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Reduced Sox9 function promotes heart valve calcification phenotypes *in vivo*

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Abstract

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, the underlying etiology of disease onset and progression is largely unknown and valve replacement remains the most effective treatment. The SRY-related transcription factor *Sox9* is expressed in developing and mature heart valves, and its function is required for expression of cartilage-associated proteins, similar to its role in chondrogenesis. In addition to cartilage-associated defects, mice with reduced sox9 function develop skeletal bone prematurely, however the ability of sox9 deficiency to promote ectopic osteogenic phenotypes in heart valves has not been examined.

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 *Sox9*$^{fl/+}$;*Col2a1-cre* mice develop calcific lesions in heart valve leaflets from 3 months of age associated with increased expression of bone-related genes and activation of inflammation and matrix remodeling processes. Consistently, ectopic calcification is also observed following direct knockdown of *Sox9* in heart valves in vitro. Further, we show that retinoic acid treatment in mature

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Disclosures
None.

heart valves is sufficient to promote calcific processes in vitro, which can be attenuated by *Sox9* overexpression.

Conclusions—This study provides insights into the molecular mechanisms of heart valve calcification and identifies reduced *Sox9* function as a potential genetic basis for calcific valvular disease.

Keywords

Heart valves; calcification; Sox9; extracellular matrix; mouse model

Introduction

Calcification of heart valve structures affects more than 27% of the US population over age 65 and is the major contributor of heart valve malfunction.1 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.[2] Normal heart valve function requires organization of differentiated cell types and specialized extracellular matrix (ECM) arranged according to blood flow within the valve leaflet.[3] This defined tissue architecture provides the mechanical resilience and compressibility required to open and close the valve orifices effectively during the cardiac cycle.4 In diseased heart valves, loss of ECM organization is associated with changes in mechanical properties, ultimately leading to dysfunction.5,6 One of the most striking alterations in valve ECM homeostasis is ectopic bone-like matrix mineralization observed in calcific valve diseae.[7,8] At the functional level, this histopathological alteration results in stiffened leaflets, narrowing of the valve opening, and impaired blood flow.[9]

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.[8] 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.[2] Recent reports have also shown that regulatory mechanisms common to osteogenesis play a major role in valvular calcification.[10] 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.[7,10–12] The mechanisms initiating ectopic osteogenic processes in heart valves are not known, but recent reports have implicated a genetic basis, and therefore developmental origins.[9,13,15,16]

The diversified cell types and ECM that form the mature valve architecture are derived during embryogenesis from undifferentiated precursor cells of endocardial cushions.[17–19] Following endothelial to mesenchymal transformation and cushion formation, valve precursor cells differentiate and secrete specialized ECM.[17,20] The molecular mechanisms that regulate this process are not well defined, but are likely essential for normal 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.[21,22] 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.[21,23] In addition to cartilage defects, mice with reduced *Sox9* function develop premature skeletal ossification, suggesting an opposing role in bone formation.[24,25] The mechanisms of *Sox9* deficiency during skeletogenesis are not clear, although SOX9 function is sufficient to inhibit RUNX2-mediated activation of osteogenic target genes.[26] Upstream regulators of *Sox9* in cartilage and bone formation include retinoic acid (RA).[27–29] RA treatment of chondrogenic cells in vitro leads to decreased *Sox9* activity, associated with reduced expression of cartilage genes[29,30] and significant increases in *Runx2* and osteogenic processes.[31,32] These studies suggest that RA and Sox9 signaling play pivotal roles in promoting cartilage and bone phenotypes. However, the potential for RA-Sox9 signaling to promote osteogenic processes in heart valve connective tissue is not known.

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 [21], and increased calcium deposition is observed on valves from viable heterozygotes (*Sox9*$^{fl/+}$;*Col2a1-cre*) .[21] However molecular, cellular and functional analyses were lacking in this previous study.[21] Here, we report that *Sox9*$^{fl/+}$;*Col2a1-cre* mice develop calcific lesions within heart valve leaflets from 3 months of age with significant increases in bone-related genes and ECM remodeling and inflammatory processes. This osteogenic phenotype is recapitulated following direct *Sox9* knockdown in heart valve explants. Further, calcification in chick valve explants is promoted by RA treatment, which can be attenuated by *Sox9* overexpression. These data suggest that Sox9 plays an important role in preventing calcific processes in normal heart valves and identifies RA-Sox9 signaling as a suitable pathway for therapeutic targets in the prevention and treatment of calcific valvular disease.

