Fibrotic Aortic Valve Stenosis in Hypercholesterolemic/Hypertensive Mice

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Objective—Hypercholesterolemia and hypertension are associated with aortic valve stenosis (AVS) in humans. We have examined aortic valve function, structure, and gene expression in hypercholesterolemic/hypertensive mice.

Approach and Results—Control, hypertensive, hypercholesterolemic (Apoe−/−), and hypercholesterolemic/hypertensive mice were studied. Severe aortic stenosis (echocardiography) occurred only in hypercholesterolemic/hypertensive mice. There was minimal calcification of the aortic valve. Several structural changes were identified at the base of the valve. The intercusp raphe (or seam between leaflets) was longer in hypercholesterolemic/hypertensive mice than in other mice, and collagen fibers at the base of the leaflets were reoriented to form a mesh. In hypercholesterolemic/hypertensive mice, the cusps were asymmetrical, which may contribute to changes that produce AVS. RNA sequencing was used to identify molecular targets during the developmental phase of stenosis. Genes related to the structure of the valve were identified, which differentially expressed before fibrotic AVS developed. Both RNA and protein of a profibrotic molecule, plasminogen activator inhibitor 1, were increased greatly in hypercholesterolemic/hypertensive mice.

Conclusions—Hypercholesterolemic/hypertensive mice are the first model of fibrotic AVS. Hypercholesterolemic/hypertensive mice develop severe AVS in the absence of significant calcification, a feature that resembles AVS in children and some adults. Structural changes at the base of the valve leaflets include lengthening of the raphe, remodeling of collagen, and asymmetry of the leaflets. Genes were identified that may contribute to the development of fibrotic AVS. (Arterioscler Thromb Vasc Biol. 2016;36:466-474. DOI: 10.1161/ATVBHA.115.306912.)

Key Words: aortic valves ▪ constriction, pathologic ▪ hypercholesterolemia ▪ hypertension ▪ plasminogen activator inhibitor 1

Aortic stenosis in humans is associated with multiple risk factors, including hypercholesterolemia and hypertension.1–4 Single risk factors have failed to consistently produce hemodynamically significant calcific aortic valve stenosis (CAVS) in mice. In the first model of CAVS, hypercholesterolemia was produced by deficiency of low-density lipoprotein receptors (Ldlr−/−) together with homozgyous expression of apo B48 (LA mice).5 On the basis of the idea that 2 hits may be needed to produce aortic stenosis, we have studied the effects of chronic hypertension, produced by activation of the renin–angiotensin system,6 combined with hypercholesterolemia (in apoE-deficient mice). Combining these 2 risk factors has led to discovery of the second experimental model that consistently develops severe aortic stenosis but in sharp contrast to the first model,5 with minimal calcification of the valve. In hypercholesterolemic mice, the amount of calcium in the valve is associated with changes in function of the valve in some,8,9 but not all,7 interventions. In humans, in some,10,11 but not all, studies,12,13 the amount of calcification in the aortic valve is a predictor of valve function. Thus, this is a new model of fibrotic aortic valve stenosis (FAVS) without significant valve calcification, findings that resemble children and some adults with aortic stenosis.12,13

Structural changes that account for aortic stenosis are not clear. In this model of FAVS, we found little change in total collagen in the valve. We now propose that collagen remodels at the base of the valve leaflets to produce a mesh, which may contribute to development of stenosis. We also propose that lengthening of the raphe of the valve leaflets may limit the opening of the valve. A method was developed to measure the length of the raphe (or seam) at the base of the valve leaflets. Another method was developed to measure the size of the valve leaflets. It has been proposed that asymmetry of leaflets in humans may lead to processes that produce aortic stenosis.14,15

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Medical treatments for aortic stenosis in humans have failed. To identify new targets for treatment of aortic stenosis, we have performed whole transcriptome profiling using RNA sequencing (RNA-Seq) of the aortic valve to identify differentially expressed genes in this new model of FAVS. Gene expression was examined before the development of aortic stenosis to avoid confounding effects of advanced disease. No other study of the aortic valve, in humans or in mice, has used RNA-Seq to compare gene expression with that of normal (control) tissue. Because this model of FAVS is characterized by only minimal calcification, one of our goals was to look with RNA-Seq for genes that promote or limit calcification and fibrosis in blood vessels.

### Materials and Methods

Materials and Methods are available in online-only Data Supplement.

