Scleraxis Is Required for Cell Lineage Differentiation and Extracellular Matrix Remodeling During Murine Heart Valve Formation In Vivo

Agata K. Levay, Jacqueline D. Peacock, Yinhui Lu, Manuel Koch, Robert B. Hinton, Jr, Karl E. Kadler, Joy Lincoln

Abstract—Heart valve structures, derived from mesenchyme precursor cells, are composed of differentiated cell types and extracellular matrix arranged to facilitate valve function. Scleraxis (scx) is a transcription factor required for tendon cell differentiation and matrix organization. This study identified high levels of scx expression in remodeling heart valve structures at embryonic day 15.5 through postnatal stages using scx-GFP reporter mice and determined the in vivo function using mice null for scx. Scx−/− mice display significantly thickened heart valve structures from embryonic day 17.5, and valves from mutant mice show alterations in valve precursor cell differentiation and matrix organization. This is indicated by decreased expression of the tendon-related collagen type XIV, increased expression of cartilage-associated genes including sox9, as well as persistent expression of mesenchyme cell markers including mst1 and snail1. In addition, ultrastructure analysis reveals disarray of extracellular matrix and collagen fiber organization within the valve leaflet. Thickened valve structures and increased expression of matrix remodeling genes characteristic of human heart valve disease are observed in juvenile scx−/− mice. In addition, excessive collagen deposition in annular structures within the atrioventricular junction is observed. Collectively, our studies have identified an in vivo requirement for scx during valvulogenesis and demonstrate its role in cell lineage differentiation and matrix distribution in remodeling valve structures. (Circ Res. 2008;103:948-956.)

Key Words: development ■ extracellular matrix ■ heart valves ■ mouse heart development ■ transcription factors

Congenital cardiovascular defects arising from abnormal formation of cardiac structures are the most common cause of infant mortality, and recent reports suggest that heart valve disease manifested in adults has origins in valve development.1-3 Heart valves are dynamic structures composed of diversified cell types and extracellular matrices (ECMs), organized to facilitate valve function.3 Valve formation initiates during embryogenesis with endotheelial-to-mesenchymal transformation in the atrioventricular (AV) canal and outflow tract regions.3-6 Consequently, swellings of endocardial cushions (ECs), composed of highly proliferative mesenchyme cells within matrix-rich cardiac jelly, form the precursor pool for future heart valve structures.6,7 Once established, ECs undergo extensive remodeling involving differentiation of mesenchyme precursor cells and spatially restricted deposition of specialized ECM.3,8 In mature valve leaflets and supporting structures, the order and integrity of connective tissue within valvular compartments is essential for valve function. This is evident in diseased or malfunctioning valves that display aberrations in connective tissue organization and cell lineage distribution.9 Despite substantial clinical implications, the regulatory processes required for valve remodeling are largely unknown, yet essential for normal heart valve formation and function.

Recent studies have elucidated that regulatory hierarchies active during heart valve remodeling are parallel with other connective tissue systems.3 Developing heart valve structures are composed of multiple cell types, including those that express the basic helix–loop–helix transcription factor scleraxis (scx) and other tendon-associated structural proteins.10 Although not exclusive to tendinous tissues, scx is predominantly expressed by tendon progenitors and differentiated tendon cells.11,12 In vivo, scx is required during stages of tenocyte differentiation and is essential for organization of tendinous matrix and, consequently, tendon function.13 Avian somite studies have shown that fibroblast growth factors (FGFs) and intermediate extracellular signal-regulated kinase (ERK)1/2 signaling regulate scx expression.14,15 However, direct downstream targets of scx signaling are largely unknown, although studies show that altered scx function leads...
to changes in expression of tendinous-structural proteins, including tenascin, type I and XIV collagen, and tenomodulin. Even though the transcriptional regulation of these potential target genes by scx have not yet been defined, these studies highlight an important role for scx in regulating cell-specific expression of matrix proteins in connective tissue systems.

