Noggin attenuates the osteogenic activation of human valve interstitial cells in aortic valve sclerosis

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Abstract

Aims Aortic valve sclerosis (AVSc) is a hallmark of several cardiovascular conditions ranging from chronic heart failure and myocardial infarction to calcific aortic valve stenosis (AVS). AVSc, present in 25–30% of patients over 65 years of age, is characterized by thickening of the leaflets with marginal effects on the mechanical proprieties of the valve making its presentation asymptomatic. Despite its clinical prevalence, few studies have investigated the pathogenesis of this disease using human AVSc specimens. Here, we investigate in vitro and ex vivo BMP4-mediated transdifferentiation of human valve interstitial cells (VICs) towards an osteogenic-like phenotype in AVSc.

Methods and results Human specimens from 60 patients were collected at the time of aortic valve replacement (AVS) or through the heart transplant programme (Controls and AVSc). We show that non-calcified leaflets from AVSc patients can be induced to express markers of osteogenic transdifferentiation and biomineralization through the combinatorial effect of BMP4 and mechanical stretch. We show that BMP4 antagonist Noggin attenuates VIC activation and biomineralization. Additionally, patient-derived VICs were induced to transdifferentiate using either cell culture or a Tissue Engineering (TE) Aortic Valve model. We determine that while BMP4 alone is not sufficient to induce osteo-genic transdifferentiation of AVSc-derived cells, the combinatorial effect of BMP4 and mechanical stretch induces VIC activation towards a phenotype typical of late calcified stage of the disease.

Conclusion This work demonstrates, for the first time using AVSc specimens, that human sclerotic aortic valves can be induced to express marker of osteogenic-like phenotype typical of advanced severe aortic stenosis.

Keywords Aortic valve sclerosis • Calcific aortic stenosis • Bone morphogenetic protein 4 • Valve interstitial cells

1. Introduction

Aortic valve sclerosis (AVSc) is a hallmark of several cardiovascular conditions, including chronic heart failure, myocardial infarction and, most prevalently, severe calcific aortic valve stenosis (AVS).1–4 This condition, present in 25–30% of patients over 65 years of age and in up to 40% of those over 75 years of age, is characterized by thickening of the aortic leaflets. This thickening has marginal effects on the mechanical properties of the valve making the presentation of the disease largely asymptomatic.5 Almost 10% of aortic sclerosis patients progress to severe calcific aortic stenosis within 10 years from the diagnosis, requiring aortic valve (AV) replacement when symptoms emerge.5 Despite its clinical prevalence, a limited number of studies have investigated the active cellular process underlying the progression of this disease. In addition, asymptomatic aortic sclerosis tissues are generally not available to investigators since these valves are not surgically replaced until moderate-to-severe stenosis occurs.

Over the last decade, several clinical trials have been performed to try to halt the progression of calcific AVS. Early enthusiastic findings, documenting a reduction in the progression of the disorder, have been questioned by later randomized studies, which show substantial equivalence between treatments and placebo.6–11 It has been proposed that the therapy may have been initiated too late in the course of the disease to be effective.6–11 An initial asymptomatic phase of valve disease is a mild thickening of the leaflets, which does not affect the mechanical properties of the valve. These patients are largely asymptomatic and, therefore, challenging to identify due to the variable and qualitative nature of aortic sclerosis description by echocardiographic evaluation.6,12,13 Once aortic sclerosis is detectable, there is an increased risk of cardiovascular events.14