### Results

Using echocardiography to examine opening of the aortic valve during systole, we measured aortic cusp separation (ACS). ACS was similar in control and hypertensive mice (Figure 1). In hypercholesterolemic mice, there was a small decrease in ACS \( (P<0.05; \text{Figure } 1E) \), which was not hemodynamically significant, based on our previous comparison of ACS and transvalvular gradient. In hypercholesterolemic/hypertensive mice, there was a significant decrease in ACS, with severe aortic stenosis (AVS, \( <0.66 \text{ mm} \)) in about half of the mice (Figure 1E). Thus, severe aortic stenosis was identified only in hypercholesterolemic/hypertensive mice. Significant aortic regurgitation was not observed in any mice.

When mice died before we planned to euthanize them at 12 months of age, the cause of death usually was not clear because deaths were not observed and were not preceded by premonitory signs. Regional wall motion abnormalities of the left ventricle (suggestive of myocardial infarction) were not detected by echocardiography. Gross examination at autopsy did not reveal myocardial infarction or blood in the thorax or abdomen, which would have suggested the rupture of aortic aneurysms.

### Histological Measurements

We used morphometry to examine structural components of the aortic valve. There was minimal calcification in the aortic valve of control mice (Figure 2A). Calcification in the valve was modest and similar in hypertensive, hypercholesterolemic, and hypercholesterolemic/hypertensive mice. There was substantial calcification in the atheroma around the base of the valve, which indicates that the Alizarin red stain was able to detect calcification when it is present (Figure III in the online-only Data Supplement). Thus, calcification of the aortic valve was minimal and not greater in hypercholesterolemic/hypertensive mice that are the group that develops AVS.

Total collagen and elastic fibers were not changed in hypertensive, hypercholesterolemic, and hypercholesterolemic/hypertensive mice compared with control mice (Figure 2B and 2C). Lipids in the aortic valve were increased in hypercholesterolemic and hypercholesterolemic/hypertensive mice (Figure 2D). Because lipids in the valve were not greater in hypercholesterolemic/hypertensive mice than in hypercholesterolemic mice, they cannot account for aortic stenosis.

### Base and Cusps of the Aortic Valve

We next focused on other structural changes that might account for AVS. A new method was developed to examine the raphe, which is the seam between cusps (Figure 3). The length of the raphe (from annulus to separation of the cusps) tended to increase (not significant) in hypertensive and hypercholesterolemic mice and increased \( >2 \text{-fold} \) \( (P<0.05) \) in hypercholesterolemic/hypertensive mice (Figure 3E). Thus, the raphe was significantly longer in the group (hypercholesterolemic/hypertensive mice) that developed aortic stenosis.

Area of all 3 cusps tended to increase in hypertensive mice (not significant; Figure 4A). Area of the right and left, but not noncoronary, cusps increased in hypercholesterolemic/hypertensive mice (Figure 4A). Asymmetry of the cusps was expressed as the ratio of the average areas of the right and
left cusps to noncoronary cusp. Aortic valve cusps were asymmetrical in hypercholesterolemic/hypertensive mice but not in other groups (Figure 4B). In addition, in scanning electron micrographs (SEMs) of hypercholesterolemic/hypertensive mice, the noncoronary cusp was smaller in hypercholesterolemic/hypertensive mice than in other groups (Figure 4C).

Thus, with echo measurements, and in SEMs, there is asymmetry of the valve cusps in hypercholesterolemic/hypertensive mice. We have described aortic stenosis in *Ldlr<sup>−/−</sup>*-*Apob<sub>100/100</sub>* (LA) mice previously<sup>5</sup> and now provide SEMs for comparison with hypercholesterolemic/hypertensive mice (Figure 4D). In SEMs, there also seems to be asymmetry of the valve in LA mice, with a small noncoronary cusp.

Although total collagen did not change in the aortic valve of hypercholesterolemic/hypertensive mice, we examined the possibility that collagen is remodeled, especially at the raphe (Figure 5). In control, hypertensive, and hypercholesterolemic mice, collagen at the base of the cusps was oriented primarily parallel or perpendicular to the long axis of the attachment site (Figure 5C). In hypercholesterolemic/hypertensive mice, there was a mesh of collagen at the base of the valve cusps, oriented around a 45° angle to the attachment of the cusps, which tethered adjacent cusps to one another (Figure 5B). Thus, although total collagen in the valve was not increased in hypercholesterolemic/hypertensive mice (Figure 2B), collagen is remodeled at the base of the valve in hypercholesterolemic/hypertensive mice to form a mesh that spans adjacent cusps, which may restrict opening of the valve.

