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Differential expression of cartilage and bone-related proteins in pediatric and adult diseased aortic valves

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Abstract

Approximately 5 million people are affected with aortic valve disease (AoVD) in the United States. The most common treatment is aortic valve (AoV) replacement surgery, however, replacement valves are susceptible to failure, necessitating additional surgeries. The molecular mechanisms underlying disease progression and late AoV calcification are not well understood. Recent studies suggest that genes involved in bone and cartilage development play an active role in osteogenic-like calcification in human calcific AoVD (CAVD). In an effort to define the molecular pathways involved in AoVD progression and calcification, expression of markers of valve mesenchymal progenitors, chondrogenic precursors, and osteogenic differentiation was compared in pediatric non-calcified and adult calcified AoV specimens. Valvular interstitial cell (VIC) activation, extracellular matrix (ECM) disorganization, and markers of valve mesenchymal and skeletal chondrogenic progenitor cells were observed in both pediatric and adult AoVD. However, activated BMP signaling, increased expression of cartilage and bone-type collagens, and increased expression of the osteogenic marker Runx2 are observed in adult diseased AoVs and are not observed in the majority of pediatric diseased valves, representing a marked distinction in the molecular profile between pediatric and adult diseased AoVs. The combined evidence suggests that an actively regulated osteochondrogenic disease process underlies the pathological changes affecting AoVD progression, ultimately resulting in stenotic AoVD. Both pediatric and adult diseased AoVs express protein markers of valve mesenchymal and chondrogenic progenitor cells while adult diseased AoVs also express proteins involved in osteogenic calcification. These findings provide specific molecular indicators of AoVD progression, which may lead to identification of early disease markers and the development of potential therapeutics.

Keywords

Aortic valve disease; Valvular interstitial cells; Calcification; Extracellular matrix

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Introduction

In the United States, aortic valve disease (AoVD) is estimated to affect 2% of the population and the prevalence increases in the aged [1,2]. Stenotic AoVD involves reduced valve cusp movement with narrowing of the effective valve opening and is often characterized by pathological calcification [3,4]. Recently, a National Heart Lung and Blood Institute working group stated that calcific aortic valve disease (CAVD) is an actively regulated disease process and recommended that further understanding of the molecular pathways involved in the progression of CAVD is necessary [5]. The natural history of AoVD is progressive and often necessitates aortic valve (AoV) replacement surgery using either a mechanical or bioprosthetic valve [6]. Unfortunately, replacement valves are associated with long-term complications. Anticoagulation therapy is necessary with mechanical replacement valves to avoid thromboemboli, and bioprosthetic valves are not durable and often require additional surgeries [6,7]. Molecular markers of AoVD progression, especially early AoVD pathogenesis, are presently unknown. In this study, the molecular characteristics of pediatric and adult diseased stenotic AoVs are examined. The goal of these studies is to define specific molecular indicators of valve pathogenesis that could lead to the development of early medical treatment to halt disease progression and prevent the need for surgery.

The mesenchymal progenitor cells, comprising the early endocardial cushions from which the heart valves develop, share a molecular profile with early chondrogenic mesenchymal precursor cells [8,9]. Transcription factors, including Twist1, Sox9, and Msx2, and the signaling molecule BMP2 (bone morphogenetic protein 2), are key factors in both early valve and bone development [10–18]. The mesenchymal progenitor cells of the endocardial cushions are the precursors of the mature valvular interstitial cells (VICs), which are the primary cellular component of adult valves [19]. After birth, there is a sharp decrease in the number of proliferating VICs and healthy mature valves are composed primarily of quiescent VICs with little to no cell proliferation or synthetic activity [19–21]. During disease, there is VIC disarray and clusters of VICs are activated, expressing myofibroblast markers and undergoing cell proliferation, ultimately leading to heterogeneous abnormalities in matrix composition [22–24]. The study of gene expression changes in activated VICs likely will provide mechanistic insights into valve pathogenesis at the molecular level. Presently, it is unclear how VIC activation leads to AoVD and whether it plays a role in early pathogenesis.

