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Insufficient Versican Cleavage And Smad2 Phosphorylation Results In Bicuspid Aortic And Pulmonary Valves

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Abstract

Bicuspid or bifoliate aortic valve (BAV) results in two rather than three cusps and occurs in 1-2% of the population placing them at higher risk of developing progressive aortic valve disease. Only NOTCH-1 has been linked to human BAV, and genetically modified mouse models of BAV are limited by low penetrance and additional malformations. Here we report that in the *Adamts5*^{-/-} valves, collagen I, collagen III, and elastin were disrupted in the malformed hinge region that anchors the mature semilunar cusps and where versican, the ADAMTS5 proteoglycan substrate, accumulates. ADAMTS5 deficient preavalvular mesenchyme also exhibited a reduction of α -smooth muscle actin and filamin A suggesting versican cleavage may be involved in TGF β signaling. Subsequent evaluation showed a significant decrease of pSmad2 in regions of preavalvular mesenchyme in *Adamts5*^{-/-} valves. To test the hypothesis that ADAMTS5 versican cleavage is required, in part, to elicit Smad2 phosphorylation we further reduced Smad2 in *Adamts5*^{-/-} mice through intergenetic cross. The *Adamts5*^{-/-};*Smad2*^{+/-} mice had highly penetrant BAV and bicuspid pulmonary valve (BPV) malformations as well as increased cusp and hinge size compared to the *Adamts5*^{-/-} and control littermates. These studies demonstrate that semilunar cusp malformations (BAV and BPV) can arise from a failure to remodel the proteoglycan-rich provisional ECM. Specifically, faulty versican clearance due to ADAMTS5 deficiency blocks the initiation of pSmad2 signaling, which is required for excavation of endocardial cushions during aortic and pulmonary valve development. Further studies using the *Adamts5*^{-/-};*Smad2*^{+/-} mice with highly penetrant and isolated BAV, may lead to new pharmacological treatments for valve disease.

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Disclosures

None declared.

Keywords

Versican; ECM; bicuspid; cardiac valves; endocardial cushions; ADAMTS

1.1 Introduction

The formation of a bicuspid or bifoliate aortic valve (BAV) is the most common cardiovascular defect and occurs in 1% to 2% of the population [1-3]. A bicuspid valve is comprised of two rather than three semilunar cusps, which are also referred to as valvar leaflets. Bicuspid pulmonary valve (BPV) is commonly associated with other congenital heart diseases, but its incidence remains unknown [4] perhaps due to the fact that the clinical course of BPV is usually benign. However there are isolated reports that BPV is associated with pulmonary artery aneurysms [5, 6].

The majority of patients that require valve replacement surgery have an underlying BAV, and surgery or catheter intervention remain the only effective options since there is no pharmacologic treatment for valve disease [7]. Although BAV is a prevalent malformation, the developmental processes and genetic components involved in cusp fusion are poorly understood [8] in part due to very few mouse models that display a highly penetrant BAV[9-13]. The only gene in humans associated with BAV is NOTCH1 [14-16] and more recent studies have shown that loss of NOTCH1 function results in a reduction of cartilage related extracellular matrix (ECM) components [17]. A role for altered ECM architecture in BAV formation is consistent with reports that patients with connective tissue disorders have an increased likelihood of BAV [18-20]. In addition, mice lacking *Alk2* in their endocardial cushion mesenchyme cells display BAV with altered ECM [13] however the mechanistic role of ECM in BAV formation remains largely unknown.

During early valve development the extracellular matrix (ECM) is critical for the formation of the endocardial cushions [21], the precursors to mature cardiac valves. However, as the endocardial cushions remodel into adult valve cusps, the ECM undergoes dramatic changes. The relatively homogeneous ECM of the endocardial cushions, comprised of aggregating proteoglycans and hyaluronan, becomes stratified in the mature cusps with the addition of fibrillar collagens (fibrosa) and elastic fibers (ventricularis) that function in part to allow both durability and mobility. Although ECM remodeling of the endocardial cushions is involved in formation of the aortic and pulmonary valves and the arterial walls, very little is known about critical ECM transitions that orchestrate coordination and divergence of these distinct tissue types during late embryonic and fetal development.

The diverse and organized ECM in adult cardiac valves generally has focused on its structural role in maintaining the strength (collagen), flexibility (elastin) and compressive ability (proteoglycans) to preserve valve shape and to maintain unidirectional blood flow. However, Dietz and his colleagues revealed that mutations in the ECM component fibrillin-1 alter the regulation of TGF β , a key growth factor involved in both normal embryonic valve development and adult valve disease [22]. Further, genetically modified mice containing mutations in transcription factors that regulate ECM production exhibit a dramatic effect on prevulvar mesenchyme growth and differentiation [23-27]. While it is increasingly clear

that ECM plays a functional role in addition to a structural role, we are in the early stages of understanding the reciprocal interactions between ECM components, intracellular signaling and biomechanical force critical for cardiac valve development.