We also used SEMs to examine the structure of the aortic valve (Figure 6). SEMs show enlargement of the base of the valve in both hypercholesterolemic and hypercholesterolemic/hypertensive mice (Figure 6C and 6D). In 1 hypercholesterolemic/hypertensive mouse (Figure 6D; with ACS=0.5 mm), there was a band of tissue between 2 cusps. In hypercholesterolemic/hypertensive mice, there was minimal atheroma/annulus abutting the base of the valve (Figure 4C). In contrast, in LA mice, there was a large amount of atheroma/annulus abutting the base of the valve (Figure 4D).

**RNA-Seq**

For RNA-Seq analysis, differential expression is defined as >2-fold increase or 50% decrease with *P*<0.01. We found statistically significant differential expression of 483 genes in hypercholesterolemic/hypertensive mice compared with control mice. Of these, only 150 genes were identified that were differentially expressed in hypercholesterolemic/hypertensive compared with hypercholesterolemic (Table II in the online-only Data Supplement). Some genes of potential interest (Table) include those related to structural organization of the valve (mineralization and collagen organization), endothelium (or endothelial dysfunction), growth factors, and inflammation. Of particular interest, expression of anticalcific (as well
as procalcific) genes was increased in hypercholesterolemic/hypertensive mice, consistent with the finding that calcification was minimal in the valve. To begin to determine whether protein, as well as mRNA, is increased in aortic valves from hypercholesterolemic/hypertensive mice, we quantified plasminogen activator inhibitor 1 (PAI-1) by immunofluorescence (Figure 7). PAI-1 protein levels were increased ≈3-fold in hypercholesterolemic/hypertensive mice (Figure 7E).

Examples of changes in gene expression for molecular signaling networks are shown in Figure V in the online-only Data Supplement. There was differential expression of many peroxisome proliferator–activated receptor-γ–linked genes in hypercholesterolemic/hypertensive mice versus control mice. When the set of genes was limited to those with differential expression in hypercholesterolemic/hypertensive mice, but not in hypertensive or hypercholesterolemic mice, which do not develop significant aortic stenosis, only 5 peroxisome proliferator–activated receptor-γ–linked genes were identified in the network.

**Discussion**

There are several major new findings in this study. First, we have discovered a second experimental model that develops severe aortic stenosis. In contrast to the first model, which is CAVS (with 10- to 100-fold greater calcification7,9), this...
new model has minimal calcification. Thus, this is the first model of FAVS. Second, although there is no change in the total amount of collagen in the valve in FAVS, a change in the arrangement of collagen at the base of the valve may knit the leaflets together and restrict opening of the valve (Figure 8). Also, the raphe at the base of the valve is longer in the group of mice with FAVS than in the other groups. In addition, there is marked asymmetry of the size of valve leaflets in the group of mice with aortic stenosis (Figure 8), a finding that is associated with valve dysfunction in humans.15,16 Third, RNA-Seq identified 150 genes that are differentially expressed in stenosis-prone valves in hypercholesterolemic/hypertensive mice; some of which are related to fibrosis.

Experimental Model of FAVS

Prolonged hypertension produced by the renin–angiotensin system, like hypercholesterolemia alone, does not produce FAVS in mice by 12 months of age. In contrast, the combination of hypertension and hypercholesterolemia produced moderate to severe FAVS. Thus, >1 risk factor was necessary to induce FAVS in mice. The observation is concordant with the apparent need for multiple risk factors to produce aortic stenosis in humans.1,2 No previous study has examined the role of chronic hypertension in the development of AVS in mice.