During valve maturation, the valve extracellular matrix (ECM) becomes stratified with increased collagen organization and increased elastic fiber deposition [20,21]. The mature aortic valves are composed of three ECM layers, the collagen-rich fibrosa layer, the ventricularis layer consisting primarily of elastic fibers, and the middle spongiosa layer with loosely arranged proteoglycans [21]. Histologically, both pediatric and adult diseased AoVs have increased ECM production and aberrant remodeling, leading to a loss of valve cusp stratification and increased valve cusp thickness [21,25–27]. Importantly, the onset of valve calcification is unusual before the fifth decade of life; however, calcification tends to occur a decade earlier in cases where the underlying AoV is congenitally malformed [28–30]. Interestingly, pediatric stenotic AoVs, the vast majority of which are malformed, do not typically calcify [28–30]. In contrast, pathologic calcification is commonly found in adult stenotic AoVD [31]. A hallmark study describing gene expression changes in adult CAVD reported that an osteoblast-like gene expression profile was associated with valve calcification [32]. Historically, valve calcification was presumed to be strictly dystrophic (passive), however, a growing body of literature suggests that genes associated with chondrogenesis and osteogenesis are expressed by activated VICs in adult CAVD [22,32–34].

The goal of this study is to compare pediatric diseased AoVs that are not calcified with adult CAVD specimens as a means to define molecular indicators of early and late AoVD and specific markers of pathological calcification. Evaluation of gene expression using markers of early valve mesenchymal progenitors, chondrogenic precursors, and osteogenic differentiation in pediatric and adult diseased AoVs will provide insight into the molecular basis for AoVD progression from non-calcified disease valves to late stage calcified valves. In this study, we observe that activated VICs in pediatric and adult diseased AoVs share expression of valve mesenchymal progenitor and chondrogenic precursor markers, whereas expression of osteogenic differentiation markers is specific to adult CAVD.

Material and Methods

Human AoV specimens

Human diseased aortic valve specimens were obtained from patients undergoing valve replacement surgery. Patients with a history of infective endocarditis, rheumatic heart disease, or a genetic syndrome were excluded. Control AoVs were obtained from age-matched individuals at the time of autopsy who died of non-cardiac causes. AoV tissues were fixed in 10% formalin, dehydrated through a graded ethanol series, washed in xylenes, and embedded in paraffin wax. Studies were approved by the Institutional Review Boards at Cincinnati Children's Hospital Medical Center and the University of Cincinnati.

Histology

Pediatric and adult AoV specimens were sectioned at 5 μ m. Pentachrome and von Kossa histological stains were performed as described previously [21]. Movat's pentachrome staining (American Master-Tech Scientific) was used to assess AoV ECM composition and trilaminar organization. Alizarin Red (2% alizarin red S in water, pH 4.2; Sigma-Aldrich) and von Kossa (Diagnostic BioSystems) stainings determined the presence or absence of pathological calcification.

Immunohistochemistry

Pediatric and adult AoV 5 μ m sections were dewaxed in xylenes and rehydrated through a graded ethanol series into distilled water. High temperature/pressure antigen retrieval using citric acid antigen unmasking solution (Vector Labs) was used for proteins with nuclear expression [35]. For ECM proteins, hyaluronidase (Sigma, H3884) at 200units/mL in 1XPBS (pH 5.4) was used to pre-treat sections for 3 hours at 37°C to degrade the hyaluronan matrix and expose the antigenic sites of collagens 2 and 10. Tissues were treated with 0.3% hydrogen peroxide and blocked in 6% normal serum (Thermo Fisher Scientific) of the secondary antibody host species (either goat or horse). The following primary antibodies were used: SMA (Sigma #IMMH2 [1:175]), PHH3 (Millipore #06-570 [1:350]), p-Smad 1/5/8 (Millipore #AB3848 [1:150]), Twist1 (Santa Cruz #sc-81417 [1:40]), Sox9 (Millipore #AB5535 [1:800]), Msx2 (Sigma #HPA005652 [1:125]), Mef2c (Sigma #HPA005533 [1:150]), collagen 2 (Developmental Studies Hybridoma Bank #11-116B3-c [1:25]), collagen 10 (Developmental Studies Hybridoma Bank #XAC9-c [1:15]), Runx2 (MBL #D130-3 [1:150]), Cleaved Caspase-3 (Cell Signaling #9664 [1:100]).

