A Novel Mouse Model of Aortic Valve Stenosis Induced by Direct Wire Injury

Shintaro Honda, Takuya Miyamoto, Tetsu Watanabe, Taro Narumi, Shinpei Kadowaki, Yuki Honda, Yoichi Otaki, Hiromasa Hasegawa, Shunsuke Netsu, Akira Funayama, Mitsunori Ishino, Satoshi Nishiyama, Hiroki Takahashi, Takanori Arimoto, Tetsuro Shishido, Takehiko Miyashita, Isao Kubota

Objective—The response-to-tissue-injury theory is currently the favorite paradigm to investigate valve pathology. To the best of our knowledge, there are currently no in vivo valve injury models. There are few calcific aortic valve stenosis (AVS) models that develop hemodynamically significant stenosis. Here, we investigated the effect of direct mechanical injury on aortic valves in vivo and developed a novel mouse model of calcific AVS.

Approach and Results—Aortic valve injury was created by inserting and moving a spring guidewire under echocardiographic guidance into the left ventricle of male C57/BL6 mice via right common carotid artery. Serial echocardiographic measurements revealed that aortic velocity was increased 1 week after injury and persistently increased until 16 weeks after injury. AVS mice showed a higher heart weight/body weight ratio and decreased left ventricular fractioning shortening 4 weeks after injury, compared with sham mice. We found remarkable proliferation of valve leaflets 4 weeks after injury. Proliferative valves showed increased production of reactive oxygen species and expression of inflammatory cytokines and osteochondrogenic factors. Alizarin red staining showed valvular calcification 12 weeks after injury.

Conclusions—We report a novel calcific AVS model to support the response-to-tissue-injury theory. This model may be a valuable tool for analyzing the mechanism of AVS and assessing therapeutic options. (Arterioscler Thromb Vasc Biol. 2014;34:270-278.)

Key Word: animal model ■ aortic valve stenosis ■ calcification

Aortic valve stenosis (AVS) has become a common disease in the elderly with a prevalence of 2% to 4% in population aged >65 years in advanced countries. It is a progressive disease characterized by a long asymptomatic phase; however, once symptoms appear, the prognosis is poor. Although the standard treatment for AVS is aortic valve replacement, it is not practical for all patients to undergo this surgery because of the risk of complications. Thus, the development of effective alternative therapies is required for patients with AVS. Although risk factors for AVS are similar to those for atherosclerosis, inhibitors of angiotensin-converting enzyme and HMG-CoA reductase show poor efficacy in AVS progression. It has been suggested that different factors from atherosclerosis are associated with the progression of AVS. Some patients with AVS do not have atherosclerotic risk factors, and ≈50% of patients with AVS do not have clinically significant atherosclerosis.

Mechanical injury caused by hemodynamic stress during the constant opening and closing of cusps is considered an important risk factor for AVS. The bicuspid valve, which contributes to a high mechanical stress, reportedly leads to a rapid progression of AVS in younger patients with low atherosclerotic risk. Moreover, the noncoronary leaflet is more likely to be affected as compared with other leaflets because of higher mechanical stress as a consequence of the absence of diastolic coronary flow. Many experimental models of AVS, for example, the hypercholesterolemic mouse model, have contributed to our knowledge of the mechanism of AVS. However, it commonly takes ≥20 weeks for these models to develop AVS, and not all animals develop hemodynamically significant stenosis with heart failure. The response-to-tissue-injury theory is one of the favorite paradigms to study valve pathology, similar to ones seen in numerous tissue and organs. Several in vitro and organ culture experiments have revealed the cellular and molecular biology of heart valve wound repair; however, these models lack physiological blood flow, hemodynamic forces, and blood constituents. Therefore, we developed a novel in vivo mouse model of AVS induced by moving a spring guidewire via right common carotid artery under echocardiographic guidance (Figure 1).
Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results

Aortic Valve Injury

The surgical procedure time, including anesthesia, needed for aortic valve injury was 21.0±5.1 minutes. Seven of 132 mice died during the operation due to sudden cardiac arrest (5 mice) and bleeding (2 mice). In the sham group, 1 of 63 mice died as a result of bleeding. After aortic valve injury, 25 of 125 mice died during the next 16 weeks. Survival curves showed that 18.7% of mice with aortic valve injury died <4 weeks, whereas none in the sham-operated mice died (Figure 2A). The causes of death after aortic valve injury were congestive heart failure with pleural effusion at autopsy (5) and unknown causes (20), most likely attributable to cardiac arrhythmia or heart failure without obvious effusion.

