Loss of β-Catenin Promotes Chondrogenic Differentiation of Aortic Valve Interstitial Cells

Ming Fang, Christina M. Alfieri, Alexia Hulin, Simon J. Conway, Katherine E. Yutzey

Objective—The Wnt/β-catenin signaling pathway has been implicated in human heart valve disease and is required for early heart valve formation in mouse and zebrafish. However, the specific functions of Wnt/β-catenin signaling activity in heart valve maturation and maintenance in adults have not been determined previously.

Approach and Results—Here, we show that Wnt/β-catenin signaling inhibits Sox9 nuclear localization and proteoglycan expression in cultured chicken embryo aortic valves. Loss of β-catenin in vivo in mice, using Periostin(Peri)Cre–mediated tissue-restricted loss of β-catenin (Ctnnb1) in valvular interstitial cells, leads to the formation of aberrant chondrogenic nodules and induction of chondrogenic gene expression in adult aortic valves. These nodular cells strongly express nuclear Sox9 and Sox9 downstream chondrogenic extracellular matrix genes, including Aggrecan, Col2a1, and Col10a1. Excessive chondrogenic proteoglycan accumulation and disruption of stratified extracellular matrix maintenance in the aortic valve leaflets are characteristics of myxomatous valve disease. Both in vitro and in vivo data demonstrate that the loss of Wnt/β-catenin signaling leads to increased nuclear expression of Sox9 concomitant with induced expression of chondrogenic extracellular matrix proteins.

Conclusions—β-Catenin limits Sox9 nuclear localization and inhibits chondrogenic differentiation during valve development and in adult aortic valve homeostasis. (Arterioscler Thromb Vasc Biol. 2014;34:2601-2608.)

Key Words: aortic valve ▪ chondrogenesis ▪ heart valve disease ▪ proteoglycans ▪ Wnt signaling pathway

Adult heart valves are stratified into extracellular matrix (ECM) compartments, defined as collagen-rich fibrosa, proteoglycan-rich spongiosa, and elastin-rich ventricularis/atrialis layers.1 In diseased heart valves, disruption of ECM layers and deposition of abnormal matrix lead to valve dysfunction. Myxomatous valve disease is characterized by excessive proteoglycan accumulation and degradation of collagen and elastin fibers, leading to valve prolapse and insufficiency.1–4 The proteoglycan-rich spongiosa is similar to the ECM of cartilage, and regulatory pathways that control chondrogenesis also are active in valve development.1,5,6 Although dysregulation and reactivation of early developmental programs has been described in heart valve disease,7–11 it remains unclear whether the expansion of proteoglycan-rich spongiosa in myxomatous valve disease is an active chondrogenic process.

See cover image

During the initiation of heart valve development, Wnt/β-catenin signaling is required for early endocardial cushion formation.12,13 At later stages, Wnt/β-catenin promotes the expression of fibrobra-related and osteogenic-like ECM genes in cultured chicken embryo valvular interstitial cells (VICs).14 Wnt/β-catenin signaling is active in normal heart valves in mice at 1 month of age and is also increased in human calcific aortic valve (AoV) disease.14,15 Together, these data suggest that Wnt/β-catenin signaling has multiple roles in valve development and disease. However, the specific regulatory requirements for Wnt/β-catenin signaling in valvular ECM stratification and maintenance have not been reported previously.1,6

Sox9, a SRY transcription factor required for cartilage lineage development,16–18 is crucial for normal valve formation and promotes expression of cartilage-associated genes.19,20 In addition, Sox9 is required to prevent calcification in adult AoVs.21,22 In diseased human and mouse heart valves,10,11,23,24 expression of Sox9 and cartilage-related genes is induced, suggesting that induction of a chondrogenic gene program contributes to heart valve disease. In endochondral bone development, Wnt/β-catenin signaling inhibits Sox9-driven chondrogenesis and promotes osteogenic differentiation.25 However, whether reduction of Wnt/β-catenin signaling directly promotes Sox9-mediated proteoglycan expansion...
in normal heart valves or in myxomatous valve disease remains untested.

Here, we used embryonic chicken aortic valve organ culture (aVOCs) to investigate the role of Wnt/β-catenin signaling in layer-specific ECM expression during valve stratification. In vivo, we used VIC-specific loss of β-catenin driven by PostnCre28 in mice to determine requirements for β-catenin in valve ECM maturation and adult homeostasis. We show that Wnt/β-catenin limits Sox9 nuclear localization and inhibits chondrogenic differentiation of aortic VIC during heart valve development and maintenance in adults. Moreover, loss of β-catenin in mice leads to the formation of hypertrophic nodules with excessive proteoglycan accumulation in adult AoV leaflets, and similarly increased nuclear localization of Sox9 and proteoglycan expression are observed in human myxomatous valve disease.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Wnt/β-Catenin Signaling Inhibits Chondrogenic Gene Expression in Cultured Chicken AoVs

Previously, we reported that Wnt3a treatment induces early osteogenic gene induction in cultured-dissociated chicken embryonic day (E)14 aortic VICs.14 However, these dissociated cells do not retain the structural architecture of stratified heart valve leaflets, and therefore ECM compartmentalization cannot be examined using this method. To analyze diversified ECM production and regulation in a more physiological context, a novel aVOC system was devised. Whole AoVs including adjacent aortic root were isolated from E14 chicken embryos and cultured in vitro as aVOCs. Although valve morphology is abnormal in cultured aVOCs, ECM compartmentalization is maintained, as shown by Movat’s pentachrome staining of proteoglycans, collagen fibers, and elastin, as well as immunostaining for elastin (ventricularis), Aggrecan and Collagen 2 (Col2; spongiosa), and also Col3 (fibrosa; Figure I A–I D in the online-only Data Supplement). Thus, this system can be used for examination of regulatory mechanisms that control ECM compartmentalization in developing valves.

