Lowering Plasma Cholesterol Levels Halts Progression of Aortic Valve Disease in Mice

Jordan D. Miller, PhD; Robert M. Weiss, MD; Kristine M. Serrano, BS; Robert M. Brooks II, BS; Christopher J. Berry, MD; Kathy Zimmerman; Stephen G. Young, MD; Donald D. Heistad, MD

Background—Treatment of hyperlipidemia produces functional and structural improvements in atherosclerotic vessels. However, the effects of treating hyperlipidemia on the structure and function of the aortic valve have been controversial, and any effects could be confounded by pleiotropic effects of hypolipidemic treatment. The goal of this study was to determine whether reducing elevated plasma lipid levels with a “genetic switch” in Reversa mice (Ldlr−/−/Apolob100/100/Mttpfl/fl/Mx1-CreH11001/H11001) reduces oxidative stress, reduces pro-osteogenic signaling, and retards the progression of aortic valve disease.

Methods and Results—After 6 months of hypercholesterolemia, Reversa mice exhibited increases in superoxide, lipid deposition, myofibroblast activation, calcium deposition, and pro-osteogenic protein expression in the aortic valve. Maximum aortic valve cusp separation, as judged by echocardiography, was not altered. During an additional 6 months of hypercholesterolemia, superoxide levels, valvular lipid deposition, and myofibroblast activation remained elevated. Furthermore, calcium deposition and pro-osteogenic gene expression became more pronounced, and the aortic cusp separation decreased from 0.85 ± 0.04 to 0.70 ± 0.04 mm (mean ± SE; P < 0.05). Rapid normalization of cholesterol levels at 6 months of age (by inducing expression of Cre recombinase) normalized aortic valve superoxide levels, decreased myofibroblast activation, reduced valvular calcium burden, suppressed pro-osteogenic signaling cascades, and prevented reductions in aortic valve cusp separation.

Conclusions—Collectively, these data indicate that reducing plasma lipid levels by genetic inactivation of the mttp gene in hypercholesterolemic mice with early aortic valve disease normalizes oxidative stress, reduces pro-osteogenic signaling, and halts the progression of aortic valve stenosis. (Circulation. 2009;119:2693-2701.)

Key Words: aortic valve stenosis ■ calcification ■ free radicals ■ hypercholesterolemia ■ valves
though the SALTIRE and SEAS trials strongly suggest that simvastatin (or combined simvastatin and ezetimibe treatment) does not slow the progression of aortic valve stenosis in patients with borderline-high cholesterol levels, patients with slightly higher blood lipid levels showed a modest benefit of rosuvastatin on the progression of aortic valve stenosis in the RAAGE trial. Although reconciling these conflicting results is difficult (slight differences in patient populations, differing pleiotropic effects of the statins, etc.), no study has determined whether initiation of lipid-lowering therapy in early stages of disease can halt or reverse the progression to hemodynamically significant aortic valve stenosis.

Progress in understanding mechanisms underlying the progression of aortic valve stenosis has been slowed by the lack of animal models. Hypercholesterolemic rabbits and single-allele knockout mice develop histological evidence of aortic valve sclerosis but rarely develop hemodynamically significant aortic valve stenosis. Recently, we reported that “apolipoprotein B100-only” low-density lipoprotein receptor–deficient mice (Apob100/100) have severe hypercholesterolemia and that approximately one third develop severe aortic valve stenosis.

In the present study, we used Ldlr/−/−/Apob100/100 mice that were also homozygous for a conditional knockout allele in microsomal triglyceride transfer protein (Mttp) and the interferon-inducible Mx1-Cre transgene (Reversa mice). The Mttp gene plays a critical role in the production of apoB-containing lipoproteins, and loss of Mttp activity dramatically reduces secretion of apoB-containing lipoproteins into the plasma. Thus, Cre-mediated inactivation of Mttp in these mice allowed us to “switch off” the severe hypercholesterolemia and test the effects on aortic valve stenosis, avoiding off-target effects of statin drug therapy. We hypothesized that lowering lipid levels with this genetic switch in the early stages of aortic valve disease would retard the progression to aortic valve stenosis.