In the chick heart, scx is expressed in a subset of valve precursor cells following EC formation. Expression is maintained during valve remodeling with high levels observed in valve-supporting structures. The in vivo function of scx during valvulogenesis has not yet been reported, but insights into its regulation have been gained from previous in vitro studies in chick. In cultured valve precursor cells, scx expression is induced by FGF4 treatment and is associated with increased phospho-ERK1/2 activity, suggesting that heart valve development shares common regulatory pathways with other connective tissue systems. Interestingly, induced expression of scx by FGF-ERK1/2 signaling is associated with downregulation of sox9, a transcription factor also expressed in valve precursor cells and common to chondrogenic tissue. Because scx- and sox9-positive cells are derived from a common population of precursor cells of the ECs, these studies suggest that valve precursor cell lineage differentiation is tightly regulated to establish and maintain a balanced distribution of ECM proteins within valve compartments required for normal valve function.

In this study, we use scx-/- mice to determine its requirement during stages of valvulogenesis and examine effects of scx loss of function on adult heart valve structure and function in vivo. Scx-/- mice display thickened heart valve structures during embryonic valve remodeling stages in association with defects in valve precursor cell lineage differentiation and ECM organization. Increased ECM production, characteristic of pathological fibrosis, is observed in valve and annular structures of scx-/- mice from postnatal stages. Heart function in juvenile scx-/- mice is subtly, but not significantly, impaired compared to wild-type littermates; however, the reduced viability of mutant mice to reach adult stages hindered our examination of valve degeneration. Collectively, data from this study reveal an important role for scx in normal embryonic heart valve remodeling in vivo.

**Materials and Methods**

Scx-/- mice back-crossed with scx-GFP reporter mice were genotyped as previously described and crossbred to generate scx--/-, scx-/-, and scx-/- control mice. Mendelian ratios were recorded at embryonic times points, as well as shortly after birth. In addition, body weights were reported in viable animals from postnatal stages. Hearts were collected at embryonic day E12.5, E14.5, E16.5, and E17.5, postnatal, and juvenile stages from E12.5, E14.5, E16.5, and E17.5 control mice. Whole hearts were fixed for in situ hybridization, RNA isolation for subsequent TaqMan low-density array (TLDA) or Western blot analyses. All animal procedures were approved and performed in accordance with institutional guidelines. An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

**Results**

**Scleraxis-GFP Is Highly Expressed From Remodeling Stages of Heart Valve Development**

Scx-GFP transgene expression recapitulates endogenous expression, and therefore reporter mice were used to indicate scx expression during stages of valve development. At E12.5, scx-GFP is detected in few valve precursor cells within the ECs (arrow, Figure 1A), as well as in the body wall and atrial septum (arrow and arrowhead respectively, Figure 1A). By E16.5, scx-GFP expression is prominent in remodeling tricuspid and mitral valve leaflets (arrows, Figure 1B), as well as in the fibrous continuity (arrowhead, Figure 1B). In postnatal hearts, scx-GFP is expressed throughout the maturing AV (arrows, Figure 1C) and aortic (arrowheads, Figure 1C) valve structures. The widespread expression pattern observed in the mouse is somewhat different from the more restricted expression pattern observed in supporting structures of chick valves. This maybe attributed to differences in the complexity of the valve at the cellular level across species. At juvenile stages, scx-GFP expression is restricted to the atrial surface of the AV valve leaflets (arrows, Figure 1D), with undetectable expression observed along the ventricular region (arrowhead, Figure 1D). Further in situ hybridization analysis confirms the specificity of the scx-GFP transgene in the AV valves (supplemental Figure IA, IC, IE, IG in the online data supplement), and extended analysis includes detection of scx in aortic valves. Scx is detected at very low levels in aortic ECs at E12.5 (supplemental Figure IB), in contrast to high levels observed during remodeling stages from E16.5 to juvenile (supplemental Figure ID, IF, and IH). Collectively, these expression studies demonstrate
that *scx* is most predominantly expressed during remodeling and maturation stages of embryonic valvulogenesis.