The role of Wnt/β-catenin signaling in heart valve ECM composition and compartmentalization was examined using the E14 chicken aVOC cultures. Cultures were treated with BIO to increase Wnt/β-catenin signaling,27 or XAV-939 to decrease Wnt/β-catenin signaling.28 When treated with BIO (Wnt activation), the proteoglycan-expressing area, as indicated by Alcian Blue staining, decreases compared with vehicle-treated controls (DMSO [dimethyl sulfoxide]; Figure II in the online-only Data Supplement). Although the area of proteoglycan expression was not increased in XAV-939–treated cultures, increased intensity of Alcian Blue staining was noted (Figure IIC in the online-only Data Supplement). In contrast, XAV-939 treatment, but not BIO, decreases the fibrillar collagen-expressing area in cultured aVOC as indicated by Saffron staining (Figure II in the online-only Data Supplement). Thus, Wnt/β-catenin signaling inhibits proteoglycan expression (spongiosa) and is required for collagen expression (fibrosa) in cultured aVOCs. Immunostaining for the chondrogenic transcription factor Sox9 demonstrates that Sox9 protein expression is decreased in cultured aVOCs with BIO treatment, whereas XAV-939 addition promotes Sox9 expression and nuclear localization (Figure IA–IC). Expression of cartilage matrix proteins Aggrecan and Col2 also are repressed by BIO (Figure IE and IH) and increased with XAV-939 (Figure IF and II). Quantification of gene expression by quantitative reverse transcription polymerase chain reaction confirms Wnt activation with BIO treatment, as indicated by increased Axin2 gene expression. Likewise, BIO treatment dampens Col2a1 expression but has no apparent effect on Sox9, Col10, or Aggrecan gene expression levels. By contrast, Wnt inhibition with XAV-939 induces chondrogenic markers Sox9 and Aggrecan, but not Col2a1 (Figure IJ). The differing effects of altered Wnt signaling on chondrogenic protein versus mRNA expression support complex downstream transcriptional and post-transcriptional regulatory mechanisms. However, together, these data support a role for Wnt/β-catenin in ECM compartmentalization by limiting nuclear localization of Sox9 and expression of chondrogenic matrix proteins.

Loss of β-Catenin in Aortic VICs Results in the Formation of Proteoglycan-Rich Nodules in Adult Mice

The role of Wnt/β-catenin signaling in adult valves in vivo was examined in mice with loss of β-catenin in VICs. Wnt/β-catenin signaling is active in semilunar valves before birth as previously reported,14 and also at 2 weeks and in adult AoVs as indicated by an Axin2-loxP/loxP reporter line20 (Figure 2A; Figure III in the online-only Data Supplement). Interestingly, Wnt/β-catenin signaling is active preferentially at the distal tip and the hinge of the AoV at 2 weeks after birth and also in adult mice. PostnCre28 was used for conditional recombination of floxed exons 2 to 6 in the β-catenin allele30 (Ctnnb1flo) and is active in AoV interstitial cells but not endothelium, as indicated by positive X-gal staining in adult PostnCre;ROSA26 mice (Figure IVB in the online-only Data Supplement).31

The efficacy of PostnCre-mediated loss of β-catenin protein was determined in semilunar valves of PostnCre;Ctnnb1lox mice at postnatal day (P)0 and 2 weeks of age. β-Catenin protein expression is reduced in the AoV interstitium, but not endothelium, of PostnCre;Ctnnb1lox mice at P0 and is not detectable at 2 weeks, compared with Cre-negative controls (Figure VA–VD in the online-only Data Supplement).
Furthermore, $\text{Axin}2$ mRNA, a downstream target of Wnt/$\beta$-catenin signaling, is significantly decreased by $\approx 70\%$ in $\text{PostnCre;}\text{Ctnnb1fl/fl}$ animals, compared with Cre-negative controls, in isolated AoV leaflets at 3 months of age (Figure VE in the online-only Data Supplement). Therefore, $\beta$-catenin protein and canonical Wnt signaling are reduced in the AoV interstitium of $\text{PostnCre;}\text{Ctnnb1fl/fl}$ mice. Although $\beta$-catenin protein is reduced, the $\text{PostnCre;}\text{Ctnnb1 fl/fl}$ mice have apparently normal semilunar valve morphology at P0 (Figure VIA–VID in the online-only Data Supplement) and initial compartmentalization of collagen and proteoglycan as indicated by pentachrome staining (Figure VIE and VIF in the online-only Data Supplement). No differences in cell density or morphometry were observed at early postnatal stages (Figure VIG and VIH in the online-only Data Supplement). In addition, $\text{PostnCre;}\text{Ctnnb1fl/fl}$ mice survive through gestation and do not exhibit obvious morbidity or mortality.

The requirement for $\beta$-catenin in valve homeostasis and ECM maintenance was determined in adult $\text{PostnCre;}\text{Ctnnb1fl/fl}$ mice. Histological analysis of $\text{PostnCre;}\text{Ctnnb1fl/fl}$ animals demonstrates that proteoglycan-rich nodules containing hypertrophic cells are present in the AoV at 2 months,
with 100% penetrance at 3 months (Figure 2B). Cre-mediated recombination occurs throughout the valve leaflets, as apparent in PostnCre;Ctnnb1fl/fl;ROSA26 lacZ reporter mice at 3 months (Figure 2B). However, the nodules are restricted to the distal tip of the AoV leaflet where Wnt/β-catenin signaling is active, as indicated by Axin2 expression (Figure 2A). The formation of proteoglycan-rich nodules is specific to the semilunar valves with a lower frequency of nodule formation in the pulmonary valve and no apparent abnormalities noted in the mitral or tricuspid valves in PostnCre;Ctnnb1fl/fl animals ≤6 months of age (Figure VII in the online-only Data Supplement).

