

Aortic regurgitation provokes phenotypic modulation of smooth muscle cells in the normal ascending aorta



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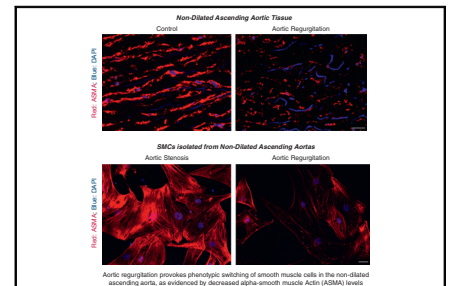
ABSTRACT

Background: Aortic complications are more likely to occur in patients with ascending aortic aneurysms and concomitant aortic regurgitation (AR). AR may have a negative influence on the aortic wall structure even in patients with tricuspid aortic valves and absence of aortic dilatation. It is unknown whether smooth muscle cell (SMC) changes are a feature of AR-associated aortic remodeling.

Methods: Nondilated aortic samples were harvested intraoperatively from individuals with normal aortic valves ($n = 10$) or those with either predominant aortic stenosis (AS) ($n = 20$) or AR ($n = 35$). Tissue from each patient was processed for immunohistochemistry or used for the extraction of medial SMCs. Tissue and cells were stained for markers of SMC contraction (alpha-smooth muscle actin), synthesis (vimentin) and senescence (p16^{INK4A} and p21^{CIP1} [p16/p21]). Replicative capacity was analyzed in cultured SMCs from AS- and AR-associated aortas. A subanalysis compared SMCs from individuals with either tricuspid aortic valves or bicuspid aortic valves to evaluate the effect of aortic valve morphology.

Results: In aortic tissue samples, AR was associated with decreased alpha-smooth muscle actin and increased vimentin, p16 and p21 compared with normal aortic valves and AS. In cell culture, SMCs from AR-aortas had decreased alpha-smooth muscle actin and increased vimentin compared with SMCs from AS-aortas. AR-associated SMCs had increased p16 and p21 expression, and they reached senescence earlier than SMCs from AS-aortas. In AR, SMC changes were more pronounced with the presence of a bicuspid aortic valve.

Conclusions: AR itself negatively influences SMC phenotype in the ascending aortic wall. This AR-specific effect is independent of aortic diameter and aortic valve morphology, although it is more pronounced with bicuspid aortic valves. These findings provide insight into the mechanisms of AR-related aortic remodeling, and they provide a model for studying SMC-specific therapies in culture. (*J Thorac Cardiovasc Surg* 2023;166:1604-16)



AR provokes loss of ASMA in SMCs in nondilated ascending aortas.

CENTRAL MESSAGE

Aortic regurgitation negatively influences SMC phenotype in nondilated ascending aortas. Contractile SMCs are replaced by either synthetic or senescent SMCs that may be primed for ECM degradation.

PERSPECTIVE

AR causes contractile SMCs to switch to synthetic/senescent phenotypes that may provoke ECM degradation. So, phenotypic modulation may be considered as a therapeutic target to prevent ascending aortic aneurysms in AR patients. SMCs retained their AR-related phenotypic properties in culture. Thus, SMC isolation from surgical aortic samples provides a medium for studying phenotype-targeted therapeutics.

Aneurysms of the ascending aorta are frequently associated with life-threatening complications; that is, aortic dissection, rupture, and sudden death. These aneurysms may derive from multiple origins, including connective tissue disorders with monogenetic causes (ie, Marfan and Loeys-Dietz syndromes)^{1,2} or bicuspid aortic valves

(BAV),³ in which the etiology is less defined. Both in aneurysms due to connective tissue disorders and in BAV disease, aortic valve regurgitation (AR) is a risk factor for aortic complications.^{4,5}

There are common features of degenerative aortic wall remodeling in ascending aortic aneurysms, including

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Abbreviations and Acronyms

AR	= aortic regurgitation
AS	= aortic stenosis
ASMA	= alpha-smooth muscle actin
BAV	= bicuspid aortic valve
ECM	= extracellular matrix
SMC	= smooth muscle cell
TAV	= tricuspid aortic valve



Scanning this QR code will take you to the table of contents to access supplementary information.



extracellular matrix (ECM) degeneration.^{3,6,7} The underlying mechanisms are incompletely understood. BAVs are associated with aortic stenosis (AS) and AR, and turbulent blood flow across the BAV is a significant cause of aneurysm formation.⁸⁻¹⁰ Irrespective of aortic valve morphology, AS is associated with turbulent blood flow, whereas AR is associated with increased stroke volume, both resulting in subsequent increases in shear stress.^{11,12} The BAV-associated aortic annulus dilates faster when AR is present,¹³ and the probability of aortic complications is higher with AR compared with normally functioning valves and AS.¹⁴ Less data are available for ascending aortic aneurysms in the absence of a BAV. We recently studied nondilated ascending aortas in individuals with tricuspid aortic valves (TAVs). We found that AR was associated with a higher degree of aortic wall remodeling compared with AS or normally functioning valves, which included elastin degradation and mucoid ECM accumulation in the medial layer.¹⁵ The underlying mechanisms of AR-induced aortic remodeling are not well defined, but our previous findings suggest that the involvement of cellular-mediated processes should be examined.

Smooth muscle cells (SMCs) are an important component of the aortic media. Contractile SMCs are embedded in the ECM, and they maintain structural and functional integrity of the aorta by circumferentially wrapping around the vessel to impart coordinated vascular tone. Previous histologic studies have identified phenotypic modulation of SMCs in aneurysmal aortas, whereby SMCs lose their contractile properties and switch to a synthetic phenotype.¹⁶ SMCs with synthetic properties are crucial during development and in response to injury, but they negatively influence the aortic wall in adults by secreting ECM-degrading enzymes.¹⁷ Others have identified noncontractile senescent SMCs in the aneurysmal ascending aorta that are capable of degrading

surrounding ECM through their senodestructive secretory phenotype.⁷ The mechanisms by which SMCs switch from the contractile phenotype to maladaptive synthetic or senescent states in the vulnerable ascending aortic wall remains uncertain. Even less is known regarding the role of medial SMCs in aortic wall remodeling in relation to AR.

We hypothesized that different flow characteristics due to aortic valve dysfunction could alter SMC phenotypes in the ascending aorta. We first histologically assessed medial cell phenotypes in the aorta of individuals with and without aortic valve dysfunction. We then isolated ascending aortic SMCs from individuals with either predominant AS or AR, and studied their phenotypic properties in culture. We exclusively studied SMCs from nondilated ascending aortas to isolate our findings from potential confounding causes of aortic wall remodeling. To evaluate whether BAVs exacerbate SMC phenotypic modulation, we compared SMCs from individuals with either TAVs or BAVs.

METHODS

The data presented in this article are available upon reasonable request from the corresponding author. This study complies with the Declaration of Helsinki, and was carried out with approval from the regional ethics committee (Ständige Ethikkommission der Ärztekammer des Saarlandes, Proposal #205/10; date of issue: August 12, 2010.). With the exception of donor controls, written informed consent was obtained from all patients. For donor samples, potential identifying data and characteristics were excluded.

Patient Enrollment and Exclusion Criteria

Fifty-five consecutive cardiac patients undergoing aortic valve surgery for either predominant severe AS (n = 20) or AR (n = 35) were enrolled in this study. For controls, aortic tissue was collected from autopsy samples (n = 10). AR patients were included only if the AR was due to cusp causes (ie, prolapse or retraction), and if their aortic root dimensions were within the normal range. Aortic valve morphology was determined preoperatively by either transthoracic or transesophageal echocardiography, and was confirmed intraoperatively by the primary surgeon. Surgical patients with TAVs (n = 21) or BAVs (n = 34) were enrolled. Before surgery, aortic dimensions were determined by computed tomography, and they were confirmed intraoperatively via transesophageal echocardiography. Aortic diameters measuring ≥ 40 mm were considered dilated,¹⁸ and were therefore excluded from this study. Patients were excluded if they had any serological evidence of chronic viral diseases (ie, HIV or hepatitis B or C) or if they showed any clinical signs of connective tissue disorders (ie, Marfan or Loeys-Dietz syndrome). Aortic samples were macroscopically analyzed and those with evidence of inflammatory disease (ie, atherosclerosis or aortitis) were also excluded. For the control group, aortic tissue samples were collected from autopsies of individuals without any macroscopic evidence of cardiac valve disease or aortic dilatation. Samples were then microscopically examined and confirmed to show no evidence of disease.