Methods

Animals
At 6 to 8 weeks of age, littermates were assigned to control, progression, or regression groups. Control mice were given 4 injections of polyinosinic-polycytidylic acid (225 μg IP) at 2-day intervals and maintained on a chow diet for 6 or 12 months. Progression mice were placed on a Western diet (Harlan Teklad No. TD86137; 42% of calories from fat; 0.25% cholesterol) for 6 or 12 months. Regression mice were placed on a Western diet for 6 months and then were given 4 injections of polyinosinic-polycytidylic acid (225 μg IP), switched to a chow diet, and followed up for an additional 6 months.

Table. Body Weight, Blood Lipid, Blood Glucose, and Insulin Levels Before and After Normalization of Blood Lipids

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Hypercholesterolemic Group</th>
<th>Reversed Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 mo</td>
<td>12 mo</td>
<td>6 mo</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>20.0±0.9</td>
<td>20.8±0.6</td>
<td>22.8±0.9*</td>
</tr>
<tr>
<td>Plasma cholesterol levels, mg/dL</td>
<td>157±20</td>
<td>145±27</td>
<td>997±87*</td>
</tr>
<tr>
<td>Whole-blood glucose, mg/dL</td>
<td>193±17</td>
<td>187±14</td>
<td>217±29</td>
</tr>
<tr>
<td>Plasma insulin, ng/L</td>
<td>175±13</td>
<td>283±20</td>
<td>263±18</td>
</tr>
</tbody>
</table>

All measurements were obtained in nonfasting animals (n=8 to 12 per group).

*P<0.05 versus the age-matched control group; †P<0.05 versus the age-matched hypercholesterolemic groups.

Measurement of Blood Lipids, Oxidative Stress, Histology, Immunohistochemistry, and Aortic Valve Function

For detailed descriptions, see the online-only Data Supplement.

Statistical Analyses

All data are reported as mean±SE. Significant differences between groups were detected with ANOVA, and Bonferroni-corrected t tests were used for posthoc testing.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Plasma Lipid Levels
Total plasma cholesterol levels in 6- and 12-month-old polyinosinic-polycytidylic acid–treated mice on a chow diet (control groups) are reported in the Table. Total plasma cholesterol levels in 6- and 12-month-old Reversa mice on a Western diet (hypercholesterolemic/progression groups) were significantly elevated compared with control mice (see the Table). Switching off Mttp expression after 6 months of hypercholesterolemia (regression groups) reduced total plasma cholesterol levels by ~75% (see the Table).

Whole-Blood Glucose and Plasma Insulin Levels
Whole-blood glucose levels were not significantly changed by any treatment in 6- or 12-month-old Reversa mice. Compared with control mice, however, plasma insulin levels were significantly increased in 6- and 12-month-old hypercholesterolemic mice (the Table). Reducing cholesterol levels after 6 months of hypercholesterolemia did not reduce plasma insulin levels compared with 6- or 12-month-old hypercholesterolemic mice (the Table).

Valvular Oxidative Stress
In the hypercholesterolemic/progression groups, superoxide levels in the aortic valve were increased markedly after 6 months of hypercholesterolemia (compared with control mice) and remained elevated at 12 months (Figure 1). Reducing cholesterol levels after 6 months of hypercholesterolemia significantly reduced valvular superoxide levels at 12 months (Figure 1).
Histological Changes in the Aortic Valve

In control mice given polyinosinic-polycytidylic acid at 6 to 8 weeks of age, lipid deposition in the aortic valve was negligible at both 6 and 12 months. In contrast, lipid deposition was significantly increased in mice in the hypercholesterolemic/progression group at 6 months and remained high at 12 months (Figure 2). In the regression group, normalizing blood lipids after 6 months of hypercholesterolemia significantly reduced valvular lipid content at 12 months ($P<0.05$ versus the 12-month hypercholesterolemic group).

In control mice, we rarely detected macrophages in the aortic valve at 6 and 12 months (Figure 3). Macrophage infiltration was significantly increased in the hypercholesterolemic/progression animals at both 6 and 12 months (Figure 3). Normalizing cholesterol levels after 6 months of hypercholesterolemia significantly reduced macrophage staining at the 12-month time point (Figure 3).