**Scx**<sup>−/−</sup> Mice Are Not Observed at Expected Mendelian Ratios Shortly After Birth and Display Thickened Heart Valve Structures by E17.5

*Scx*<sup>−/−</sup> pups were not observed at expected Mendelian ratios shortly after birth; however, ratios during embryogenesis appear normal (Table). Notably, 16% (compared to the expected 25%) of *scx*<sup>−/−</sup> mice were observed soon after birth, and only 47% of viable null mice survived to 2 months of age despite efforts to maximize longevity (see expanded Materials and Methods section in the online data supplement). The cause of early lethality is not known and warrants further investigation. In our hands, *scx*<sup>−/−</sup> mice recapitulate previously described phenotypes including limited use of all paws and reduced functionality of back muscles (data not shown).

We also report a significant reduction in body weight of *scx*<sup>−/−</sup> mice at 6 weeks (53%) and 2 months of age (31%) compared to *scx*<sup>+/+</sup> control littermates (Figure 2A). To determine the effects of *scx* loss of function on valve formation, we examined gross morphology of developing valve structures in *scx*<sup>+/+</sup> and *scx*<sup>−/−</sup> mice. ECs at E12.5 and valve primordia at E16.5 from *scx*<sup>−/−</sup> mice are indistinct from *scx*<sup>+/+</sup> mice (data not shown). However, pentachrome staining at E17.5 indicates that *scx*<sup>−/−</sup> mice (Figure 2C, AV valve shown) display significantly thickened valve structures compared to *scx*<sup>+/+</sup> control mice (arrows, Figure 2B). In addition the overall shape of the heart appears “globular” in *scx*<sup>−/−</sup> mice (arrowheads, Figure 2C compared to 2B). Comparisons of tissue sections from *scx*<sup>−/−</sup> and *scx*<sup>+/+</sup> mice back-crossed with *scx*-GFP mice demon-

**Table. Mendelian Ratios of *scx*<sup>−/−</sup> Mice**

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<td>Birth</td>
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Expected Mendelian ratios are observed during embryogenesis; however, expected ratios are not seen shortly after birth.

**Figure 2.** Valve structures from *scx*<sup>−/−</sup> mice have increased area from E17.5. A, At postnatal stages, *scx*<sup>−/−</sup> mice display smaller body weights. Pentachrome staining at E17.5 (C and D) and GFP expression at postnatal stages (D and E) show thickened heart valve structures in *scx*<sup>−/−</sup> (B and D) animals compared to *scx*<sup>+/+</sup> (C and E). Arrows in D and E show valve leaflet structures, and arrowheads indicate the “globular-shaped” myocardium in B and C and chordae tendineae in D and E. F, Quantitative analysis to show increased aortic (Ao), tricuspid (tv), and mitral (mv) valve area in *scx*<sup>−/−</sup> mice. G and H, Cell number counts (shown by red dots), based on nuclear DAPI staining, show decreased cell number per 50 μm<sup>2</sup> area in septal mitral valve (smv) leaflets from *scx*<sup>−/−</sup> mice at E17.5 compared to controls (l). LV indicates left ventricle.
strate an increase in valve area likely indicative of thickness in \textit{scx}^{-/} mice at postnatal stages (arrows, Figure 2D and 2E). Two-dimensional morphometric analysis from tissue sections collected from \textit{scx}^{-/} and \textit{scx}^{+/+} mice quantitatively illustrate a significant increase in mitral, tricuspid, and aortic valve area in null animals compared to controls that significantly worsens over time (Figure 2F). Because increased valve area was first observed at E17.5 in \textit{scx}^{-/} mice, subsequent molecular analyses was performed at this time point to determine primary effects of \textit{scx} loss of function on valvulogenesis.

To propose a possible explanation for the increase in valve area, changes in cell proliferation and apoptosis were determined in \textit{scx}^{+/+} and \textit{scx}^{-/} mice at E17.5; however, no significant differences were observed (data not shown). To further support the lack of observed changes in cell number, the average number of cells within a 50 \mu m^2 area of the septal mitral valve leaflet of E17.5 \textit{scx}^{-/} (Figure 2G and 2I) and \textit{scx}^{+/+} (Figure 2H and 2I) mice were counted from tissue sections. Interestingly, the average number of cells per area, as depicted by DAPI (4',6-diamidino-2-phenylindole) nuclear stain, was significantly decreased in \textit{scx}^{-/} mice (boxed areas, Figure 2G and 2H). This further suggests that an increase in cell number did not contribute to the increased valve area phenotype but more likely an increase in matrix deposition. These findings show that from stages of valve remodeling, heart valve area in \textit{scx}^{-/} mice is increased despite no change in cell number.