For colorimetric immunohistochemistry, the following secondary antibodies were used: goat anti-mouse peroxidase (Jackson ImmunoResearch #115-035-062), goat anti-rabbit peroxidase (Jackson ImmunoResearch #111-035-045), goat anti-rabbit biotin (Thermo Fisher Scientific, kit #32054), and horse anti-mouse biotin (Thermo Fisher Scientific, #32052). A streptavidin-horseradish peroxidase ABC reagent (Thermo Scientific, #32054) was used in conjunction with biotin conjugated secondary antibodies. Staining was detected using the metal enhanced diaminobenzidine (DAB) substrate kit (Thermo Fisher Scientific,

#34065). Most tissue sections were subsequently counterstained with nuclear fast red (Vector Labs) according to manufacturer's instructions. Staining was imaged using an Olympus BX51 microscope, SPOT digital camera (Diagnostic Instruments) and SPOT software (version 4.5).

Immunofluorescent alpha SMA/Mef2c double labeling was performed using the secondary antibodies, goat anti-mouse alexa-568 (Invitrogen, A11004) and goat anti-rabbit alexa-488 (Invitrogen, A11008). The nuclear counterstain ToPro3-642/661 (Invitrogen, T3605) was used according to manufacturer's instructions. Sections were imaged using a Zeiss LSM 510 confocal microscope and LSM version 3.2 SP2 software.

Statistics

A Fisher's exact test was used to compare the number of positively stained specimens in control versus diseased groups (Table 1). A p-value < 0.05 was considered significant (Table 1).

Results

Study population of pediatric and adult human AoV specimens

Pediatric diseased AoVs from patients aged 1 to 12 years, with a mean age of 5.2 years, (n = 5) were compared to pediatric control valves, aged 1 to 16 years with a mean age of 7.6 years (n = 4) (Table 1). All of the pediatric diseased specimens had isolated AoV stenosis and 4/5 (80%) had bicuspid AoV. None of the pediatric patients had additional cardiovascular diseases, eg. coronary artery disease. Pediatric diseased specimens also had evidence of ECM disorganization and valve thickening; importantly, no calcification was observed (Figure 1). However the oldest (12 years) and most severely diseased pediatric specimen had distinct noncalcified fibrotic foci. Adult AoV specimens included affected valves from individuals aged 53 to 83 years, with a mean age of 72 years, (n = 7), and age-matched control valves (n = 6). Among adult diseased valves, 1/7 (14%) had an underlying BAV. Unlike pediatric patients, adult patients had other significant disease presentation, including coronary artery disease (3/7), systemic hypertension (3/7), diabetes mellitus (2/7), aortopathy (2/7), and renal failure (1/7); some had more than one comorbid condition. All adult patients had CAVD with aortic stenosis evident in macroscopic calcified nodule formation, ECM disorganization, and valve thickening (Figure 1). Tissue heterogeneity was apparent in the adult diseased specimens, in that regions of the valves appeared thickened and possessed calcified nodules, whereas other regions appeared relatively normal. Adult control AoVs did not possess calcific nodules; however, there was minimal diffuse von Kossa staining near the AoV hinge region in 5 out of 6 control valves, consistent with fine mineralization previously reported with aging [27].

Histological assessment reveals ECM disorganization in both pediatric and adult AoVD and pathological calcification in adult CAVD

In order to determine the specific molecular mechanisms underlying AoVD progression and calcification, pediatric and adult diseased and control AoV specimens were subjected to extensive immunohistochemical analysis. ECM organization and the presence of pathological calcification were assessed using pentachrome and alizarin red histological stains. A collagen-dense fibrosa layer, proteoglycan-rich spongiosa layer, and ventricularis layer composed of elastin are apparent in both pediatric and adult control AoVs (Figure 1A, E). In contrast, pentachrome staining reveals a loss of valve cusp stratification, increased collagen deposition, and fragmented elastin in pediatric and adult diseased AoVs (Figure 1B, F). Alizarin red staining is an indicator of calcium deposition and has been used previously to identify pathologic valve calcification [22]. Cusp calcification is not apparent

in pediatric and adult control AoVs (Figure 1C, G). Calcification is also not observed in diseased pediatric AoVs (Figure 1D), whereas large pathologic calcified nodules, indicated by alizarin red staining, are readily apparent in adult diseased AoVs (Figure 1H). These studies indicate that the ECM is disorganized, with similar changes in ECM composition and gross architecture in both pediatric and adult diseased AoVs. In addition, adult diseased AoVs have large nodules of pathologic calcification, whereas calcification is not observed in pediatric diseased AoVs.