In control mice, both valvular mineralization (von Kossa; Figure 1 of the online-only Data Supplement) and calcification (alizarin red; Figure 4) were negligible at the 6- and 12-month time points. In the hypercholesterolemic/progression mice, valvular mineralization and calcification were significantly elevated after 6 months of hypercholesterolemia and increased further after 12 months of hypercholesterolemia (Figure 1 of the online-only Data Supplement and Figure 4). Reducing cholesterol levels with the genetic switch after 6 months of hypercholesterolemia prevented the increases in valvular mineralization and calcification at 12 months ($P<0.05$ versus 12-month hypercholesterolemic/progression mice; Figures I and IV of the online-only Data Supplement).

Profibrotic Signaling, Myofibroblast Activation, and Fibrosis in the Aortic Valve

Levels of phospho-Smad2 were very low in valves from control mice (Figure 5). After 6 and 12 months of hypercholesterolemia, phospho-Smad2 was markedly increased (Figure 5). The increased phospho-Smad2 immunofluorescence was attenuated by reducing cholesterol levels after 6 months of hypercholesterolemia (Figure 5).

Expression of $\alpha$-smooth muscle actin was rarely detected in aortic valves from control mice at 6 or 12 months (Figure 5). After both 6 and 12 months of hypercholesterolemia, significant expression of $\alpha$-actin was found in the aortic valve (Figure 5). Normalizing cholesterol levels after 6 months of hypercholesterolemia virtually eliminated $\alpha$-smooth muscle

![Image](https://example.com/image1.png)

**Figure 1.** Superoxide in aortic valve before and after normalization of blood lipids. A and B, Six- and 12-month control mice; C and D, 6- and 12-month hypercholesterolemic (Hchol) mice; E, mice reversed 12 months; F, mean data for all mice (8 to 12 per group). Note that superoxide levels were markedly increased in both 6- and 12-month hypercholesterolemic animals and were completely normalized by reduction of blood lipids in the reversed animals. Arrows highlight areas of positive staining in aortic valve tissue. $^{*}P<0.05$ vs the time-matched control group; $^{#}P<0.05$ vs the 12-month hypercholesterolemic group.

![Image](https://example.com/image2.png)

**Figure 2.** Lipid (red staining) in aortic valve before and after normalization of blood lipids. A and B, Six- and 12-month control mice; C and D, 6- and 12-month hypercholesterolemic (Hchol) mice; E, mice reversed 12 months; F, mean data for all mice (8 to 12 per group). Valvular lipid deposition was significantly increased in hypercholesterolemic mice at both 6 and 12 months. Arrows indicate aortic valve tissue. Normalizing blood lipids produced significant reductions in valvular lipid in reversed animals. $^{*}P<0.05$ vs the time-matched control group; $^{#}P<0.05$ vs the 12-month hypercholesterolemic group.
actin immunofluorescence in the aortic valve at 12 months (Figure 5).

In control mice, collagen staining was restricted to a narrow band in the fibrosa of the valve. After 6 and 12 months of hypercholesterolemia, collagen deposition extended beyond the fibrosa layer and was found in association with lipid-laden plaques (Figure 5). The increased collagen staining was not detectably altered by reducing cholesterol levels after 6 months of hypercholesterolemia.

**Procalcific Signaling in the Valve**

Phospho-Smad1/5/8 levels were relatively low in control mice at 6 and 12 months and were dramatically increased in hypercholesterolemic mice at 6 and 12 months (Figure 6). Phospho-Smad1/5/8 immunofluorescence was markedly reduced by normalizing cholesterol levels after 6 months of hypercholesterolemia (Figure 6).

Immunofluorescence of Msx2, CBFA1, and Osterix was relatively low in control animals at 6 and 12 months (Figure 6). Immunofluorescence of all 3 pro-osteogenic genes was modest in hypercholesterolemic animals at 6 months but was markedly increased in hypercholesterolemic mice at 12 months (Figure 6). Increases in Msx2, CBFA1, and Osterix immunofluorescence were nearly abolished by normalizing cholesterol levels at 6 months (Figure 6).

**Aortic Valve Function**

In control mice, aortic valve cusp separation distance averaged 0.84±0.04 mm at 6 months and 0.83±0.03 mm at 12 months (Figure 7). Aortic valve cusp separation distance did not differ significantly between control and hypercholesterolemic mice at 6 months. Cusp separation distance decreased significantly from 6 to 12 months in the hypercholesterolemic group (Figure 7), and the prevalence of hemodynamically significant aortic valve stenosis increased from 14% to 50% (see Figure II of the online-only Data Supplement). Reducing cholesterol levels with the genetic switch at 6 months of age completely prevented the reductions in aortic valve cusp separation (see Figure 7).