**Type XIV Collagen Expression Is Downregulated and Collagen Fiber Organization Is Disrupted in Heart Valves From \textit{scx}^{-/} Mice at E17.5**

Type XIV collagen is highly expressed in tendon tissue\(^{24}\) and previous studies have shown that \textit{type XIV collagen} expression is lost in tendon forelimbs in \textit{scx}^{-/} mice.\(^{13}\) Type XIV collagen expression has not yet been shown in heart valve structures. Therefore, to determine whether it is present in valvular structures, and to examine similar downregulation in heart valves of \textit{scx}^{-/} mice, we performed immunohistochemistry. In \textit{scx}^{+/+} mice, type XIV collagen is highly expressed throughout the valve leaflets (arrows, Figure 3A), with notably less expression detected on the leaflet surface (arrowheads, Figure 3A). In contrast, heart valves from \textit{scx}^{-/} mice show significantly decreased levels of type XIV collagen expression in both AV (arrows, Figure 3B) and aortic valve regions (data not shown). Downregulation of type XIV collagen expression is restricted to valvular structures, because regions that do not express \textit{scx}, including the great vessels, continue to express high levels of type XIV collagen (arrows, Figure 3C and 3D). Interestingly, other tendon-associated collagens, including \textit{type XI collagen}, did not change (data not shown). Ultrastructural analyses using transmission electron microscopy of postnatal aortic valve leaflets reveals highly organized bundles of parallel collagen fibers running longitudinal (arrows, Figure 3E and 3G) and perpendicular (arrowheads, Figure 3E and 3G) within the valve leaflet of \textit{scx}^{-/+} mice. In contrast, collagen fiber organization is in disarray in \textit{scx}^{-/} mice (Figure 3F and 3H). Organized bundles of parallel collagen fibers are not apparent in mutant animals and fibers appear fragmented and randomly oriented (arrows, Figure 3F and 3H). Collectively, these studies show that heart valves from \textit{scx}^{-/} mice have reduced expression of the tendon-associated collagen type XIV and disorganized collagen fiber alignment.

**Mesenchyme Cell Markers Are Persistently Expressed in Heart Valves From \textit{scx}^{-/} Mice at E17.5**

Undifferentiated valve precursor cells within the ECs express cell markers associated with mesenchyme cells.\(^{25-27}\) As
leaflets from scx<sup>−/−</sup> mice. In summary, mesenchyme cell markers are persistently expressed in scx<sup>−/−</sup> mice during stages of heart valve remodeling.

**Cartilage-Associated Gene Markers Are Increased in scx<sup>−/−</sup> Mice at E17.5**

Cartilage- and tendon-like cell types that contribute to mature valve structures are derived from a common population of valve precursor cells within ECs. To examine effects of scx loss of function on valve precursor cell differentiation into cartilaginous cell types, we used TLDA analyses to quantitatively measure changes in gene expression of cartilage-associated genes previously observed in heart valves at E17.5. At E17.5, heart valves from scx<sup>−/−</sup> mice show increased expression of sox<sub>5</sub>, sox<sub>9</sub>, cartilage oligo matrix protein (COMP), and cartilage link protein (CLP) compared to scx<sup>+/+</sup> mice (Figure 5A). Increased expression of CLP and sox<sub>9</sub> in heart valves from scx<sup>−/−</sup> mice is also seen by immunohistochemistry. In scx<sup>−/−</sup> mice at E17.5, CLP is more highly expressed toward the ventricular region of the valve leaflets (arrows, Figure 5B), with lower levels of expression observed along the atrial surface (arrowhead, Figure 5B), whereas in scx<sup>+/+</sup> mice, CLP is expressed at high levels throughout the valve leaflet (arrows, arrowhead, Figure 5C). Similarly, sox<sub>9</sub> expression is also notably higher in valve leaflets from scx<sup>−/−</sup> mice (arrows, Figure 5E) compared to scx<sup>+/+</sup> mice (Figure 5E). Increased sox9 expression in heart valves from scx<sup>−/−</sup> mice is also detected by Western blot (Figure 5F). In summary, heart valves from scx<sup>−/−</sup> mice express higher levels of cartilage-associated genes at E17.5 compared to control mice.

