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-leaflets, 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% H₂O₂ 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 1:1000 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**

calcifying smooth muscle cells promotes migration and osteoclastic differentiation of macrophages. *Arterioscler Thromb Vasc Biol.* 2011;31:1387-1396