Procurement of Ascending Aortic Tissue

For each of the AS and AR patients, a circumferential fragment of aortic tissue (4-5 mm width) was excised from the anterior circumference of the thoracic aorta, 5 to 10 mm above the sinotubular junction. The excised tissue was divided in the operating room, and a portion was immediately fixed in 4% phosphate-buffered formalin for histological studies. The remaining aortic tissue was carefully transferred in phosphate buffered saline to a

sterile tissue culture hood for immediate extraction of medial SMCs. For autopsy samples, full circumferential aortic rings were excised and immediately fixed in 4% phosphate-buffered formalin. Due to the nature of tissue retrieval from autopsy samples, fresh tissue was unavailable for SMC isolation.

SMC Isolation and Culture

SMCs were isolated from the medial layer of the convex wall of the aorta through enzymatic digestion with Liberase (Roche), a purified collagenase blend. First, to ensure that only medial cells would be isolated, we separated the medial layer from the aortic tissue. This was done by gently scraping away the endothelial cell layer with a scalpel and then peeling away the adventitia and the outermost medial layers with forceps. The remaining medial tissue fragment was washed in phosphate buffered saline and then cut into $\sim 1 \times 1$ mm pieces to be used for the enzymatic digestion.

Medial tissue fragments were added to 1.5 mL Eppendorf tubes containing the enzymatic digestion media (830 μ L M199 Media; ThermoFisher Scientific, 11,150,059; + 0.5% FBS + 1% penicillin/streptomycin), 150 μ L Liberase (05,401,020,001) and 30 μ L DNase. The sample was incubated for 2h (37 °C, 5% carbon dioxide, and 95% humidity), and then the digested supernatant was collected through a cell strainer and maintained on ice until the end of the final digestion. The undigested aortic medial tissue was transferred to a new 1.5 mL Eppendorf tube containing fresh enzymatic digestion media. The digestion process was repeated for a second round with an incubation period of 1.5 hours. The collected supernatant from both digestions were combined and then centrifuged at 750 RPM for 6 minutes at 4 °C. The resulting cell pellet was washed with phosphate buffered saline, and then reconstituted in M199 media containing 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were plated on 0.4% gelatin-coated 60-mm culture dishes (Gelatin Type B Powder; Sigma-Aldrich, G9391; ThermoFisher Scientific Cell Culture Petri Dishes, 150,340), and incubated at 37 °C, 5% carbon dioxide, and 95% humidity. Media was changed 24 hours after the SMC isolation was completed, and then every 48 hours until confluence was reached. At this point, the cells were detached from the culture dishes (Trypsin/EDTA solution; ThermoFisher Scientific, R001100) and were either re-plated on coverslips for immunocytochemistry (cell passage 1), or were replated on fresh gelatin-coated dishes, repeatedly until growth was arrested and cells no longer reached confluence. The maximum cell passage achieved by the SMC culture from each patient was deemed as the replicative capacity. For immunocytochemistry, the SMCs were serum-starved (0.5% fetal bovine serum) once 80% confluence was reached. The cells were fixed 72 hours after serum withdrawal with 4% paraformaldehyde.

Immunostaining of Aortic Tissue and Cultured SMCs

Formalin-fixed aortic tissue samples were embedded in paraffin, and were sectioned at 1 μ m thickness. Aortic tissue sections and paraformaldehyde-fixed aortic SMCs were immunolabeled with rabbit polyclonal antibodies against either alpha-smooth muscle actin (ASMA) (1:100, ab5694; Abcam), a contractile protein,¹⁹ or vimentin (1:100, ab137321; Abcam), an intermediate filament protein that is abundantly expressed in synthetic SMCs.²⁰ Cellular senescence was evaluated in aortic tissue and SMCs by immunolabeling for cyclin-dependent kinase inhibitors, p16^{INK4a} (monoclonal mouse, 1:50, MA5-17054; Invitrogen) and p21^{Cip1} (1:50, MA1-33926; Invitrogen), which demarcate 2 core senescence pathways.^{21,22} Negative controls were incubated with rabbit or mouse immunoglobulin G. Bound primary antibodies were visualized with fluorescently conjugated secondary goat antibodies targeted against rabbit or mouse (Alexa-594). Aortic tissue samples were counter-stained with 4',6-diamidino-2-phenylindole and then were mounted onto microscopy slides. SMC-containing coverslips were mounted onto microscopy slides with a 4',6-diamidino-2-phenylindole-containing mounting media (Vectashield, H-1200-10).

Microscopy and Image Analysis

Fluorescently labeled aortic tissue samples and SMCs were imaged with a laser scanning confocal microscope (Zeiss LSM, Plan Apochromat). Aortic tissue samples were imaged at 40 \times with a 1.3 oil objective, and SMCs were imaged at 20 \times with a 0.8 M27 objective. For each patient, 5 to 10 regions of interest were captured per slide for both aortic tissue and SMC samples by a blinded evaluator. The fluorescence intensity of ASMA and vimentin were measured in ImageJ (National Institutes of Health) and normalized to the background intensity of each image. The percent positivity of p16 and p21 were also measured in ImageJ by counting the number of positive nuclei in each region of interest, and normalizing by the total number of nuclei in the image.

Statistics

All statistical analyses were carried out using Prism 9 (Graphpad Software). All datasets were tested for normality using the D'Agostino and Pearson omnibus test. In the case of normally distributed data, comparisons between groups were made with the Student *t* test or the 1-way analysis of variance with the Bonferroni-adjusted post hoc test. If at least 1 data set did not pass the normality test, data sets were compared with the Mann-Whitney *U* test or the Kruskal-Wallis test with Dunn multiple comparisons post hoc test. Linear regression analyses were used to assess for relationships between continuous variables. Patient age data are presented as mean \pm SD. Cell passage data are presented as median \pm SD.

RESULTS

Patient Characteristics

Ascending aortic tissue was collected intraoperatively from 55 consecutive patients undergoing aortic valve surgery for either predominant AS (*n* = 20) or predominant AR (*n* = 35). Control ascending aortic tissue was collected from autopsy samples with no evidence of aortic valve disease or dilatation (*n* = 10). The mean age of patients with predominant AS was 57.9 ± 11.7 (minimum: 19, maximum: 70) years, and in patients with predominant AR it was 59.4 ± 13.8 (minimum: 15, maximum: 84) years. Patients from the control group were significantly younger than those from the AS and the AR groups (mean: 39.7 ± 6.1 , minimum: 28, maximum: 50 years; *P* = .0001). There was no significant difference in age between the AS and AR groups (*P* = .89). Furthermore, there was no significant difference in the degree of SMC loss in the histological samples between the AS and the AR groups (*P* = .23). Aortic dimensions were similar between AS and AR groups (aortic sinus: *P* = .10, sinotubular junction: *P* = .23, midascending aorta: *P* = .33). Due to the nature of tissue retrieval from autopsy samples, in vivo patient data were not available for the control group. Clinical characteristics are presented in [Table 1](#).

For a subanalysis, AS and AR patients were further divided into groups based on aortic valve morphology. Of the patients with predominant AS, 11 had a TAV and 9 had a BAV. There was no significant difference in patient age between TAV and BAV subgroups (*P* = .07). In the AR group, 10 patients had a TAV and 25 had a BAV. The AR patients with a BAV were significantly younger than those with a TAV (*P* = .02). Clinical characteristics based on aortic valve morphology are presented in [Table 2](#).