**Heart Valves and Annular Structures From Juvenile scx<sup>−/−</sup> Mice Display Characteristics of Pathological Fibrosis**

Immunohistochemistry and TLDA analyses were used to determine changes in expression of markers associated with pathological matrix remodeling in scx<sup>−/−</sup> mice at 2 months of age. Heart valves from juvenile scx<sup>−/−</sup> control mice show low levels of tenascin-C expression (Figure 6A), whereas expression is detected at high levels in thickened valves from scx<sup>−/−</sup> mice, especially in ventricular regions (arrowhead, compared to arrow, Figure 6B). Likewise, periostrin is expressed at low levels in juvenile heart valves from scx<sup>−/−</sup> mice (Figure 6C), in contrast to high levels observed in scx<sup>−/−</sup> mice (arrows, Figure 6D). These findings are supported by increased tenascin-C and periostrin expression in scx<sup>−/−</sup> mice observed at the transcript level (Figure 6E). In addition, increased expression of matrix remodeling enzymes mmp3 and timp3 (Figure 6F) are also detected. Masson’s trichrome staining was used to examine collagen deposition as a measure of fibrosis in scx<sup>−/−</sup> and scx<sup>+/−</sup> juvenile mice. Increased staining was observed in the annular regions of AV and aortic valves in scx<sup>−/−</sup> mice (mitral valve, arrow, Figure 6H). In summary, loss of scx function is associated with increased deposition of remodeling-associated ECM in valve and annular regions.
Discussion

There are increasing reports to suggest that heart valve disease manifested later in life has origins during embryonic development. Diseased valves are often characterized by excessive ECM production, matrix disorganization, and altered cell distribution, leading to increased thickness and ultimately malfunctioning valves. The genetic etiology underlying these pathological phenotypes are largely unknown, but may be related to defects in signaling pathways important for cell lineage differentiation and matrix deposition programs during embryonic valve remodeling. Scx is expressed in tendon progenitor cells and required for formation, organization, and function of tendon tissue. This study and others report additional expression in developing heart valve structures. The requirements for scx during mouse heart valve development, and EC formation appears normal in scx mice. Scx valve development were examined in vivo using mice null for scx. Scx expression is not detected during initial stages of valve development, and EC formation appears normal in scx mice, suggesting that scx is not required at this time. However, by E17.5, valve structures from scx−/− mice are significantly thickened and show defects in cell lineage differentiation and matrix organization. This is marked by decreased expression of the tendon-associated collagen type XIV, and increased expression of cartilage-associated genes, as well as persistent expression of mesenchyme cell markers. Electron microscopy of postnatal valves reveals disorganized connective tissue architecture, including random orientation of shortened collagen fibers in valves from scx−/− mice. Juvenile scx−/− mice display progressively thickened valve structures with increased expression of fibrosis-associated ECM proteins periostin and tenascin-C, as well as remodeling proteases associated with pathological matrix remodeling. Increased collagen deposition was also observed in annular regions. These data provide evidence for a role for scx during remodeling of heart valve structures in vivo and provide insights into molecular mechanisms required for normal heart valve formation and maintenance.

Scx Is Required for Cell Lineage Differentiation and Matrix Distribution During Heart Valve Remodeling