Activated VICs undergoing cell proliferation are observed in human pediatric and adult diseased AoVs

Activated VICs were identified in pediatric and adult human AoV specimens by immunostaining for smooth muscle alpha actin (SMA) and phospho-histone H3 (PHH3). SMA is expressed in a few sporadic cells in pediatric and adult control valves (Figure 2A, E), but is widespread and abundant in both pediatric and adult diseased AoV specimens (Figure 2B, F). Cell proliferation was detected in pediatric and adult diseased AoVs by immunostaining for PHH3, which marks mitotic proliferating cells. In contrast to normal VICs that are not proliferating (Figure 2C, G), abundant PHH3 staining is observed in both pediatric and adult diseased AoVs (Figure 2D, H; Table 1). Together, these analyses demonstrate that a subpopulation of the VICs in both pediatric and adult diseased AoVs is activated and proliferating.

Mef2c is co-expressed with SMA in a subpopulation of activated VICs and in pathological neovessels in diseased AoVs

The transcription factor myocyte enhancer factor 2c (Mef2c) is expressed in a number of different cell types related to heart and vasculature formation and is crucial for myocardial, endocardial, and vascular development [36,37]. In addition, Mef2c is expressed in prehypertrophic and hypertrophic chondrocytes during development and is necessary for chondrocyte hypertrophy and endochondral bone formation [38]. Robust widespread Mef2c expression is observed in adult CAVD specimens (Figure 3D, F), whereas no staining is detectable in adult control specimens (Figure 3C, E; Table 1; $p < 0.05$). Mef2c expression is variable in pediatric diseased AoV specimens with increased expression in 3/5 specimens (Figure 3B), whereas no Mef2c expression is observed in pediatric control valves (Figure 3A, Table 1). In order to determine whether Mef2c is expressed in activated myofibroblasts, double immunofluorescent labeling for Mef2c and SMA was performed in adult specimens. Widespread Mef2c expression is observed in adult diseased AoVs in comparison to control valves (green nuclei) and Mef2c is co-expressed with SMA in activated myofibroblasts and in the smooth muscle-like cells of pathologic neovessels (Figure 3E–F, white arrowheads). In addition, Mef2c is expressed in a separate population of cells that are not SMA-positive (Figure 3F, open arrow). Taken together, these findings indicate that Mef2c is expressed in activated myofibroblasts in both pediatric and adult diseased AoVs, and is expressed in the neovasculature of adult CAVD specimens, consistent with a role in both angiogenic remodeling and calcification. Mef2c is also present in a distinct SMA-negative population of VICs with unknown function during AoVD.

Transcription factors common to valve mesenchymal progenitor cells and chondrogenic precursors are expressed in pediatric and adult AoVD

The transcription factors Twist1, Sox9, and Msx2 have well-established roles in the development of the heart valves and in the progression of endochondral bone formation [10–14,16,17]. Expression of these transcription factors was investigated in pediatric and adult AoV specimens. Abundant widespread expression of Twist1 (Figure 4B, H), Sox9 (Figure 4D, J), and Msx2 (Figure 4F, L) is observed in the majority of pediatric and adult diseased AoVs in comparison to control specimens (Figure 4A, C, E, G, I, K; Table 1). In pediatric

diseased AoVs, the extensive staining is not localized to a particular region of the valve (Figure 4B, D, F). In adult diseased AoVs, staining is evident both in areas of calcification and regions of ECM disorganization (Figure 4H, J, L). The observed expression of the transcription factors Twist1, Sox9, and Msx2 in both human pediatric and adult diseased AoVs is consistent with roles in VIC activation and proliferation. The shared protein expression of Twist1, Sox9, and Msx2 between pediatric and adult diseased AoVs suggests a common mechanism underlying valve pathogenesis.

Downstream effectors of BMP signaling are active in adult CAVD but not in pediatric noncalcific AoVD

Bone morphogenetic protein (BMP) signaling is active in early valvulogenesis as well as during chondrogenesis and osteogenic bone formation [15,18]. BMP pathway activation in pediatric and adult diseased AoVs was assessed by detection of phosphorylated Smads 1, 5, and 8 (p-Smad1/5/8), which are downstream effectors of BMP signaling [39]. Immunohistochemical analysis demonstrates a significant increase in p-Smad1/5/8 expression in adult CAVD specimens compared to no detectable expression in controls (Figure 5C–D, Table 1; $p < 0.05$). In contrast, p-Smad1/5/8 expression was not observed in 4/5 pediatric diseased specimens, and the one pediatric AoVD specimen with p-Smad1/5/8 expression was the oldest and most severely diseased. P-Smad 1/5/8 expression was not observed in pediatric control specimens (Figure 5A–B, Table 1). These observations suggest that BMP signaling, via p-Smad1/5/8 activation, is active in adult CAVD but is absent in the majority of pediatric AoVD specimens. Thus, BMP signaling activation may be a crucial step in the progression of AoVD from early matrix abnormalities to late calcification thereby contributing to the initiation of pathologic valve calcification.

