Online Supplementary Methods

Animals. At 6–8 weeks of age, "Reversa" (ldlr$^{-/-}$/apoB$^{100/100}$/mttp$^{fl/fl}$/Mx1-Cre) littermates were assigned to either "control", "progression," or "regression" groups, as described previously[1]. Control mice were given 4 injections of polyinosinic-polycytidylic acid (pI-pC, 225 μg, i.p.) at two-day intervals and maintained on a chow diet for 14 months. Progression mice were placed on a Western diet (Harlan Teklad #TD88137, 42% of calories from fat, 0.25% cholesterol). After 12 months of hypercholesterolemia/progression, a subset of mice were selected for this study based on the presence of moderate to severe stenosis (cusp separation distance less than 0.7mm), and randomized to two groups: 1) a group which underwent sustained progression/hypercholesterolemia for 2 months, and 2) a group which underwent normalization of lipids by a genetic switch for 2 months (i.e., "regression"/"reversal" group). As described previously, the regression/reversed mice were given 4 injections of pI-pC (225 μg, i.p.) over the course of 8 days to drive Cre recombinase expression and conditionally inactivate the microsomal triglyceride transfer protein and were switched to a chow diet. All data are the average of 5-8 animals per group.

Measurement of whole blood glucose, plasma cholesterol, and plasma insulin levels. Animals were anesthetized with an overdose of inhaled sevoflurane, and the chest cavity was rapidly opened. The inferior vena cava was severed, and blood was taken for analysis. Plasma cholesterol was measured using a colorimetric kit (Wako Diagnostics).

Measurement of histological changes in the valve. Serial sections (10 μm thickness) were taken from tissue frozen in OCT. Lipid deposition was measured using Oil Red O (Sigma, France). Tissue calcification was measured using Alizarin Red staining. Images were obtained using light microscopy at 4x and 10x magnification (Olympus BX51 Digital Light Microscope, Olympus, Japan). For analysis, Adobe Photoshop CS2 (version 7, Adobe Systems Inc. San Jose, CA) was used to select only pixels expressing red histological staining. Valve cusps were traced to obtain a measurement of valve area. Data are expressed as the percentage of valve area that displays positive staining.

Measurement of valvular oxidative stress. Superoxide in the valve was evaluated using *ex vivo* staining for dihydroethidium fluorescence (DHE, Molecular Probes, Inc.). Tissue samples were frozen in OCT compound and 10 μm transverse sections were cut through the aortic valve using a cryostat. Sections were incubated in 0.002 mmol/L DHE and protected from light for 30 minutes at room temperature. Images were obtained using a Bio Rad MRC-1024 laser scanning confocal microscope at 4x magnification to detect fluorescence (Ex/Em: 488/585 nm). To examine specificity of the stains for superoxide, adjacent sections were incubated with polyethylene glycol superoxide dismutase (PEG-SOD). To evaluate the fluorescent intensity of positively stained areas, only positively stained cells/nuclei were thresholded using Image J software (version 1.42; National Institutes of Health, Bethesda, MD) and expressed data as the difference between the inhibited and non-inhibited fluorescent images (peg-SOD-inhibitable DHE; mean relative light units (RLU'S)/pixel).

Immunohistochemistry. Immunohistochemistry was used to detect the pro-osteogenic markers phospho-Smad1/5/8, CBFA1, and β-catenin. Immunohistochemical detection of proteins was carried out as follows: P-Smad1/5/8 (Cell Signaling Technologies, #9511, 1:300 + TSA kit), CBFA1 (Santa Cruz, #C-19, 1:50), and β-catenin (BD Transduction # 610153, 1:200). Fluorescent images were acquired using a confocal microscope. Iimmunofluorescence was quantified using ImageJ software as described previously[1, 2]. Background immunofluorescence/autofluorescence was calculated from sections taken from a subset of animals in each group which were not treated with primary antibodies. Immunofluorescent data are expressed as background-corrected fluorescent intensity. .

Echocardiographic evaluation of aortic valve function. Aortic valve function was evaluated as described previously[1]. Briefly, mice were sedated with midazolam (0.15 mg subcutaneously); each mouse was cradled in the left lateral recumbent position while a 15-MHz linear-array probe was applied horizontally to the chest. The imaging probe was coupled to a Sonos 5500 imager (Philips Medical Systems, Bothell, Wash), generating 180–200 two-dimensional frames per second in both short- and long-axis left ventricular (LV) planes.