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onset of early symptoms of calcific aortic stenosis, the survival curve deviates even more from expected, with a dramatic decline in the case of severe symptomatic stenosis. Despite its high prevalence, little is known about the early stages of development of AV disease, from the initiating pathogenic mechanisms determining the thickening of the leaflets to the activation of valve interstitial cells (VICs) and their transdifferentiation into osteoblastic-like cells. The AV leaflets are a highly specialized structure consisting mostly of VICs and complex extracellular matrix (ECM) structures.\textsuperscript{16–18} The leaflet is divided into three functionally specific layers. The fibrosa layer, facing the aorta, is primarily composed of type I collagen fibres with a strong preferred circumferential orientation. The ventricularis, facing the left ventricle, mainly consists of elastin and collagen. The spongiosa, located between the fibrosa and the ventricularis, is largely composed of glycosaminoglycans.\textsuperscript{12} It has been speculated that VICs maintain valve tissue homeostasis through regulated ECM biosynthesis.\textsuperscript{17} VICs appear to be phenotypically plastic as they transdifferentiate during valve development, disease and remodelling from a quiescent to an osteogenic-like phenotype.\textsuperscript{19,20} The activation of VICs results in the expression of specific markers such as osteopontin (OPN), osteonectin (ON), runt-related transcription factor 2 (RUNX2), α-smooth muscle actin (SMA), and alkaline phosphatase (ALP). Furthermore, activated VICs express and actively remodel fibronectin (FN), which is a major component of the insoluble ECM.\textsuperscript{21} We and others have recently investigated VIC plasticity during different valve disease.\textsuperscript{16,22,23} Bone Morphogenic Protein 2 and 4 (BMP2 and BMP4) are known to be potent osteogenic morphogens and to be present in ossified valves and directly affects osteogenic marker expression.\textsuperscript{19,20,24–26} Furthermore, AV leaflets are exposed to large cyclical stresses. During each cardiac cycle, the normally functioning AV interacts closely with the surrounding environment and it is exposed to a myriad of mechanical forces such as transvalvular pressure, axial, shear and bending stresses, and cyclic flexure.\textsuperscript{13,17,27–30} While normal hemodynamic forces have been shown to cause constant tissue renewal in AV, altered mechanical forces are believed to induce changes in AV biology that could possibly lead to valve disease.\textsuperscript{16–18,31,32} Here, we investigate for the first time the mechanisms of human VIC activation in the very early stage of the disease using surgically resected AV tissues and patient-matched derived cells.

2. Methods

2.1 Patient population and definition of AVSc

Patients were enrolled in the study following Institutional Review Board approved guidelines of University of Pennsylvania Perelman School of Medicine (protocol no. 809349) and according to the Declaration of Helsinki. Subject enrolment was carried out based on echocardiographic evaluation and patient’s charts review. Enrolment table and patient demographics are described in Table 1. Control and aortic sclerosis tissues were obtained through collaborations with the heart transplant research program at the University of Pennsylvania Perelman School of Medicine and with The Gift of Life Program. (More details are provided in Supplementary material online). Aortic sclerosis was defined as irregular, non-uniform thickening of portions of the AV leaflets and/or commissures thickened portions of the AV with or without an appearance suggesting calcification (i.e. bright echoes), non-restricted or minimally restricted opening of the aortic leaflets, and peak continuous-wave Doppler velocity across the valve <2 m/s. Fourteen controls, 17 AVSc, and 29 AVS patients were selected based on echocardiographic evaluation and exclusion criteria (Supplementary material online, Table S1, Videos S1–3, Figure S1).

2.2 RT² Profiler PCR Array and RT–qPCR

RT² Profiler PCR Array was used to analyse the expression of ECM gene and cell adhesion molecules (CAMs; Supplementary material online, Table S2). Data analysis was performed with software provided by SABiosciences. Validation of genes from PCR array was carried out by real-time PCR (RT–qPCR) using gene-specific primers and fold change was used to describe the variation from baseline control (Supplementary material online, Table S3).

2.3 Isolation of aortic VICs

Isolation of aortic VICs was performed using a modification of the method described by Branchetti et al.\textsuperscript{23} All the experiments were performed with cultured cells between the second and fifth passage. Phenotypic stability was tested as described in the Supplementary material online.

2.4 BMP4-mediated activation of human native tissue and human-derived VICs

Ex vivo and in vitro experiments were performed using human recombinant BMP4 protein (Peprotech, Rocky Hill, NJ, USA; 100 ng/mL), human recombinant Noggin (NKG; Peprotech, Rocky Hill, NJ, USA; 500 ng/mL), and osteogenic media (OS; DMEM/F12, 10% fetal bovine serum, 4 mM l-glutamine, 1% penicillin/streptomycin, 50 mg/mL ascorbic acid, 10 nM dexamethasone, and 10 mM β-glycerophosphate).

2.5 Bioreactor design and tissue preparation

The tension bioreactor used in this study is similar to the previously described flexure bioreactor used for flexural stimulation of engineered valve tissue as described by Merryman et al.\textsuperscript{17,33}

2.6 Scaffold preparation and recellularization

Scaffolds were prepared using porcine AV leaflets. They were decellularized and recellularized with VICs isolated from aortic sclerosis patients and used for the treatment with BMP4 in static or dynamic controls in OS (Supplementary material online).

2.7 Statistical analysis

The data were analysed with the SPSS software using suitable methods for parametric and non-parametric analyses (Student’s t-test, ANOVA test, and Kruskal–Wallis test).