At least 30 previous studies have examined the aortic valve in apoE-deficient mice, usually in mice that were fed a high-fat diet. Many studies demonstrated aortic valve disease, but none has reported hemodynamically significant stenosis (ie, a large decrease in pressure across the aortic valve). Although severe aortic stenosis has been observed with echocardiography in apoE-deficient mice, the prevalence is low (1 of 64 mice with Doppler velocity of >4.0 m/s).20 We observed statistically significant reduction in ACS in hypercholesterolemic mice, but the physiological effect of that reduction would be expected to be minimal, based on previous hemodynamic correlation studies in mice.5

We and others have assumed that calcification or fibrosis mediate hemodynamically significant FCAVS21 and that global measurements of calcification and fibrosis in the valve would predict hemodynamically significant aortic stenosis. We have not found consistent evidence to support this assumption. During development of CAVS, calcification increases in the valve.21,22 Reduction of calcium in the valve by 70% during treatment of hypercholesterolemia, however, failed to reduce stenosis.5 Furthermore, in children11 and adults,12 aortic stenosis often occurs in the absence of gross calcification. Thus, associations between calcification of the aortic valve and valve function are highly variable, in humans as well as mice, reflecting heterogeneity of underlying disease processes.

In this study, calcification of the aortic valve was minimal with hypertension or hypercholesterolemia alone and did not produce hemodynamically significant aortic stenosis. Furthermore, the amount of calcification of the valve was not greater in hypercholesterolemic/hypertensive mice with stenosis. Thus, calcification of the valve does not account for stenosis in this experimental model of severe aortic stenosis.

Calcification and collagen content in the valve do not account for AVS in this model. It is theoretically possible that neighboring annulus atheromata could restrict valve function. On the basis of SEMs (Figure 4C and 4D), however, atheromatous tissue abutting the valve is minimal in hypercholesterolemic/hypertensive mice, which differs from CAVS in LA mice. Thus, generalized encroachment of atheroma/annulus on cusp excursion is unlikely as an explanation for valve stenosis. Second, although total collagen in the valve did not increase in hypercholesterolemic/hypertensive mice, there was reorganization of collagen in regions that critically influence cusp mobility.

The experimental model that we described previously5 are LDLr<sup>−/−</sup> Apob<sup>100/100</sup> mice. The mice develop CAVS. The current model is Apoe<sup>−/−</sup> REN<sup>−</sup> AGT<sup>+</sup>, and the mice develop FAVS. Mechanisms that account for the strikingly different phenotype are not clear, but one might speculate that activation of...
the renin–angiotensin system in REN+ AGT+ mice may contribute to fibrosis observed in Apoe−/− REN+ AGT+ mice.

One likely explanation for the failure of pharmacological treatments to slow the progression of AVS in humans is that underlying mechanisms may be heterogeneous. This possibility is supported by findings in humans10–13 and now in mice that the amount (and perhaps role) of calcification differs in human adults and children and in 2 experimental models.

**Base and Cusp of Aortic Valve**

Two lines of evidence indicate that changes at the base of valve leaflets occur in hypercholesterolemic/hypertensive mice, which may account for FAVS. First, in hypercholesterolemic/hypertensive mice, the length of the raphe more than doubles. Second, with polarized light, there is a mesh of cross fibers at the base of leaflets in hypercholesterolemic/hypertensive but not in control, hypertensive, or hypercholesteremic mice. On the basis of change in orientation of collagen fibers in hypercholesterolemic/hypertensive mice, we conclude that there is reorganization of collagen fibers and structural remodeling. If fibrosis is viewed as a process where extracellular matrix remodeling leads to reduced tissue distensibility or mobility, then our novel findings are consistent with the concept of FAVS. Our finding of severe aortic stenosis in the absence of gross increases in valve collagen is analogous to findings in some patients with aortic stenosis. Côté et al23 found at least 25-fold variability in valve collagen among patients with severe aortic stenosis and also found that ≈20% of explanted valves demonstrated minimal valve collagen. This new finding, taken together with studies in CA VS mice, demonstrates that mechanisms leading to severe aortic stenosis can be diverse in mice, as is the case in humans.

The stimulus for remodeling, with formation of a mesh between leaflets in hypercholesterolemic/hypertensive mice,