Qualitatively similar changes in aortic valve function were observed by magnetic resonance imaging in a small subset of mice, and significant reductions in aortic valve orifice area were observed in some of the hypercholesterolemic mice (Figure 7). Reductions in aortic valve orifice area were
markedly attenuated by normalizing cholesterol levels at 6 months (Figure 7).

Discussion

The major novel findings of this study are that (1) in hypercholesterolemic mice, superoxide is increased and myofibroblasts are activated in early stages of valve disease, preceding stenosis of the aortic valve; (2) after 6 months of hypercholesterolemia, normalization of cholesterol levels with a genetic switch decreases oxidative stress, myofibroblast activation, and pro-osteogenic signaling in the aortic valve; and (3) normalization of cholesterol levels in mice with early aortic valve disease halts the progression of valve calcification and prevents adverse changes in cusp mobility.

Oxidative Stress During Progression and Regression of Aortic Valve Disease

Marked increases in superoxide levels were found in hypercholesterolemic mice with early aortic valve disease (ie, after 6 months of hypercholesterolemia). Previous work from our laboratory and other groups has revealed that oxidative stress is increased in both humans8,9 and mice10 with aortic valve stenosis. The present data suggest that increases in oxidative stress occur well before the development of aortic valve stenosis and are not merely epiphenomena related to hemo-

Figure 5. Phospho-Smad2 immunofluorescence, α-smooth muscle actin immunofluorescence, and collagen (blue) staining in the aortic valve before and after normalization of lipid levels. P-Smad2 levels were markedly increased in the 6- and 12-month hypercholesterolemic (Hchol) mice. Normalizing blood lipids significantly reduced the amount of P-Smad2 immunofluorescence in reversed mice. α-Smooth muscle actin immunofluorescence was increased in 6- and 12-month hypercholesterolemic mice and virtually nondetectable in reversed mice. Collagen deposition also progressively increased in hypercholesterolemic mice from 6 to 12 months but was not reduced in reversed mice. Arrows denote areas of aortic valve tissue when immunofluorescence is very faint.

Figure 6. Procalcific proteins in the aortic valve before and after normalization of lipid levels. P-Smad1/5/8 was markedly increased in 6- and 12-month hypercholesterolemic (Hchol) animals and was substantially reduced in the reversed group. Immunofluorescence of the pro-osteogenic genes Msx2, CBFA1, and Osterix was increased in both the 6- and 12-month hypercholesterolemic animals and markedly reduced by normalizing blood lipid levels. Arrows denote aortic valve tissue when fluorescence is very faint or point to valvular tissue when nonvalvular tissue is present in the micrograph.
of our knowledge, this is the first study to show that reducing cholesterol levels after the development of early pathological changes in the aortic valve results in reduced valvular lipid content and inflammatory cell infiltrate.

We also observed marked increases in myofibroblast activation in early aortic valve disease, as judged by increased numbers of α-smooth muscle actin–positive cells in the valve. Activated valvular myofibroblasts not only may increase valvular collagen content but also could have the potential to differentiate to an osteoblast-like phenotype. Studies of calcifying vascular smooth muscle in vitro demonstrated that loss of α-smooth muscle actin expression is an important event during differentiation to osteoblast-like cells. Data from humans with aortic valve stenosis, however, suggest that some osteoblast-like cells may retain their contractile properties even in advanced stages of the disease. The data reported here also suggest that a concomitant upregulation exists of α-smooth muscle actin and osteogenic genes in mouse valves. Additional work is needed to clarify the differences in gene expression during differentiation of these cell types (ie, valvular myofibroblasts versus vascular smooth muscle cells).

We observed significant increases in valvular cusp mineralization (von Kossa) and calcium deposition (alizarin red) in hypercholesterolemic mice at both 6 and 12 months. These changes were associated with increases in phospho-Smad1/5/8, which are typically associated with bone morphogenetic protein signaling. In 12-month hypercholesterolemic animals, we also observed substantial increases in immunofluorescence of Msx2, CBFA1, and Osterix, which are associated with differentiation of cells to an osteoblast-like phenotype. After normalization of cholesterol levels, we observed marked reductions in phospho-Smad1/5/8, Msx2, and Osterix immunofluorescence, along with corresponding reductions in valvular calcium. These reductions in valvular calcium are somewhat surprising because calcium burden typically is not reduced in regression studies that examined calcified atherosclerotic plaques. Several groups, however, have identi-
fied osteoclast-like cells in the aortic valve (which are less common in atherosclerotic plaques) that may promote a microenvironment that is conducive to resorption of bone-like matrices during regression of valve disease.