3. Results

3.1 Analysis of AVSc microstructure and ECM

H&E and modified Movat’s Pentachrome staining were used to analyse the microstructure of the AV leaflets and the changes in AV thickness. Figure 1A–C shows representative images of the histological analysis for controls (n = 5), AVSc (n = 5), and AVS (n = 5) patients. ECM components are distinguishable in the Movat’s staining as proteoglycan (bluish green), collagen (yellow), and elastin (dark violet); nuclei are stained dark red. In control tissues, we observed a distinctly modified structure, with increased ECM deposition and slight reduction in the total number of cells (Figure 1B). Tissues from aortic stenosis patients showed increased structure disarray, deposition of collagen and proteoglycan, and the presence of large fibrotic and calcified areas in the fibrosa (Figure 1C). We then investigated quantitative differences in ECM components among the
Table 1 Patient enrolment and demographics

<table>
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<tr>
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<th>Controls (N = 14)</th>
<th>Aortic sclerosis (N = 32)</th>
<th>Aortic stenosis (N = 48)</th>
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<tr>
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<td>Rheumatic heart disease</td>
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<td>5 (10.4%)</td>
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<td>Analysed</td>
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<td>17</td>
<td>29</td>
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**(B) Demographics**

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<th>Aortic stenosis</th>
<th>P-value</th>
</tr>
</thead>
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<td>Age</td>
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<td>57.8 ± 12.3</td>
<td>75.8 ± 7.9</td>
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<td>Male subjects</td>
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<td>13 (76.5%)</td>
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<td>Smokers</td>
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<td>8 (47.1%)</td>
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<td>Diabetes</td>
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<td>7 (24.1%)</td>
<td>23 (79.3%)</td>
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<td>Hypertension</td>
<td>2 (16.7%)</td>
<td>9 (52.9%)</td>
<td>23 (79.3%)</td>
<td>&lt;0.001</td>
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<tr>
<td>Cerebral vascular accident</td>
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<td>7 (24.1%)</td>
<td>23 (79.3%)</td>
<td>&lt;0.001</td>
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<td>Coronary artery disease</td>
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<td>Congestive heart failure</td>
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<td>4 (13.8%)</td>
<td>18 (62.1%)</td>
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<tr>
<td>Hyperlipidaemia</td>
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<td>6 (35.3%)</td>
<td>18 (62.1%)</td>
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</table>

Figure 1 Analysis of AVSc microstructure and ECM. (A–C) Representative images (n = 5/group) for control, AVSc, and AVS tissues: H&E staining; Movat’s Pentachrome staining; ECM components are distinguished as proteoglycan (bluish green), collagen (yellow), and elastin (dark violet); VIC nuclei are stained dark red. Alizarin red was used to visualize calcium content. F, fibrosa; S, spongiosa, and V, ventricularis. Bar represents 0.5 mm.

(D–F) Bar graph shows fold change gene expression of Decorin (DCN), Biglycan (BGN), and Collagen Type I (Col1A1) transcripts by RT–qPCR, respectively. Data were normalized against 18S gene expression and represented as fold change ± dCt SE. *P < 0.01.
three groups, Decorin was found up-regulated 17.8 ± 1.1-fold in aortic sclerosis vs. control (P < 0.01) and 19.3 ± 0.9-fold in aortic stenosis vs. control (P < 0.01). Biglycan was up-regulated 18.1 ± 1.9-fold in aortic sclerosis vs. control (P < 0.01) and 22.9 ± 1.2-fold in aortic stenosis vs. control (P < 0.01). Collagen type I was up-regulated 7.5 ± 0.1-fold in aortic sclerosis vs. control (P < 0.01) and 12.2 ± 0.1-fold in aortic stenosis vs. control (P < 0.01; Figure 1D–F).