### Table. Change in Selected Genes by Category

<table>
<thead>
<tr>
<th>Gene Name or Function</th>
<th>Fold Change vs Control</th>
<th>Fold Change HC/HT vs HC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mineralization</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPP1 Osteopontin</td>
<td>4.3</td>
<td>95</td>
</tr>
<tr>
<td>BMP2 BMP</td>
<td>...</td>
<td>3.2</td>
</tr>
<tr>
<td>GDF10 BMP3</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>ASPN Asporin (tissue mineralization)</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>LRRC17 Osteoclast differentiation</td>
<td>0.37</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Collagen organization</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERPINE1 PAI-1</td>
<td>...</td>
<td>5.5</td>
</tr>
<tr>
<td>OGN Osteoglycin</td>
<td>...</td>
<td>0.12</td>
</tr>
<tr>
<td>LGALS3 Galectin 3: myofibroblast proliferation</td>
<td>...</td>
<td>10.0</td>
</tr>
<tr>
<td>COL8A1 Collagen 8</td>
<td>3.2</td>
<td>6.5</td>
</tr>
<tr>
<td>FLNB Filamin-β</td>
<td>...</td>
<td>2.4</td>
</tr>
<tr>
<td>EFEMP1 EGF-containing fibulin-like</td>
<td>0.46</td>
<td>...</td>
</tr>
<tr>
<td>ADAMTS8 Disintegrin-like and MMP</td>
<td>2.8</td>
<td>...</td>
</tr>
<tr>
<td>ADAMTS5 Disintegrin-like and MMP</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>MIFAP4 Microfibrillar associated</td>
<td>0.34</td>
<td>...</td>
</tr>
<tr>
<td>TNC Tenascin</td>
<td>...</td>
<td>5</td>
</tr>
<tr>
<td><strong>Endothelium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GUCA1A3 Guanylate cyclase</td>
<td>...</td>
<td>0.26</td>
</tr>
<tr>
<td><strong>Growth Factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGFβ Platelet-derived growth factor</td>
<td>3.8</td>
<td>2.9</td>
</tr>
<tr>
<td>FGF14 Fibroblast growth factor 14</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFH Modulates C3</td>
<td>...</td>
<td>0.46</td>
</tr>
<tr>
<td>IL33 Interleukin 33</td>
<td>0.44</td>
<td>...</td>
</tr>
<tr>
<td>IL11RA Interleukin I/α-subunit</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>C1S Complement</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>C3 Complement</td>
<td>...</td>
<td>5.9</td>
</tr>
<tr>
<td>CYTL1 Cartilage destruction</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td><strong>Ion channels</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CACNG7 Calcium channel, voltage dependent</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>TRPV4 Transient receptor potential cation channel</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>ATP1A2 ATPase, Na+/K+ transporting</td>
<td>0.45</td>
<td>...</td>
</tr>
<tr>
<td>KCNJ8 Potassium inwardly rectifying channel</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>GPML6A (Ca&lt;sup&gt;2+&lt;/sup&gt; transport) glycoprotein</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>RYR2 Ryanodine receptor 2, cardiac</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

*P<0.01 for all numeric values. BMP indicates bone morphogenetic protein; EGF, epidermal growth factor; HC, hypercholesterolemia, HT, hypertension; MMP, matrix metalloproteinase; and PAI-1, plasminogen activator inhibitor 1.*
is not clear. Remodeling may be driven by cells within the leaflets or possibly because of remodeling or changes in stress of the aortic wall.

Changes in collagen and proteoglycans have been observed in aortic stenosis, which seem to be related to calcific nodules in valve cusps. Because the observations were made in valves removed from humans, the base of the valves was not examined. We have previously measured collagen in valves from mice but did not examine the base of the valves specifically.

**RNA-Seq**

A unique aspect of our RNA-Seq approach is that expression is compared in an experimental model that is documented to develop severe aortic stenosis versus disease models that do not consistently develop severe AVS. No other study, in humans or mice, has used RNA-Seq to compare valves with aortic stenosis or are diseased but not yet stenotic versus controls. In addition, we examined expression before development of aortic stenosis to avoid reporting epiphenomena associated with end-stage disease.

An advantage of RNA-Seq compared with arrays is the greater potential for gene discovery. Whereas arrays examine genes that are selected and limited, RNA-Seq covers the entire transcriptome. This approach could result in identification of genes that have not been fully annotated.

A recent study used RNA-Seq to compare tricuspid and bicuspid valves from humans. The study illustrates a productive approach to examine the aortic valve. Seven of 10 genes that differed in the 2 groups produce or degrade extracellular matrix, which implies an important role of fibrosis.