Valve and Ventricular Function

Immediately after injury, aortic regurgitation was not observed on 2-dimensional color Doppler and pulse-wave Doppler imaging, and aortic velocity did not increase. Mice with aortic valve injury had significantly higher aortic velocity and smaller aortic valve area compared with sham-operated mice 1 week after surgery. The elevated velocity persisted for 16 weeks without improvement. Left ventricular outflow tract velocity was not significantly increased at all time points. Left ventricular fractional shortening was significantly decreased 4 weeks after surgery, and left ventricular end-diastolic diameter was increased 8 weeks after injury (see Table). Heart weight/body weight ratio gradually increased (Figure 2C). Additionally, real-time PCR revealed that the expression of brain natriuretic peptide in the left ventricle was upregulated in mice with aortic valve injury compared with sham-operated mice 4 weeks after surgery (Figure 2D).

Histological Changes in the Aortic Valve

Thrombus formation on both sides of the valve and destruction of collagen and elastin alignment in the leaflets of injured valves were observed immediately after aortic valve injury on microscopic examinations (Figure 3A and 3B). Injured collagen and elastin layers were significantly thickened compared with sham-operated mice (Figure 3C and 3D). Sham-operated aortic valves showed no thrombus and no destruction of collagen and elastin alignment. Stereoscopic microscopy showed obvious thickening of the injured leaflets 4 weeks after surgery (Figure 3E and 3F). The average thickness of the injured trileaflet was significantly greater than that of the sham (168.6±26 versus 59.6±17.5 μm; P<0.01; Figure 3G). There was no obvious damage and obstruction in the ascending aorta and left ventricular outflow tract on histological and echocardiographic study (Figure I in the online-only Data Supplement). Microscopic examination revealed that most of injured valves showed osteochondrogenic changes, including precartilaginous-like aggregation and cartilage-like structure with mucus production, basophilic stained matrix, and small cavities, which were characterized in cartilage on the aortic side of the valve, 4 weeks after surgery (Figure 3F). Immunofluorescence revealed the expression of type 2 collagen in the injured valve, consistent with the chondrogenic-like changes on HE stain (Figure 3H and 3I). These

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<table>
<thead>
<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
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<tr>
<td>AVS</td>
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<tr>
<td>BMP-2</td>
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<tr>
<td>Runx2</td>
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<td>Sox9</td>
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**Figure 1.** 2-Dimensional echocardiogram of the parasternal long-axis view. **A**, The wire was observed in the ascending aorta. **B**, The wire was inserted into the left ventricle under echocardiographic guidance. **C** and **D**, Schematic illustrations corresponding to **A** and **B**. **E**, Aortic valve was scratched with the body of the wire. **F**, Tip of the wire was positioned on the left ventricular side of the valve and spun to injury. Ao indicates aorta; LA, left atrium; and LV, left ventricle.
osteochondrogenic changes gradually progressed and matured with time, and distinct calcification on HE was observed in the leaflet 16 weeks after injury (Figure 3J; Figure II in the online-only Data Supplement). Although injured valves were not stained with Alizarin red before 8 weeks postinjury, calcium deposition was observed 12 weeks after injury. Injured valves with more osteochondrogenic changes showed strongly positive staining 16 weeks after surgery (Figure 3K and 3L).

**Fibro-Proliferative Change and Prolonged Inflammation in the Valve**

As shown in Figure 4A, large amounts of collagen deposition and obvious neovascularization were observed in injured valves compared with sham-operated valves. Immunofluorescence showed myofibroblast activation as α-smooth muscle actin (Figure 4B) and CD31-positive cells as endothelial cells (Figure 4C) in proliferative valves 4 weeks after injury. Mac3-positive macrophage accumulation and vascular cell adhesion molecule-1–positive cells as activated endothelial cells were observed in injured valve for 16 weeks (Figure 4D and 4E). SMA expression and Mac3-positive macrophage accumulation were observed in the whole of injured valve, which was consistent with fibrogenic changes caused by inflammation induced by wire injury. The mRNA expression of tumor necrosis factor-α, interleukin-1β, and interleukin-6 were remarkably increased in injured valves compared with sham mice 1 week after surgery and remained significantly elevated 4 weeks after injury (Figure 4F–4H). Transforming growth factor-β1 was also upregulated until ≥4 weeks after injury (Figure 4I).

