</td>
<td>2.49 ±0.54</td>
<td>0.581</td>
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</tbody>
</table>

Echocardiography reveals no significant differences in cardiac structure and function in Sox9flox/+,Col2a1-cre mice compared to Sox9flox/+ controls at 12 months of age. IVS, interventricular septum; LVID, left ventricular internal diameter; LVPW, left ventricular posterior wall thickness; LV Vol, left ventricle volume (µL); RV Vol, right ventricle volume (µL); EF, ejection fraction (percent); FS, fractional shortening (percent); IVRT, isovolumetric relaxation time (ms); MV E, mitral valve E wave peak velocity (mm/s); MV A, mitral valve A wave peak velocity (mm/s); AV Peak Vel, aortic valve peak velocity (mm/s); AV Peak Grad, aortic valve peak pressure gradient (mmHg); d, diastole; s, systole. Values are in mm unless otherwise noted, ±standard deviation.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Symbol</th>
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<th>6 months</th>
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<th>12 Months</th>
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<td>Bone morphogenetic protein 10</td>
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<td>-1.53</td>
<td>0.330</td>
<td>1.87</td>
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</table>
Online Table II. Relative gene transcript levels in Sox9fl/+;Col2a1-cre mice as compared to Sox9fl/+ littermate controls at 3, 6 and 12 months of age. Quantitative changes in target gene expression following TLDA. Fold changes are calculated as $2^{(-\text{deltaCT})}$, with decreases (fold changes smaller than 1) transformed by dividing from -1 to allow more intuitive interpretation of the data. P-values given were generated by Student’s t-test, p<0.05 is considered significant. “References” lists citations from published studies indicating significant changes in homologous gene transcript or corresponding protein level in diseased human heart valves, or changes in gene expression in other published mouse models of calcific valve disease.