Previous studies have shown that valve remodeling requires differentiation of several cell types including tendon- and cartilage-like cell lineages from multipotent valve precursor cells, as well as deposition of specific ECM. This process is tightly regulated to establish and maintain normal valve structure and function. In this study, we show that in the absence of the tendon-associated gene scx, cell differentiation and matrix deposition are altered in many ways. Firstly, the contribution of a tendon-like connective tissue to the developing valve structures is attenuated, marked by a dramatic decrease in the tendon-associated collagen type XIV; these findings are consistent with previous tendon studies in scx−/− mice. Secondly, valve precursor cells in scx−/− mice appear to be preferentially promoted toward the chondrogenic-like differentiation pathway, indicated by increased expression of sox9 and cartilage-associated structural proteins. Thirdly, it appears that without scx, a population of valve precursor cells remain somewhat undifferentiated and continue to express high levels of mesenchymal cell markers including snai1, mxi1, and tbx20. In addition to gross valve defects, the ventricles of scx−/− mice appear spherical in
shape, a feature associated with myocardial remodeling in the failing heart. Because scx expression was not observed in the ventricular myocardium, this structural defect is likely an indirect effect from scx loss of function. Collectively, these studies identify a requirement for scx in cell lineage differentiation and matrix deposition during stages of heart valve remodeling in vivo. In addition, these findings provide insights into potential target genes of scx during valvulogenesis that warrant further investigation.

**Figure 6.** Increased ECM deposition and remodeling is observed in the AV region of juvenile scx<sup>−/−</sup> mice. A through D, Immunohistochemistry was used to determine expression levels of tenascin-C (Tn-C) (A and B) and periostin (C and D) in scx<sup>−/−</sup> mice compared to scx<sup>+/+</sup> mice at 2 months of age. Tn-C (A and B) and periostin (C and D) expression is significantly increased in valve leaflets from scx<sup>−/−</sup> mice. B, Note increased expression of Tn-C in ventricular regions of valve leaflet from scx<sup>−/−</sup> mice (arrowhead, compared to arrow). E and F, TLDA analysis shows significantly increased expression of Tn-C and periostin at the transcript level in scx<sup>−/−</sup> mice compared to scx<sup>+/+</sup> mice, as well as mmp3 and timp3. G and H, Trichrome staining to show collagen deposition (blue) in AV annular structures. Arrows indicate the posterior paraseptal annulus structure at the left AV groove. Note increased collagen deposition in scx<sup>−/−</sup> mice (arrows). mv indicates mitral valve; mmv, mural mitral valve.

**Scx Is Required for Connective Tissue Organization and Homeostasis**

Scx<sup>−/−</sup> mice show significant defects in ECM organization and distribution. Alterations in the organization of collagen fiber bundles observed in these mutant mice may be attributable to the loss of type XIV collagen, a network-forming collagen that has previously been shown to modulate fibril assembly and organization in several connective tissue systems. Because changes in cell proliferation or apoptosis
were not detected in scx\(^{-/-}\) mice at E17.5, it seems likely that increased matrix deposition is the cause of increased valve tissue thickness. This is recognized by an increase in cartilaginous matrix at E17.5, as well as excessive deposition of pathological matrix remodeling proteins in valves from scx\(^{-/-}\) mice by juvenile stages. In addition, excessive collagen deposition is observed in the annular structures around the AV junction. These observations at later juvenile stages are likely secondary to the primary effects on cell lineage differentiation and matrix organization resulting from the loss of scx function. Nonetheless, findings from juvenile scx\(^{-/-}\) mice support the notion that scx is required for connective tissue homeostasis.

**Scx and Heart Valve Disease**

Heart valve disease is associated with defects in connective tissue remodeling and homeostasis including ECM disorganization and increased deposition.\(^1\) However, the signaling pathways important for normal heart valve remodeling are not clear. Mice null for scx exhibit pathological criteria common to diseased valves from embryonic stages and, by juvenile stages, express high levels of fibrosis-associated genes and matrix proteases previously observed in human valve pathology.\(^2-10\) Echocardiography revealed subtle, but insignificant, differences in cardiac function between scx\(^{-/-}\) and scx\(^{+/+}\) mice (data not shown). However, the premature lethality of null mice prevents examination of degenerative valve disease in adult animals. Therefore, we can only speculate that the observed increase in valve area over time, in association with extensive ECM defects, will have detrimental effects on valve structure and function in older viable animals. Utilization of cell-specific loss-of-function models would be appropriate to gain further insights into the long-term effects of scx knockdown on valve pathology and function, as well as determine additional functional roles for scx during valve development and maturation. In conclusion, this study has identified that scx is required for stages of valvular remodeling and adds to increasing evidence that valve disease associated with alterations in ECM has its origins in valve development.