**Oxidative Stress in Injured Valves**

To evaluate the production of reactive oxygen species, we performed dihydroethidium staining 4 weeks after injury. Mac3-positive macrophage accumulation and vascular cell adhesion molecule-1–positive cells as activated endothelial cells were observed in injured valve for 16 weeks (Figure 4D and 4E). SMA expression and Mac3-positive macrophage accumulation were observed in the whole of injured valve, which was consistent with fibrogenic changes caused by inflammation induced by wire injury. The mRNA expression of tumor necrosis factor-α, interleukin-1β, and interleukin-6 were remarkably increased in injured valves compared with sham mice 1 week after surgery and remained significantly elevated 4 weeks after injury (Figure 4F–4H). Transforming growth factor-β1 was also upregulated until ≥4 weeks after injury (Figure 4I).

**Table. Time Course of Serial Echocardiographic Parameters**

<table>
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<tr>
<th></th>
<th>Control</th>
<th>Immediately</th>
<th>1 wk</th>
<th>2 wk</th>
<th>3 wk</th>
<th>4 wk</th>
<th>8 wk</th>
<th>12 wk</th>
<th>16 wk</th>
<th>Trend</th>
<th>P</th>
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<tr>
<td>HR, bpm</td>
<td>574±32</td>
<td>588±30</td>
<td>576±26</td>
<td>591±16</td>
<td>590±45</td>
<td>597±39</td>
<td>577±35</td>
<td>591±23</td>
<td>565±23</td>
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<td>IVS, mm</td>
<td>0.69±0.06</td>
<td>0.71±0.07</td>
<td>0.76±0.06*</td>
<td>0.88±0.08*</td>
<td>0.88±0.08*</td>
<td>0.91±0.05*</td>
<td>0.98±0.08*</td>
<td>1.01±0.08*</td>
<td>1.02±0.06*</td>
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<tr>
<td>PW, mm</td>
<td>0.69±0.04</td>
<td>0.71±0.05</td>
<td>0.78±0.08*</td>
<td>0.88±0.08*</td>
<td>0.85±0.11*</td>
<td>0.93±0.07*</td>
<td>1.03±0.10*</td>
<td>1.04±0.07*</td>
<td>1.03±0.06*</td>
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<td>LVESD, mm</td>
<td>2.98±0.15</td>
<td>2.98±0.11</td>
<td>3.15±0.24</td>
<td>3.21±0.25</td>
<td>3.24±0.09</td>
<td>3.35±0.50</td>
<td>3.55±0.46*</td>
<td>3.65±0.64*</td>
<td>4.21±0.97*</td>
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<tr>
<td>LVESD, mm</td>
<td>1.46±0.09</td>
<td>1.51±0.10</td>
<td>1.54±0.18</td>
<td>1.63±0.20</td>
<td>1.77±0.17</td>
<td>1.84±0.67*</td>
<td>2.01±0.35*</td>
<td>2.12±0.52*</td>
<td>2.60±1.00*</td>
<td>&lt;0.01</td>
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<tr>
<td>FS, %</td>
<td>51.0±1.2</td>
<td>49.3±2.4</td>
<td>51.2±3.6</td>
<td>49.2±2.7</td>
<td>45.4±4.0</td>
<td>46.2±8.7*</td>
<td>43.2±4.0*</td>
<td>42.4±4.6*</td>
<td>36.5±9.8*</td>
<td>&lt;0.01</td>
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<tr>
<td>LVOT velocity, cm/s</td>
<td>136±24</td>
<td>128±7</td>
<td>147±18</td>
<td>152±18</td>
<td>151±20</td>
<td>139±17</td>
<td>140±27</td>
<td>144±20</td>
<td>160±11</td>
<td>0.09</td>
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<td>Aortic velocity, cm/s</td>
<td>150±29</td>
<td>136±10</td>
<td>371±48*</td>
<td>381±35*</td>
<td>370±52*</td>
<td>380±71*</td>
<td>383±88*</td>
<td>419±82*</td>
<td>427±51*</td>
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<tr>
<td>AVA, mm²</td>
<td>1.56±0.12</td>
<td>1.60±0.77</td>
<td>0.67±0.13*</td>
<td>0.66±0.12*</td>
<td>0.66±0.10*</td>
<td>0.57±0.13*</td>
<td>0.56±0.12*</td>
<td>0.57±0.13*</td>
<td>0.65±0.06*</td>
<td>&lt;0.01</td>
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Data are mean±SD from 7 to 15 mice for each group. AVA indicates aortic valve area; FS, fractional shortening; HR, heart rate; IVS, interventricular septum; LVESD, left ventricular (LV) end-systolic diameter; LVESD, LV end-systolic diameter; LVOT, LV outflow tract; and PW, posterior wall.