To visualize the morphology and stratification of the AoV, Movat’s pentachrome and Masson’s trichrome staining were performed. In controls, the AoV is stratified with defined layers of proteoglycans and collagen (Figure 2C and 2E). However, all 3-month-old PostnCre;Ctnnb1fl/fl mice exhibit large nodules almost exclusively in the distal tip of the AoV (Figure 2D and 2F). These nodules in PostnCre;Ctnnb1fl/fl AoVs are densely stained by Alcian Blue, indicating strong proteoglycan deposition (Figure 2D). By contrast, fibrillar collagen deposition is apparently reduced in the nodules (Figure 2F), but accumulation is obvious surrounding the nodules, as indicated by Masson’s trichrome staining. Strikingly, the nodular cells also exhibit dramatic morphological changes including the presence of hypertrophic cells (Figure 2B, 2D, and 2F). However, cell death was not apparent in the nodules of PostnCre;Ctnnb1fl/fl animals, as indicated by cleaved caspase-3 immunoreactivity (Figure VIII in the online-only Data Supplement). In addition, the PostnCre;Ctnnb1fl/fl nodules do not calcify at 1 year of age (data not shown). The increased proteoglycan deposition and hypertrophic cellular morphology of the nodular cells in PostnCre;Ctnnb1fl/fl AoVs at 3 months are similar to hypertrophic chondrocytes. Thus, β-catenin is required for normal ECM maintenance, and loss of β-catenin leads to the formation of hypertrophic cartilage-like nodules in the adult AoV.

**AoV Nodules Express Chondrogenic Genes as a Result of Loss of β-Catenin**

Spongiosa and fibrosa ECM compartmentalization, as well as induction of chondrogenic regulatory mechanisms, were examined in PostnCre;Ctnnb1fl/fl AoV nodules. Sox9 is required for chondrogenesis, and for the expression of hyaluronic and proteoglycan link protein 1 (Hapln1) in developing heart valves. Hapln1 is normally restricted to the ventricularis side of Cre-negative control AoVs (Figure 3A and 3C), but its expression is expanded throughout the proteoglycan-rich nodules in PostnCre;Ctnnb1fl/fl AoVs, as detected by immunofluorescent staining (Figure 3B and 3C). Likewise, the transcription factor Sox9 is strongly expressed in the nuclei of nodular cells at the distal tip of AoV in PostnCre;Ctnnb1fl/fl animals (Figure 3B and 3C), and the percentage of Sox9-positive nuclei is higher in the nodules compared with either perinodular areas or entire AoV leaflets in Cre-negative controls (Figure 3D). Thus, the loss of β-catenin leads to increased nuclear Sox9 expression in proteoglycan-rich nodular cells in the adult AoV. Similarly, nuclear localization of Sox9 and increased Hapln1 expression are also apparent in human myxomatous mitral valve disease (Figure IX in the online-only Data Supplement).

The expression of Sox9 target genes Aggrecan (Acan), Col2, and ColX, which are expressed in cartilage, was examined in AoV lacking β-catenin. Both Aggrecan and Col2 are strongly expressed in the nodules of PostnCre;Ctnnb1fl/fl AoVs (Figure 4B and 4E), in contrast to no detectable expression in the distal tip of AoV in Cre-negative controls (Figure 4A and 4D). Consistent with the morphology similar to hypertrophic cartilage (Figure 2F and 2H), the nodular cells in PostnCre;Ctnnb1fl/fl AoVs also express ColX (Figure 4H), which is a hypertrophic chondrocyte marker and is not normally expressed in heart valves (Figure 4G). Likewise, quantitative reverse transcription polymerase chain reaction analysis of gene expression demonstrates that Acan is increased by 33-fold (Figure 4C), Col2α1 by 260-fold (Figure 4F), and Col10a1 by 700-fold (Figure 4I) in PostnCre;Ctnnb1fl/fl AoVs compared with controls. Thus, the loss of β-catenin leads to induction of a chondrogenic gene program apparent in increased nuclear localization of Sox9 and induced expression of downstream target genes Acan, Col2α1, Hapln1, and Col10a1.
The proteoglycan composition and timing of induction were further examined in PostnCre;Ctnnb1fl/fl AoVs. Versican (Vcan) is normally the predominant proteoglycan in the spongiosa layer of AoVs (Figure 4J), but is dramatically reduced in the nodules of PostnCre;Ctnnb1fl/fl AoVs, demonstrating a switch in the predominant proteoglycan to Aggrecan, with loss of β-catenin (Figure 4K). Consistent with the transition to a chondrogenic ECM, the expression of Hapln1 expands markedly to encompass the entire nodule in PostnCre;Ctnnb1fl/fl AoVs (Figure 4K). Quantitative reverse transcription polymerase chain reaction analysis also demonstrates that Hapln1 is increased by 8-fold and Vcan is decreased by 60% (Figure 4L). The initiation of nodule formation and proteoglycan alterations were examined at early postnatal stages. At P0 and 2 weeks, Aggrecan, not normally expressed in mouse AoVs, is detected in PostnCre;Ctnnb1fl/fl AoVs (Figure XB and XD in the online-only Data Supplement). Other chondrogenic ECM markers, including Col2 and ColX, are not expressed in PostnCre;Ctnnb1fl/fl AoVs at this early stage, indicating that formation of the hypertrophic nodules develops over time. Thus, the loss of β-catenin leads to induction of proteoglycans and collagens characteristic of cartilage, beginning with Aggrecan. Together, these data suggest that loss of β-catenin drives VICs in PostnCre;Ctnnb1fl/fl AoVs to undergo progressive chondrogenic differentiation and maturation after birth.