Many genes were differentially expressed in the aortic valve of hypercholesterolemic/hypertensive mice (summarized in the Table). Expression of bone morphogenetic protein 2 (which stimulates bone formation) was greater in hypercholesterolemic/hypertensive mice, but this difference may be offset by enormous increases in osteopontin (which protects against calcification), decreases in bone morphogenetic protein 3 (which negatively regulates bone density), and decreased expression of asporin (which may induce collagen mineralization). The balance of these changes may contribute to the finding that the mice do not develop the calcific type of aortic stenosis (CAVS). Expression of some other genes (LDLr-related protein 6 and TNFRSF1IB [which encodes osteoprotegerin]) that inhibit calcification in blood vessels is not increased in the aortic valve of hypercholesterolemic/hypertensive mice.

In the aortic valve of hypercholesterolemic/hypertensive mice, there are prominent changes in genes associated with collagen organization. Expression of osteoglycin, which contributes to collagen organization (and also induces ectopic bone formation), was reduced >60% in hypercholesterolemic/hypertensive versus hypercholesterolemic mice. Differential expression of galectin 3, which is profibrotic, increased. There were large changes in matrix metalloproteinases.

RNA-Seq, in addition to providing information about genes related to calcification and collagen organization, also
demonstrated differential regulation of genes that modulate endothelial dysfunction and ion channels (Table). Expression of guanylate cyclase, an effector of endothelial function,1 is decreased in hypercholesterolemic/hypertensive mice. In valves from hypercholesterolemic/hypertensive mice, there is differential expression of several genes that are associated with ion channels, including TRPV4. In vascular endothelium, TRPV4 is activated by laminar shear and regulates endothelial permeability.28

After RNA-Seq, we chose to examine PAI-1 protein based on 2 criteria. First, expression of the gene is increased in valves from hypercholesterolemic/hypertensive mice and not in the 3 other groups. Second, PAI-1 is profibrotic and procalcific in other organs and is highly upregulated by inflammation.29

Expression of PAI-1 also correlates with calcification in human aortic valves.30 Thus, increased expression of PAI-1, demonstrated with RNA-Seq and protein, supports the hypothesis that PAI-1 contributes to development of FAVS in hypercholesterolemic/hypertensive mice. The findings provide evidence for the potential utility of this approach for identification of new therapeutic targets.

Limitations

The degree to which some novel findings in hypercholesterolemic/hypertensive mice, for example, raphe length, collagen fiber orientation, and gene expression patterns, can be extrapolated to humans with aortic stenosis is not yet known.

Several mice (primarily hypercholesterolemic/hypertensive mice) died before the time at which we planned to euthanize the groups. Although immediate cause of death was usually not known, it is likely that the mice that died were more severely affected and that we underestimated the synergy of hypertension and hypercholesterolemia in induction of FCASV.

RNA-Seq revealed differential expression of a number of genes that are known to be operative in other settings in the processes of endothelial dysfunction, calcification, fibrosis, and inflammation. We confirmed increased levels of 1 gene product, PAI-1. Determination of the pathophysiologic effect of many newly identified candidate gene products is beyond scope of this initial report of a new model of FAVS.

In summary, we used novel approaches to examine structural changes in the aortic valve and their relationship to valve function. First, the length of the intercusp raphe nearly doubled in the group (hypercholesterolemic/hypertensive) that developed stenosis. A small increase in length of the intercusp raphe would be expected to profoundly restrict opening of the valve. Second, a collagen mesh was observed at the base of the valve in hypercholesterolemic/hypertensive mice, which may lengthen the raphe. In addition, there was asymmetry of the cusps, which may contribute to initiation of processes that produce dysfunction of the aortic valve. Finally, RNA-Seq identified novel candidates, including PAI-1, during the prestenotic disease phase in hypercholesterolemic/hypertensive mice.

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Disclosures

None.

References

This study describes a new experimental model of aortic valve stenosis. In striking contrast to the first model, calcification is minimal, a feature that resembles children and some adults with aortic stenosis. Remodeling of collagen at the base of the valve leaflets may restrict opening of the valve. RNA sequencing identified molecular targets during the developmental phase of stenosis.

Significance
Fibrotic Aortic Valve Stenosis in Hypercholesterolemic/Hypertensive Mice
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Materials and Methods Supplement

All studies were approved by the Institutional Animal Care and Use Committee of the University of Iowa (PHS Animal Welfare Assurance #A3021-01). Male and female mice were fed normal chow.