Materials and Methods

Sox9$^{fl/fl}$ female mice[33] were bred with *Col2a1-cre* males (Jackson Laboratories)[34] to generate heterozygous offspring at expected Mendelian ratios. *Sox9*$^{fl/+}$;*Col2a1-cre* mice and *Sox9*$^{fl/+}$ littermate controls were aged and subject to echocardiography as described22. Following functional analysis, hearts were removed for RNA extraction from atrioventricular regions (containing the mitral and tricuspid valves) or whole hearts fixed, cryo-embedded, and sectioned for histological staining, in situ hybridization, and immunofluorescence as described. 21 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.35 For in vitro studies, neonate mouse or embryonic day 10 (E10) chick mitral valve explants were treated with DMSO, 1μmol/L RA, adenovirus- (Ad-) GFP, Ad-Sox9 or Ad-Cre. Expanded Materials and Methods are 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.[21] Using immunofluorescence, we also detect Sox9 expression in the mitral (mv) (Figure 1A), tricuspid, pulmonic (data not shown), and aortic (Ao) valve (Figure 1B) leaflets during remodeling stages at E17.5. In order to determine the role of *Sox9* in murine heart valves we employed 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 (mv) (Figure 1C), aortic (Ao) (Figure 1D), tricuspid and pulmonic valve leaflets (data not shown) from E15.5, consistent with Sox9 expression (Figures 1A, B).[21] In heart valves from 3 month old *Sox9*$^{fl/+}$;*Col2a1-cre*$^{-/-}$ (*Sox9*$^{fl/+}$) mice, Sox9 is expressed in cells throughout the valve leaflet (Figure 1E). However, following recombination in *Sox9*$^{fl/+}$;*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 non-recombined cells (arrowheads, Figure 1F) and cells along the edges of valves from *cre*-negative *Sox9*$^{fl/+}$ littermate controls (Figure 1E). Pecam staining indicates the endothelial cell layer (Figure 1E, F). These data validate successful recombination of Sox9 in targeted cells.

Increased calcium deposition in heart valve leaflets from adult *Sox9*$^{fl/+}$;*Col2a1-cre* mice

As determined by von Kossa reactivity, calcium deposits are observed in aortic (Ao) (Figure 2A, B), mitral (mv) (Figure 2C, D), and tricuspid (data not shown) valve leaflets of *Sox9*$^{fl/+}$;*Col2a1-cre* mice from 3 months of age (Figure 2B, D), but not in leaflets from control *Sox9*$^{fl/+}$ mice (Figure 2A, C). 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 *Sox9*$^{fl/+}$;*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 as calcification.[36] Notably, calcified lesions were observed only in leaflets and not other valvular structures. For quantitative comparisons, the area of von Kossa reactivity relative to the valve leaflet area as defined by Alcian blue counterstain was determined in tissue sections from mutant and control mice at 3, 6, and 12 months of age (Figure 2E, F). Heart valves from *Sox9*$^{fl/+}$;*Col2a1-cre* mice have significantly increased von Kossa reactivity compared to littermate *Sox9*$^{fl/+}$ mice at 3 months of age. Control *Sox9*$^{fl/+}$ mice consistently had little or no reactivity (0.22%±0.24). Due to the increased variability in von Kossa reactivity in 6 and 12 month old *Sox9*$^{fl/+}$;*Col2a1-cre* mice (Figure 2F), the area of calcium deposition did not significantly change with age, and differences compared to control mice were not observed (Figures 2E, F). However there is a trend toward increased lesion size with older age in *Sox9*$^{fl/+}$;*Col2a1-cre* mice (Figure 2E). Survival is comparable to controls at 12 months of age. These findings suggest that reduced *Sox9* function in *Col2a1*-derived cells during development promotes calcium deposition in adult heart valve leaflets.

Heart valves from *Sox9*$^{fl/+}$;*Col2a1-cre* mice show increased osteogenic signaling and inflammatory and ECM remodeling processes at 12 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.[37,7] To identify similar processes in *Sox9*$^{fl/+}$;*Col2a1-cre* mice, high throughput quantitative real-time PCR was performed using custom TaqMan low-density array (TLDA) cards designed to identify changes in genes characteristic of advanced human heart valve calcification.[10,38–44]

In heart valves from 12 month old *Sox9*$^{fl/+}$;*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 (ON)* (1.8-fold), *Osteopontin (OP)* (8.7-fold) *Osteoprotegerin (OPG)* (3.9-fold) and *Smpd3* (1.9-fold) compared to *Sox9*$^{fl/+}$ littermate controls (Figure 3A).[12,45] Online table II shows results for all the genes examined in *Sox9*$^{fl/+}$;*Col2a1-cre* mice at 3, 6, and 12 months of age. *In situ* hybridization confirms increased *ON* transcript in 12 month old *Sox9*$^{fl/+}$;*Col2a1-cre* valve leaflets over *Sox9*$^{fl/+}$ control (Figure 3B,C).

Consistent with previous observations in calcified human valves[38,45,48–50], inflammatory responses appear active in heart valves from 12 month old *Sox9*$^{fl/+}$;*Col2a1-cre* mice compared to controls. This is indicated by increased expression of *Vascular adhesion molecule-1 (Vcam-1)* (1.9-fold), a cell adhesion molecule involved in pro-inflammatory signal transduction[51], *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[52,53] (Figure 4A). Spatially, increases in Vcam-1 expression by immunostaining were observed throughout the valve leaflet (data not shown). Further, increases in inflammation-related transcripts observed in *Sox9*$^{fl/+}$;*Col2a1-cre* mice are significantly higher at 12 months compared to 3 months of age.

Valvular disease is frequently associated with ECM disorganization and excess collagen deposition, leading to fibrosis.[3] In *Sox9*$^{fl/+}$;*Col2a1-cre* mice, transcript levels of genes associated with tissue fibrosis[46,47] 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.[14] In valves from *Sox9*$^{fl/+}$;*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 *Sox9*$^{fl/+}$;*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 *Sox9*$^{fl/+}$;*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 *Sox9*$^{fl/fl}$ mice were infected with adenovirus (Ad) 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*, explants infected with Ad-Cre display 28% von Kossa reactivity (28%) (Figure 5B, C), compared to Ad-GFP controls (<5%) (Figure 5A, C), or explants from *Sox9*$^{+/+}$ mice infected with Ad-Cre (data not shown). This increase in calcium deposition is associated with a significant increase in *Osteopontin* and a trend towards increased *Runx2* expression. These data support a direct role for reduced *Sox9* function in promoting calcific heart valve phenotypes.