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**Disclosures**

None.

**References**


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Supplementary Materials and Methods

Generation of mice

$\textit{Scx}^{+/−}$ mice $^1$ backcrossed with $\textit{scx}-\textit{GFP}$ mice $^2$ were sacrificed at post-natal day 2 (PND2) and 2 months of age. In addition, timed embryonic (E) litters were collected at E12.5 and E16.5, counting day E0.5 by evidence of a copulation plug. Alternatively $\textit{scx}^{+/−}$ mice were intercrossed to generate $\textit{scx}^{−/−}$ mice carrying the $\textit{scx}-\textit{GFP}$ transgene. At 4 weeks of age, $\textit{scx}^{+/−}$ and $\textit{scx}^{+/+}$ mice were weaned, while $\textit{scx}^{−/−}$ mice were left with their mother, and food and water in the form of gel packs were added to the bottom of the cage to promote viability. Viable $\textit{scx}^{−/−}$ mice and respective $\textit{scx}^{+/−}$ littermate controls, were sacrificed at 2 months of age, PND2, and between E12.5-E17.5 stages. Hearts, or whole embryos were collected and fixed in 4% paraformaldehyde overnight at 4°C for histological analysis or in situ hybridization (see below). Alternatively atrioventricular (AV) valve tissue was dissected from unfixed hearts at PND2 and 2 months old and RNA was extracted $^3$. Genotyping for $\textit{scx}^{−/−}$, $\textit{scx}^{+/−}$ and $\textit{scx}^{+/+}$ mice was performed as previously described $^1$.

Histological Analysis

Following fixation, embryos and hearts were processed for frozen or paraffin embedding as previously described $^3$. Frozen sections from $\textit{scx}^{+/−}$ mice backcrossed with $\textit{scx}-\textit{GFP}$ mice, were cut at 12µm, mounted on Permafrost slides and the GFP visualized. In addition, frozen sections were stored at −20°C and subjected to in situ hybridization (see below). Alternatively, heart tissue from $\textit{scx}^{−/−}$ and $\textit{scx}^{+/−}$ littermates was paraffin-embedded and 5µm tissue sections were subjected to Haematoxylin and Eosin (H&E), Gomori’s Trichrome or Pentachrome staining, or immunohistochemistry (IHC). Procedures for H&E, Trichrome and Pentachrome staining have been previously reported $^4$. IHC for Perostin (1:200, a kind gift from Dr. Stanley Hoffman), Phospho-histone H3 (1:100, Upstate), tenascin-C (1:100, Chemicon), type XIV collagen (1:250, a kind gift from Dr. Manuel Koch), msx1 (1:200, Santa Cruz), snai1 (1:500, Santa Cruz), sox9 (1:2000, courtesy of Dr. Michael Wegner) and cartilage link protein (CLP) (1:5, Developmental
Hybridoma Bank) were performed using immunofluorescence as reported. Chondroitin ABC (Sigma) antigen retrieval treatment for 30 minutes at 37°C was required to detect expression of tenascin-C and CLP. All fluorescent staining was visualized using Olympus BX51 microscope and captured using Olympus DP71 camera.

In situ hybridization

Prepared 12µm-thick frozen sections of AV and aortic valves from E12.5, E16.5, PND3 and 2.5 juvenile scx+/− mice were subjected to in situ hybridization as previously described. The scx-specific riboprobe was a kind gift from Dr. Ronen Schweitzer. Following detection of scx expression tissue sections were counterstained with 15% nuclear fast red for 15 minutes.

Morphometric analysis

Changes in area of the mitral, tricuspid and aortic valve structures were determined from 2-D histological tissue sections collected from at least 3 scx−/− and scx+/− mice at E17.5, PND2 and 2 months of age. The area of each valve structure was measured in 15 tissue sections taken from comparable regions throughout the hearts and quantitatively analyzed using Image ProPlus 6.2 software. The valve area included the region at which the valve leaflet began to protrude from the mural or septal side, to where the leaflet rejoins the myocardium. This area included regions of chordae tendineae in the AV valves. The average area of the valve structures was calculated and reported as a fold change in scx−/− mice over scx+/− controls. Statistical significance of observed changes in valve area was determined using Student’s t-test (P<0.05).