### 3.2 Osteogenic-like gene expression and Bone Morphogenetic Protein 4 are up-regulated in asymptomatic AVSc-derived tissues

We performed an RT2 PCR Array analysis of 84 ECM related and CAMs to characterize the cellular physiology of aortic sclerosis-derived tissues and to identify the signalling pathways associated with early-stage transdifferentiation of VICs and ECM remodelling (Figure 2A). The complete list of ECM and CAM genes analysed is reported in Supplementary material online, Table S2. Among the genes up-regulated more than two-fold, we validated the differential expression of genes involved in VIC osteogenic-like transdifferentiation in a total of 24 patients. OPN (Figure 2B and C), ON (Figure 2D and E), Thrombospondin 1 (THBS1), and Thrombospondin 2 (THBS2) (Figure 2H and I) were significantly up-regulated in both aortic sclerosis and aortic stenosis tissues compared with healthy controls. RUNX2 (Figure 2F and G) was significantly up-regulated in aortic stenosis tissues compared with controls. Accordingly, western blot and immunofluorescence staining for SMA (Figure 2J and K) revealed an increased number of biosynthetically active VICs in the fibrosa of aortic sclerosis and aortic stenosis tissues when compared with healthy controls.
healthy control tissues. Finally, since BMP4 is an inducer of osteoblastic phenotype, we tested whether BMP4 was also up-regulated in aortic sclerosis specimens. Figure 2L and M shows that BMP4 is up-regulated in both aortic sclerosis and aortic stenosis and shows a side-specific expression correlating with fibrosa-specific susceptibility to undergo cellular and extracellular remodelling typical of late-stage disease. Our analysis surprisingly revealed that osteogenic-like gene up-regulation is not statistically significantly different between asymptomatic aortic sclerosis and severe aortic stenosis (P = 0.593). On the other hand, aortic sclerosis and aortic stenosis are statistically different than controls (P < 0.01). Based on these results, we carried out in vitro and ex vivo experiments to determine the role of BMP4 in the transdifferentiation of human AVSc-derived VICs.

### 3.3 Bone Morphogenetic Protein 4 induces osteogenic markers expression and biomineralization of human AVSc tissue

To test the impact of BMP4 on AVSc-derived tissues, we selected patients with calcium score 1 (no calcium) as described by Rosenhek et al. and applied 15% stretch at 1 Hz using a tensile bioreactor to

**Figure 3** Bone Morphogenetic Protein 4 induces biomineralization of human AVSc tissue. (A) Preparation of leaflet tissue strips. (B) Configuration of testing sample in tension bioreactor. (C) TUNEL assay using native tissue analysed after the excision of the tissue. (D) TUNEL assay using native tissue incubated in the bioreactor under dynamic condition after 6 days. (E) TUNEL assay using native tissue incubated in the bioreactor under dynamic condition in the presence of BMP4 (100 ng/mL). (F) Immunohistochemistry staining with Alizarin red (to visualize calcium content) in aortic valve sclerosis (AVSc) tissue cultured in static and dynamic conditions in the presence of OS + BMP4 (100 ng/mL). *Represent the location of the fibrosa layer. (G) ALP expression by RT–qPCR in native tissue cultured under dynamic condition in the presence of OS + BMP4 (100 ng/mL). Data were normalized against 18S gene expression and represented as fold change ± dCt SE. *P < 0.05.
mimic the stress on the valve during the cardiac cycle. As an internal control, native tissues were treated statically in the presence or absence of BMP4 (100 ng/mL) in OS. The tension bioreactor, as described by Merryman et al.,33 consists of two chambers, each with multiple media baths; each bath has stationary pins press-fit into the bottom for tissue anchorage. The opposing pins are fixed to an actuating arm that is attached to a cross-arm, which is connected to a central motorized piston (Figure 3A and B). Cyclic stretch of the leaflets does not damage leaflet morphology and maintains native ECM structure and cellular composition.33,35 Initially, we tested VIC survival within the native tissue in the bioreactor for 6 days under 15% of stretch either at 1 Hz or statically. Before and after the stretch or under static conditions, a TUNEL assay showed unvaried apoptotic rate in control and AVSc-derived tissues (Figure 3C and E). Interestingly, the combination of mechanical stimulation and BMP4 resulted in the accumulation of calcium (Alizarin Red staining) in surgically resected tissues derived from asymptomatic patients diagnosed with AVSc (Figure 3F). To confirm this finding, we measured the expression of ALP in the native tissue under dynamic condition in the presence or absence of BMP4. We observed that ALP levels were up-regulated in the dynamic + BMP4 condition by 26.9 ± 0.7-fold (P<0.05) compared with the tissue exposed to dynamic condition only (Figure 3G).