Collagen 2 and collagen 10 are expressed in adult CAVD but not pediatric AoVD

In the heart, collagen 2 is robustly expressed in the developing endocardial cushions and is downregulated during the later stages of valve remodeling, whereas collagen 10 is not known to be expressed in the developing AoVs [40]. During limb development, collagen 2 is expressed primarily in less mature cartilage precursors, whereas collagen 10 is expressed in the mature hypertrophic chondrocytes in the transition to endochondral bone formation [41–44]. Immunohistochemistry was used to investigate the expression of collagen 2 and collagen 10 during human pediatric and adult AoVD. Very little collagen 2 staining is observed in pediatric diseased specimens, and expression is found significantly more often in pediatric control specimens (Figure 6A–B, Table 1; $p < 0.05$). Furthermore, collagen 10 expression is not observed in pediatric diseased and control AoVs (Figure 6C–D, Table 1). In contrast, collagen 2 expression is significantly increased in adult CAVD specimens in comparison to adult controls, which normally express a low level of collagen 2 (Figure 6E, F, Table 1; $p < 0.05$). Additionally, localized areas of collagen 10 expression are observed in adult CAVD specimens near heavily calcified regions of the valve (Figure 6H, Table 1). No collagen 10 staining was observed in adult control AoVs (Figure 6G, Table 1). Increased expression of collagen 2 and collagen 10 in the valve matrix of adult CAVD specimens suggests that adult diseased, but not pediatric diseased, AoVs have shared characteristics with the ECM during the transition from cartilage to bone. Evidence of this type of ECM is consistent with active osteogenic mechanisms underlying valve calcification in the adult valves.

Runx2, an osteogenic marker, is expressed in adult but not pediatric AoVD

The transcription factor Runx2 is expressed in bone progenitors during embryonic limb development and is necessary for osteoblast maturation and mineralization [45]. Runx2 expression has been previously demonstrated in adult diseased AoVs, however expression of this osteogenic transcription factor has not been investigated in pediatric AoVD [33]. To

determine whether Runx2 expression is specific to calcified adult valves, immunohistochemistry was used to compare Runx2 expression in adult versus pediatric diseased AoVs. Strikingly, Runx2 expression is not detected in pediatric diseased or control valves (Figure 7A, B; Table 1). In contrast, Runx2 expression is observed in nuclei surrounding and throughout nodules of pathologic calcification in adult diseased AoVs, but its expression is not apparent in regions of the valve with no calcification (Figure 7F, Table 1). Runx2 expression is not detected in adult control AoV specimens (Figure 7E). Von Kossa histological staining confirms the presence of pathologic calcification in adult CAVD specimens (Figure 7H), but not in pediatric diseased AoVs (Figure 7D). No calcific mineralization is detected in control AoVs (Figure 7C, G). Thus the osteogenic transcription factor Runx2 is expressed during CAVD and is not present in pediatric AoV where calcification is absent.

Dystrophic valve calcification has been compared to atherosclerosis where there is a disruption of the valve endothelial layer, accumulation of lipids, infiltration of inflammatory cells, VIC apoptosis, and passive accumulation of mineralized calcium [27,34,46]. To ascertain if apoptosis is associated with calcification, the extent of cleaved caspase-3 was examined in adult AoV specimens. Cleaved caspase-3 is observed in a very few sporadic cells in adult control AoVs, whereas there is a modest increase in adult diseased AoVs. However, widespread apoptosis was not observed (Figure 7I, J). Thus, apoptotic-induced dystrophic calcification could account for only a portion of the observed pathologic valve calcification. In contrast, the increased expression of Runx2 and other osteogenic regulatory proteins is evidence for an active osteogenic process as a predominant mechanism of valve calcification in adult CAVD. Overall, the data presented herein suggest an active osteogenic-like process of valve calcification is occurring in adult diseased AoVs that is not observed in pediatric diseased valves.