Retinoic acid treatment reduces *Sox9* expression and promotes osteogenic-like processes in mature chick heart valves

Retinoic acid (RA) has previously been identified as an upstream regulator of *Sox9* during cartilage and bone development.[28,29] In order to determine similar roles in mature heart valves intact avian mitral valve explants were subject to RA treatment in vitro. At embryonic day 10 (E10), avian heart valves express high levels of *Sox9*27 and exhibit highly organized ECM patterning indicative of maturation3. Following 1μmol/L RA treatment for 48 hours, von Kossa staining reveals significantly increased calcium deposition (21.7%) compared to DMSO controls (2.9%)(Figure 6A, B, G). Further, 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 1μmol/L RA were infected with adenovirus (Ad) expressing full length mouse *Sox9* (Ad-Sox9) or *GFP* (Ad-GFP). Consistent with Figure 6A, negligible von Kossa reactivity is observed in DMSO-treated explants infected with Ad-GFP (Figure 6C) or Ad-Sox9 (Figure 6E). In DMSO treated explants, endogenous chicken *Sox9* expression does not change, although 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, H). 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 *Sox9$^{fl/+}$;Col2a1-cre* mice. Further, these calcification processes are associated with activation of genes characteristic of ECM remodeling and inflammation. Despite these pathological phenotypes, valve function in adult *Sox9$^{fl/+}$;Col2a1-cre* mice is comparable to *Sox9$^{fl/+}$* littermate 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. Further, 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 valvular 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. [3,4,37]. Previous studies have shown that mature valve leaflets are rich in proteoglycans and express markers characteristic of cartilage tissue.[4] Further, our group has shown that cartilage-associated gene expression in developing heart valves requires Sox9, similar to findings in chondrogenic systems.[21,23,54,55] These findings highlight an important role for Sox9 in establishing the desired connective tissue composition in normal heart valves. In this current 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 misregulation 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

Increased Sox9 expression has previously been observed in myxomatous valves associated with increased proteoglycan production.[11] Conversely, *Sox9$^{fl/+}$;Col2a1-cre* mice develop calcific lesions on the surface of heart valve leaflets adjacent to blood blow. These findings 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 due to 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..[7,53] The underlying etiology that promotes pathological bone signaling in heart valves are not understood, but recent studies have identified *NOTCH1* mutations in patients with bicsupid aortic valve (BAV) and aortic valve calcification. [15] Interestingly, both *NOTCH1* and *SOX9* have been shown to repress RUNX2 activity.[15, 26],. Therefore one might predict that the osteogenic phenotypes observed in *Sox9$^{fl/+}$;Col2a1-cre* mice are due to lost repression of Runx2. However *Notch1* expression was not significantly

different in heart valves from *Sox9*$^{fl/+}$;*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 *Sox9*$^{fl/+}$;*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 aggravated phenotypes with additional exposure to known clinical risk factors including hypercholesterolemia and renal dysfunction, highlighting the multifactorial nature of this disease, and therefore exposing *Sox9*$^{fl/+}$;*Col2a1-cre* mice to such factors would be a plausible approach for future studies.[14,56,57]

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 retinoic acid inhibitors as anti-osteogenic 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.

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 current 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 pathologic state is associated with increased signaling of genes active during bone development, and activation of inflammatory and matrix remodeling process in calcified valves from *Sox9* mutant mice. Further, we have identified retinoic acid as an upstream repressor of *Sox9* function in promoting calcification in vitro. This study has 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard Abbreviations and Acronyms

Ao	aortic valve
Ad	adenovirus
DMSO	dimethyl sulfoxide
E	embryonic day
EC	endocardial cushions
ECM	extracellular matrix
ON	osteonectin
OP	osteopontin
OPG	osteoprotegrin
m	months
mv	mitral valve
tv	tricuspid valve
RA	retinoic acid
TLDA	TaqMan Low Density Array