Based on these results, we tested the impact of BMP4 on VIC activation, biomineralization, and the expression of SMA, FN, OPN, ON, and ALP (Figure 4). FN was chosen since it is involved in bone mineralization process and is an indicator of myofibroblast activation.21,36 The explanted tissues cultured in static conditions for 6 days did not show any up-regulation of SMA, OPN, and ON (Figure 4A). In contrast, the tissues subjected to the combinatory effect of BMP4 and dynamic stimulation show increased expression of SMA, FN, OPN, and ON (P<0.05; Figure 4B–D). To provide supporting evidence for a direct functional role of BMP4 in inducing osteogenic activation of VICs, we repeated the previous experiments in the presence of BMP antagonist NOG. As shown in Figure 4C and D, NOG reduces BMP4-mediated up-regulation of SMA, FN, and OPN by 117%, 163%, and 64.3%, respectively (P<0.05). Notably, we also observed a reduction in the ALP expression by 44.4% (P<0.05; Figure 4E).

3.4 NOG attenuates BMP4-induced activation of human VIC in asymptomatic AVSc

Our experiments show that we can induce VIC osteogenic-like transdifferentiation of human isolated AV leaflets from asymptomatic patients with aortic sclerosis. Taking this a step further, we sought to promote VIC activation on isolated patient-derived cells. Since it has been reported that ECM substrate induces the expression of differentiation markers,36,37 we conducted the experiment using collagen type I-coated (Figure 5) and uncoated plates (see Supplementary material online, Figure S2). Treatment of VICs isolated from healthy controls...
with BMP4 induced the expression of OPN, RUNX2, and ON by 7.63 ± 0.6-, 5.36 ± 0.6-, and 3.44 ± 0.2-fold, respectively (P < 0.05). Conversely, AVSc- and AVS-derived VICS plated on collagen type I were unresponsive to BMP4 treatment in OS (Figure 5A–C). BMP4 antagonist NOG had a reducing effect on the expression of OPN, RUNX2, and ON only in VICs isolated from controls by 24.37 ± 0.4-, 24.28 ± 0.2-, and 23.27 ± 0.4-fold, respectively (P < 0.05; Figure 5A–C).

In order to test the impact of the combinatory effect of BMP4 and mechanical stimulation (Figures 3 and 4) on isolated cell, we implemented a TE model based on decellularized porcine AV scaffold repopulated with human AVSc-derived cells. Complete decellularization and subsequent recellularization with human cells were confirmed by H&E and immunohistological studies (Figure 5D and E). Porcine AV scaffolds repopulated with human aortic sclerosis cells were placed in the tensile stretch bioreactor and exposed to either mechanical stretch alone, in the presence of BMP4 or in presence of both BMP4 and NOG for 6 days. Leaflets were then processed (as described previously) and analysed for SMA, OPN, ON, and RUNX2 expression. Contrary to the expression profile analysed in 2D culture (both collagen coated or uncoated), all osteogenic markers were significantly up-regulated in comparison with the engineered leaflets with no BMP4 treatment (Figure 5F and G). SMA was up-regulated by 1.94 ± 0.1-fold, OPN by 2.28 ± 0.3, ON by 3.17 ± 0.3, and RUNX2 by 3.22 ± 0.2 (P < 0.05). Finally, NOG reverts the effect of BMP4 on SMA, ON, and RUNX, and further reduced the expression of OPN by 24.11 ± 0.2-folds (P < 0.05).

4. Discussion

4.1. AV dysfunction progress from early asymptomatic sclerosis to severe calcific AVS

To impact the progression, we need to understand the earliest stages of disease to be able to measure the effects of targeted therapy on the microscopic processes in the valve leaflets. Despite recent efforts, progress to understand, diagnose, and treat calcific AV disease have been hindered by our inability to resolve the cellular mechanisms.

Figure 5 Noggin attenuates BMP4-induced activation of human VIC in asymptomatic AVSc. (A–C) Bar graphs represent fold change gene expression of OPN, RUNX2, and ON in vitro treatment in the presence of OS ± Bone Morphogenetic Protein 4 (BMP4) (100 ng/mL) ± NOG (500 ng/mL) in control, AVSc and AVS-derived VICS plated on collagen type I. *P < 0.05. (D) Decellularized porcine AV scaffold. (E) H&E staining showing the presence or absence of VICS in the native porcine AV (Native), after decellularization (Decell) and after reseeding of human VICS (Recell). (F) Immunofluorescence staining showing expression of α-SMA, OPN, ON, and RUNX2 in AVSc tissue cultured in dynamic conditions in the presence of OS ± BMP4 (100 ng/mL). (G) SMA, OPN, ON, and RUNX2 expression by RT–qPCR in TE tissue cultured under dynamic condition in the presence of OS ± BMP4 (100 ng/mL) ± Noggin (NOG) (500 ng/mL). All RT–qPCR analysis were normalized against 18S gene expression and represented as fold change ± dCt SE. *P < 0.05. OS was used to calculate the basal gene levels.
leading VIC activation towards an osteogenic-like phenotype.\textsuperscript{2,4,38} Here, for the first time, we performed an analysis of AV leaflets and patient-matched derived VICs using control, AVSc, and calcific AVS patients. Notably, the valve mechanical functions of aortic sclerosis are largely unaffected; patients with aortic sclerosis have similar AV area and Doppler velocity values compared with controls. Accordingly, aortic sclerosis patients are largely asymptomatic and are not indicated for AV replacement. Therefore, we established collaboration with the heart transplant programme that allowed us the use of either hearts of donors that were not used for transplantation or from recipients that, although transplanted, had a normally functioning AV.