Recently, we discovered that mice deficient in the ECM 'proteoglycanase' ADAMTS5 develop myxomatous cardiac valves with 100% penetrance due to significant accumulation of intact versican, a proteoglycan substrate for this protease [28]. Importantly, the *Adamts5*^{-/-} myxomatous valve phenotype is rescued by *in vivo* reduction of versican, suggesting that versican accumulation occurs in the absence of its cleavage [28]. Here we investigated the hypothesis that clearance of versican via ADAMTS5 is required for differentiation of preavalvular mesenchymal cells that generate and organize fibrillar ECM components during aortic and pulmonary valve development and in adult valve homeostasis. Since versican cleavage is one of the first ECM mechanisms identified for post-EMT endocardial cushion remodeling, we took an 'outside-in' approach to determine how versican accumulation in the ECM ultimately impacts intracellular signaling in preavalvular mesenchyme. These studies focused on the morphogenetic processes involved in development of the hinge, a valvular structure, that is dependent on excavation of the endocardial cushions, a poorly understood process that is critical for aortic and pulmonary valve formation, and that we have discovered is dependent on ADAMTS5 versican cleavage.

Materials and Methods

2.1 Gene-targeted mice

All mouse experiments were done under protocols approved by the Medical University of South Carolina IACUC. The *Adamts5*^{-/-} mice used in this study were the *Adamts5*^{tm1Dgen/J} (Jackson Laboratories, Bar Harbor, ME) that were bred into C57Bl/6 (> 10 generations) and maintained as previously described [29, 30]. Genotyping of *Adamts5* mice was performed using PCR as previously published [30]. *Smad2*^{+/-} mice were also maintained on a pure C57/Bl6 background. Developmental tissue evaluated in this study was obtained from *Adamts5*^{+/-}/*Smad2*^{+/-} × *Adamts5*^{+/-}/*Smad2*^{+/+} intercross matings.

2.2 Histology and Immunohistochemistry

Standard histological procedures were used [31]; tropoelastin (Abcam, AB21601), ADAMTS5, pSmad2 (Abcam, AB47083), GAGβ [31], and NOS3 (Thermo Scientific, RB-9279) staining utilized embryos or isolated hearts that were fixed in 4% paraformaldehyde. Collagen I (mdbioproducts, 203002), α smooth muscle actin (Sigma, A 5228), and Collagen III (Abcam, AB6310) immunohistochemistry (IHC) was performed using Amsterdam fixed tissue [28] and fluorophore conjugated secondary antibodies were purchased from Jackson ImmunoResearch.

2.3 Quantification of Immunofluorescence and Valve Anomalies

Three-dimensional reconstructions were generated using Amira™ 5.3.3 (Visage Imaging, Andover, MA) as previously described [28]. Approximately 60, 5μm-thick paraffin sections were used to generate each aortic and pulmonary valve reconstruction.

Quantification of valve thickness (histological sections): The widest portion of the cusps was measured. An average of > 18 measurements were taken over a minimum depth of 60µm per heart. The same strategy was also performed for the hinge, defined as the attachment point of the cusp to the transient myocardium (**Fig. 1A, D**) defined as α sarcomeric actin positive tissue, originating from the cardiac outflow tract and secondary heart field that ‘disappears’ through multiple mechanisms as the arterial tissue is formed and aortic (AV) and pulmonary valves (PV) mature. The measurements were taken from the base of the ventricle and moved anteriorly in the developing aortic and pulmonary arteries. An Olympus BX40 microscope with DP2-BSW (v1.4, build 2743) software was used to obtain measurements. A minimum number of 3 animals were used per genotype from internally controlled litters for statistical analysis of morphometric data.

Quantification of IHC data: To quantify expression from IHC data, digital images of *Adamts5*^{-/-} and WT heart sections were acquired at identical confocal settings using the Leica TCS SP5 AOBS Confocal Microscope System (Leica Microsystems Inc., Exton, PA). Internally controlled littermates, coordinately processed tissue within the same IHC experiment qualify as n=1, per genotype. Percent positive pSmad2 was obtained by determining positive pixels for pSmad2 IHC that was normalized to total nuclei. A minimum of three separate experiments with four different litters of matched *Adamts5*^{-/-} and WT littermates was used for immunohistochemistry quantification and representative images of IHC ECM localization.

2.4 Bicuspid phenotypic scoring of murine aortic and pulmonary valves

The bicuspid phenotype from *Adamts5*^{+/-}/*Smad2*^{+/-} × *Adamts5*^{+/-}/*Smad2*^{+/-} crosses was scored when only two cusps were noted after examination of all serial sections (late fetal stages) and/or when there were only two arterial wall-anchoring sites (or commissures) found in the valve (sections and whole mount dissected hearts were used). Valves with residual cusps (defined as less than 20µm thick) that appeared to be raphes, were scored as bicuspid and represented less than 10% of the cohort. In the age ranges of post natal day 1 (P1) to 6 months the PV and AV phenotype, i.e tricuspid or bicuspid, was determined after the aortic and pulmonary valves were dissected by removing the ascending artery and ventricular tissue; i.e. turret dissections; turrets were also bisected to better visualize additional cusp anomalies.

2.5 Electron Microscopy

Transmission electron microscopy (Hitachi 7600) of mouse valve tissue was performed as previously described [32].