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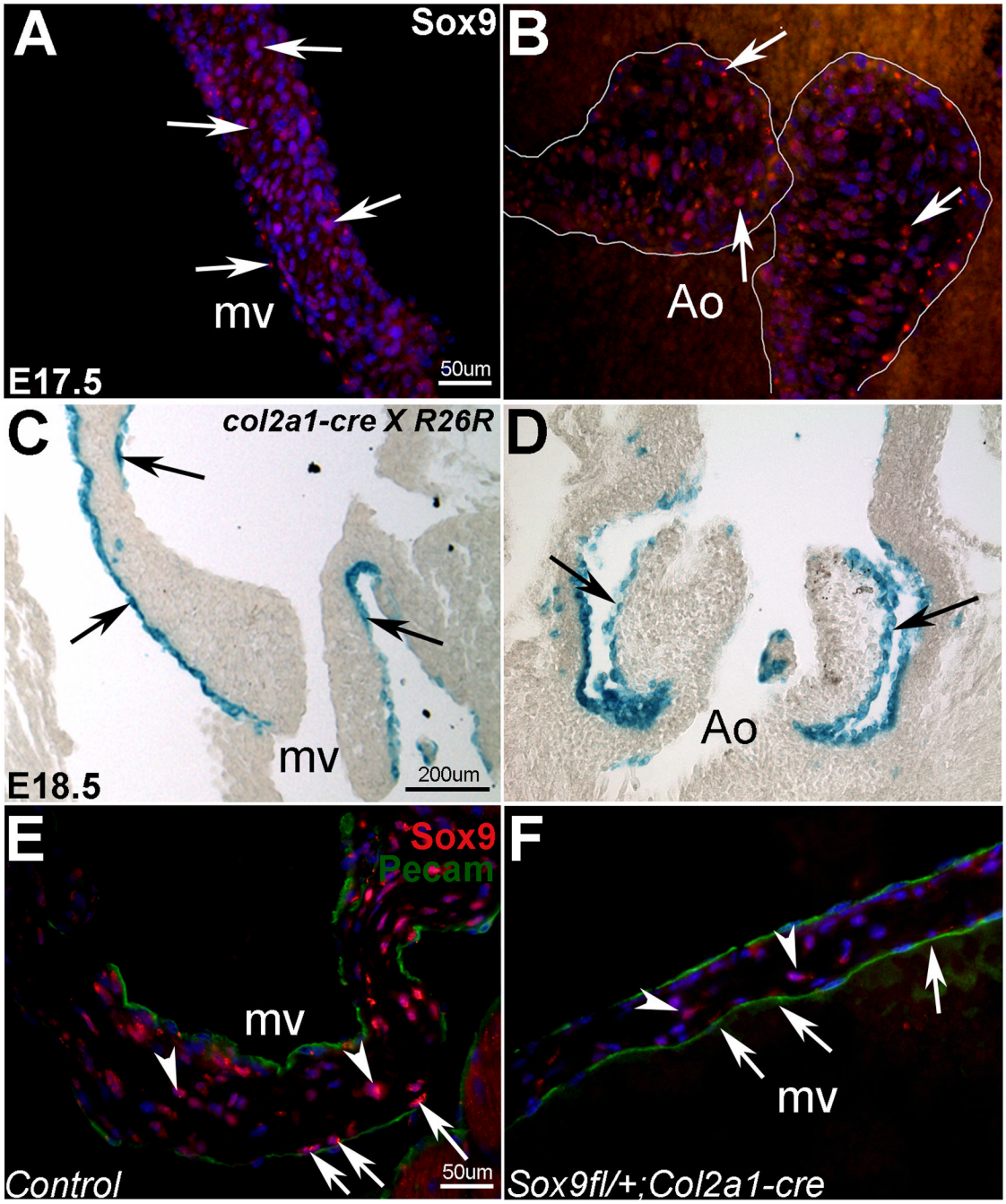

Figure 1. Sox9 expression is detected in developing and mature heart valve leaflets

Sox9 (red) is expressed in the mitral (mv) (A) and aortic valve (Ao) leaflets at E17.5 (arrows) (B). X-gal staining shows *Col2a1*-cre specific recombination (blue) in a subset of cells of the mitral (C) and aortic valves (D) in E18.5 *Col2a1-cre* × *Rosa26R* reporter mice. In $Sox9^{fl/+}$;*Col2a1-cre* mice (F), Sox9 expression (red) is reduced in recombined cells along the edge of the valve leaflets (arrows) compared to adjacent valve interstitial cells (arrowheads) not expressing the *Col2a1-cre* transgene. In control leaflets (E), cells with strong Sox9 expression are observed both at the leaflet surface and in the interstitium. Sox9 signal appears reduced in cells at the leaflet surface in $Sox9^{fl/+}$;*Col2a1-cre* mice (F). Pecam (green) indicates endothelial cells (E, F). Nuclei are stained with DAPI (A,B,E,F).