**Fibroa ECM Is Dysregulated in Adult AoV Lacking β-Catenin**

The initial formation and compartmentalization of the fibroa layer relative to the spongiosa was examined in PostnCre;Ctnnb1fl/fl AoVs at P0 and 2 weeks. The initial organization of collagen and proteoglycan-rich regions of semifumar valves is apparently normal, as indicated by Movat’s pentachrome staining (Figure VIE and VIF in the online-only Data Supplement). Localized expression of Col1 and Hapln1 also is apparently normal at 2 weeks in both PostnCre;Ctnnb1fl/fl animals and Cre-negative controls (Figure XG and XH in the online-only Data Supplement). In adult mice, Col1 expression normally is restricted to the fibroa layer (Figure 5A). However, this well-defined layer is lost in PostnCre;Ctnnb1fl/fl AoVs, in which Col1 is absent from the nodules but is apparent in the perinodular areas (Figure 5B). Likewise, Col3 is predominantly expressed in the fibroa layer in Cre-negative control AoVs (Figure 5C). However, in PostnCre;Ctnnb1fl/fl AoVs, Col3 expression is expanded throughout the thickened leaflets but is only minimally expressed in the proteoglycan-rich nodules (Figure 5D). Consistent with the expression of Col1 and Col3, endogenous Postn is not detected within the nodules of PostnCre;Ctnnb1fl/fl animals but is expressed in the thickened distal tip in the collagen-rich region (Figure 5E and 5F). In addition, Colla1 and Postn transcripts are significantly decreased in PostnCre;Ctnnb1fl/fl AoVs, compared with Cre-negative controls (Figure 5G), at 3 months. Thus, the proteoglycan-rich nodules lose the expression of fibrillar collagens and matricellular Postn, whereas the fibroa layer is disrupted in adult PostnCre;Ctnnb1fl/fl AoVs. Together, these data demonstrate that the loss of β-catenin signaling disrupts normal ECM maintenance and compartmentalization in the adult AoV.

**Discussion**

β-Catenin is required for endothelial-to-mesenchymal transition at the early endocardial cushion stage in heart valve development, and upregulation of Wnt/β-catenin signaling has been implicated in heart valve disease. Here, we show that β-catenin signaling is required for normal ECM maintenance and heart valve homeostasis in cultured embryonic chicken AoVs and adult mice. In vitro and in vivo analyses demonstrate increased nuclear localization of Sox9 and...
induction of downstream chondrogenic gene expression when Wnt/β-catenin signaling is reduced. Thus, Wnt/β-catenin signaling limits nuclear localization of Sox9 in aortic VICs and is required for maintenance of ECM compartmentalization. These regulatory interactions are intrinsic to VICs, because PostnCre is not active in endothelial cells and β-catenin expression is maintained on the surface of PostnCre;Ctnnb1floflo AоЙs. Together, these studies demonstrate a critical role for Wnt/β-catenin signaling in heart valve ECM homeostasis with implications for myxomatous valve disease mechanisms (see model, Figure XI in the online-only Data Supplement).

Embryonic aVOC cultures treated with the Wnt/β-catenin activator BIO have decreased proteoglycan deposition, whereas treatment with the inhibitor XAV leads to increased chondrogenic matrix protein expression and nuclear localization of Sox9. Likewise, the treatment of isolated embryonic aortic VICs with Wnt3a induces fibrosa-like gene expression consistent with a role in heart valve ECM compartmentalization.14 However, the initial stratification of PostnCre;Ctnnb1floflo AоЙs is normal at 2 weeks of age, indicating that Wnt/β-catenin signaling is not required for initiation of valve stratification, but rather for maintenance of normal ECM compartmentalization. Further evidence for active Wnt/β-catenin signaling in adult valve homeostasis is the expression of an Axin2lacZ reporter in the distal regions of adult AoV and reduction of Axin2 transcripts in PostnCre;Ctnnb1floflo AоЙs. Interestingly, the formation of hypertrophic cartilage-like nodules in PostnCre;Ctnnb1floflo mice also occurs at the distal tips of the AoV leaflets. The lack of an initial effect on valve stratification and the localization of the chondrogenic nodules to the distal tips of the AoV leaflets in PostnCre;Ctnnb1floflo mice is in contrast to the results observed in cultured embryonic aVOCs and suggests that Wnt/β-catenin signaling is modulated by physiological factors in vivo. It has been reported that mechanical tension and compression stimulate the expression of Wnt10b, and coreceptor Lrp5 in cultured osteoblast cells.34 The intracellular mechanosensing RhoA/ROCK cascade also affects Sox9 expression and activity.15,36 Thus, mechanical stimulation may have a role in the interaction between Wnt/β-catenin signaling and Sox9-mediated chondrogenic differentiation in the aortic VICs.