2.6 Statistical Analysis

In graphs the data was presented as the mean ± StdDev (error-bars). An independent samples t-test or one-way ANOVA was conducted to evaluate one-way data. If a significant difference was observed, Bonferroni’s post-hoc test was performed to identify groups with significant differences. *P* values with *p* < 0.05 were considered significant. Details of how original data were obtained are included in their respective experimental methods section.

Results

3.1 Formation of the cusps of the pulmonary and aortic valves involves changes in the extracellular matrix

During both PV and AV development endocardial cushions, rich in the proteoglycan versican, directly interface with α sarcomeric actin positive ventricular myocardium (**Figure 1 A, D**). At early stages the pulmonary valve cusps interface with more myocardium than aortic cusps. The outlined myocardial cuff (**Fig. 1A**), which is also referred to as transient myocardium, (**Fig. 1A, D**) disappears by E17 (**Fig. 1B**). As development progresses (E17) the endocardial cushions sculpt into mature valve cusps, through a process referred to as excavation. Thinning of the endocardial cushions generates the semilunar appearance of the cusps and creates the space referred to as the valvulosinus (**Fig. 1, A-C, E, F, arrowheads**). Developmental ECM transitions associated with excavation generate a fibrous rich structure, termed the annulus that connects the hinge of the valve cusps to the ventricular myocardium (**Fig. 1 C, F**). Experiments in this manuscript evaluate the role of ADAMTS5 dependent versican cleavage in excavation of the endocardial cushions and formation of the hinge and fibrous rich ECM that anchors the valve cusps to the myocardium at the base of the ventricles.

3.2 Myxomatous ADAMTS5 deficient valves show dysregulation of extracellular matrix and cytoskeletal proteins associated with human valve disease

We have previously shown that developing cardiac valves deficient in the ECM protease ADAMTS5 result in an increase in its aggregating proteoglycan substrate, versican. Here we expand this observation to focus on the developing hinge region, denoted by the asterisks in **Figure 2**, which also shows an increase in versican in the *Adamts5*^{-/-} mice compared to WT littermates (**Fig. 2A, B; E15.5**). We examined ECM components collagen type I (COL1A1), elastin (ELN) and collagen type 3 (COL3A1), which are linked to human myxomatous valve disease, [18-20, 33] in both the *Adamts5*^{-/-} and WT littermates. Collagen I was readily detected and appeared to be organized into fibers in the hinge regions of PV in the WT (**Fig. 2C, asterisk; P1**), and was continuous within the adjacent ventricular myocardium (**Fig. 2C, arrow**). In contrast, collagen I in the ADAMTS5 deficient myxomatous valves was diffuse and poorly organized in the hinge region (**Fig. 2D, asterisk; P1**) and was not integrated into the adjacent myocardial tissue like the WT (**Fig. 2D, arrow**). During normal aortic and pulmonary valve development, elastic fiber organization occurs relatively late (E17 and forward) [34] and was detected along the ventricular aspect of the valve cusps continuous with the arterial wall lining the valvulosinus at post-natal day 1 (P1), (**Fig. 2E, open arrow; P1**). Elastic fiber formation was disrupted or delayed at P1 in the *Adamts5*^{-/-} valves (**Fig. 2F, open arrow; P1**) and was dramatically altered at the juncture of the hinge. Collagen III, which is upregulated in myxomatous disease [35], was sequestered in the spongiosa region of WT aortic and pulmonary valve cusps at E15.5 (**Fig. 2G, line; E15.5**); however in *Adamts5*^{-/-} mice collagen III was found throughout the myxomatous *Adamts5*^{-/-} cusp (**Fig. 2H, line; E15.5**). In the narrow hinge region of WT mice at E15.5 there was very little collagen III detected (**Fig. 2G, asterisk**), while in the expanded hinge region of the *Adamts5*^{-/-} PV, collagen III immunolocalization was pronounced and indistinguishable from the myxomatous cusp (**Fig. 2H, asterisk**). Changes

in fibrous ECM components were the most pronounced in the *Adamts5*^{-/-} PV hinge region, results were similar in the AV, but not as dramatic (data not shown). Collectively, these data demonstrated that ECM proteins associated with human myxomatous valve disease and bicuspid valve malformations were disrupted in the ADAMTS5 deficient aortic and pulmonary valves. Therefore a consequence of versican accumulation in the *Adamts5*^{-/-} compared to the WT was the loss of fibrous ECM organization. In addition to ECM genes, mutations in cytoskeletal proteins filamin A [36, 37] and α SMA [38] have been associated with mitral valve prolapse (MVP) and BAV. By E17 in normal PV and AV development, filamin A was strongly expressed in the narrowing hinge region where cells were condensed and collagen was organized (**Fig. 2I, arrow; E17.5**). In contrast, the ADAMTS5 deficient mice showed reduced staining intensity in the hinge (**Fig. 2J, arrow; E17.5**). The arterial wall that showed strong positive staining served as an internal control for filamin A (**Fig. 2J, L; open arrow**). The dramatic reduction of filamin A immunodetection in the *Adamts5*^{-/-} compared to the WT was also pronounced in post-natal valves with myxomatous morphology (**Fig. 2K, L; P8**). We identified that α SMA was transiently expressed and detected in Amsterdam fixed cardiac WT valve tissue concomitant with cell compaction and collagen-rich ECM production (**Fig. 2M, arrowhead, P1**) in late gestation and at perinatal time points. Immunodetection of α SMA was reduced in the *Adamts5*^{-/-} valves (**Fig. 2N**) subjacent to the endocardium (**Fig. 2M, N; white outline, P1**); the reduction of α SMA in this developmental time point correlated with reduced mesenchymal cell compaction [28].