Discussion

Diseased AoVs, both pediatric and adult, share common pathologic features such as increased valve thickness, loss of valve leaflet stratification, and VIC activation, suggesting that similar disease processes occur in both. However, a major difference between pediatric and adult AoVD is that pathologic calcification is observed only in adults (Figure 1) [28–30]. The pathological differences are also apparent on the molecular level with adult diseased valves expressing markers of osteogenesis that are not apparent in pediatric valves (Summarized in Figure 8). Activated VICs in pediatric and adult diseased AoVs express transcription factors including Twist1, Sox9, and Msx2, which are markers of mesenchymal-type progenitor cells characteristic of developing valves and skeletal elements [10–14,16,17]. Expression of these progenitor cell markers in both pediatric and adult diseased AoVs indicates that both early noncalcific and late calcific diseased valves share common molecular mechanisms involved in VIC activation and ECM dysregulation. However, the expression of osteochondrogenic markers is specific for adult CAVD. Increased BMP signaling, abundant expression of the immature cartilage ECM molecule collagen 2 and the hypertrophic chondrocyte marker collagen 10, and expression of the osteogenic marker Runx2 are limited to adult CAVD, providing evidence for an osteogenic process in valve calcification. These results indicate that genes expressed by mesenchymal-type progenitor cells and differentiating osteogenic cells are expressed in AoVD and contribute to an active disease process evident in pediatric and adult diseased AoVs. Taken together, these findings define a progressive spectrum of disease, which may begin to facilitate the identification of biomarkers of early disease and new treatment approaches for late disease.

VIC proliferation sharply decreases perinatally and is not observed in normal mature valves [20,21]. However, VIC proliferation and activation is observed in both pediatric and adult

AoVD (Figure 2). Inflammatory cells have been reported in human CAVD and are thought to release potent cytokines and chemokines, which may promote VIC activation and valve calcification [33,34,47,48]. The population of activated VICs may be derived predominantly from resident VICs, but may also include infiltrating cells such as bone marrow derived cells [49]. Although the initiating events for VIC activation are unclear, common expression of the transcription factors Twist1, Sox9, and Msx2 in pediatric and adult diseased AoVs suggests that a developmental gene program common to chondrogenesis and valve morphogenesis is activated. Expression of Twist1, Sox9, and Msx2 has been previously demonstrated in human adult CAVD [12,33,50]. Here we show that these transcription factors are also induced in pediatric diseased AoVs, suggesting common molecular mechanisms underlying both pediatric non-calcific and adult late stage AoVD. Increased expression of Twist1, Sox9, and Msx2 may promote the production of a cartilage-type matrix and induce VIC proliferation in both pediatric and adult AoVD leading to valve sclerosis/thickening.

The transcription factor Mef2c, which has a known function in chondrocyte maturation and bone formation, is abundantly expressed in pediatric and adult diseased AoVs [38]. Mef2c expression co-localizes with SMA in activated myofibroblast-like VICs in pediatric and adult AoVD, but is also observed in a large population of SMA-negative VICs. Mef2c expression also is apparent in smooth muscle cells of pathologic neovessels in adults, but comparable Mef2c staining in neovessels was not observed in pediatric specimens. The expression of Mef2c in AoVD suggests a role in VIC activation, neovascularization, and osteochondrogenic-like ECM dysregulation. The data support that pediatric and adult diseased AoVs share increased expression of the transcription factors Twist1, Sox9, Msx2, and Mef2c, suggesting roles in VIC activation and chondrogenic-like matrix changes. Identification of these common markers may serve as potential clinical markers for early valve pathogenesis.

The expression of p-Smad1/5/8 in adult diseased AoVs represents an important divergence in the molecular profile between pediatric and adult diseased AoVs and implicates active BMP signaling in the progression of CAVD. BMP treatments can induce an osteoblast-like differentiation in isolated adult human AoV VICs, indicating that BMP signaling may be a necessary step in osteogenic-like valve calcification [51,52]. Adult CAVD specimens also express the osteogenic transcription factor Runx2 and the hypertrophic chondrocyte ECM molecule collagen 10 which were not observed in pediatric disease specimens [33]. These findings suggest a molecular basis to the pathologic valve calcification in adult CAVD related to bone formation. The data presented support a role for an active osteogenic-like process during AoVD, however, dystrophic calcification may also contribute to valve calcification. A slight increase in VIC apoptosis was observed in adult diseased AoVs, suggesting that apoptosis-induced dystrophic calcification could occur in late stage adult diseased valves. However, abundant expression of molecules involved in active bone formation, such as p-Smad1/5/8, Runx2, and collagen 10 in late-stage calcified adult diseased AoVs, which were not observed in the majority of non-calcified pediatric diseased AoVs, suggests that an osteogenic-like process is a predominant mechanism underlying pathologic valve calcification. Based on the current findings, there is an actively regulated disease process contributing to the progression of calcification in diseased AoVs. Currently there is no pharmacologic treatment for heart valve disease and the standard treatment for stenotic AoVD is valve replacement surgery. Based on these studies, a specific molecular profile predicting the progression of AoVD to pathological calcification is defined, which may advance efforts to identify medical treatments for AoVD.