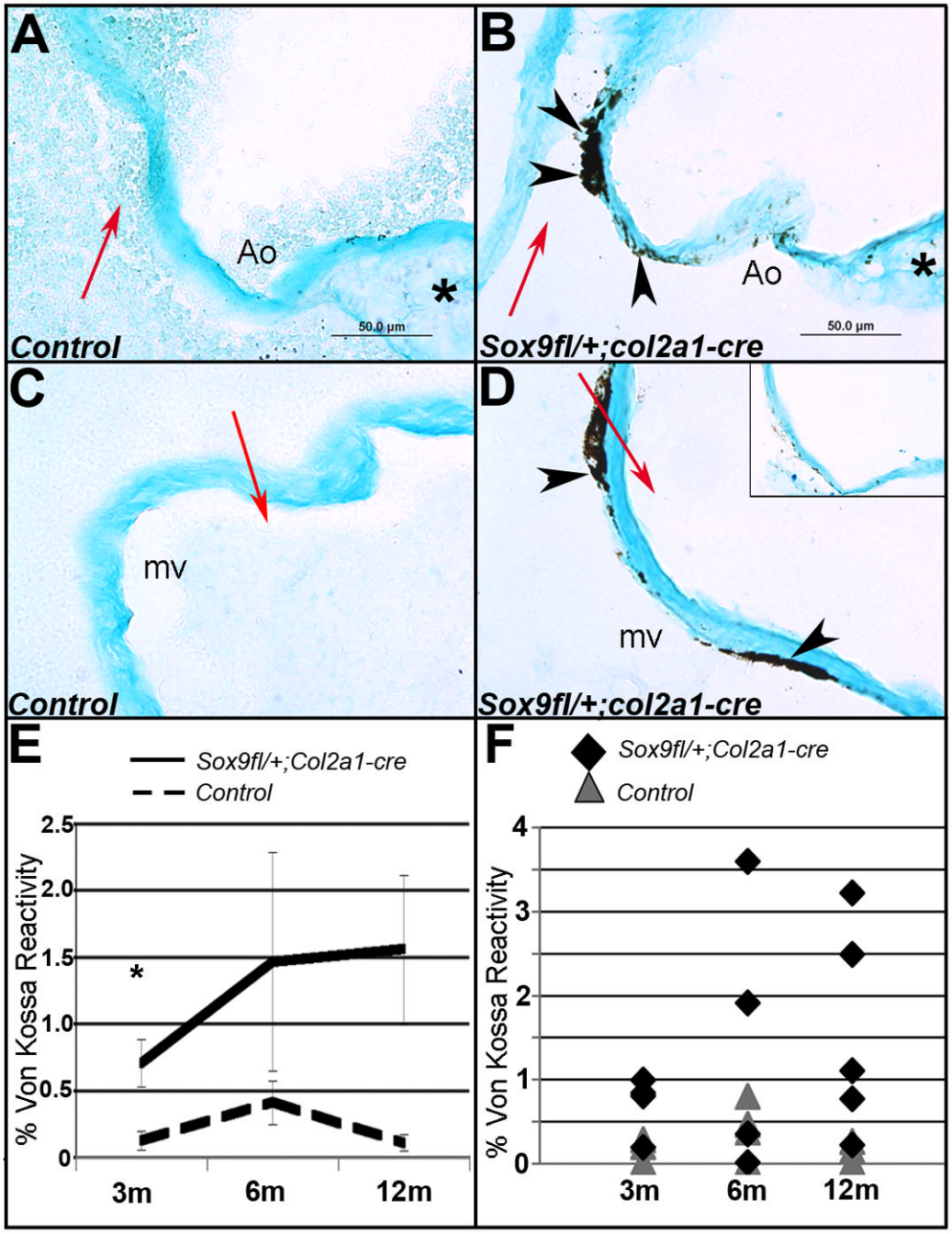

Figure 2. Von Kossa reactivity reveals increased calcium deposition in valve leaflets from Sox9$^{fl/+}$;*Col2a1-cre* mice

Von Kossa reactivity indicates calcium deposits (black, arrowheads) in aortic (Ao) (B) and mitral valve (mv) (D) leaflets adjacent to blood flow (red arrows) in tissue sections from *Sox9*$^{fl/+}$;*Col2a1-cre* mice at 12 months of age. Von Kossa reactivity was not significantly detected in littermate Sox9$^{fl/+}$ controls (A, C). Inset (D), no von Kossa treatment. Alcian blue counterstain defines the valve area (A–D), * indicates the base of the aortic valve, adjacent to the myocardium (A, B). Quantification of von Kossa reactivity as a percentage of valve area demonstrates significant increases in calcium deposits in *Sox9*$^{fl/+}$;*Col2a1-cre* mitral valve

leaflets with a trend toward increasing severity and variability with age (E, F). m, months. Low magnification images (A–D) are available in Online Figure I.