Electron microscopy revealed that in WT valves at P8, differentiating mesenchymal cells of the hinge were aligned with very little ECM space (**Fig. 2O, arrowhead**), consistent with effective compaction. In contrast, the hinge region of *Adamts5*^{-/-} PV showed disorganized cells with considerably more ECM space than WT (**Fig. 2P, arrowhead**), consistent with cell-cell compaction and cell-matrix abnormalities as well as myxomatous change. The disruption of ECM components and cytoskeletal proteins associated with human valve disease suggests that cardiac valves of ADAMTS5 deficient mice may serve as a relevant mouse model to elucidate signaling pathways involved in the etiology of valve disease.

3.3 Smad2 phosphorylation is dependent on versican clearance via ADAMTS5 in early stages of endocardial cushion remodeling during aortic and pulmonary valve development

Because both filamin A and α SMA are associated with activation of TGF β through Smad receptor mediated signaling [39-41], due to the fact that filamin A deficient cells showed impaired Smad2 phosphorylation [41] we examined pSmad2 expression in *Adamts5*^{-/-} valves to determine the potential role of TGF β in ECM and cytoskeletal abnormalities. Since the focus of the changes investigated here lie within tissue-tissue interfaces in early valves, rather than global changes within the entire heart, an immunohistochemical approach was used to investigate the normal pSmad2 reactivity in developing WT valves in comparison to ADAMTS5 deficient littermates. At E12.5-E14.0 we observed a significant reduction of pSmad2 in outflow tract mesenchyme and remodeling arterial tissue (**Fig. 3A-D, G**) in the *Adamts5*^{-/-} hearts compared to WT. Importantly, the reduction of pSmad2 showed an inverse correlation with the immunolocalization of intact versican (GAG β) in sister sections (**Fig. 3E, F**), i.e. ECM that exhibited intense staining for intact versican was essentially devoid of pSmad2 nuclear staining (**Fig. 3A-F**). However, at E15.5 and older we did not

find a consistent or statistically significant difference in pSmad2 nuclear staining in cardiac valve sections of *Adamts5*^{-/-} compared to WT littermates (**Fig. 3H**); this observation is consistent with a temporally restricted mechanism which corresponds with structural maturity of the hinge region.

3.4 Reduction of Smad2 via intergenetic cross with *Adamts5*^{-/-} results in high penetrance of myxomatous bicuspid pulmonary and aortic valves

To determine if versican cleavage by ADAMTS5 was playing a role in the initiation of Smad2 signaling we reduced Smad2 in the *Adamts5*^{-/-} mice. We reasoned that further reduction of Smad2 would exacerbate the *Adamts5*^{-/-} aortic and pulmonary valve phenotype and may reveal additional anomalies during valve development where versican cleavage and TGFβ intersect. Prior to the *Adamts5*;*Smad2* intercross we evaluated the *Smad2*^{+/-} mice and did not detect an aortic or pulmonary valve phenotype. However in the context of ADAMTS5 deficiency i.e. *Adamts5*^{-/-};*Smad2*^{+/-} (KO-Het) we observed a high frequency of bicuspid pulmonary and aortic valves (not seen at high penetrance in control littermates (**Fig. 4J**)). In post natal hearts fine dissections of PV and AV were used to evaluate morphology and cusp arrangement (**Fig. 4 and SFig. 1**). There were protrusions on the ventricular face of the cusp in the *Adamts5*^{-/-};*Smad2*^{+/-} valves (**Fig. 4I**). A Raphe (**SFig. 1H, asterisk**), was visible in the open turret, in an adult PV of the *Adamts5*^{-/-};*Smad2*^{+/-} heart. The graph (**Fig. 4J**) depicts the frequency of bicuspid PV and AV in the *Adamts5* × *Smad2* intercross as well as control genotypes. There was approximately 60% bicuspid PV and 75% bicuspid AV with approximately 50% of *Adamts5*^{-/-};*Smad2*^{+/-} mice had both bicuspid PV and AV (**Fig. 4J, grey overlay (-/-;+/-)**).

Representative histological sections, of the *Adamts5*^{-/-};*Smad2*^{+/-} (KO-Het) valves revealed increased width in both the hinges and cusps of the PV and AV compared to the ADAMTS5 KO-WT as well as other control littermates (WT-WT; Het-Het; WT-Het; **Fig. 5A-F'**). However, the only statistically significant difference was in the PV cusp where the average KO-Het cusps were wider than the KO-WT cusps and showed statistical significance when compared to the WT-WT (**Fig. 5N**). Although the morphometrics did not always reveal statistical significance, there was a strong correlation with both the increased hinge and cusp size of the KO-Het and the bicuspid valve anomaly i.e. the PV morphometrics demonstrated an increase in the hinge size of the KO-Het compared to the KO-WT (p<0.16) (**Fig. 5M**). The increases in width and cusp of the KO-Het were also evident in the AV (**Fig. 5O, P**). Analysis of histological sections and subsequent 3D reconstructions (**Fig. 5G-L**) revealed that in the PV, the left (L) cusp was always involved in fusion while in the AV the non-coronary cusp (N) was fused either with the left coronary (L) or right (R) coronary cusp (**SFig. 2**).