*P<0.05 vs control.
Figure 3 Continued.
aortic valve injury. Superoxide production was detected in injured valves but not in sham-operated valves (Figure 5A). Immunofluorescence revealed that the expression of p-22phox, which is functionally important for NADPH oxidase activation, was significantly increased in injured valves compared with sham-operated valves (Figure 5B).

### Osteochondrogenic Signaling in the Injured Valve

We performed immunofluorescence to investigate osteochondrogenic signals for all time points after valve injury. Bone morphogenetic protein-2 (BMP-2), an important growth factor for osteochondrogenic response, was strongly expressed and gradually increased over all the injured valves (Figure 6A).

The expression of chondrocyte-specific transcription factor Sry-related HMG box 9 (Sox9) and of osteochondrogenic transcription factor runt-related transcription factor 2 (Runx2) was widely increased in proliferative valves compared with sham-operated valves, especially consistent with histological osteochondrogenic changes observed under microscopy (Figure 6B and 6C). Moreover, osteocalcin (specifically secreted by osteoblasts) was detected and gradually increased in the injured valves (Figure 6D). We observed colocalization of BMP-2, Sox9, and Runx2 with histological osteochondrogenic changes. Especially, Sox9 expression was remarkably consistent with cartilage-like structure. Osteocalcin secreted by osteoblasts, a marker of osteogenesis, colocalized with...
Figure 4. Activated myofibroblasts and upregulated expression of inflammatory cytokines after murine aortic valve injury. A, Collagen deposition with Masson trichrome staining 4 weeks after valve injury and quantification of collagen deposition. An arrow in the injured valve shows neovascularization. B to E, α-Smooth muscle actin (αSMA; B; green), CD31 (C; red), Mac3 (D; red), vascular cell adhesion molecule-1 (VCAM-1; E; green), and DAPI (blue) immunostaining of aortic valves 4 weeks after surgery and quantification of immunofluorescent data. Data are mean±SD from 5 mice for each group. #P<0.05 vs control. F to I, Quantitative analysis of mRNA expression for tumor necrosis factor-α (TNFα; F), interleukin (IL)-6 (G), IL-1β (H), and transforming growth factor-β1 (TGFβ1) (I). Data are mean±SD from 7 to 10 mice for each group. #P<0.05 vs control.
 osteochondrogenic changes, and furthermore, osteocalcin secretion increased with time. Double immunofluorescence staining revealed that Mac3-positive macrophages did not show osteochondrogenic markers (Figure IIIA–IIIC in the online-only Data Supplement). α-Smooth muscle actin–positive myofibroblasts expressed osteochondrogenic markers (Figure IID–IIIF in the online-only Data Supplement). Osteocalcin expression colocalized with chondrocyte-specific marker Sox9 (Figure IIIIG in the online-only Data Supplement). Quantitative and statistical analyses revealed that the expression of BMP-2, Runx2, and osteocalcin gradually increased for 16 weeks. The peak expression of Sox9 was at 12 weeks (Figure 6A–6D).