Loss of β-catenin in VICs leads to increased expression and nuclear localization of Sox9, as well as increased expression of Col2a1, Acan, and Col10a1, in cultured aVOC and adult AoVs. This same regulatory hierarchy is active in hypertrophic cartilage,37–39 and loss of β-catenin in mesenchymal progenitors leads to increased expression of Sox9 and ectopic cartilage formation in the developing skull.40,41 Here, we demonstrate that the loss of Wnt/β-catenin signaling leads to increased nuclear localization of Sox9 and chondrogenic gene induction in valve progenitors and adult valves. Although Sox9 and β-catenin proteins have been demonstrated to interact in cartilage42 and gonad development,43 the mechanism by which loss of β-catenin leads to increased nuclear localization of Sox9 is not known. In developing valves, Sox9 is required for expression of cartilage-related ECM genes, including Hapln1 and Col2a1.20 Haploinsufficiency of Sox9 in heart valves is sufficient to promote ectopic calcification in adult mice,24 and Sox9 suppresses Osteopontin expression preventing matrix mineralization in postnatal mouse heart valve explants.22 Increased Wnt/β-catenin signaling has also been implicated in human calcific AoV disease, potentially by promoting active osteogenesis in the fibrosa layer.15,44 Here, we show that the loss of β-catenin leads to abnormal chondrogenic differentiation of aortic VICs with excessive accumulation of proteoglycans in mice. Likewise, nuclear Sox9 and Hapln1 protein expression are increased in human myxomatous valve disease. Together, these data provide further evidence for shared regulatory mechanisms in skeletal lineages and in heart valve development and disease.1,5,6
Excessive proteoglycan accumulation is one of the characteristics of myxomatous valve disease.\textsuperscript{1–4} Although its cause seems complex, mutations in the cytoskeletal protein Filamin A (FLNA) and a variety of ECM protein mutations have been linked to human myxomatous valve disease.\textsuperscript{5,3,4,4–47} Additional causes include congenital malformation, increased serotonin signaling, or infective endocarditis.\textsuperscript{49} In each case, the myxomatous valve exhibits increased proteoglycan and decreased collagen composition leading to insufficiency and regurgitation. However, the molecular mechanism(s) of disease pathogenesis and progression are still poorly understood.\textsuperscript{3,4} Expression of Aggrecan, Col2, and Sox9 is induced in human myxomatous valve disease, suggesting that chondrogenic differentiation of VICs is integral to disease pathogenesis.\textsuperscript{24} Similarly, in mice lacking endogenous \textit{Postn}, inappropriate differentiation of mesenchymal cushion cells and abnormal Aggrecan expression were detected in \textit{Postn}\textsuperscript{−/−} valves.\textsuperscript{46,47} Here, we demonstrate that VICs directly undergo chondrogenic differentiation with excessive proteoglycan accumulation as a result of loss of β-catenin. The chondrogenic nodules observed in mouse AoVs are not a characteristic feature of human valve pathology. However, predominant nuclear Sox9 localization in regions of increased Hapln1 protein expression was observed in both \textit{Postn}\textsuperscript{Cre;Cntnb1}\textsuperscript{L00} AoV and human myxomatous mitral valve disease, supporting a role for this regulatory interaction in human valve pathogenesis. It remains to be determined whether Wnt/β-catenin signaling is repressed by the multiple causes of myxomatous valve disease or contributes to pathogenesis that ultimately leads to valve insufficiency. If this is the case, maintaining or increasing Wnt/β-catenin signaling could be exploited therapeutically as an alternative to surgical repair, which is the current standard of care for severe myxomatous valve disease.\textsuperscript{3}

Acknowledgments
We thank Dr. A. Colige from the Laboratory of Connective Tissues Biology (GIGA, Sart-Tilman, Belgium) and Dr. M. Radermecker from the Department of Cardiovascular and Thoracic Surgery and Human Anatomy (CHU-Sart-Tilman, Belgium) for sharing human mitral valve samples.

Sources of Funding
This work is supported by the National Institutes of Health Grant R01HL094319 (to K.E. Yutzey), American Heart Association Predoctoral Fellowship 13PRE16410009 (to M. Fang) and R01HL60714 (to S.J. Conway).

Disclosures
None.