Images of the aortic valve were acquired in M mode, at a nominal sampling rate of 1000 frames/second, with two-dimensional images used for guidance. Pulse-wave Doppler tracings were obtained with depth gates near the ventricular aspect of the mitral valve to measure heart rate. All images were acquired by an operator blinded to the treatment groups.

Statistical analyses. All data are reported as mean ± SE. Significant differences between groups were detected using an analysis of variance, and Bonferroni-corrected T-tests were used for *post hoc* testing.

Online Supplementary Data

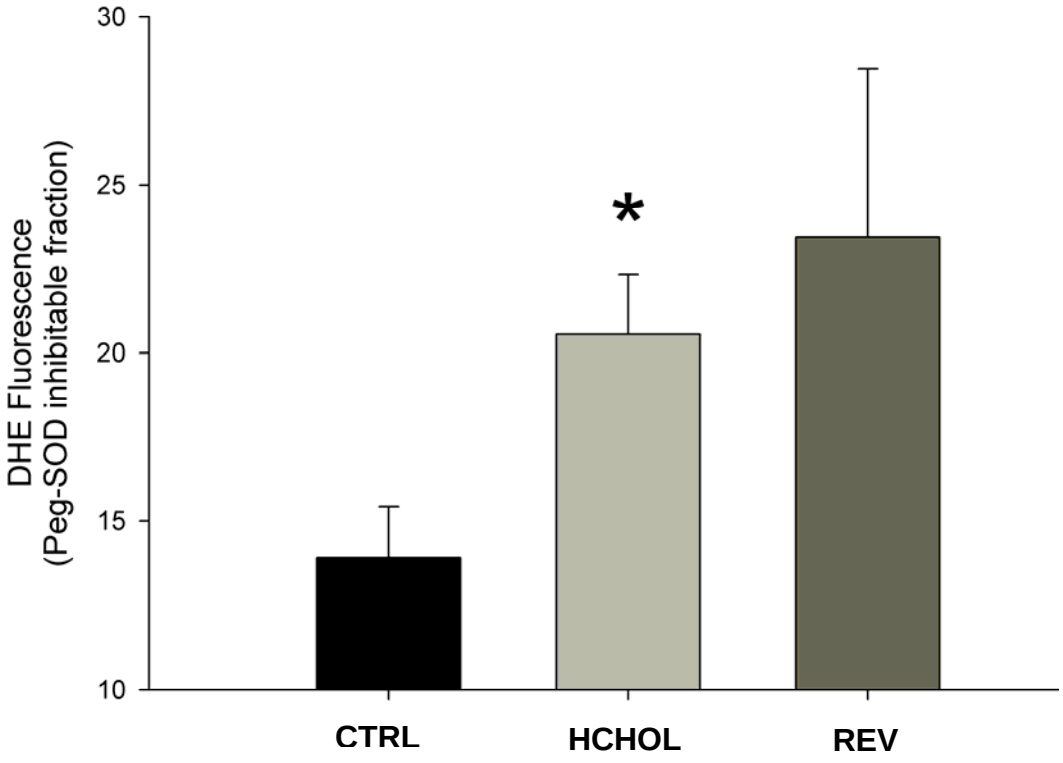

Figure I. Effects of reducing plasma lipids on superoxide levels in the aortic valve. Note that valvular superoxide is not altered by reduction of blood lipids. CTRL = control, HCHOL = high fat/hypercholesterolemic group, REV = "reversed" group.

REFERENCES

1. Miller JD, Weiss RM, Serrano KM, Brooks RM, 2nd, Berry CJ, Zimmerman K, Young SG, Heistad DD. Lowering plasma cholesterol levels halts progression of aortic valve disease in mice. *Circulation.* 2009;119(20):2693-2701.

2. Miller JD, Chu Y, Brooks RM, Richenbacher WE, Pena-Silva R, Heistad DD. Dysregulation of antioxidant mechanisms contributes to increased oxidative stress in calcific aortic valvular stenosis in humans. *Journal of the American College of Cardiology.* 2008;52(10):843-850.