AR Is Associated With Actin Loss and Increased Vimentin in the Ascending Aortic Wall

We first aimed to determine whether phenotypic switching of SMCs is a feature of aortic valve disease-associated aortic remodeling. Nondilated ascending aortic tissue was assessed for the expression of ASMA (contractile phenotype) and vimentin (synthetic phenotype). The expression of ASMA was decreased in AR-associated aortas compared with AS-associated aortas ($P = .03$) and control aortas ($P < .0001$) (Figure 1, A and B). Conversely, the expression of vimentin was increased in AR-associated aortas compared with AS-associated aortas and control aortas (all P values $< .0001$) (Figure 1, A and B). ASMA was decreased in AS-associated aortas compared with control ($P = .03$), and there was a trend toward increased vimentin ($P = .06$) (Figure 1, A and B). Lower magnification images of ASMA and vimentin are displayed in Figure E1. Assessing for a switch from contractile to synthetic SMCs, linear regression analyses revealed that ASMA expression negatively correlated with vimentin expression within aortic samples for all 3 groups (Control: $R^2 = 0.42$, $P = .04$; AS: $R^2 = 0.29$, $P = .01$; AR: $R^2 = 0.4$, $P < .0001$) (Figure 1, C). Furthermore, ASMA expression inversely correlated with patient age for all three groups (Control: $R^2 = 0.45$, $P = .03$; AS: $R^2 = 0.35$, $P = .007$; AR: $R^2 = 0.4$, $P < .0001$) (Figure 1, D). Vimentin expression positively correlated with patient age for the AR group ($R^2 = 0.36$; $P = .0001$), whereas no significant relationship was detected for the control or AS group (Control: $R^2 = 0.22$, $P = .69$; AS: $R^2 = 0.13$, $P = .12$) (Figure 1, D).

AR Is Associated With Cellular Senescence in the Ascending Aortic Medial Layer

To determine whether cellular senescence is a feature of AS- or AR-associated aortic remodeling, we analyzed ascending aortic tissue for the expression of the cell cycle inhibitors, p16^{INK4a} and p21^{Cip1}. The expression of both p16^{INK4a} and p21^{Cip1} was increased in AR-associated aortas compared to AS-associated aortas ($P < .0001$ and $P = .0001$, respectively) and controls ($P = .0005$ and $P = .0011$, respectively) (Figure 2, A and B). There was no significant difference in either p16^{INK4a} or p21^{Cip1} expression between AS-associated aortas and control aortas (all P values = .99) (Figure 2, A and B). There was no significant relationship between p16^{INK4a} and patient age in either group (Control: $R^2 < 0.0001$, $P = .95$; AS: $R^2 = 0.17$, $P = .07$; AR: $R^2 = 0.008$, $P = .60$) (Figure 2, C). On the other hand, p21^{Cip1} expression increased with patient age for both the AS ($R^2 = 0.3$; $P = .03$) and AR ($R^2 = 0.16$; $P = .02$) groups (Figure 2, D). There was no significant association between patient age and p21^{Cip1} expression for the control group ($R^2 = 0.009$; $P = .79$) (Figure 2, D).

TABLE 1. Patient characteristics

Patient characteristics	Aortic stenosis		Aortic regurgitation
	Control		
No. of patients	10	20	35
Patient age (y)	28-50	19-70	15-84
Patient age (y)	39.7 ± 6.1*	57.9 ± 11.7	59.4 ± 13.8
Ascending aortic diameter range (cm)	–	2.4-3.9	2.6-4.0
Ascending aortic diameter	–	3.2 ± 0.5	3.4 ± 0.4
Comorbidities			
Hypertension	–	6 (30)	10 (29)
Hyperlipidemia	–	5 (25)	11 (31)
Smoker	–	3 (15)	5 (14)
Diabetes	–	1 (5)	2 (6)
Medications			
β-Blocker	–	4 (20)	4 (11)
ACE inhibitor	–	2 (10)	7 (20)
Diuretic	–	3 (15)	4 (11)
Calcium channel blocker	–	1 (5)	5 (14)
Statin	–	2 (10)	3 (9)
Cardiovascular Parameters			
Stroke volume	–	59.4 ± 27.5	78.1 ± 28.8
Aortic wall thickness	–	0.4 ± 0.2	0.5 ± 0.2
LV thickness: Septum	–	1.5 ± 0.3	1.4 ± 0.4
LV thickness: Posterior wall	–	1.5 ± 0.3	1.4 ± 0.4
LV internal diameter: Systole	–	3.1 ± 0.9	3.7 ± 0.7
LV internal diameter: Diastole	–	4.6 ± 1.2	5.3 ± 0.8

Values are presented as n, range, mean ± SD, or n (%). ACE, Angiotensin converting enzyme; LV, left ventricle. * $P < .05$.

AR-Associated Changes in the Aortic Wall Are More Pronounced in the Presence of a BAV

Because BAVs are associated with ascending aortic aneurysms, we next sought to determine whether the presence of a BAV aggravates AS- or AR-associated changes in the aortic wall. Among patients with predominant AS, ASMA expression was decreased in the BAV-associated ascending aorta (BAV-AS) compared with TAV aortas (TAV-AS) ($P = .003$) (Figure 3, A). No significant changes were

TABLE 2. Clinical characteristics based on aortic valve morphology

Aortic valve morphology	Aortic stenosis		Aortic regurgitation	
	TAV	BAV	TAV	BAV
No. of patients	11	9	10	25
Patient age, y (range)	22-70	19-68	22-84	15-79
Patient age, y (mean ± SD)	60.4 ± 8.4	55.4 ± 9.6	65.3 ± 9.1	53.5 ± 8.8*
Ascending aortic diameter (cm)	2.4-3.9	2.4-3.8	2.6-4.0	2.6-4.0
Ascending aortic diameter	3.1 ± 0.4	3.3 ± 0.6	3.4 ± 0.3	3.4 ± 0.5

Values are presented as n, range, or mean ± SD. TAV, Tricuspid aortic valve; BAV, bicuspid aortic valve; SD, standard deviation. * $P < .05$ versus aortic regurgitation-TAV.