References
The role of Wnt/\(\beta\)-catenin signaling in heart valve stratification and subsequent extracellular matrix maintenance is not known. In this study, we found that Wnt/\(\beta\)-catenin signaling is required for heart valve homeostasis because loss of \(\beta\)-catenin leads to increased chondrogenic differentiation of aortic valvular interstitial cells in adults. In the absence of \(\beta\)-catenin, valvular interstitial cells are susceptible to ectopic chondrogenic differentiation, leading to the formation of nodules containing proteoglycan-rich hypertrophic cartilage-like cells, with nuclear localization of the chondrogenic transcription factor Sox9 and loss of fibrillar collagen. This chondrogenic phenotype is similar to human myxomatous heart valve disease characterized by increased nuclear Sox9 localization and excessive proteoglycan accumulation.
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Arterioscler Thromb Vasc Biol. 2014;34:2601-2608; originally published online October 23, 2014;
doi: 10.1161/ATVBAHA.114.304579
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Figure SI. Chicken aortic valve organ cultures (aVOC) maintain compartmentalized ECM in vitro. Aortic valves isolated from E14 chicken embryos were cultured and sectioned for histology and immunostaining. A. The compartmentalized ECM is visualized by Movat’s pentachrome staining, with collagen/fibrosa in yellow, proteoglycan/spongiosa in blue and elastin in black. B-D. Immunofluorescent staining (arrows) of serial sections from the same cultured aVOC tissue show: B. Elastin protein (green, arrows) C. Aggrekan (green, arrows) and Col2 (red, right arrow) expression and D. Col3 (green, arrows) corresponding to Movat’s stained regions. Nuclei are counterstained with Topro3 in blue.
Figure SII. Wnt/β-catenin signaling is required for collagen deposition and inhibits proteoglycan expression in aVOC cultures. A-C. ECM compartmentalization, as indicated by Movat’s Pentachrome staining, is shown for sectioned aVOCs treated with A. DMSO, B. BIO or C. XAV. Representative images are shown for each group. D. The percent positive pixels for each stain individually was quantified for aVOCs treated with DMSO (n=5), BIO (n=4) and XAV (n=5). Student’s t-tests were used to determine significant differences between stained areas for BIO or XAV treated explants compared to DMSO-treated controls. # indicates p<0.01. Error bars represent SEM.
Figure SIII. $Axin2^{LacZ/+}$ expression, indicative of active Wnt/β-catenin signaling, is localized to distal regions of aortic and pulmonic semilunar heart valves in 2 week-old mice. A, B. LacZ reporter activity was detected in sectioned $Axin2^{LacZ/+}$ semilunar valves by X-gal staining. Arrows indicate X-gal staining (blue) prevalent at the distal tip of an aortic valve (AoV) and pulmonary valve (PV) at 2 weeks of age.
Figure SIV. PostnCre is active non-cardiomyocyte lineages in adult PostnCre;ROSA26 mice. A, E. Ventral and dorsal views of a whole mount X-gal-stained (blue) heart from a 5-month-old PostnCre;ROSA26 mouse are shown. B. In histological X-gal-stained sections, interstitial cells in the AoV are labeled by X-gal. C. Few epicardial cells (Epi, arrows) in proximity to atrioventricular junction exhibit recombination indicated by X-gal. D. A subpopulation of cardiac fibroblasts (CF, arrows) in the LV free wall near the root of mitral valve parietal leaflet also is X-gal positive. F. Most, but not all, of the interstitial cells in the pulmonary valve (PV) are labeled. G. Cells (arrows) around major vessels (v) and H. The majority of the VICs in mitral valve (MV) are X-gal positive.
Figure SV. β-catenin protein expression is reduced in valve interstitial cells, and Wnt/β-catenin signaling is significantly reduced in the AoV of PostnCre;Ctnnb1^{flo/fl} mice. A, B. β-catenin protein (green, arrows) is expressed in Cre-negative controls (Ctrl) and is reduced (arrowheads) in PostnCre;Ctnnb1^{flo/fl} (LOF) AoV at P0, as detected by immunofluorescence staining. C, D. β-catenin protein (red, arrows) is expressed in controls, but is reduced in the AoV of LOF mutants at 2 weeks (D, arrowheads). Note that β-catenin protein is expressed in endothelial surface cells in both control and LOF AoV. E. At 3 months, Axin2 mRNA, a transcriptional target of Wnt/β-catenin signaling, is significantly decreased in LOF AoV as detected by qRT-PCR. Expression is shown relative to the level in controls set to 1.0. Statistical significance was determined by Student’s t-test (n=3; # p<0.01).
Figure SVI. *PostnCre;Ctnnb1*<sup>fl/fl</sup> AoV do not exhibit morphological defects at P0-P14. A-D. The morphology and stratification of *PostnCre;Ctnnb1*<sup>fl/fl</sup> (LOF) and Cre-negative control (Ctrl) AoVs was evaluated in histological sections stained with Masson’s Trichrome (A, B) or Movat’s Pentachrome at P0 (C, D) and 2 weeks (E, F). Arrows indicate formation of the spongiosa layer (blue) in Pentachrome staining in LOF AoVs. E, F. At 2 weeks compartmentalized proteoglycan (blue, arrowhead) and collagen (yellow, arrows) are apparent by Movat’s Pentachrome staining in control and LOF AoVs. G. The cell density within the LOF AoV is not statistically different (n.s., p=0.846) from controls at P0 as determined by Student’s t-test (n=5). H. The width of LOF AoV leaflets at distal (n.s., p=0.097), medial (n.s., p=0.176) or proximal (n.s., p=0.167) regions is not different from that in controls at 2 week. Statistical analysis was performed using Student’s t-test (n=4).
Figure SVII. *PostnCre;Ctnnb1<sup>fl/fl</sup>* mice do not show apparent abnormalities in mitral valves or myocardium. A-D. The stratification of mitral valves (MV) is demonstrated by Movat’s Pentachrome staining is apparently normal in *PostnCre;Ctnnb1<sup>fl/fl</sup>* (LOF) hearts and Cre-negative controls at 2 months (A, B) and 6 months (C, D) of age. E, F. The myocardium stained red with Masson’s trichrome is comparable in LOF mutants (F) and Cre-negative controls (E).
Figure SVIII. Cell death is not detected in the PostnCre;Ctnnb1^{fl/fl} nodules as determined by immunofluorescent staining of cleaved caspase-3. A, B. Cleaved caspase-3 (CC3, red) is not detected in PostnCre;Ctnnb1^{fl/fl} (LOF) AoV at 6 months (inset, B) by immunofluorescence. The proteoglycan-rich nodule is visualized by immunostaining of Hapln1 (arrowhead in B, green). Arrows in A indicate normal expression of Hapln1. C. Hapln1 expression (green) in condensing cartilage and CC3 (red) expression in the interdigit region (arrows in high magnification inset) are detected in E14.5 mouse limb as a positive control.
Figure SIX. Sox9 nuclear localization and Hapln1 expression are increased in human myxomatous mitral valve disease. A, B. Increased Sox9 (red) and Hapln1 (green) expression is detected in myxomatous mitral valves (MMV) compared to control mitral valves (Ctrl). The expression of Sox9 and Hapln1 is very low in the control (A), whereas nuclear Sox9 (red) staining is apparent in the Hapln1-positive (green) area in MMV (B). Higher magnifications of indicated areas are shown in A’ (Ctrl) and B’ (MMV). The arrowhead in B indicates strong nuclear Sox9 expression (red) where Hapln1 (green) is expressed. Nuclei are counterstained in blue by DAPI. Staining shown is representative of n=6 Ctrl and n=6 MMV specimens analyzed.
Figure SX. Induced expression of Aggrecan is observed in the AoV of PostnCre;Ctnnb1 fibril mice at P0 and compartmentalization of Col1 and Hapln1 expressing cells is normal at 2 weeks. A, B. Aggrecan (green) expression is shown PostnCre;Ctnnb1 fibril (LOF) and Cre-negative control AoVs at P0. A. Arrows indicate normal AoV without Aggrecan expression at P0. B. Arrowheads indicate aberrant expression of Aggrecan in P0 LOF AoV. C, D. Aggrecan (green) expression is shown in LOF and control AoVs at 2 weeks. Arrows indicate normal AoV without Aggrecan expression in controls, whereas arrowheads indicate abnormal expression of Aggrecan in LOF AoV at 2 weeks of age. E, F. The expression of Col1 (green) and Hapln1 (red) is shown in LOF and control AoV at 2 weeks. Arrows indicate Col1 expression in the fibrosa, and arrowheads indicate Hapln1 expression on the ventricularis side of the AoV in both LOF and controls.
Figure SXI. Model for Wnt/β-catenin signaling in heart valve ECM maintenance and disease. In normal valvular interstitial cells, Wnt/β-catenin signaling promotes collagen deposition in the fibrosa layer, while inhibiting Sox9-activated proteoglycan expression in the spongiosa. Loss of β-catenin in mouse AoV interstitial cells promotes Sox9 expression and nuclear localization, and also induces expression of chondrogenic ECM proteins, including Acan and ColX, resembling myxomatous valve disease. Color keys for fibrosa, spongiosa and ventricularis are provided in yellow, blue and black, respectively. nSox9, nuclear Sox9; PG, proteoglycan.
**Supplemental Table**