**Discussion**

Mechanical injury is considered one of the most important risk factors for AVS because of the characteristic structure and function of the aortic valve. We have demonstrated aortic valve response to direct mechanical injury in vivo and developed a novel calcific AVS mouse model with hemodynamically significant stenosis with heart failure. Injured valves showed myofibroblast proliferation, production of cytokines and growth factors, as well as evoked inflammation, neovascularization, and calcification, all consistent with the typical features of clinical AVS. To the best of our knowledge, this is the first report of an in vivo heart valve wound repair model for AVS. Panizzi et al. reported an in vivo mouse aortic valve injury model for aortic valve endocarditis, which was caused by blindly inserting suture material via the right carotid artery into the heart. Although their methodology is similar to our AVS model, their model could not have possibly developed AVS because aortic valve damage caused by a suture material would be smaller than that caused by a guidewire. Wire injury, if insufficient, did not cause AVS in our preliminary experiments.

The murine aortic valve was selectively injured with wire under echocardiographic guidance, with no injury to any other part of the heart, in 20 minutes. Although 20% of the mice with valve injury died <4 weeks, the survival rate of AVS mice was higher compared with transverse aortic constriction and myocardial infarction models. Hypercholesterolemic mice and other models have been used to clarify the calcification mechanism of AVS; however, these mice do not always develop hemodynamically significant stenosis with respect to long feeding period. However, almost all the mice with aortic valve injury showed rapid valve proliferation and ventricular hypertrophy with increased aortic velocity. Calcium deposition with coarse osteocartilaginous structure was also upregulated at relatively early stages compared with other models. The injured aortic valves showed histological changes that were more chondrocytic than osteoblastic in the early phases, followed by more mature osteochondrogenic changes with calcium deposition in the late phase. Our results also showed that the expression of BMP-2, Runx2, and osteocalcin gradually increased for 16 weeks, and the peak expression of Sox9 was at 12 weeks. The proceeding osteochondrogenic changes were mainly observed in the aortic side of the valve, consistent with the pathology of human AVS. The results suggest that these developing processes of calcific valves seemed to be associated with endochondral ossification. Because it has been reported that surgically excised human aortic valves showed simultaneous chondrification and ossification, endochondral ossification may be important or essential for the development of valve calcification caused by mechanical injury.

Our study showed that aortic valve wire injury induced severe AVS in healthy C57/BL6 mice fed normal chow, suggesting mechanical injury to the aortic valve and supporting the response-to-tissue-injury theory. Inflammatory cytokines, re-active oxygen species, growth factors, and osteogenic factors have been observed in human diseased valves and are considered to play important roles in the development of AVS. Our results show that inflammatory cytokines (tumor necrosis factor-α, interleukin-1β, and interleukin-6) and transforming growth factor-β1 were upregulated 1 week after injury, and this was sustained until 4 weeks. Additionally, reactive oxygen species and osteogenic factors increased 4 weeks after injury. Increased macrophage and vascular cell adhesion molecule-1–positive cells for 16 weeks suggested sustained inflammation and endothelial damage. Sustained inflammation, increased growth factors and endothelial damage induced by hemodynamic stress, and turbulent flow through remodeled valves may contribute to the progression of AVS, including calcification.

**Figure 5.** Generation of reactive oxygen species 4 weeks after murine aortic valve injury. A, In situ detection of superoxide with dihydroethidium (DHE) fluorescence (red) in valve. B, Immunofluorescent staining for p22phox (red) and DAPI (blue) and quantification of immunofluorescent data. Data are means±SD from 5 mice for both groups.
The results show that osteochondrogenic factors gradually increased, and histological osteochondrogenic changes with calcification were clearly detected. However, further studies are needed to elucidate the mechanism by which abnormal wound repair to injured valves occurs.

There are 2 limitations to the study that need a mention here. First, we could not perform Western blot analysis for quantitative protein measurement because tissue samples from murine aortic valves were limited. Second, we could not get direct hemodynamic data by cardiac catheterization; however, we were able to successfully measure elevated aortic velocities, which were consistent with histological findings using echocardiography. The process of injuring the aortic valve under echocardiography is not difficult if the parasternal long-axis view on echocardiography can be held while moving the wire to prevent losing sight of the wire and injuring other parts of the heart.

We demonstrated that direct wire injury to the murine aortic valve induced severe AVS in vivo. We suggest that our calcific AVS model has pathological features similar to human AVS. This mouse model may be valuable for analyzing the mechanism of AVS and developing therapeutic strategies.