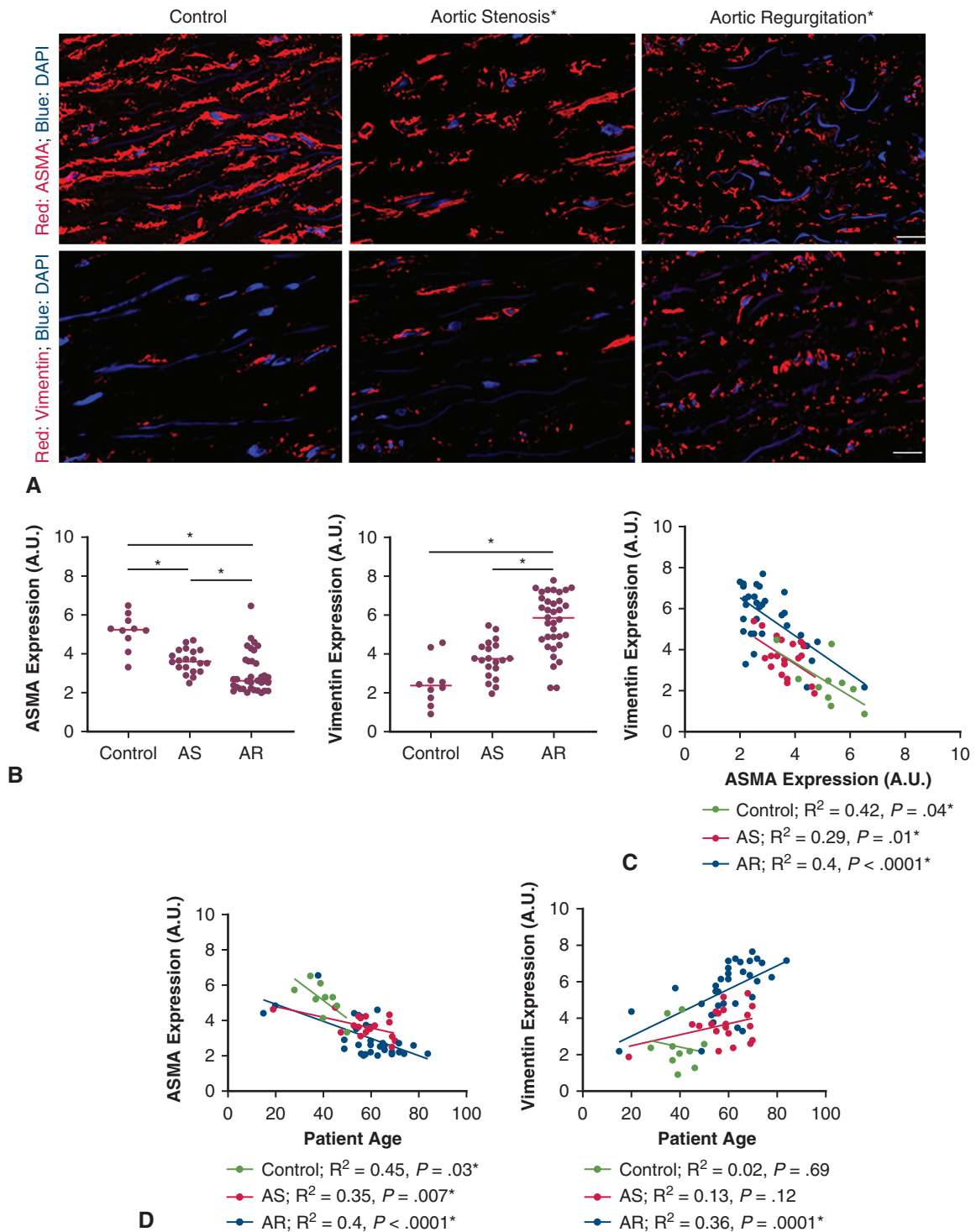


FIGURE 1. Alpha-smooth muscle actin (ASMA) is decreased while vimentin is increased in aortic regurgitation (AR)-associated aortas. A, Fluorescent micrographs of ASMA (top) and Vimentin (bottom) in control (left), AS- (middle), and AR- (right) associated ascending aortic tissue. B, Graphs depicting ASMA (top) and vimentin (bottom) expression in the ascending aorta from each group. Horizontal bars represent median values. C, Graph depicting the relationship of ASMA and vimentin for each group. D, Graphs depicting the relationship between ASMA (left) or vimentin (right) and patient age for each group. Scale bar = 20 μ m. A.U., Arbitrary units; DAPI, 4',6-diamidino-2-phenylindole; AS, aortic stenosis. *Statistically significant.

observed in either the expression of vimentin ($P = .96$) (Figure 3, B) or the senescence markers when comparing TAV-AS to BAV-AS aortas ($p16^{\text{INK4a}}$: $P = .17$; $p21^{\text{Cip1}}$: $P = .30$) (Figure 3, C and D).

For patients with predominant AR, the expression of ASMA was decreased in the ascending aorta of BAV patients (BAV-AR) compared with those with a TAV (TAV-AR) ($P = .0001$) (Figure 3, E). On the other hand, vimentin expression was increased in BAV-AR ascending aortas compared to that of TAV-AR ($P = .002$) (Figure 3, F). Despite being biologically younger in age, cellular senescence was exacerbated by the presence of BAV as $p16^{\text{INK4a}}$ ($P = .005$) and $p21^{\text{Cip1}}$ ($P = .003$) expression was increased in BAV-AR aortas compared with TAV-AR aortas (Figure 3, G and H).

AR Is Associated With Phenotypic Switching of SMCs Isolated From the Normal Ascending Aorta

To determine if disruptive SMC changes underlie AR-associated ascending aortic remodeling, we isolated medial SMCs from fresh ascending aortic tissue and studied their

phenotypic properties in culture. There was a significant decrease in ASMA expression in SMCs from AR-associated aortas compared with those from AS-associated aortas ($P = .0001$) (Figure 4, A and B). On the other hand, vimentin expression was increased in AR-associated SMCs versus AS-associated SMCs ($P < .0001$) (Figure 4, A and B). Similar to our findings in aortic tissue, there was an inverse correlation between ASMA and vimentin expression for both groups (AS: $R^2 = 0.40$, $P = .003$; AR: $R^2 = 0.12$, $P = .02$) (Figure 4, C). While patient age increased, ASMA expression decreased (AS: $R^2 = 0.33$, $P = .009$; AR: $R^2 = 0.24$, $P = .003$) and vimentin expression increased in SMCs from both groups (AS: $R^2 = 0.25$, $P = .02$; AR: $R^2 = 0.24$, $P = .003$) (Figure 4, D).

Cellular Senescence Is Accelerated in SMCs Isolated From AR-Associated Ascending Aortas

We measured cellular senescence in isolated SMCs by analyzing the expression of $p16^{\text{INK4a}}$ and $p21^{\text{Cip1}}$, and by assessing the replicative capacity of SMCs in culture. There was a trend toward increased expression of $p16^{\text{INK4a}}$ in AR

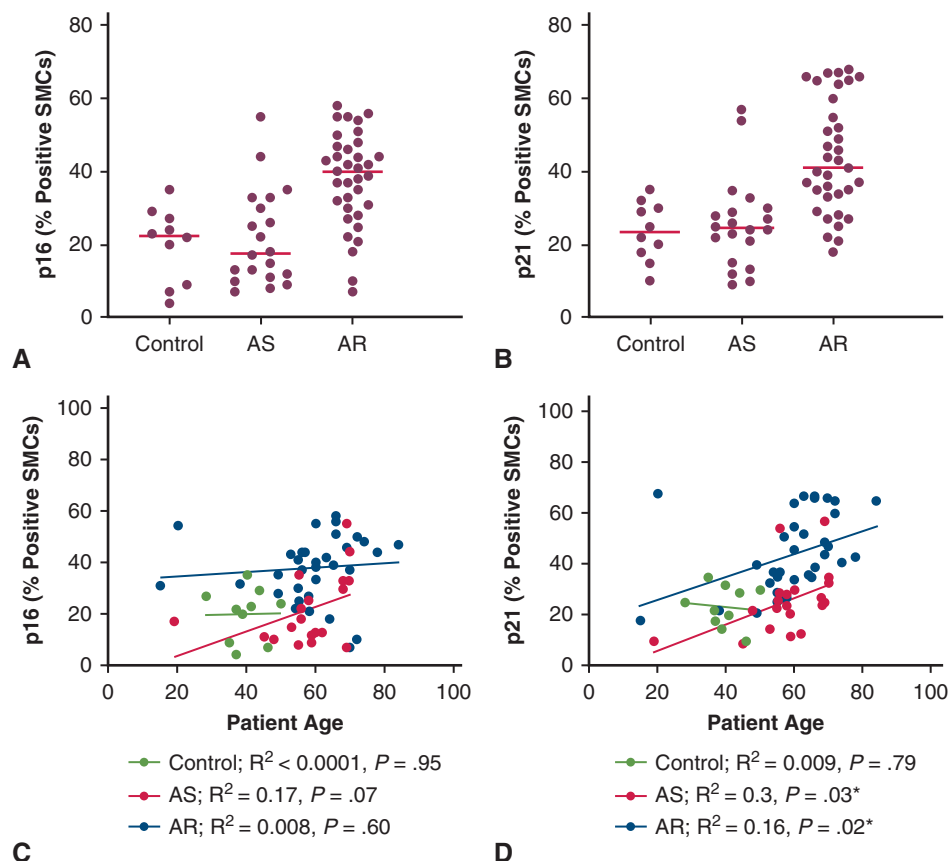


FIGURE 2. Senescence markers $p16^{\text{INK4a}}$ and $p21^{\text{Cip1}}$ are increased in aortic regurgitation (AR)-associated aortas. A and B, Graphs depicting $p16^{\text{INK4a}}$ (A) and $p21^{\text{Cip1}}$ (B) expression in the ascending aorta from control patients, or from patients with aortic stenosis (AS) or AR. Horizontal bars represent median values. C and D, Graphs depicting the relationship between $p16^{\text{INK4a}}$ (C) or $p21^{\text{Cip1}}$ (D) expression and patient age for each group. SMCs, Smooth muscle cells. *Statistically significant.