Table SI. Primers used for quantification of chicken mRNA expression by qRT-PCR.

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SUPPLEMENTAL MATERIAL
Detailed Methods for Fang et al.: Chicken aortic valve organ culture. Chicken aortic root and aortic valves were dissected from embryonic (E)14 white leghorn chicken embryos and cultured at 37°C with 5% CO₂ in M199 (CellGro) supplemented with 10% Fetal Bovine Serum (Hyclone) and 1% penicillin/streptomycin (Life Technologies). The following treatments were used to manipulate the Wnt/β-catenin signaling pathway: XAV-939 (10μM, SelleckChem) and BIO (6-Bromoindirubin-3'oxime; 5μM, Cayman Chemical). DMSO (Sigma) was added at 0.1% as the vehicle control in complete medium consisting of M199 (CellGro) supplemented with 10% Fetal Bovine Serum (Hyclone) and 1% penicillin/streptomycin (Life Technologies). Media was replaced every 2 days for up to 1 week when the aVOCs were taken for RNA, histological analysis, or immunofluorescent staining.

Transgenic and mutant mice. Mice were sacrificed under CO₂ inhalation at the specified time point for each experiment. All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Research Foundation. PostnCre transgenic mice have been described previously.1 Mice with a β-catenin allele containing loxp-sites (Ctnnb1fl/fl), Axin2lacZ/+ and ROSA26 reporter mice were previously described.2-4 Genotyping was performed using genomic DNA extracted from clipped tails with primers as previously described.1-4

Human tissue collection. P2 segments of mitral valve posterior leaflets were collected from control hearts rejected for transplantation (n=6) and from myxomatous mitral valves (n=6) obtained during surgery to correct severe mitral regurgitation as described in Hulin et al.5 The control group is composed of 4 men and 2 women aged 59.7 ± 9.8 years old. The MMV group is composed of 6 men aged 60.8 ± 8.2 years old. The study conforms to the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of Liege University Hospital (B70720071262), with written consent obtained from the informed patients.

Histology and immunofluorescence. Hearts or organ cultures were fixed in 4% paraformaldehyde in PBS, dehydrated through gradient ethanol washes, embedded in paraffin, and sectioned. Masson’s Trichrome staining and Movat’s Pentachrome staining were performed according to manufacturer’s (American MasterTech) protocols. For immunostaining, antigen retrieval was performed using antigen unmasking solution (Vector Laboratories) in a pressure cooker as described previously.6 Enzymatic antigen retrieval was performed per primary antibody manufacturer’s instructions. For ColX (Abcam), tissues were incubated at 37°C for 3 min with 0.25% trypsin (Sigma). For Versican or Aggrecan single staining, tissues were treated with chondroitinase (200mU/ml, Sigma) at 37°C for 1 hr. For Col2 and Aggrecan costaining, tissues were treated with hyaluronidase (200U/ml, Sigma) for 3 hrs at 37°C. The following primary antibodies were used: anti-Aggrecan (1:500, Millipore, #AB1031), anti-β-catenin (1:100, Invitrogen, #138400), anti-cleaved caspase-3 (1:200, Cell Signaling, #9664), anti-Col1
(1:50, Millipore, #AB765P), anti-Col2 (1:25, DSHB, #II-Il6B3), anti-Col3 (1:50, Rockland, #600401105), anti-ColX (1:50, Abcam, #AB58632), anti-Elastin (1:100, Abcam, #AB9519-500), anti-Hapln1 (1:75, DSHB, #9/30/8-A-4), anti-Periostin (1:100, Abcam, #AB14041), anti-Sox9 (1:200, Millipore, #AB5535) and anti-Vcan (1:500, Millipore, #AB1033). Alexa Fluor-488 or 568 conjugated secondary antibodies (Life Technologies) were used. Nuclei were counterstained with Topro3 (Life Technologies) or DAPI (Life Technologies). Slides were sealed with Vectashield mounting medium (Vector Laboratories) for confocal microscopy (Nikon). For all histology, a minimum of n=4 controls and mutants were stained and visualized with consistent results for each group.