Our study provides several new insights into the early pathogenesis and the progression of AVSc. First, our data show that AV leaflets form AVSc patient shows extensive ECM remodelling even in the absence of biomineralization (Figure 1). These events are associated with activation of VICs towards an osteogenic-like phenotype associated with up-regulation of BMP4 pathway (Figure 2). Taken together, these results show that aortic sclerosis could be considered, from the cellular and molecular point of view, a pathological stage. Secondly, the results demonstrate that non-calcified AV leaflets from asymptomatic patients can be induced to express markers of osteogenic transdifferentiation under the combinatory effect of biological (BMP4 pathway) and mechanical (tensile stretch) forces (Figures 3 and 4). Recent studies on AV disease have described the expression of developmental genes in areas of valve calcification; thereby supporting the idea that valve calcification is not a passive deposition of calcium, but is actively regulated by a hierarchy of signalling pathways and transcription factors. Among these, BMPs signalling is important for valvulogenesis and osteogenesis and it is increased during calcific AVS.\textsuperscript{39,40} Recent manuscripts suggest a regulatory mechanisms of the BMP family in controlling osteogenic phenotype under mechanotransduction stimulation.\textsuperscript{41–43} BMP4 is essential in the osteochondrogenic gene programme associated with vascular and valve calcification.\textsuperscript{44,45} However, the factors that regulate mineralization in aortic diseases are poorly understood. Here, we reported the up-regulation of SMA, OPN, RUNX2, and ON during the progression of calcific AVS. Furthermore, we observe the fibrosa-layer susceptibility of non-calcified aortic sclerosis tissue to undergo biomineralization similarly to the natural course of the pathology. The deposition of calcium on the aortic side of aortic sclerosis tissue is likely due to the presence of nucleation centres, which provide the starting point for calcium nodule formation.\textsuperscript{46} Notably, VIC transdifferentiation towards an osteogenic-like phenotype characterized by SMA, FN, OPN, and ALP overexpression are reduced by BMP4 antagonist NOG. Conversely, uncoupled stimulations using BMP4 on cultured cells or static treatment on tissues are insufficient to drive AVSc-derived specimens to express osteogenic markers (Figure 4). Finally, using the TE model, we demonstrated that the combinatory effect of BMP4 and mechanical stress induces the transdifferentiation of VICs. Of note, when using the TE model, we do not see the accumulation of calcium deposition, which could be explained by the short duration (6 days) of the experiment and by the lacking of centre of nucleation (Figure 5).

Several limitations are associated with this study. First, quantitative knowledge of how VICs respond to loading translated from the surrounding tissue is necessary to understand how organ-level forces affect VIC homeostasis. Therefore, a limitation of this model is that proper interpretation of phenotypic changes within the AV leaflet requires knowledge of how VICs respond to specific local tissue strains in different layers. Another limitation is that, in our model, AV leaflets (both native and TE model) were stretched under uniaxial conditions rather than the more natural-mimicking biaxial conditions, but this was unavoidable under the bioreactor experimental setup.

It is concluded that despite normal mechanical function, AV leaflets from aortic sclerosis patients show accumulation of side-specific activation of VIC. It is also shown that AVSc tissue can be induced to bio-mineralize and undergo osteogenic-like transdifferentiation under the combinatory effect of BMP4 and mechanical stretch. Finally, we demonstrate that patient-isolated VICs can be induced to express markers of osteogenic-like phenotype using a TE AV model. The proposed model for the transdifferentiation of VICs from asymptomatic patients could be used to investigate the plasticity of the cellular components and to test therapeutic approaches to control the transition to the pathological phenotype.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**Conflict of interest:** none declared.

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