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Disclosures

None.

References

There are few experimental models of aortic valve stenosis (AVS) that develop hemodynamically significant stenosis with heart failure. In this study, we report a novel calcific AVS model to support the response-to-tissue-injury theory. We found that aortic valve wire injury induces severe AVS in male C57/BL6 mice fed normal chow. Proliferative valves showed increased production of reactive oxygen species and expression of inflammatory cytokines and osteochondrogenic factors, which accompanied valvular calcification 12 weeks after injury. This model may be a valuable tool for analyzing the mechanism of AVS and assessing therapeutic options. Furthermore, this model may also be used for pressure-overload heart failure model that does not require open chest surgery, because the surgical procedure is easy to perform and surgical invasion is much less compared with transverse aortic constriction surgery.
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Supplemental Figure I.
Echocardiographic image of aortic valve with high echoic lesion and without subvalvular or supravalvular obstruction 12 weeks after injury. An arrow shows aortic valve with high echoic lesion. Ao, aorta; LV, left ventricle; LA, left atrium.
Supplemental figure II.
Supplemental Figure III. Immunofluorescence double staining to locate cells expressing osteochondrogenic markers (Sox9, Runx2 and osteocalcin) 8 weeks after injury. A-C, Immunofluorescent study of osteochondrogenic markers (green), Mac3 (red) and DAPI (blue). D-F, Immunofluorescence of αSMA (green), osteochondrogenic markers (red) and DAPI (blue). G, Immunofluorescent staining of Sox9 (green), osteocalcin (red) and DAPI (blue).
Supplemental Methods

Animals
A total of 194 (132 mice for aortic valve injury and 63 for sham surgery) male C57/BL6 mice aged 8-10 weeks old were used. All experimental procedures were performed according to the animal welfare regulations of Yamagata University School of Medicine, and the study protocol was approved by the Animal Subjects Committee of Yamagata University School of Medicine. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.

Process of Aortic Valve Injury
The animals were anesthetized by intraperitoneal injection of a solution comprising tribromoethanol (250 mg/kg; Sigma-Aldrich, Japan), and the mouse chest was shaved and treated with a chemical hair remover to reduce ultrasound attenuation on echocardiography. The right carotid artery was exposed by blunt dissection, and a spring wire for angioplasty (0.36-mm diameter) bent at a 15-degree angle, was introduced into the artery. The wire inserted into the ascending aorta was easily recognized by echocardiography in the parasternal long-axis view (Figure 1A and 1C), and was slowly rotated and carefully inserted into the left ventricle (Figure 1B and 1D). Aortic valve injury was induced by scratching the leaflets with the body of the wire for 20 times (Figure 1E) and spinning the tip of the wire correctly positioned on the left ventricular side of the valve for 50 times (Figure 1F). After the wire was removed, the right carotid artery was ligated. Sham-surgery was performed in the same way but with no wire insertion into the left ventricle.

Functional Assessment of the Aortic Valves by Echocardiography:
We serially measured aortic velocity and aortic valve area by echocardiogram until 16 weeks after aortic valve injury. Two-dimensional and Doppler transthoracic echocardiography was performed with a Philips HD11 ultrasound machine (Philips Medical Systems, Bothell, WA, USA) under anesthesia with an intraperitoneal administration of tribromoethanol (250 mg/kg). Adequacy of anesthesia was monitored at all times by assessment of skeletal muscle tone, respiration rate and rhythm, and response to tail pinch. Left ventricular wall thickness and internal dimensions were measured and the left ventricular fractional shortening was calculated as previously described. The measurements of the left ventricular outflow tract and aortic valve were recorded to evaluate the maximum velocity and velocity time integral by pulse-wave Doppler ultrasound with linear probe in the parasternal long axis view. Sampling point was set in the center of left ventricular outflow tract and aortic valve. The Doppler incident angle was 51° and angle-correction was performed in individual mouse within 60 degrees. The parasternal long axis view was used to measure the diameter of the left ventricular outflow tract. Aortic valve area was measured by continuity equation. Two-dimensional color Doppler
and pulse wave Doppler imaging was used to evaluate the presence of aortic regurgitation.