**Study Groups.** We studied hypertensive mice that express a transgene for human renin (REN+ mice) and human angiotensinogen (AGT+ mice) as described previously.1, 2 Heterozygous human REN+ were bred with heterozygous human AGT+ mice, to obtain about ¼ of the mice that were double transgenic (REN+ AGT+). Mice with human REN+ or AGT+ alone are normotensive.1, 2 Thus, wild-type mice, REN+ mice, and AGT+ mice were included in the Control group.

Apolipoprotein E deficient mice (Apoe−/−), which are hypercholesterolemic (HC), were obtained originally from JAX and bred over > 20 generations on C57BL/6J background. We bred female Apoe−/− mice with male REN+AGT+ mice, to obtain mice that are hypercholesterolemic and hypertensive (HC/HT) 3 (Table S-I).

**Echocardiography.** Mice were sedated with midazolam (0.15 mg SC). Echocardiograms were obtained with a 30 MHZ linear array transducer coupled to a Vevo 2100 imager (FUJIFILM Visual Sonics, Toronto, Canada). Images of short and long axes were obtained in the left lateral position, with a frame rate of ~180-250 Hertz. Image analysis was performed offline using Vevo 2100 analysis software (Version 1.5). All echo and histological measurements were made by a person who was blinded with respect to genotype.

Aortic cusp separation (ACS) was determined as described previously.4 A hemodynamically validated ACS threshold of less than 0.66 mm was used to define severe aortic stenosis.4 When it became clear that many HC/HT mice died before they would be euthanized at 12 months (Figure S-I), frequent echos were performed on this group beginning at 6 months of age. Thus, echocardiograms were performed in HC/HT mice every 2 weeks starting at 6 months of age, until the mice developed severe aortic stenosis or reached 12 months. Other genotypes were echoed at 6, 9, and 12 months.

Aortic cusp asymmetry was analyzed from the short- axis 2D view of the aortic valve by tracing each cusp during systole (Figure S-II). The cusp area was measured as the area within a line connecting two adjacent cusp attachment sites and the outer edge of the aortic annulus. The presence of aortic valve regurgitation was assessed using color Doppler.5

**Valve histology.** Mice were euthanized with pentobarbital sodium when they developed severe aortic stenosis, determined by echo, or at 12 month of age in 6-7 mice of each group, aortic valves were frozen for histologic staining. Tissues were cut on a cryostat (10 µm sections) and placed on glass slides. Sections were stained for calcification with Alizarin red (Lifeline Cell Technology), lipid with oil red-O (Sigma), Movat’s pentachrome (Scy Tek) to measure elastic fibers and raphe length), and picrosirius red (Scy Tek).4, 6, 7

To measure components of the entire valve, we traced the valve cusps and base of the valve (Figure S-III). Annulus and atheroma, which had substantial calcification (S-IV), were excluded. Histologic quantitation was performed using an automated pixel-counting algorithm (Adobe Photoshop) and reported in mm². Raphe length, representing the distance from aortic annulus to the point at which adjacent valve cusps separate, was measured using digital calipers, utilizing images stained with Movat’s Pentachrome. For measurement of elastic fibers, valve annulus was included in the analysis, because elastic fibers are located almost exclusively in the annulus.

Sections were stained with picrosirius red, which binds to collagen, aligns longitudinally with collagen fibers, and produces birefringence. Slides were imaged using polarized light. Total collagen and orientation of fibers were measured. A reference angle was determined for each attachment site along the long axis of the cusp attachment site at the annulus. Using this
reference axis, orientation of visualized collagen bundles in each attachment site was
determined by using polar co-ordinates, yielding a range of orientation from 0 to 90°.

**Scanning Electron Microscopy.** Valves were fixed with 2.5% glutaraldehyde in 0.1 M sodium
cacodylate buffer for 1 h, post-fixed with 1% osmium tetroxide for 1 h, dehydrated through
sequential incubation in 25%, 50%, 75%, 95% and 100% ethanol, and dried using the CO2
Sorvall Critical Point Drying System (DuPont, Wilmington, DE). Samples were mounted on
aluminum stubs, sputter coated with gold-palladium and examined with an S-4800 scanning
electron microscope (Hitachi High Technologies America Inc., Pleasanton, CA).

**RNA-Seq Library Preparation and Sequencing.** RNA was obtained from aortic valves from
four groups of 5-6 month old mice (n=4 in each group): Control (normotensive/\textit{Apoe}^{+/+}
littermates), hypertensive, hypercholesterolemic, and HC/HT. None of the mice had aortic
stenosis, with echoes obtained before euthanasia for collection of RNA samples.