To determine changes in valve cell number, the average number of DAPI-positive nuclei in 10, 50µm² regions, in 5 tissue sections from 3 independent scx−/− and scx+/− mice at 2 months of age were calculated. Statistical significance was determined using Student’s t-test (P<0.05).

Electron Microscopy
Hearts subject to transmission electron microscopy were collected from $scx^{-/-}$ and $scx^{+/+}$ mice at PND2 and fixed in 2% glutaraldehyde:100mM phosphate buffer (pH7.0) for 30 minutes at room temperature. Bisected hearts were placed in fresh 2% glutaraldehyde for a further 2 hours at 4°C, followed by a wash in 200mM phosphate buffer containing 0.1% sodium azide. Further processing and image capturing was performed as previously described $^5$.

**Western Blotting**

Protein lysates from AV valve regions were collected from E17.5 $scx^{-/-}$, $scx^{+/+}$ and $scx^{+/+}$ mice. Samples were lysed in 1x buffer (20 mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM Sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na$_3$VO$_4$, supplemented with complete EDTA free protease inhibitor cocktail). 30µg of total protein was run on a 12% SDS PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked in 5% fat free dried milk for 1 hour (hr) and probed with antibodies against Sox9 (1:1000, 1 hr, a kind gift from Dr. Michael Wegner) and actin (1:5000, 1 hr, Chemicon/Millipore), followed by incubation with anti-rabbit-horseradish peroxidase-conjugated secondary antibody (1:5000, 1 hr, Cell Signaling). Membranes were then washed three times in 1x TBST for 10 minutes. Western blots were developed using Super Signal West Femto Substrate (Pierce) and BioMax MR film (Eastman Kodak).

**Taqman Low Density Array (TLDA) analysis**

The Taqman gene expression array (Applied Biosystems) was custom designed to quantitatively analyze changes in expression of 96 genes in up to 4 experimental cDNA samples. Following RNA extraction, 1µg of total RNA was subject to cDNA synthesis following the manufacturer’s instructions (Applied Biosystems). Consequently 100ng of each cDNA sample was loaded onto the TLDA array and PCR amplification was performed as recommended (50°C for 2 minutes, 94.5°C 10 minutes, 97°C for 30 seconds and 59.7°C for 1 minute x 40 cycles) using the ABI
7900HT Fast Real-Time PCR System. The average cycle count for each target gene was normalized to 18s to give the average delta count (ΔCt) using the RQ SDS manager software (Applied Biosystems). Then for each target gene, the average ΔCt reading from each experimental cDNA was subtracted from the average ΔCt from the comparative 18s endogenous control (ΔΔCt). The average fold change in gene expression of the experimental sample (scx−/−) compared to the control (scx+/-) sample was calculated by 2−ΔΔCt. Statistical significance in fold changes in gene expression between control and experimental samples was determined using Student’s t-test (P<0.05).

Echocardiogram

Transthoracic echocardiography was performed on 2 month old scx−/− (n=7) and scx+/- (n=6) mice using the VisualSonics 770 (Toronto, Canada). 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. The average reading for each parameter measured was recorded from at least ten frames from each animal and the standard deviation calculated. Statistical significance was determined using Student’s t-test (P<0.05).

References


Supplementary Figure 1. Endogenous scx is highly expressed in remodeling atrioventricular and aortic valves. (A) Scx expression is detected in a small number of valve precursor cells (vpc) within the AV endocardial cushion (EC) at E12.5, as well as in the body wall (bw). (B) Expression in aortic ECs is observed at very low levels. (C, D) By E16.5, scx is highly expressed in remodeling AV (C) and aortic (D) heart valve structures (arrows). High levels of expression continue in mitral (mv), tricuspid (tv) (arrows) (E), and aortic (AoV) (arrows) (F) valves of postnatal animals. (G, H) Diminished expression levels are observed in juvenile mice. Arrowheads indicate melanocytes present in the valves at this time.

RV, right ventricle; Counterstain with Nuclear Fast Texas Red.

Scale bars = 200µm.