Finally, we observed marked increases in valvular fibrosis in hypercholesterolemic mice at 6 and 12 months. Valvular fibrosis was associated with increases in immunofluorescence for phospho-Smad2, which is generally coupled to transforming growth factor-β signaling. Interestingly, transforming growth factor-β is markedly increased in patients with severe aortic valve stenosis and has been implicated as a contributor to calcification of valvular interstitial cells in vitro. Increased transforming growth factor-β signaling also has been reported in atherosclerotic plaques, where it may promote collagen synthesis and formation of a fibrous cap.

After the normalization of cholesterol levels, we observed reductions in both phospho-Smad2 levels and myofibroblast activation. Reducing blood lipids, however, did not result in reductions in valvular fibrosis. Similar observations have been made during the early stages of regression in atherosclerotic monkeys, in which reductions in lipid content were associated with increased collagen content. These increases in plaque collagen content can persist even after long-term regression and could serve an important role in stabilizing plaques. Our data suggest, however, that increases in valvular fibrosis—at least to the extent observed in the present study—are not likely to be a primary determinant of the severity of aortic valve stenosis induced by hypercholesterolemia. Additionally, our data suggest that lipid-lowering therapy is not likely to slow the progression of valve disease when valvular fibrosis and/or leaflet fusion is the primary cause of cusp restriction.

Reducing Cholesterol Levels Halts the Progression of Aortic Valve Stenosis

The major finding of this study was that normalizing cholesterol levels with a genetic switch halts the progression of aortic valve stenosis in hypercholesterolemic mice. Clinical trials examining the effect of statin therapy on progression of aortic valve stenosis have yielded conflicting results, and retrospective studies suggest a poor correlation between LDL levels and hemodynamic progression of aortic valve stenosis. Further complicating matters is the fact that animal models rarely manifest progression to hemodynamically significant aortic valve stenosis (transvalvular gradient >20 mm Hg). Thus, to the best of our knowledge, this is the first study to demonstrate that lowering blood lipids in the presence of early changes in the aortic valve prevents reductions in cusp mobility and valve orifice area.

Our findings contrast with some recent reports from human trials that demonstrated no beneficial effect of lipid-lowering therapy on progression of aortic valve stenosis. These studies initiated lipid-lowering therapy after moderate to severe aortic valve stenosis was present. In the present investigation, we reduced blood lipids by >60% at a time that preceded any detectable impairment in valve function. Collectively, these data suggest that intervening in the early stages of the disease holds the most promise for finding that lipid lowering slows the progression of aortic valve disease in humans.

Determining the mechanisms underlying the beneficial effects of HMG-CoA reductase inhibitors (statins) is difficult as a result of off-target or pleiotropic effects of these drugs. We chose not to administer statins for 2 reasons. First, depending on the dosage and compound used, statins may not reduce cholesterol levels in Ldlr-deficient mice. Second, our goal was to examine effects of lowering cholesterol per se independently of pleiotropic effects of drugs. By inactivating Mttp, we avoided any confounding pharmacological effects of statins and were able to show that lipid lowering is responsible for attenuating pro-osteogenic signaling and retarding the progression of aortic valve disease.

Study Limitations

An important limitation in our study design is that lipid lowering was initiated at 6 months, which represents an early stage of valve disease. However, pro-oxidative, proinflammatory, and procalcific signaling pathways were already activated at that time. Whether normalization of lipid levels would stop the progression or induce the regression of more advanced disease is not clear. Effectiveness of lipid lowering on progression of advanced aortic valve stenosis would likely depend on the ability of this intervention to change macrophage emigration, lipid resorption, osteoblast-like cell activity, and osteoclast-like cell recruitment and activity within the valve.

Because of limited tissue availability in mouse aortic valves, we were not able to assess DNA binding activity of the pro-osteogenic factors reported in this study. Our immunohistochemical measurements of pro-osteogenic protein levels, however, may actually underestimate the amount of pro-osteogenic activity because data from cell culture experiments have demonstrated that increased nuclear binding of CBFA1 can occur in the absence of changes in protein levels.