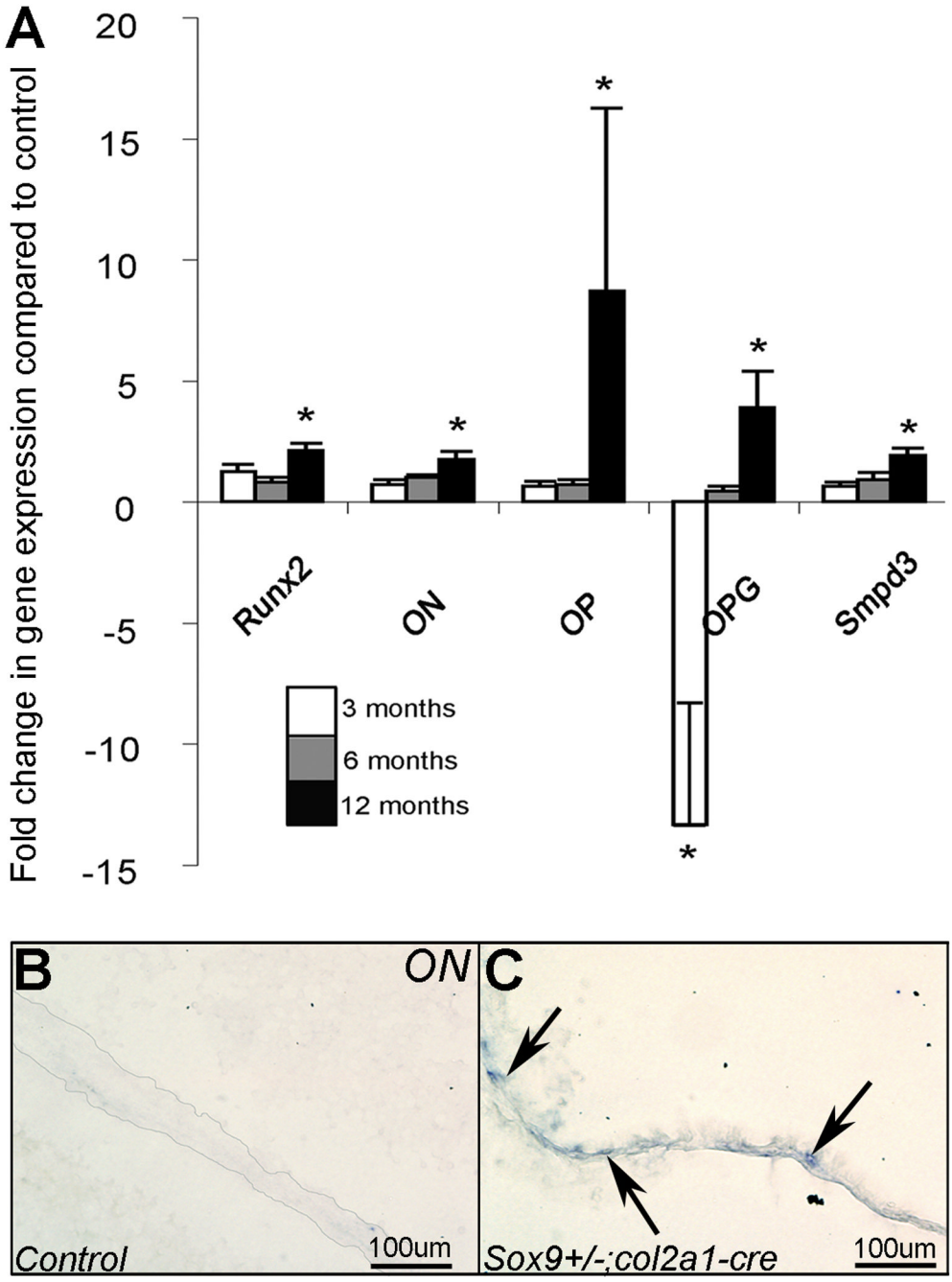

Figure 3. Transcript levels of bone-related genes are increased in 12 month old *Sox9$^{fl/+}$;Col2a1-cre* mice

(A) Normalized fold changes in transcripts levels of osteogenic-signaling genes *Runx2, Osteonectin (ON), Osteopontin (OP), Osteoprotegrin (OPG)* and *Smpd3* in *Sox9$^{fl/+}$;Col2a1-cre* versus *Sox9$^{fl/+}$* control mice at 3, 6, and 12 months as determined by TaqMan Low Density Array (TLDA). *p<0.05. *Osteonectin*, undetected by *in situ* hybridization in control mitral valve leaflets (B), is detected in a 12 month *Sox9$^{fl/+}$;Col2a1-cre* mouse mitral valve leaflet (arrows, C). Low magnification images (B, C) are available in Online Figure II.