3.5 Endothelial nitric oxide synthase (NOS3) expression was not significantly altered in the valvular endothelium but showed a decrease in the hinge region of developing SLV cusps in the ADAMTS5 deficient hearts

Since NOS3 is activated by pSmad2 [42, 43] and is associated with bicuspid valve malformations, specifically right coronary cusp (R)-N fusion, [11, 44] we examined the WT

and ADAMTS5 deficient hearts to determine if there was a reduction of NOS3 expression. There was no detectable difference in the endothelial expression of NOS3 in the PV or the AV in ADAMTS5 deficient mice compared to WT littermates (**Fig. 6A-G**, arrowheads). However, there was a reproducible decrease in IHC NOS3 staining in the hinge mesenchyme (boxes in **Fig. 6C-E, F, G and F', G'**) and the developing aortic and pulmonary arterial walls (**Fig. 6C-E, F, G**, open arrows), areas of high ADAMTS5 expression and versican cleavage [28]. Although we did not detect a difference in valvular endothelial NOS3 expression in *Adamts5*^{-/-} mice there was reduced NOS3 in hinge mesenchyme where ADAMTS5 is normally expressed (**Fig. 6**).

4.0 Discussion

Data presented in this manuscript were the first to demonstrate that abnormal proteoglycan accumulation and reduced pSmad2 lead to BAV and BPV. To determine the role of the ECM 'proteoglycanase' ADAMTS5 in remodeling of the outflow tract cushions, we have employed an *in vivo* outside-in experimental approach. Here we revealed that versican clearance was required for the organization of fibrous ECM in the forming hinge and commissures, structures of the valves that are malformed in the *Adamts5*^{-/-} mice. The width of the hinge is also a measure of excavation, or thinning of the endocardial cushions that give rise to the semilunar appearance of adult valve cusps. Although integral to the formation of the valve cusps, versican cleavage is the first ECM molecular mechanism that we are aware of that contributes to the regulation of excavation of the endocardial cushions. In *Adamts5*^{-/-} aortic and pulmonary valves versican accumulation also disrupted α SMA and filamin A, cytoskeletal proteins associated with valve anomalies in human disease [39-41]. This correlated with a loss of mesenchymal cell-cell condensation. We had previously published that prevalvular mesenchyme is condensed and has rounded cells when surrounding ECM has predominantly cleaved versican. In contrast mesenchyme that is well-spaced has stellate cells in association with ECM rich in 'intact' versican [28, 45]. Therefore, changing the state of versican from 'intact' to 'cleaved' appeared to be a requirement for the induction of both mesenchymal cell condensation and a more rounded shape, which we hypothesize are early indicators of prevalvular mesenchyme differentiation. Data presented in this manuscript showed that the morphogenetic consequence of reduced mesenchymal cell differentiation and collagen formation in aortic and pulmonary valve development was aberrant excavation i.e. thinning of the hinge region (**Fig. 7**). The exacerbated phenotype of the *Adamts5*^{-/-}; *Smad2*^{+/-}, where both the hinge and distal cusp regions were larger than single *Adamts5*^{-/-}, demonstrated a previously unknown connection between excavation of the cusps and malformations that include bicuspid aortic and pulmonary valves.

Although additional experimentation is necessary to determine the cell types that require ADAMTS5 for normal aortic and pulmonary valve development, these data and our previously published work [28], suggest that in early stages of endocardial cushion remodeling both endocardium and transient myocardium secrete ADAMTS5 and initiate versican cleavage that may influence mesenchymal cell differentiation. The fusion pattern identified, namely the non-coronary (N) cusp with either the right or the left coronary cusp is consistent with the NOS3 deficient mouse model of BAV where endothelial-mesenchymal

signaling is impaired [11], (**SFig. 3**) identifying involvement of the intercalated outflow tract (OFT) cushions. The Syrian hamster model of BAV undergoes a different cusp fusion pattern. In the Syrian hamster BAV the R-L cusp fusion arises due to a malformation of the initial septal structure of the outflow tract, presumably due to excessive fusion of the major outflow tract cushions (**SFig. 3**). Interestingly, tenascin a fibrous ECM protein, is disrupted in the abnormal septal structures of the Syrian hamster bicuspid valves [8]. Notably this initial septal structure of the outflow tract, dependent on the migration of cardiac neural crest, disappears prior to the formation of the adult heart. We speculate that versican cleavage may be required to remodel the basal lamina to distinguish valvular endothelium, from the endothelium of the major outflow tract cushions which fuse and initially septates the common OFT [46-48]. Alternatively, the expanded endocardial cushion size in the *Adamts5*^{-/-}; *Smad2*^{+/-} hearts, due to lack of versican clearance and loss of cell-cell condensation, may 'push' the remodeling cusps in close proximity and/or alter blood flow that ultimately results in cusp fusion. The fact that we observed decreased NOS3 within the hinge mesenchyme of *Adamts5*^{-/-}; *Smad2*^{+/-} valves suggests NOS3 deficiency may not only implicate endothelial cell dysfunction but also aberrant mesenchymal differentiation. Collectively these observations also allow for reciprocal interactions between insufficient ECM remodeling of the endocardial cushions and altered hemodynamics that might contribute to BAV formation.