Fresh aortic valves were embedded in OCT and 80-micron sections were cut; the valve
leaflet and base were dissected with a 26g needle under a microscope to exclude aorta, and
transferred into TriZol. Dissection of the valve was successful in excluding aorta, because \(\alpha\) smooth muscle actin (which is expressed in aorta but not valve), was 50-fold greater in tissue
samples of aorta vs aortic valve. Following isolation, the RNA was further purified using
Qiagen RNeasy column with DNase digestion. Using Nugen Ovation RNA-Seq System v2,
cDNA was generated from 1 ng of total RNA.

Indexed sequencing libraries were generated with the KAPA hyper prep kit. Pooled
samples were run on the Illumina HiSeq 2500 genome sequencer, and quality of the sequence
reads was assessed using the Fast-QC program. Paired end reads (50-bp) were aligned to the
mouse transcriptome (UCSC known genes, genome build mm10, Dec. 2011) with Bowtie\textsuperscript{®}.
Counts between samples were normalized and statistical comparisons of groups were made
with edgeR (an R/Bioconductor package). MetaCore\textsuperscript{®} software was used to build gene
networks.

Data from the RNA-Seq experiment have been deposited at the NCBI Gene Expression
Omnibus (accession GSE71534).

To validate findings from RNA-Seq, protein expression of PAI-1 in the valve was
measured. Immunofluorescence was performed as described previously.\textsuperscript{7,8}

Immunofluorescence for PAI-1 used goat antibody sc-6644 from Santa Cruz Biotech; rat
antibody 553370 from BD Pharmigen was used for CD31. Images were photographed digitally
and quantified without knowledge by the observer of the group of mice.

**Statistical Analysis.** Group data are reported as mean ± SE. Significant differences (\(p <
0.05\)) between groups were detected using one-way ANOVA followed by Student-Newman-
Keuls tests.
Supplement References


Fig S-I. Survival of Mice. Mice that died without premonitory signs are summarized in the graph. * = p < 0.05 vs Control, † = p < 0.05 vs HC, and ** = p < 0.05 vs HT.
Fig S-II. Method for echocardiographic measurements of size of valve leaflets. Valve contours were determined using echo cine clips, using back-and-forth frame-by-frame iterative viewing. As shown (A-D), still frames do not translate well enough for off-line determinations. Thus, valve identification, electronic planimetry, and area determination were performed (E-H) by a reader blinded to genotypes.

- Con
- HT
- HC
- HC/HT

Cusp free border, which forms the valve orifice (O)
Cusp annular border
**Fig S-III.** Method for outlining aortic valve leaflets and base of cusps, with exclusion of annulus and atheroma (a). Histological sections were stained with Alizarin red; a section is enlarged to illustrate tracing of valve to exclude annulus and atheroma. Calcification in the atheroma was not included in valves for aortic valve. Calcification was minimal in aortic valve and base of HT/HC mice.
**Figure S-IV.** Calcification of atheroma. Histological sections from Control (A), HT (B), HC (C), and HC/HT (D) mice, stained with Alizarin Red. There is prominent calcification in the atheroma (red arrow) of HC (C) and HC/HT (D) mice, with minimal calcification of the aortic valve (blue arrow). Note: black staining is lipofuscin. Also, note that the scale is different in D, because the annulus and valve are larger.
## Data Supplement

### Table S-I. Baseline characteristics of mice.

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<td>380 ± 49⁺</td>
<td>151 ± 6⁺</td>
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Values are mean ± SE in 8-10 mice, += p<0.05 vs Control
Table S-II. Genes that are differentially expressed in HC/HT vs. HC valves

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**Those with a decrease in HC/HT (97 genes)**

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Figure S-V. Differential expression of PPARγ-linked genes in mice which are not stenosis-prone (Control on left, HC on right) vs. mice prone to develop aortic stenosis (HC/HT). Differential expression of individual genes that are putatively linked to PPARγ are assembled into a network, using MetaCore databases. In HC/HT mice vs. Control mice (left), the analysis indicates many PPARγ-linked candidate genes. In HC/HT mice vs. HC mice (which do not develop significant aortic stenosis), the number of PPARγ-linked candidate genes in the network is reduced to 5. See MetaCore website, https://portal.genego.com/ (Metacore version 6.2 of Thomson Reuters) for key to symbols.