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Original article

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 the ADAMTS5 proteoglycan substrate versican, accumulates. ADAMTS5 deficient prevalvular mesenchyme also exhibited a reduction of α-smooth muscle actin and filamin A suggesting versican cleavage may be involved in TGFβ signaling. Subsequent evaluation showed a significant decrease of pSmad2 in regions of prevalvular 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.

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 BAV patients require valve replacement surgery or catheter intervention 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 mesenchymal 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

Abbreviations: BAV, bicuspid aortic valve; BPV, bicuspid pulmonary valve; ECM, extracellular matrix; ADAMTS5, a disintegrin-like and metalloprotease domain with thrombospondin 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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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.

Investigation of 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 collogues revealed that mutations in the ECM component fibrillin-1 alter the regulation of TGFβ, 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 prevalvular 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 prevalvular 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 prevalvular mesenchyme. These studies focused on the morphogenetic processes involved in development of the hinge, a valvular structure, that anchors the cusps to adjacent tissues. Hinge formation 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.

2. 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, (Abcam, AB41037) 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 flourophore 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, 5 μm-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 60 μm 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). Transient myocardium was 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 were 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 20 μm 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 sample 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.