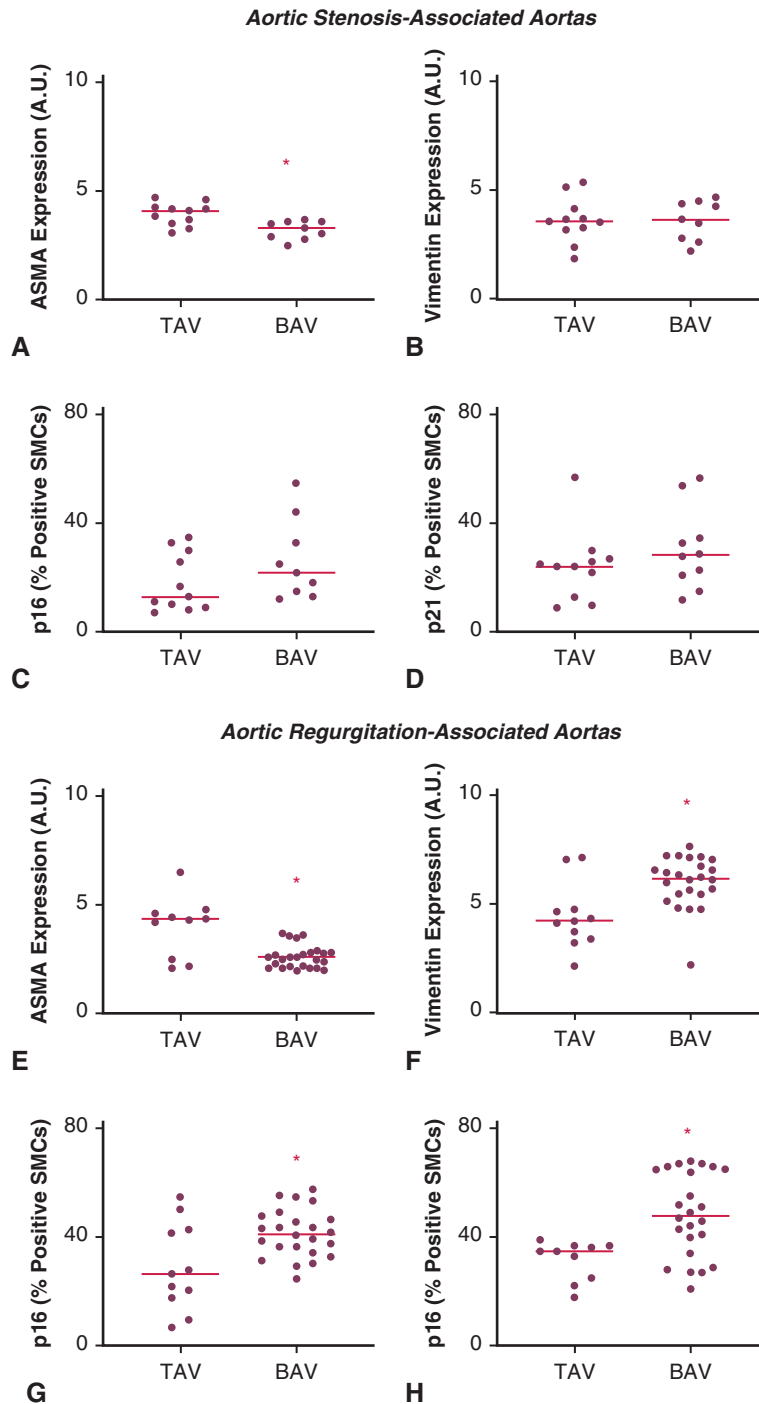


FIGURE 3. Bicuspid aortic valve (BAV) morphology aggravates phenotypic changes in aortic regurgitation (AR)-associated aortas. A through D, Graphs depicting the expression of alpha smooth muscle actin (ASMA) (A), vimentin (B), p16 (C), and p21 (D) in the aorta of individuals with aortic stenosis and either tricuspid aortic valves (TAV) or BAVs. E through H, Graphs depicting the expression of ASMA (E), vimentin (F), p16 (G), and p21 (H) in the aorta of individuals with aortic regurgitation and either TAVs or BAVs. Horizontal bars represent median values. A.U., Arbitrary units; SMCs, smooth muscle cells. *Statistically significant.

SMCs versus AS SMCs ($P = .06$) (Figure 5, A). The expression of p21^{Cip1} was significantly increased in SMCs isolated from AR-associated aortas compared to that of AS-

associated aortas ($P = .006$) (Figure 5, A). Patient age correlated with p21^{Cip1} expression in both groups (AS: $R^2 = 0.25$, $P = .03$; AR: $R^2 = 0.40$, $P < .0001$), whereas

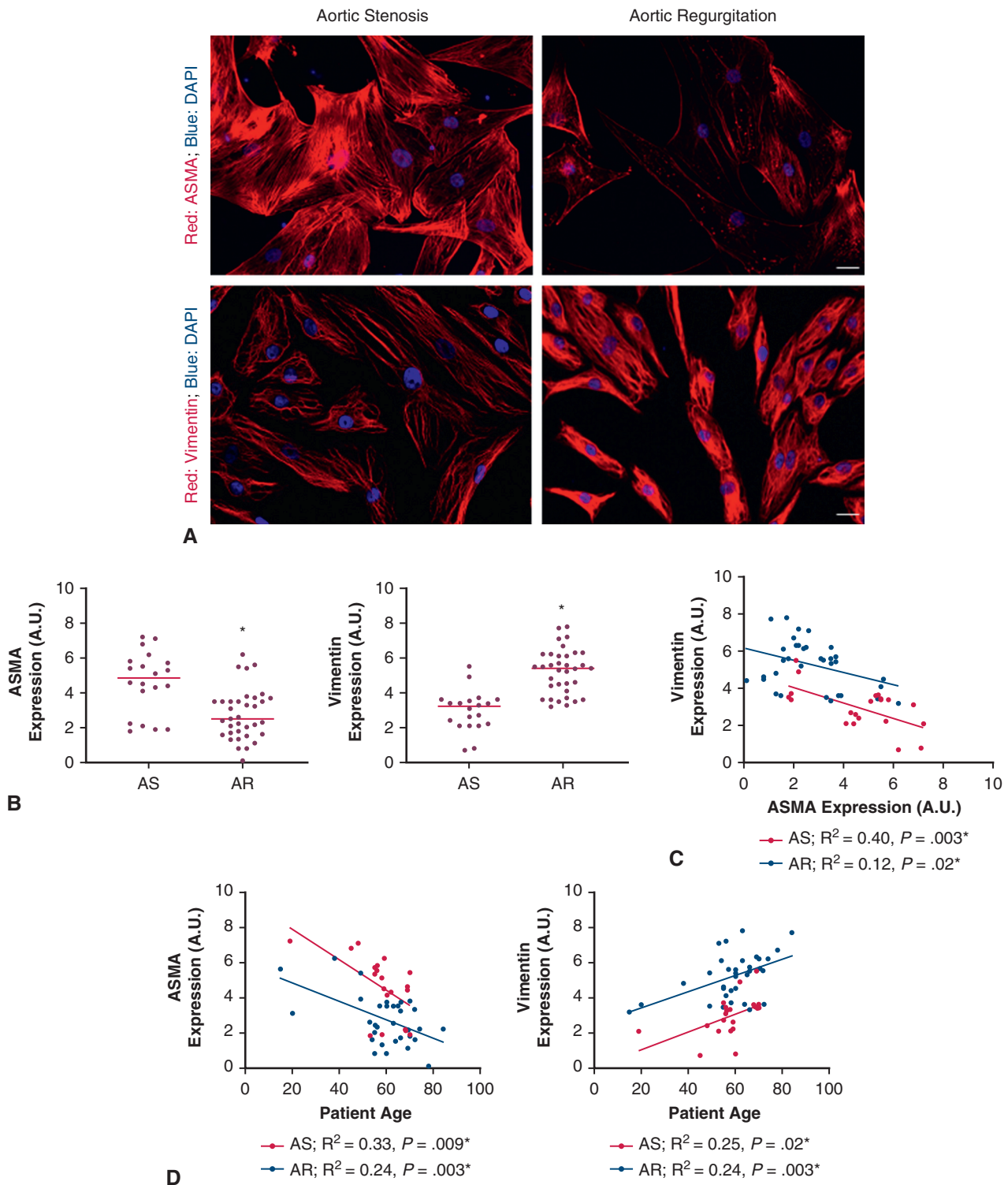


FIGURE 4. Alpha-smooth muscle actin (ASMA) is decreased while vimentin is increased in smooth muscle cells (SMCs) isolated from aortic regurgitation (AR)-associated aortas. A, Fluorescent micrographs of ASMA (top) and vimentin (bottom) in aortic stenosis (AS)- (left) and AR- (right) associated SMCs. B, Graphs depicting ASMA (top) and vimentin (bottom) expression in SMCs from each group. Horizontal bars represent median values. C, Graph depicting the relationship between ASMA and vimentin in SMCs for each group. D, Graphs depicting the relationship between ASMA (left) or vimentin (right) and patient age in SMCs for each group. Scale bar = 20 μ m. All SMC cultures were fixed and stained at cell passage 1. DAPI, 4',6-diamidino-2-phenylindole; A.U., arbitrary units. *Statistically significant.