**Measurement of histological changes in the valve:**

The mice were killed by intraperitoneal administration of an overdose of tribromoethanol 4, 8, 12 and 16 weeks after aortic valve injury. After perfusion fixation with saline and 4% paraformaldehyde, the aortic valve was post-fixed in 4% paraformaldehyde overnight at 4 °C, embedded in paraffin and sectioned (4-μm thickness). All samples were routinely stained with hematoxylin and eosin and Masson’s Trichrome. Changes in elastin immediately after injury were detected by Elastica-van Gieson staining. Tissue calcification was measured using Alizarin red staining. Images were obtained using light microscopy at 40x and 200x magnification (BX50, Olympus, Japan). The thickness of valvular tri-leaflet, collagen and elastin layers in the leaflets were measured using ImageJ software (version 1.45q; National Institutes of Health), as previously reported. The extent of collagen deposition stained blue with Masson’s Trichrome stain was calculated as the percentage of the total leaflet area. The relative calcified area stained with Alizarin red was calculated as the percentage of the total leaflet area using ImageJ software.

**Immunofluorescence:**

Endogenous peroxidase was quenched in methanol containing 1.5% H$_2$O$_2$ for 20 minutes at room temperature. After rinsing, sections were immersed in citric acid buffer (pH 6.0) and subjected to microwave irradiation for 10 minutes under a boiling state. Sections were blocked with 5% defatted dry milk in phosphate-buffered saline (PBS) for 10 minutes. Incubations with primary antibodies were carried out overnight at 4 °C at the following dilutions: type 2 collagen (Abcam, ab21291 1:100), Mac3 (BD Pharmingen, 550292, 1:200), α smooth muscle actin (Sigma Aldrich, F3777, 1:50), CD31 (Abcam, ab28364, 1:50), vascular cell adhesion molecule 1 (Santa Cruz, sc-1504), p22-phox (Santa Cruz, sc-20781, 1:50), bone morphogenetic protein-2 (BMP-2; Abcam, ab14933, 1:50), Sry-related HMG box 9 (Sox9; Santa Cruz, sc-20095, 1:50), runt-related transcription factor 2 (Runx2; Santa Cruz, sc-8566, 1:50), and osteocalcin (Santa Cruz, sc-23790, 1:200). After primary antibody incubation, sections were washed in 1xPBS and incubated with respective Alexa 488 anti-goat (Invitrogen), Alexa 555 anti-rat (Cell Signaling), or Alexa 568 anti-rabbit (Invitrogen) secondary antibodies at 1:100 dilution for 1 hour at room temperature, washed, and incubated with 4’,6-diamidino-2-phenylindole (DAPI, Lonza) for identification of nuclei before mounting. The slides were observed under an immunofluorescence microscope (DP-70, Olympus, Japan). Fluorescent intensity corrected by background intensity was quantified using ImageJ software as described previously reported.

**Superoxide Detection:**

After perfusion fixation, the aortic valve was embedded in OCT compounds (Tissue-Tek; Sakura Finetechnical, Japan). Frozen 5-μm-thick sections of sham-operated and injured valves were incubated at the same time with dihydroethidium (Sigma Aldrich, Japan) (10 μmol/L) in PBS for 30 minutes at
37 °C in a humidified chamber and protected from light, as previously reported. Images were analyzed using Image J software as described previously reported.

Quantitative Real-Time Polymerase Chain Reaction (RT-PCR):
Immediately after microscopically controlled dissection from the heart, each aortic valve and left ventricle was frozen in liquid nitrogen for RNA extraction. Total RNA was extracted using Trizol reagent (Invitrogen, Tokyo, Japan), and 400 ng valve RNA and 1000 ng ventricle RNA were used to generate cDNA. Real time PCR amplification was performed as previously described. Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers were designed based on GenBank sequences: brain natriuretic peptide, NM-008726; transforming growth factor-β1, NM-011577; tumor necrosis factor-α, NM-013693; interleukin-1β, NM-008361; interleukin-6, NM-031168; and GAPDH, NM-001001303.

Statistical Analyses:
All data are presented as mean ± SD. Statistical significance was determined by one-way ANOVA followed by the Bonferroni post-hoc test. Two groups were compared using the Student t test. For the survival study, Kaplan-Meier analysis was used. Probabilities of 0.05 or less were considered to be statistically significant.

References
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