Abbreviations

AoV	Aortic Valve
AoVD	Aortic Valve Disease
BMP	Bone Morphogenetic Protein
CAVD	Calcific Aortic Valve Disease
DAB	Diaminobenzidine
ECM	Extracellular Matrix
Mef2c	Myocyte enhancer factor 2c
PHH3	Phospho-histone H3
SMA	Smooth Muscle Alpha Actin
VICs	Valvular Interstitial Cells

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Figure 1. ECM disorganization is apparent in pediatric and adult diseased aortic valves (AoVs) and pathologic calcification is identified in adult diseased AoVs

(A, E) Movat's pentachrome staining delineates the fibrosa (F), spongiosa (S), and ventricularis (V) layers in control specimens. (B, F) Loss of valve cusp stratification is observed in pediatric and adult diseased AoVs. (B) Elastin fiber fragmentation is apparent in diseased valves (white arrow). (C, D, G) Calcification, detected by alizarin red staining, is not observed in control valves or in pediatric diseased valves. (H) Large calcific nodules (red staining) are present in adult diseased valves. (F, H) Calcified regions are indicated by dotted lines and asterisks. Scale bars represent 100µm.

Figure 2. Activated valvular interstitial cells (VIC) are present in pediatric and adult human aortic valve disease (AoVD)

Smooth muscle alpha actin (SMA) immunostaining is used to identify activated myofibroblast-like VICs. (A, E) Minimal SMA staining (brown cytoplasmic staining; black arrows) is observed in pediatric and adult control specimens. (B, F) Widespread SMA staining is observed in both pediatric and adult diseased AoVs. (C, D, G, H) Immunoreactivity for Phospho-histone H3 (PHH3), marking mitotic proliferation, is abundant in both pediatric and adult diseased AoVs, relative to control specimens, which do not have evidence of proliferation (dark brown nuclei; black arrows). Tissues were counterstained with nuclear fast red (pink nuclei). Scale bars represent 100µm.

Figure 3. Mef2c expression is induced in diseased aortic valves and is co-expressed with SMA in a subset of activated VICs and neovessels
 (A–D) Immunoreactivity for myocyte enhancer factor 2c (Mef2c) is abundant in both pediatric and adult diseased AoVs (brown nuclei in B and D; black arrows), but is not found in control specimens (A, C). (E, F) Co-immunofluorescence of Mef2c and SMA reveals SMA positive cells in both adult control and diseased AoVs (red cytoplasmic staining; white arrows). (F) SMA identifies activated myofibroblast-like VICs and neovessels (white arrowheads) in adult diseased AoVs. Mef2c expression is not observed in control specimens, but is widely expressed in adult diseased AoVs (green nuclei; F). Mef2c co-localizes with

SMA in activated myofibroblasts and neovessels (white arrowheads), but also stains a population of SMA negative cells (green nuclei; open arrow). Scale bars represent 100 μ m.

Figure 4. Expression of the transcription factors Twist1, Sox9, and Msx2 is increased in pediatric and adult aortic valve disease (AoVD)

(A–F) Pediatric AoVs. (G–L) Adult AoVs. Immunohistochemistry of Twist1 (A, G), Sox9 (C, I), and Msx2 (E, K) demonstrates very little positive staining in pediatric and adult control valves. In comparison, widespread increased expression of Twist1 (B, H), Sox9 (D, J), and Msx2 (F, L) is observed in pediatric and adult diseased AoVs (brown nuclear staining; black arrows). Tissues are counterstained with nuclear fast red (pink nuclei). (J, L) Areas of calcification are delineated with dotted lines and asterisks. Scale bars represent 100µm.