In the *Adamts5*^{-/-} hearts, changes in mesenchymal differentiation, including the lack of cell-cell compaction, correlated with a loss of nuclear pSmad2. However, the mechanism by which accumulation of versican inhibits pSmad2 remains to be determined since we are at the advent of determining how the ECM microenvironment influences cell behavior and signaling in the context of valve development and disease. It has been suggested that glucosaminoglycans (GAGs) attached to the versican core protein bind TGFβ ligand and sequester it from signaling. Toward this end an ECM lacking intact versican is devoid of TGFβ3 in chondrocyte differentiation [49]. Versican also binds key ECM signaling components at its N (hyaluronan) and C termini (fibrillin-1) [50] therefore its proteolytic cleavage may release ECM constituents that ultimately effect Smad2 phosphorylation. In ADAMTS5 deficient newborn skin fibroblasts Smad2 is not phosphorylated in response to TGFβ1 like WT fibroblasts [51]. However in dermal fibroblasts of a high passage number, ADAMTS5 deficiency increases pSmad2 and αSMA [52]. Therefore the specific *in vivo* and *in vitro* context is also critical when determining the role of ADAMTS activity on TGFβ signaling. Given that ADAMTS versican cleavage separates the N-terminal HA binding domain from the GAG-binding-G3 domain, and that the N-terminal fragment appears to be stable while the GAG-G3 domains are cleared, poses many pathway interactions that could profoundly influence Smad2 phosphorylation when the ratio of intact to cleaved versican is altered through ADAMTS cleavage.

The requirement of TGFβ is well established during endocardial cushion formation, however its role during endocardial cushion remodeling is not well understood (reviewed [53]). Recently the analysis of *Tgfb2*^{-/-} embryos revealed loss of TGFβ/Smad2 signaling resulted in a lack of ECM heterogeneity and an increase in cartilage associated ECM including versican and hyaluronan [54], similar to ADAMTS5 deficient valves where decreased

pSmad2 correlated with versican accumulation and a loss of fibrous ECM. These data also indicate the potential for reciprocal (ECM-TGF β -ECM) interactions that may facilitate TGF β progression in valve development; where an abnormal increase in cartilage related ECM components inhibits induction of TGF β , (i.e. ADAMTS5 deficiency; ECM-TGF β), while a reduction in TGF β signaling pathway components (i.e. *Tgfb2*^{-/-} embryos; TGF β -ECM) results in accumulation of 'cartilage (versican)-rich ECM'. A developmental model that invokes reciprocal ECM-TGF β signaling suggests an efficient means of amplifying differentiation during normal development. However, the converse is also true, that disruption at any point within the ECM-TGF β cycle blocks the propagation of TGF β signaling required for normal aortic and pulmonary valve development and exacerbates the developmental anomaly.

The fact that the bicuspid valves of the combinatorial mutant *Adamts5*^{-/-};*Smad2*^{+/-} correlate with proteoglycan accumulation and reduced TGF β signaling, brings up an interesting dichotomy with respect to the typical clinical manifestation of adult bicuspid valve disease. Generally, human BAV disease is a result of fibrosis and calcification (reviewed [4, 55, 56]). More specifically, NOTCH1 loss of function results in an up-regulation of the ADAMTS proteases and a reduction of SOX9 [17, 57, 58], the exact opposite of the ADAMTS5 deficient BAV etiology. These data suggest antagonism between the TGF β and NOTCH signaling pathways during development and in adult homeostasis may be in a fine-tuned balance to generate and to maintain the stratified ECM of adult valves. As more human mutations linked to BAV emerge and additional mouse models with BAV are characterized, the interdependence of ECM composition and TGF β /NOTCH signaling in BAV formation and disease will be further elucidated.

Although not specifically addressed, but likely a key factor in determining aortic and pulmonary valve morphology, are the reciprocal interactions between biomechanical forces and ECM composition that remain a difficult variable to investigate *in vivo*. For example, NOS3 is upregulated by pSmad2 [42, 43], but it is also increased in response to biomechanical force [59, 60]; therefore it is reasonable to expect that both changes in pSmad2 and biomechanical force within the myxomatous ADAMTS5 deficient valve cusps ultimately influence NOS3 expression and impact mesenchymal differentiation. The viable double mutant *Adamts5*^{-/-};*Smad2*^{+/-} mice with high penetrant BAV/BPV, provides an important new model to study BAV malformation and its progression through adult disease. The ultimate goal of these studies is to identify potentially novel therapeutic targets that will allow effective pharmacological treatment for patients whose only current option to repair cardiac valve dysfunction is replacement surgery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard abbreviations and acronyms

BAV	bicuspid aortic valve
BPV	bicuspid pulmonary valve
ECM	extracellular matrix
ADAMTS5	<u>A</u> <u>D</u> isintegrin-like and <u>M</u> etalloprotease domain with <u>T</u> hrombo <u>S</u> pondin type motifs
EMT	epithelial to mesenchymal transition
WT	wild type
OFT	outflow tract
αSMA	alpha smooth muscle actin
VIC	valvular interstitial cells
PV	pulmonary valve
AV	aortic valve

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Highlights

Accumulation of versican inhibits mature ECM formation in the hinge of semilunar valves.