Quantification of ECM compartmentalization. For analysis of ECM compartmentalization, individual reagents from Movat’s Pentachrome staining kit (American MasterTech) were used on deparaffinized aVOC tissues to quantify collagen, proteoglycan and elastin content. Each stain was performed separately on serial sections of the same tissue explants. For collagen content, samples were dehydrated in 100% ethanol, placed in alcoholic saffron solution for 15 minutes, and then rinsed with 100% ethanol. For proteoglycan content, samples were placed in 3% glacial acetic acid for 3 minutes, and transferred into 1% Alcian Blue solution for 30 minutes, rinsed in distilled water, and dehydrated through alcohol series. For the Elastin stain, samples were incubated in Verhoeff’s elastic stain for 20 minutes, rinsed in tap water for 5 minutes, briefly decolorized in 2% ferric chloride, and rinsed in water. Tissues were subsequently incubated in 5% sodium thiosulfate for 1 min, rinsed in tap water for 5 minutes, and dehydrated through an alcohol series. All samples were cleared in xylene and mounted with Cytoseal (Electron Microscopy Sciences). Quantification of color pixel intensity was determined using NIS Elements Basic Research software (Nikon). Pixel color intensity cut-off values were set for each stain and then applied to determine the percent of positive pixels in the region of interest (ROI), which included only the aVOC part of the image. For each stain evaluated individually, the average of color-positive pixels over the total pixels was calculated from at least 3 slides per aVOC, and 3 sections per slide for a total of 9 sections per aVOC.

Quantification of nuclear Sox9. For control mouse AoVs, the percentage of Sox9-positive nuclei was calculated as the number of Sox9-positive nuclei divided by the total number of nuclei within the AoV leaflets. The percentage of Sox9-positive nuclei also was calculated for the PostnCre;Ctnnb1fl/fl (LOF)-perinodular region, as defined by nuclei outside of the Hapln1-stained nodule, and for the LOF-nodular region, defined by nuclei within the Hapln1-stained nodule. A minimum of 2 sections was used per animal, and 6 LOF animals with 8 Cre-negative controls were analyzed.

X-gal staining. β-gal expression in Axin2lacZ/+, PostnCre;ROSA26 and PostnCre;Ctnnb1fl/fl;ROSA26 mice was detected by X-gal staining of histological sections as previously described.6
Morphometric analysis. Morphometric analysis, including quantification of cell density and widths of different regions within the AoV, in histological sections has been previously described.\textsuperscript{7, 8} For the cell density measurement in the AoV at P0, nuclei were counterstained by Topro3 (Invitrogen) according to manufacturer’s instructions. The total nuclei were counted and the area of AoV leaflets was measured using NIS Elements Basic Research software (Nikon). The cell density was then calculated as total nuclei divided by the area of the AoV leaflets. 2-3 sections per animal were analyzed, and the average cell density from 5 animals was then compared between \textit{PostnCre;Ctnnb1\textsuperscript{fl/fl}} mice and controls. For the width measurement at 2 weeks, the distal width was measured as the widest part in the distal tip of the AoV, the medial width was measured at the thinnest part of the mid-leaflet, and the proximal width was measured as the widest part of the hinge. A total of 4 sections were analyzed per animal, and the average widths from 4 animals were then compared between \textit{PostnCre;Ctnnb1\textsuperscript{fl/fl}} and controls.

Quantitative RT-PCR (qRT-PCR). For mouse aortic valve samples, total RNA was purified using a RNeasy Mini Kit (Qiagen) following manufacturer’s instructions. AoVs from 6-8 mice were pooled together per biological sample, and a total of three biological samples were used for RNA isolation. 200~400 ng total RNA was used for cDNA synthesis using SuperScript II First-Strand Synthesis Kit (Invitrogen) with oligo(dT) primers, and quantitative real-time PCR analysis was performed using Taqman probes (Applied Biosystems). The relative copy number was calculated using \textit{ΔΔC}_{T} method,\textsuperscript{9, 10} and B2M was used for normalization. For \textit{Acan}, \textit{Col10a1} and \textit{Col2a1}, the Ct value in controls was arbitrarily set to 35, as the expression level of these genes in normal mouse valves is extremely low.\textsuperscript{11} For aVOC samples, 5 aVOCs were pooled together and RNA was isolated using Trizol reagent (Invitrogen). A total of 500ng of RNA was reverse-transcribed and used for quantitative real-time PCR (qRT-PCR) analysis with SYBR green reagent (Applied Biosystems). The copy number was calculated using a relative standard curve,\textsuperscript{10} and \textit{Gapdh} was used for normalization. Chicken-specific qRT-PCR primers are listed in Table SI.

Statistics. Student’s \textit{t}-tests were performed to determine statistically significant differences for paired comparisons. For comparison of the percentage of nuclear Sox9 among control, LOF-perinodule and LOF-nodule, One-way analysis of variance (ANOVA) was performed with Bonferroni’s post-hoc analysis to test the difference between specific groups using PRISM6 software package (GraphPad). Data are reported as mean ± SEM, and statistically significant differences are reported where \textit{p}<0.05 or \textit{p}<0.01.

SI References