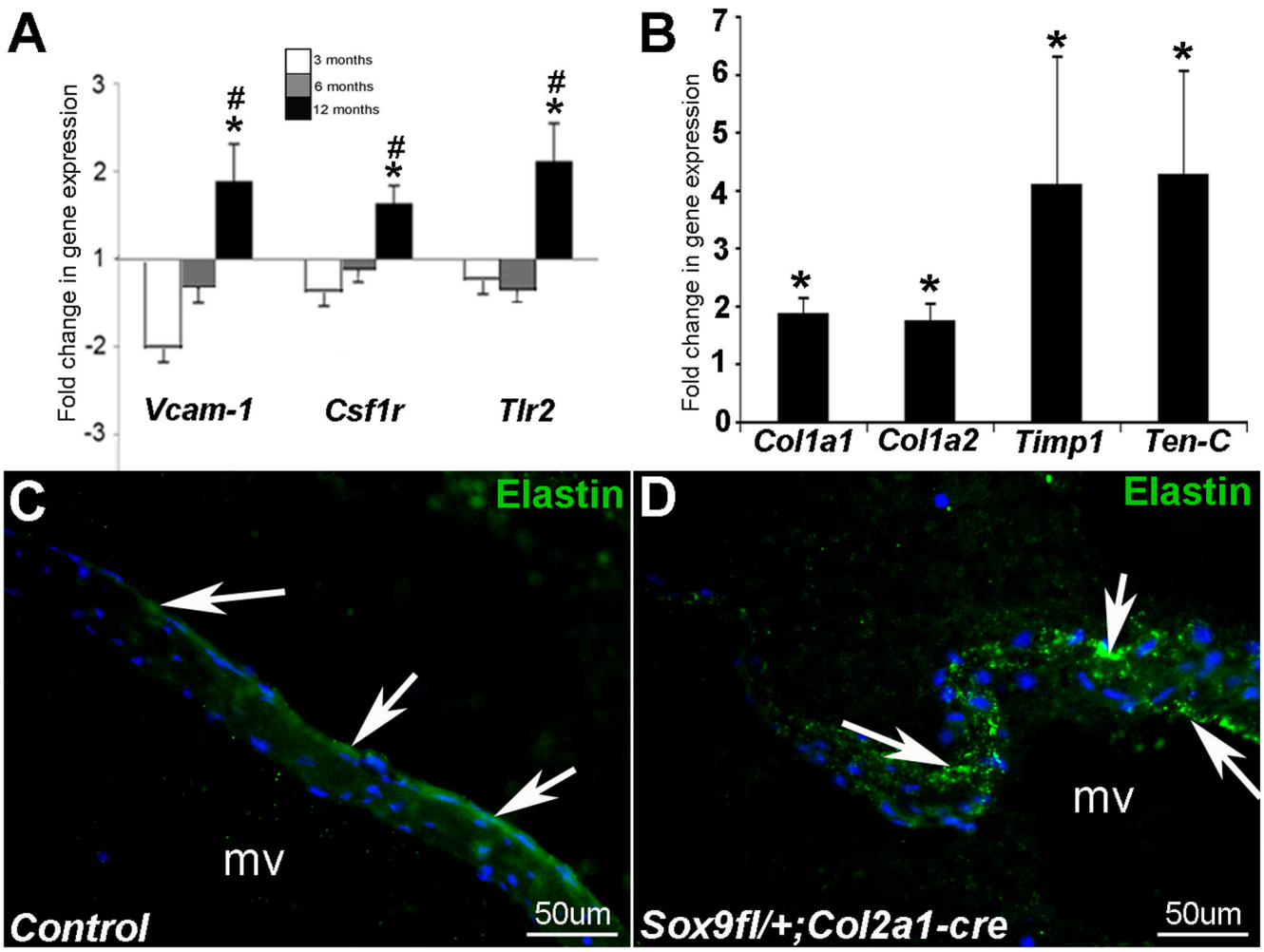

Figure 4. Inflammation and ECM remodeling-related genes are increased in 12 month old *Sox9$^{fl/+}$;Col2a1-cre* mice

(A) Inflammation-associated genes *Vcam-1*, *Csfr1*, and *Tlr2* are increased in*Sox9$^{fl/+}$;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$^{fl/+}$;Col2a1-cre* mice. Elastin (green) is detected on the atrial surface of the mitral valve in control *Sox9$^{fl/+}$* mice (arrows, C). Elastin fibers are fragmented and disorganized in *Sox9$^{fl/+}$;Col2a1-cre* mitral valve leaflets (arrows, D).

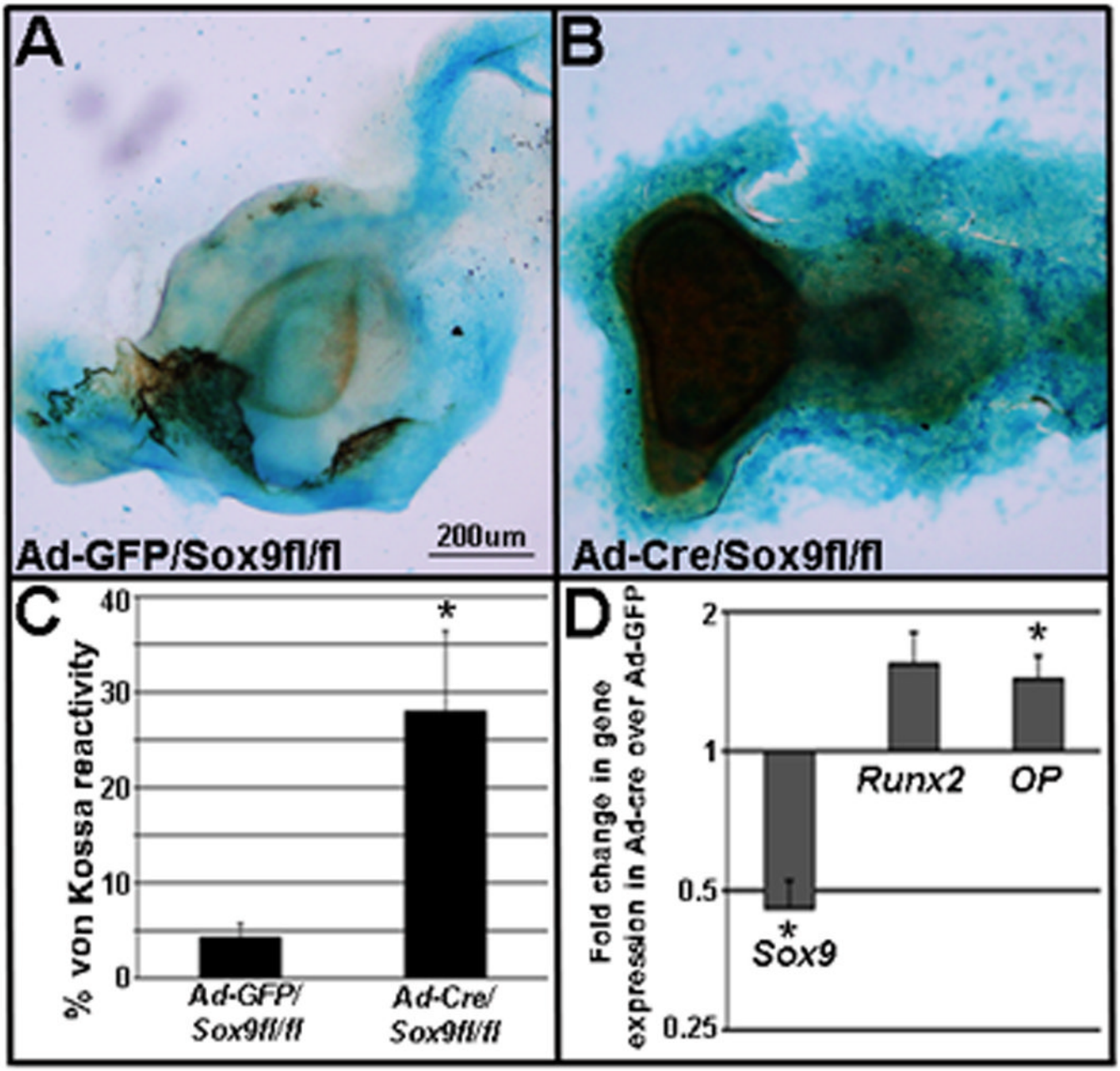

Figure 5. *Sox9* knockdown increases calcification phenotypes in mouse valve explants

(A, B) von Kossa reactivity in $Sox9^{fl/fl}$ 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, while *Runx2* and *Osteopontin (OP)* are increased relative to GFP-treated controls. *p>0.05.