no relation was detected between age and p16^{INK4a} (AS: $R^2 = 0.09$, $P = .19$; AR: $R^2 = 0.05$, $P = .20$) (Figure 5, B). By allowing SMC cultures from each patient to grow indefinitely, we found that the replicative capacity of SMCs from AR-associated aortas (cell passage 3.0 ± 1.1) was decreased compared with that of SMCs from AS-associated aortas (cell passage 5.0 ± 1.6 ; $P < .0001$) (Figure 5, C). This suggests that AR SMCs enter cellular senescence at an accelerated rate compared with SMCs from AS-associated aortas.

AR-Associated Phenotypic Switching of SMCs Is More Pronounced in the Presence of a BAV

Like in tissue, we assessed phenotypic switching of SMCs from AS- and AR-associated aortas in patients with

either a TAV or a BAV. For patients presenting with predominant AS, ASMA expression was decreased in BAV-AS SMCs compared with TAV-AS SMCs ($P = .001$) (Figure 6, A). The expression of vimentin was increased in BAV-AS SMCs compared with TAV-AS SMCs ($P = .02$) (Figure 6, A). No differences in SMC senescence were observed between TAV-AS and BAV-AS SMCs because the expression of p16^{INK4a} and p21^{Cip1} was similar between groups ($P = .78$ and $P = .66$, respectively) (Figure 6, A). Furthermore, SMC replicative capacity did not differ between groups ($P = .12$) (Figure 6, B).

Among patients with predominant AR, the expression of ASMA was decreased in SMCs from BAV-AR aortas compared to TAV-AR aortas ($P < .0001$) (Figure 6, C). Vimentin expression was increased in BAV-AR SMCs

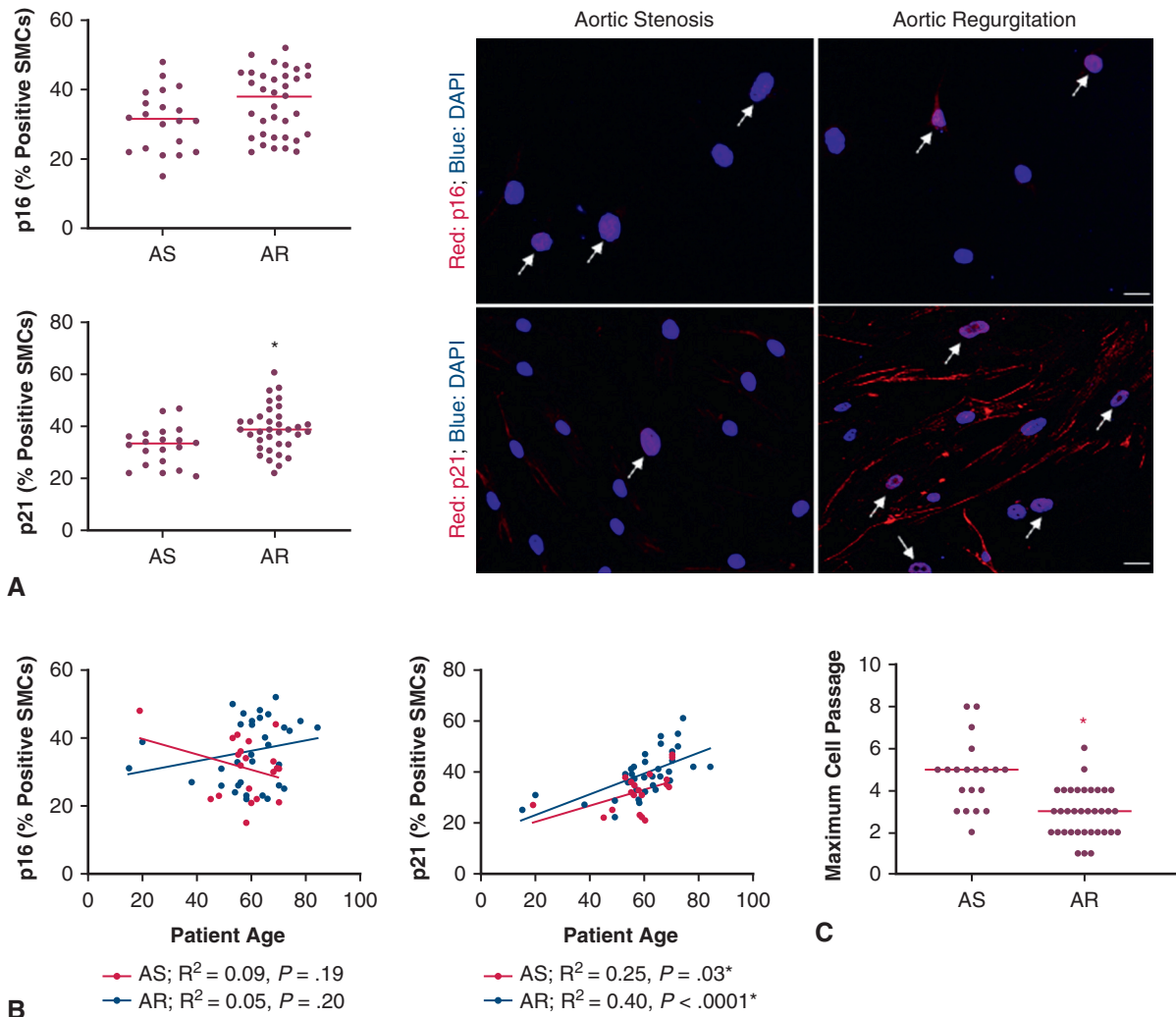


FIGURE 5. Senescence markers p16^{INK4a} and p21^{Cip1} are increased in smooth muscle cells (SMCs) isolated from aortic regurgitation (AR)-associated aortas. A, Graphs (left) depicting p16^{INK4a} (top) and p21^{Cip1} (bottom) expression in aortic SMCs isolated from patients with aortic stenosis (AS) or AR, with corresponding fluorescent micrographs (right). Arrows indicate p16 (top)- or p21 (bottom)- positive SMCs. Scalebar = 10 μm. B, Graphs depicting the relationship between p16^{INK4a} (left) or p21^{Cip1} (right) expression and patient age in SMCs for each group. C, Graph depicting the maximum cell passage of cultured SMCs isolated from AS- or AR-associated aortas. Horizontal bars represent median values. All SMC cultures were fixed and stained at cell passage 1. *Statistically significant.