Dysregulation of proteoglycan cleavage inhibits pSmad2 in preavalvular mesenchyme.

Versican accumulation inhibits cusp excavation, and correlates with a bicuspid anomaly.

ADAMTS5 and Smad2 deficiency leads to a highly penetrant model of bicuspid aortic valves.

Figure 1. Excavation of the endocardial cushions that give rise to the cusps of the pulmonary and aortic valves involves changes in the extracellular matrix

'Intact versican' GAG β (green) in the endocardial cushions and forming cusps of the pulmonary (A, B) and aortic (D, E) valves. Collagen I expression shown at E17 in the pulmonary (C) and aortic (F) valves. Open arrowheads (A-C, E, F) denote excavation of the endocardial cushions that generate the valvulosinus. Boxes (B, E) show hinge regions of the pulmonary valve (PV; B, C) and in the aortic valve (AV; E, F). Blue-myocardial marker, α sarcormeric actin; Red- propidium iodide; Red outline-transient myocardium (A, D) or myocardial cuff of the PV (A); Yellow outline -PV annulus (B, C) and AV annulus (E, F).

Figure 2. ECM and cytoskeletal proteins associated with human valve disease are disrupted in myxomatous *ADAMTS5* deficient valves compared to WT
 'Intact versican' GAG β (green) in WT E15.5 (A) and the *Adamts5*^{-/-} pulmonary valves (B). Collagen I localization at P1 in WT (C) and in *Adamts5*^{-/-} valves (D, green; arrows (C, D) collagen I in the hinge region denoted by asterisk). Tropoelastin (green) localization at P1 in the ventricular aspect of the hinge in the WT (E) in contrast to the *Adamts5*^{-/-} (F; open arrows (E, F) -elastin organization). Collagen III (green) at E15.5 is restricted to the spongiosa in the WT (G, line); in the *Adamts5*^{-/-} valves collagen III is expanded in the cusp (H, line). Filamin A (green) in the hinge region of WT (I, K) and *Adamts5*^{-/-} deficient valves at E17 and P8 respectively (J, L; arrows-differential staining of filamin A). α SMA expression (M, N, green) in P1 valves (arrowheads-differential α SMA expression). Electron

micrographs of WT (O) and *Adams5*^{-/-} (P) valve hinge (P8; arrowheads- ECM intercell space). * (A-D, G-H)-hinge regions at the cusp-transient myocardial interface; Red-propidium iodide; blue- (A, B), α SMA; blue (G, H), α Sarcomeric actin. Scale bar in A = 100 μ m applies to B-H; I= 200 μ m applies to J-L; M = 50 μ m applies to N; O = 2 μ m applies to P. n = 4; each genotype.

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Figure 3. Smad2 is reduced in areas of versican accumulation in remodeling outflow tract cushions of ADAMTS5 deficient mice
pSmad2 (green) immunolocalization at E13.5 (A, B) and E12 (C, D) in WT (A, C) and *Adamts5*^{-/-} (B, D) mesenchyme. ‘Intact versican’ GAGβ (green) (E, F) at E12 in sister sections of C and D respectively. Vertical bars denote areas with inversed correlation of versican and pSmad2. Arrows-subendocardial mesenchyme; asterisks-myocardium subjacent to the hinge; m-myocardium; D-distal outflow tract; P-proximal outflow tract. Red-propidium iodide; blue - (A-D) α-sarcomeric actin; blue - (E, F) αSMA. Quantification of pSmad2 nuclear staining E12-14 (G; n=4, WT, 4 different litters and *Adamts5*^{-/-} hearts (n=4, *Adamts5*^{-/-}, 4 different litters; * P < 1.5 × 10⁻⁷) normalized to WT. Quantification of pSmad2 nuclear staining E15.5-P8 (H; n=8, WT, 7 different litters and n=8, *Adamts5*^{-/-}, 7 different litters; p<0.2950) normalized to WT. Scale Bar in A= 100μm and applies to B; C = 200μm and applies to D-F.

Figure 4. *Adamts5*^{-/-}; *Smad2*^{+/-} exhibit a high penetrance of bicuspid pulmonary and aortic valves

Dissected aortic valves (AV) at postnatal day 8 (P8; A-C) and 1 month (1mo; D-F). Arrowheads denote commissures (A-F) and highlight the anomalous and asymmetric commissure formation in *Adamts5*^{-/-}; *Smad2*^{+/+} (B, E) and two versus three commissures in the *Adamts5*^{-/-}; *Smad2*^{+/-} (C, F). Opened AV (G-I) from adult hearts. Arrows (I) denote myxomatous bulges on the ventricular face of the bicuspid *Adamts5*^{-/-}; *Smad2*^{+/-} cusps. Graph (J) depicting phenotypic penetrance of bicuspid pulmonary and aortic valves of *Adamts5*^{-/-}; *Smad2*^{+/-} compared to intergenetic cross control genotypes. Dotted - BPV percentage; open bars - BAV percentage. Grey overlay -percentage of *Adamts5*^{-/-}; *Smad2*^{+/-} hearts that had both BPV and BAV. Scale bar in A = 350µm applies to B, C; D = 300 µm applies to E, F; G = 250µm applies to H and I.