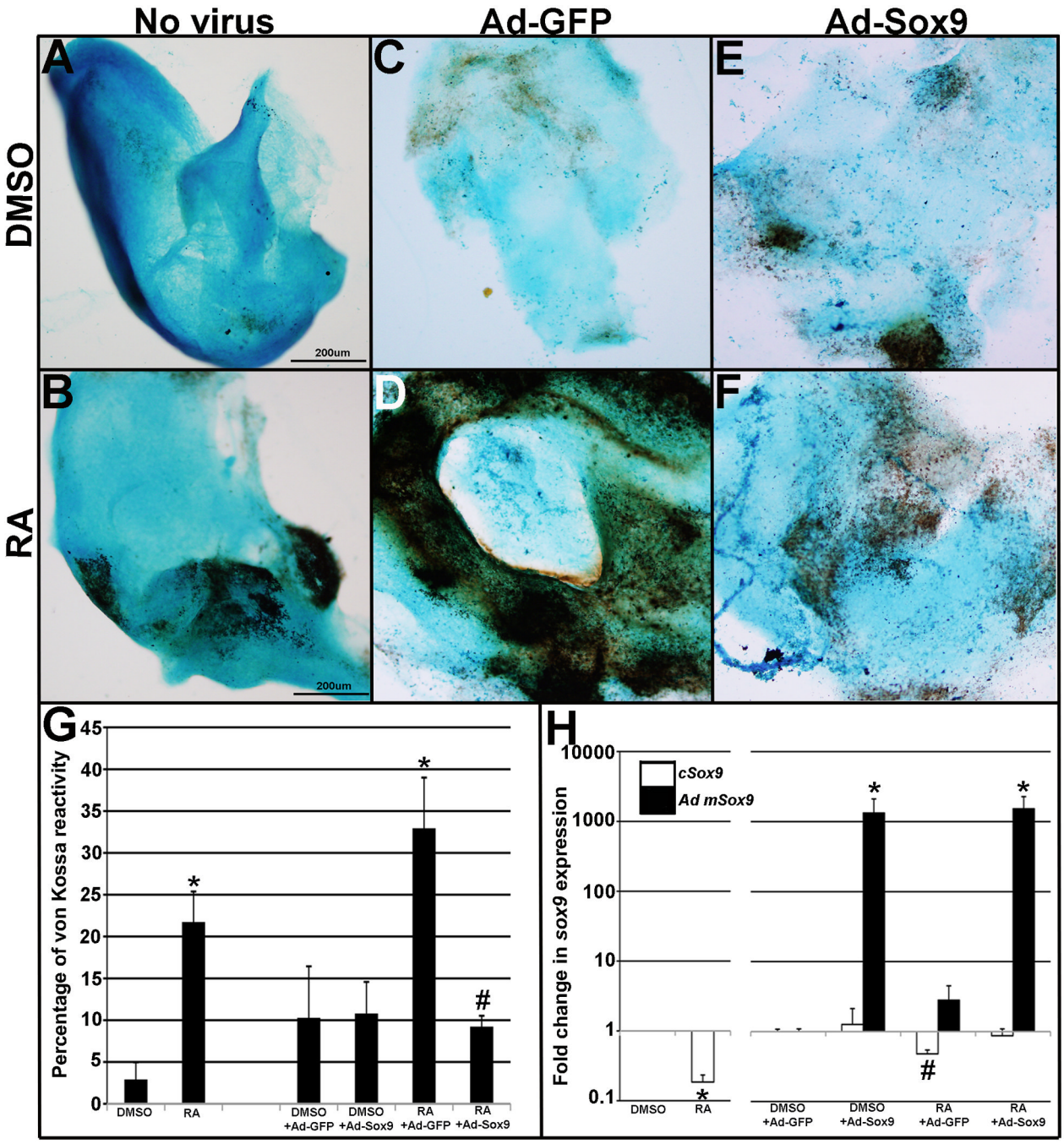

Figure 6. Retinoic acid treatment reduces *Sox9* expression and promotes matrix mineralization

Cultured E10 chick mitral valve explants were treated with DMSO (A, C, E) or RA (B, D, F) and subject to infection with Ad-GFP (C, D) or Ad-Sox9 (E, F), or cultured without virus (A, B). (G) Quantitation of von Kossa reactivity in treated explants, normalized to area (Alcian blue staining). *p<0.05 compared to relative DMSO control, #p<0.05 compared to RA and Ad-GFP infection. (H) Normalized fold change in endogenous chicken *Sox9 (cSox9)* and adenoviral mouse *Sox9 (Ad mSox9)*. *p<0.05 compared to respective DMSO- or RA-treated controls. #p<0.05 compared to DMSO+Ad-GFP.