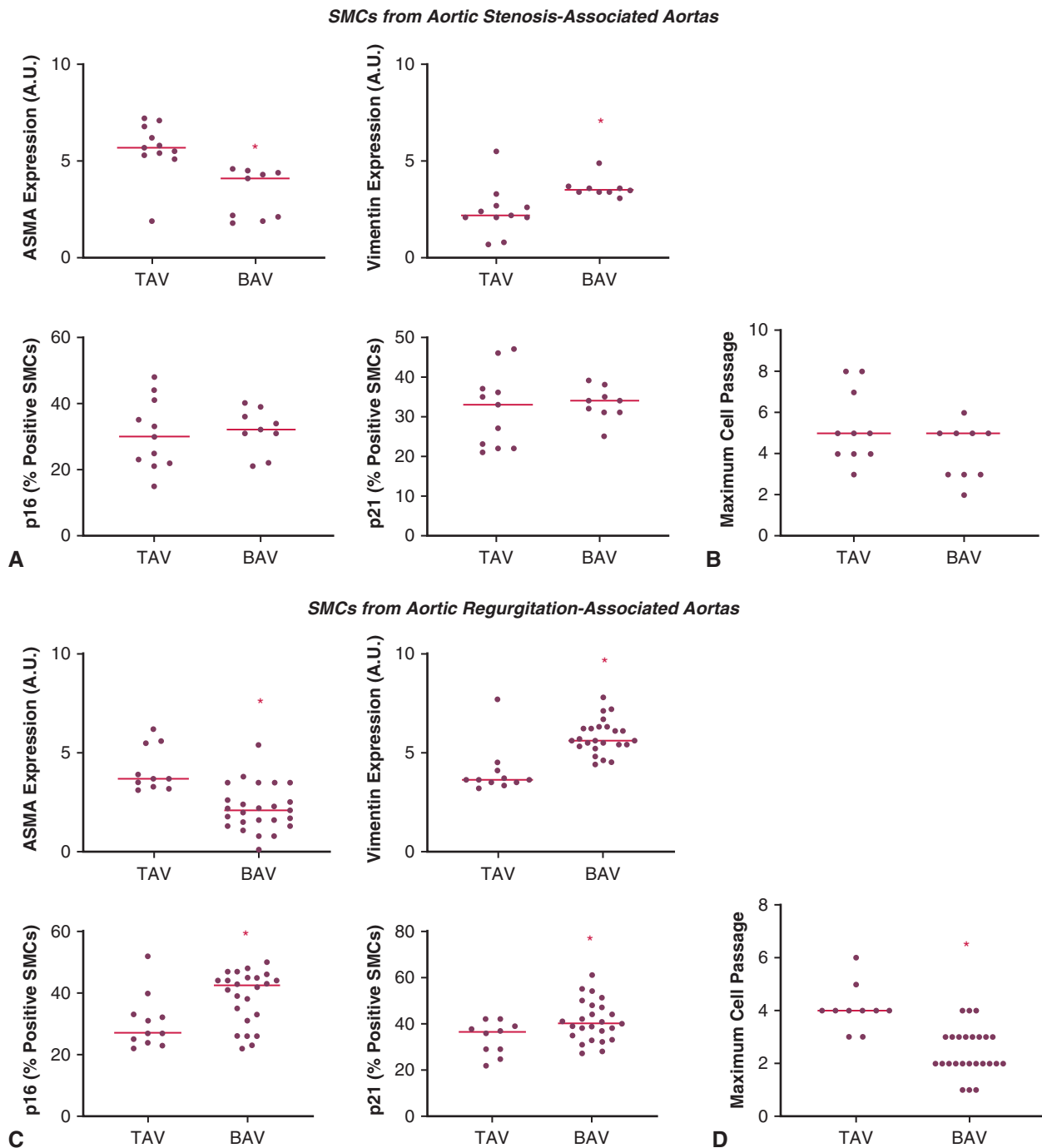
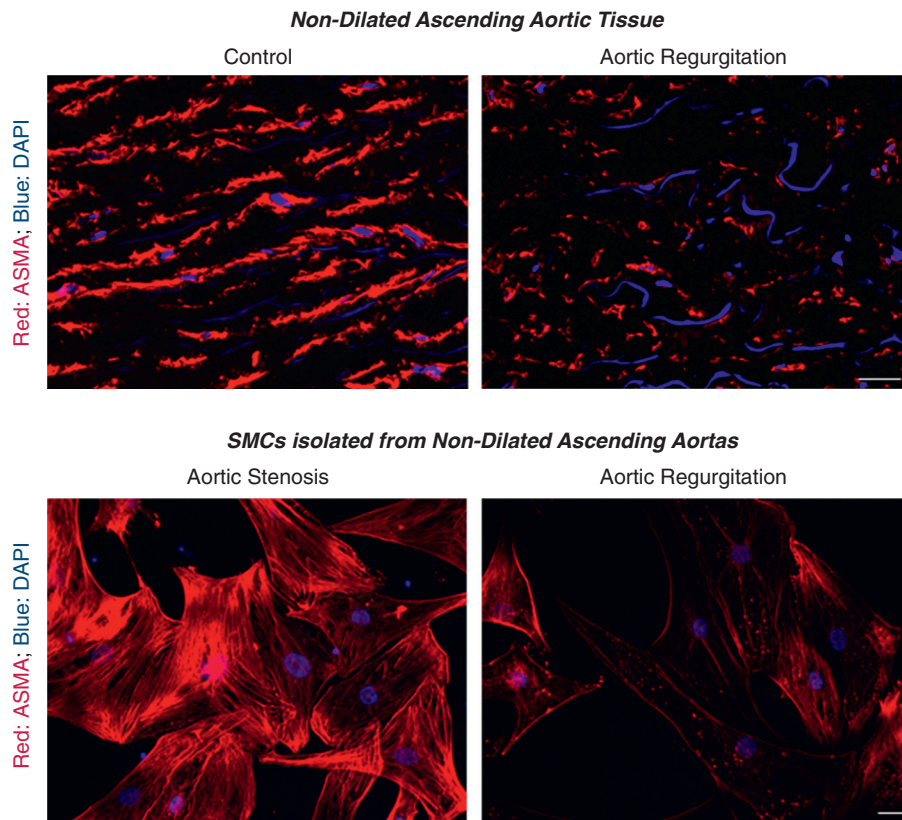


FIGURE 6. Bicuspid aortic valve (*BAV*) morphology aggravates phenotypic changes in smooth muscle cells (*SMCs*) isolated from aortic regurgitation-associated aortas. **A**, Graphs depicting the expression of alpha smooth muscle actin (*ASMA*), vimentin, p16, or p21 in aortic *SMCs* from individuals with aortic stenosis and either tricuspid aortic valves (*TAV*) or *BAVs*. **B**, Graph depicting the maximum cell passage of cultured *SMCs* isolated from individuals with aortic stenosis and either *TAVs* or *BAVs*. **C**, Graphs depicting the expression of *ASMA*, vimentin, p16, or p21 in aortic *SMCs* from individuals with aortic regurgitation and either *TAVs* or *BAVs*. **D**, Graph depicting the maximum cell passage of cultured *SMCs* isolated from individuals with aortic regurgitation and either *TAVs* or *BAVs*. Horizontal bars represent median values. All *SMC* cultures were fixed and stained at cell passage 1. *A.U.*, Arbitrary units. *Statistically significant.

compared with *TAV-AR SMCs* ($P < .0001$) (Figure 6, C). As opposed to AS, valve morphology appears to have influenced AR-associated cellular senescence, as the expression

of p16^{INK4a} and p21^{Cip1} were significantly increased in *BAV-AR SMCs* compared with *TAV-AR SMCs* ($P = .02$ and $.03$, respectively) (Figure 6, C). Moreover, the



Aortic regurgitation provokes phenotypic switching of smooth muscle cells in the non-dilated ascending aorta, as evidenced by decreased alpha-smooth muscle Actin (ASMA) levels

FIGURE 7. Aortic regurgitation provokes loss of alpha-smooth muscle actin (ASMA) in smooth muscle cells (SMCs) in a nondilated ascending aorta. DAPI, 4',6-diamidino-2-phenylindole.

replicative capacity of BAV-AR SMCs was significantly reduced compared with that of TAV-AR SMCs ($P < .0001$) (Figure 6, D). Therefore, despite being younger in age, SMCs from patients with BAV-AR exhibit accelerated senescence compared with TAV-AR SMCs.

DISCUSSION

It has long been recognized that AR has prognostic implications in diseases with ascending aortic involvement. With Marfan syndrome, for instance, the presence of AR increases the risk of aortic dissection and rupture.^{4,23} With BAV and AR, the rate of aortic dilatation and the risk of aortic events is higher compared with BAV with AS¹⁴ or normally functioning BAVs.¹³ These pathological consequences remain even after aortic valve replacement.^{24,25} These findings suggest that an underlying intrinsic factor involving both aortic degeneration and AR may be present in ascending aortic aneurysm disease. In the setting of the aneurysmal aorta, it is difficult to determine whether AR has an independent prognostic effect, or whether AR and aortic degeneration are separate entities of a common origin. This is even more complex in the presence of a

BAV, in which genetic factors and turbulence are assumed to contribute to aortic degeneration.