Figure 5. Phospho-Smad 1/5/8 activation is predominant in adult diseased AoVs and not pediatric diseased AoVs
 (A–B) Pediatric AoVs. (C–D) Adult AoVs. (A–D) Immunohistochemistry reveals abundant p-SMAD1/5/8 expression in adult diseased AoVs (D, brown nuclear staining; black arrows). Little to no expression of p-SMAD1/5/8 was observed in pediatric diseased tissues or in adult and pediatric control specimens (A–C). Tissues were counterstained with nuclear fast red (pink nuclei). Scale bars represent 100 μ m.

Figure 6. Collagen 2 and collagen 10 are expressed in adult but not pediatric diseased AoVs (A–D) Pediatric AoVs. (E–H) Adult AoVs. (A–B) Immunohistochemistry of collagen 2 (characteristic of pre-mature chondrocytes) demonstrates a low level of collagen 2 expression in pediatric control AoVs, which is undetectable in pediatric diseased AoVs. (E–F) In contrast, collagen 2 expression is increased in adult diseased AoVs in comparison to control valves (dark brown extracellular staining; black arrows). The hypertrophic chondrocyte marker collagen 10 is not detectable by immunohistochemistry in pediatric and adult controls specimens or in pediatric diseased AoVs (C, D, G) and is only observed near calcification in adult diseased AoVs (H, light brown extracellular staining; black arrows). Areas of calcification are outlined with dashed lines and marked by asterisks. Scale bars represent 100µm.

Figure 7. Valve mineralization and expression of Runx2 is specific to adult diseased AoVs (A–D) Pediatric AoVs. (E–J) Adult AoVs. (A–B, E–F) Immunohistochemistry demonstrates Runx2 expression in adult diseased AoVs surrounding and throughout areas of calcification (F, brown nuclei; black arrows). Runx2 expression is not observed in adult and pediatric control AoVs or in pediatric diseased AoVs (A, B, E). (C–D, G–H) Calcified mineral deposits, detected by von Kossa staining, were not observed in control (C, G) and pediatric diseased AoVs (D), and were only detected in adult diseased AoVs (H, dark brown staining). (I–J) Apoptosis, detected by cleaved caspase-3 immunoreactivity, is minimal in control AoVs (I) and is slightly increased in adult diseased AoVs (J) (brown nuclei; black arrows). Widespread apoptosis was not observed. (C–D, G–H) Tissues were counterstained with nuclear fast red (pink nuclei). (F, J) Areas of calcification are outlined with dashed lines and marked by asterisks. Scale bars represent 100µm.

Figure 8. Osteochondrogenic gene regulatory mechanisms are activated in pediatric and adult AoVD progression

(A) A normal mature valve is composed of three ECM layers, the fibrosa (light blue), spongiosa (gray), and ventricularis (navy blue), and contains quiescent valvular interstitial cells (VIC, white circles). (B) Pediatric diseased valves do not typically calcify, but have increased valve thickness, activated VIC (orange stars), disorganized ECM (heterogeneous mixture of light blue, navy blue, and gray), and increased expression of the transcription factors Mef2c, Sox9, Twist1, and Msx2. (C) In addition to the changes that occur in pediatric AoVD, adult diseased AoVs often have large pathologic calcified nodules (distorted black shapes), increased active BMP signaling (p-SMAD1/5/8 expression), and increased expression of the osteogenic transcription factor Runx2. Valve matrix changes are also apparent in adult CAVD with increased expression of collagen 2 and collagen 10. Green half-circle = valve annulus, Ao = aorta.

Table 1

Expression analysis of human pediatric and adult diseased AoVs.

	Pediatric AoV		Adult AoV	
	Controls	Diseased	Controls	Diseased
ECM disorg.	0/4	5/5	0/6	7/7
Calcification	0/4	0/5	0/6	7/7
Antibody				
Phospho-histone H3	1/4	4/5	1/6	7/7
Mef2c	0/4	3/5	0/6	6/7
Twist1	0/4	2/5	1/6	5/7
Sox9	1/3	5/5	3/6	7/7
Msx2	2/3	5/5	3/6	7/7
Phospho-SMAD 1/5/8	0/3	1/5	0/6	6/7
Collagen 2	3/4	0/5	Present in 5/6 ^b	Increased in 5/7 ^b
Collagen 10	0/3	0/5	0/6	4/7
Runx2	0/4	0/5	0/6	4/7

^aNumbers in "Controls" and "Diseased" columns represent positively stained specimens/total numbers of specimens immunostained.

^bCollagen 2 expression is normally present at low levels in adult control AoV, whereas 5/7 adult diseased AoVs exhibit greatly increased collagen 2 expression.

^cP-values < 0.05 are considered significant.