Conclusions

Reduction of blood lipids in a hypercholesterolemic mouse model of aortic valve stenosis reduces oxidative stress, lipids, and calcium burden in the valve and attenuates pro-osteogenic signaling pathways. We speculate that reducing blood lipids in hypercholesterolemic humans with early aortic valve disease could elicit similar changes and slow the rate of disease progression.

Acknowledgments

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Disclosures

Dr Heistad owns stock in Merck (> $10,000).

References


CLINICAL PERSPECTIVE

Hypercholesterolemia is a major risk factor for calcific aortic valve stenosis. Calcium deposition and ossification of the aortic valve cusps appears to be an active biological process rather than a passive precipitation of calcium. However, clinical trials of lipid-lowering therapy on the progression of moderate to severe aortic valve stenosis have yielded disappointing results. In this study, we examined aortic valve stenosis in a severely hypercholesterolemic mouse model in which the hyperlipidemia could be eliminated with a genetic switch. In the severely hypercholesterolemic mice, we observed progressive aortic valve stenosis and increases in both oxidative stress and calcium deposition in the aortic valve. We also observed evidence of transforming growth factor-β and bone morphogenetic signaling. Switching off the hypercholesterolemia during early stages of valve disease (ie, before any reductions in the opening of the aortic cusps) completely prevented the development of aortic stenosis and reduced oxidative stress, pro-osteogenic signaling, and valvular calcium burden. These data suggest that calcification of the aortic valve during hypercholesterolemia is an active process, and aggressive lipid-lowering therapy, when initiated very early in the course of disease, can prevent the development of aortic valve stenosis.
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SUPPLEMENTARY MATERIAL
ONLINE SUPPLEMENTARY METHODS

Animals. In this study, we used female LDL receptor–deficient mice that were homozygous for apolipoprotein B100–only allele, a conditional knockout allele of \( \text{Mttp} \), and an Mx1-\textit{Cre} transgene (\( \text{Ldlr}^{+/+}/\text{Apob}^{100/100}/\text{Mttp}^{fl/fl}/\text{Mx1Cre}^{+/+} \)) \(^1\). At 6–8 weeks of age, mice were assigned to either “control”, “progression,” or “regression” groups. Control mice were given 4 injections of polyinosinic-polycytidylic acid (\( \text{pl-pC} \), 225 \( \mu \text{g} \), i.p.) at two-day intervals and maintained on a chow diet for 6 or 12 months. Progression mice were placed on a Western diet (Harlan Teklad #TD88137, 42% of calories from fat, 0.25% cholesterol) for 6 or 12 months. Regression mice were placed on a Western diet for 6 months, and then were given 4 injections of \( \text{pl-pC} \) (225 \( \mu \text{g} \), i.p.), switched to a chow diet, and followed for an additional 6 months.

Measurement of whole blood glucose, plasma cholesterol, and plasma insulin levels. Animals were heavily anesthetized with an overdose of inhaled sevoflurane, at which point the chest cavity was rapidly opened via a full-length sternotomy. The inferior vena cava and aorta were severed immediately superior to the diaphragm, and blood was taken for analysis. Whole blood glucose levels were immediately analyzed using a glucometer (AccuChek), and the remainder of the blood was centrifuged for the isolation of the plasma fraction. Aliquots of blood plasma were stored at -20°C or -80°C until analysis. Plasma cholesterol was measured using a colorimetric kit (Wako Diagnostics), and plasma insulin levels were analyzed using an ELISA specific for mouse insulin (Mercodia).

Measurement of histological changes in the valve. Serial sections (10 \( \mu \text{m} \) thickness) were taken from the frozen and perfusion fixed tissue blocks described above. Lipid deposition was measured using Oil Red O (Sigma, France). Tissue mineralization
and calcification were measured using Von Kossa and Alizarin Red stains, respectively. Images were obtained using light microscopy at 4x and 10x magnification (Olympus BX 51 Digital Light Microscope, Olympus, Japan). Changes in valvular collagen burden were assessed using Masson’s trichrome stain. For analysis, we used Adobe Photoshop CS2 (version 7, Adobe Systems Inc. San Jose, CA) to select only pixels expressing specific red or black histological staining. We subsequently traced the valve leaflet 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.** To evaluate levels of superoxide in the valve, we used ex vivo staining for dihydroethidium fluorescence (DHE, Molecular Probes, Inc.). Tissue samples were frozen in OTC compound and 10 μm transverse sections were cut through the aortic valve using a cryostat. Sections were then incubated in 0.002 mmol/L DHE or 0.001 mmol/L DCF 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, we thresholded only positively stained cells/nuclei using Image J software (version 1.32j; National Institutes of Health, Bethesda, MD) and expressed data as the difference between the inhibited and non-inhibited fluorescent images (SOD-inhibitable DHE; mean relative light units (RLU’S)/pixel).