We have recently shown AR-specific ascending aortic degeneration, independent of aortic dilatation, aortic valve malformations, or known connective tissue disorders.¹⁵ The most profound degenerative alterations were localized in the medial layer, which included elastin degradation and decreased fibrillin and collagen expression. To this point, the underlying mechanisms have not been investigated. SMCs in the aortic media regulate the turnover of elastin and ECM proteins.²⁶ Thus, these degenerative findings raise the question of whether SMC-mediated changes drive degeneration in the ascending aortic wall in individuals with AR. SMCs are known to exist in different phenotypes that relate to different functional properties.²⁷ We therefore analyzed SMC phenotypes in nondilated aorta because switching from the contractile to synthetic or senescent states primes SMCs for ECM remodeling.^{7,28,29}

Phenotypic switching of SMCs to a synthetic state has been observed in isolated SMCs³⁰ and tissue samples from aneurysmal ascending aortas.³¹⁻³³ Synthetic SMCs are capable of secreting ECM-degrading enzymes^{28,29} and

may, thus, play a role in medial degeneration in the aneurysmal aorta. We recently found a similar phenotypic switch of SMCs in the normal ascending aorta, which was related to increasing patient age, but independent of aortic dilatation.³⁴ Here, phenotypic modulation of SMCs was observed in association with AR, and again independent of dilatation. This was confirmed by decreased ASMA expression, which is the main contractile protein in SMCs, and an increase in vimentin, which promotes SMC migration. Such changes were minimal with AS and absent in the case of no discernable aortic valve disease. In line with our previous report,³⁴ phenotypic modulation was more pronounced with increasing age. BAV morphology further aggravated phenotypic modulation, both with AS, and even more so with AR. The BAV patients were significantly younger than the TAV patients in the AR group, suggesting that BAV morphology may be more pathogenic than age. We observed similar phenotypic changes in isolated SMCs, demonstrating that aortic valve disease-related SMC alterations are retained in culture.

SMC differentiation can be influenced by signals from the ECM and intimal endothelial cells.³⁵ By isolating SMCs from the aortic wall, we were able to clarify that our results are SMC-specific, and independent of recurrent influences of surrounding aortic wall components. Taken together, these findings suggest that AR, patient age, and BAV morphology may independently or codependently influence SMC phenotypes in the ascending aorta before dilatation.

More recently it was found that SMCs may enter an irreversible state of cell cycle arrest known as senescence in response to certain injury-related triggers, like unrepaired DNA damage and overwhelming oxidative stress.²¹ Although this particular phenotypic switch is believed to be a protective mechanism, senescent SMCs have the ability to compromise surrounding tissue through their senescence-associated secretory phenotype and can reside in the aorta for an extended period of time.^{7,22} Senescent SMCs have been identified in aneurysmal aortas from mice³⁶ and humans.⁷ In this study, we show that senescence is also associated with AR in the nondilated ascending aortic wall, suggesting that SMC senescence does not rely on aortic dilatation. This was evidenced by increased p16/p21, which are cell cycle inhibitors that are required for growth arrest. We also found that cultured SMCs isolated from AR-associated aortas entered senescence earlier than those from AS-associated aortas, confirming the specificity of a pathogenic AR-effect. It is important to note that SMC senescence was increased in both AS and AR aortas in the presence of a BAV. This is in line with a previous study that showed that SMCs isolated from BAV-associated aneurysms had significantly shorter telomeres than that of TAV aneurysms.³⁷ Whether factors underlying AR and BAV independently influence SMC senescence, or whether there

is interplay between the 2 pathogenic factors remains to be elucidated.

The findings herein confirm for the first time that AR has a negative influence on the ascending aortic wall, independent of aortic diameter, aortic valve morphology, and aortic loading conditions. We showed that AR itself negatively influences SMC phenotype, such that contractile SMCs are replaced by either synthetic or senescent SMCs that may be primed for ECM degradation (Figure 7). Based on our findings, the mechanisms underlying AR may have a genetic origin that also predispose to SMC changes, which should be further evaluated; it might be considered as a therapeutic target in the future to prevent or delay ascending aortic aneurysm formation in patients with AR. Furthermore, we found that SMCs isolated from surgical samples retain their AR-related phenotypic properties in culture. Therefore, SMC isolation from surgical aortic samples provides a medium for studying phenotype-targeted therapeutics.

Limitations

It is important to note that control samples are challenging to come by for human surgical studies. We utilized autopsy specimens that were macro- and microscopically confirmed to show no signs of aortic valve disease or ascending aortic dilatation. We recognize that by using autopsy specimens for controls, this results in the possibility of postmortem artifacts. In this study, autopsy samples had less-pronounced SMC changes in the aortic media compared with AS and AR aortic samples. It would be beneficial to compare our findings with other control samples in future studies, such as those from heart donors.

Conflict of Interest Statement

The authors reported no conflicts of interest.

The *Journal* policy requires editors and reviewers to disclose conflicts of interest and to decline handling manuscripts for which they may have a conflict of interest. The editors and reviewers of this article have no conflicts of interest.

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Key Words: smooth muscle cells, alpha-smooth muscle actin, ascending aorta, bicuspid aortic valve, senescence

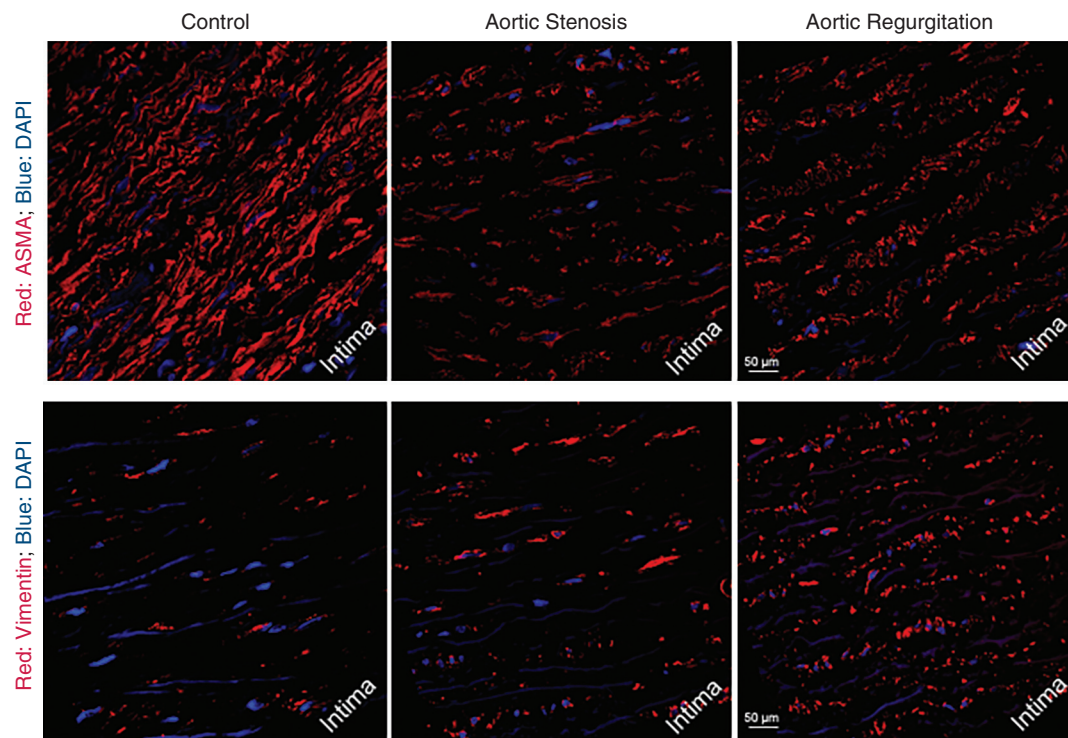


FIGURE E1. Alpha-smooth muscle actin (ASMA) is decreased when vimentin is increased in aortic regurgitation (AR)-associated aortas. Florescent micrographs of aortic cross-sections stained for ASMA (*top*) and vimentin (*bottom*) in control (*left*), AS- (*middle*), and AR- (*right*) associated ascending aortic tissue. Scale-bar = 50 μm. Intima is labeled. DAPI, 4',6-diamidino-2-phenylindole.