Figure 5. *Adamts5*^{-/-} mice with *in vivo* reduction of Smad2 display enlarged pulmonary and aortic valve cusps and hinge regions

Hematoxylin and eosin stained frontal sections of E17.5 pulmonary (PV) and aortic (AV) valves (A-F). ' -denotes section of the same heart, approximately 40 μm dorsally. Black arrowheads-hinge regions. Amira™ 3D reconstructions of E19.5 PV and AV cusps (G-L). L, red-left cusp of the PV; R, blue- right cusp of the PV; An, yellow- anterior cusp of the PV; purple-fusion of the R and L in the bicuspid PV; N, blue- non-coronary cusp of the AV; L, yellow-left coronary cusp of the AV; R, white- right coronary cusp of the AV; green-fusion of N and L of the bicuspid AV. Graph of average PV hinge (M) and cusp (N) width. Average width of AV hinge (O) and AV cusp (P). n=3, each genotype; p values as marked. *-denotes statistical significance. Scale bar in A = 150μm applies through F'; G = 200μm applies to H-L.

Figure 6. Endothelial nitrous oxide synthase (NOS3) is not significantly altered in the valvular endothelium of ADAMTS5 deficient mice

Expression of NOS3 was examined in histological sections of WT, *Adamts5*^{-/-} and *Adamts5*^{-/-};*Smad2*^{+/-} developing valves (A,B,C-E,F-G', green). NOS3 staining in valvular endothelium at E11.5 (A, B), E13.5 (C-E), and E14.5 (F, G), is depicted by arrowheads. Open arrowhead (B) shows altered pattern but not altered intensity of NOS3 in *Adamts5*^{-/-};*Smad2*^{+/-}. Boxes (C-E; F, G) of the forming hinge region (mesenchymal-myocardial interface) shown in higher magnification in the inset (C-E) or in the adjacent panel (F' and G') and reveal mesenchymal NOS3 staining of the WT. NOS3 expression was noted in the arterial walls (open arrows C-E; F, G) Cleaved versican, i.e. DPEAAE staining (A' and B', green) present in *Adamts5*^{+/+};*Smad2*^{+/-} and not detected in *Adamts5*^{-/-};*Smad2*^{+/-} littermate. Intact versican (C'-E', green) correlates with the hinge width and subjacent to the myocardium (C'-E', blue). αSMA- alpha smooth muscle actin; αSarc-alpha sarcormeric actin. NOS3 immunohistochemistry involved E11.5-E15.5, n=4 each genotype. Scale bar in A = 100μm applies to A', B, B'; C = 100μm applies to D, E and

C, ' D' and E'; Inset C = 20 μm and applies to all inset boxes; F = 200 μm applies to G. Bar in F' = 20 μm applies to G'.

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Figure 7. Schematic depicting versican cleavage in normal aortic and pulmonary valve development and its disruption in *Adamts5*^{-/-} and *Adamts5*^{-/-}; *Smad2*^{+/-} mouse models with valve malformations

Whole hearts (A) depict the relative orientation of models in B and C; blue squares (A) show cross section (E11, E12), and oblique orientation (E13-E15) for models in B and C. Models depict WT (B) and ADAMTS5 deficient (C) development of outflow tract cushions. After septation (B, C, E13-E15) the predominant form of versican, either intact (royal blue) or DPEAAE (cleaved versican) (cyan) is shown in normal valve development (B), with a loss of DPEAAE (C). Malformations of valve development at specific stages are depicted (C). The bicuspid malformation, a predominant phenotype of the *Adamts5*^{-/-}; *Smad2*^{+/-} (C, E12) and delayed or insufficient excavation (C, E13, E15) resulting in enlarged hinge regions (C, E15) in *Adamts5*^{-/-} and more severe in *Adamts5*^{-/-}; *Smad2*^{+/-} mice. Pink-neural crest cells (NCC) involved in outflow tract septation; Yellow- collagen fiber formation during ECM stratification (B, E15), reduced in *Adamts5*^{-/-}; *Smad2*^{+/-} and *Adamts5*^{-/-} (C, E15) mice. White arrows (C, E12) show fusion of endocardial cells (EC) in bicuspid malformations of ADAMTS5 deficiency. Black arrowheads depict regions of normal excavation (B, E13, E15) or reduced excavation (C, E15); the developmental process of excavation creates the valvular sinus regions, which is the space between the valve cusps and the ascending arterial walls. Red-transient myocardium that expresses ADAMTS5; Orange- arterial wall tissue, defined as collagen-rich, α smooth muscle actin positive and α sarcomeric actin (myocardial marker) negative. LC- lateral outflow tract cushion; IC- intercalated cushion. Note: for simplicity we have left out NCC and endocardial derived cells however, a subset of these cells remain in the cusps at stages represented in these models.