**Immunohistochemistry.** Immunohistochemistry was used to detect inflammatory cell infiltrate (macrophages), pro-osteogenic markers (phospho-Smad1/5/8, Msx2,
Osterix, CBFA1), and pro-fibrotic markers (phospho-Smad2). Immunohistochemical detection of proteins was carried out as follows: P-Smad2 (Cell Signaling Technologies, #3108, 1:50), P-Smad1/5/8 (Cell Signaling Technologies, #9511, 1:300 + TSA kit), Msx2 (Sigma-Aldrich, #1069, 1:10), Osterix (Abcam, #22552, 1:75), alpha-smooth muscle actin (Sigma-Aldrich, #A2547,1:200), CBFA1 (Santa Cruz, #C-19, 1:50).

Echocardiographic evaluation of aortic valve stenosis. Aortic valve function was evaluated as described previously 3. 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. Doppler interrogation of blood velocity through the aortic valve was not possible because the imaging probe does not have continuous-wave Doppler capability. All images were acquired by an operator blinded to the treatment groups.

Magnetic resonance imaging evaluation of aortic valve stenosis. Cardiac magnetic resonance imaging was performed in a subset of mice using a 4.7 Tesla Varian UNITY/Inova scanner (Varian Inc., Palo Alto, CA) and dedicated 38 mm diameter birdcage radiofrequency coil. In brief, animals were sedated using a subcutaneous injection of midazolam and morphine, and subcutaneous electrodes were placed for
electrocardiograph monitoring and cardiac gating of images. Mice were subsequently placed in a plastic mouse holder to minimize motion. A 2-dimensional cardiac-gated cine spoiled gradient echo pulse sequence was applied with the following parameters: Repetition time = 6 ms, Echo time = 3.4 ms, flip angle = 15°, in-plane resolution 0.22 mm x 0.22 mm with a slice thickness of 0.6 mm for aortic valve analysis. The number of cine frames per cardiac cycle varied between 15 and 20 depending on heart rate.

Analysis of images was performed at an off-site workstation. Aortic stenosis is assessed by measuring in-plane aortic valve area during peak systole.

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.
Figure S1. Mineralization of aortic valve (assessed with Von Kossa staining) before and after normalization of lipid levels. A) 6 month control; B) 12 month control; C) 6 months of hypercholesterolemia; D) 12 months of hypercholesterolemia; E) 12 month “reversed”; F) mean data for all animals studied (8-12 per group). Mineralization was significantly increased in hypercholesterolemic mice at 6 and 12 months. Normalizing blood lipids produced significant reductions in valvular mineralization in “reversed” mice.
Figure S2. Distribution of aortic valve cusp separation distances before and after normalization of lipid levels. The first column shows control mice at 6 and 12 months of age; the second column shows hypercholesterolemic mice at 6 and 12 months of age; the last column shows “reversed” mice at 12 months of age, and mean data depicting the percentage of animals with hemodynamically significant aortic valve stenosis in each group (i.e., maximum cusp separation distance < 0.6 mm).
Figure S3. Top panel: near-normal aortic valve function in a 12 month-old Reversa mouse that received injections of pI-pC to normalize cholesterol levels at age 6 months. The cusp separation distance for this mouse was 0.85 mm. Bottom panel: moderate aortic stenosis in a 12 month-old hypercholesterolemic Reversa mouse. The cusp separation distance for this animal was 0.5 mm. Annotations: Ao W: aortic wall; vertical white line demarcates the point at which cusp separation distance was measured.
Figure S4. Image illustrating the plane in which the MRI images were obtained for the determination of aortic valve orifice area (corresponding to Figure 7B in the manuscript).
SUPPLEMENTARY REFERENCES

