Association Between Shear Stress and Platelet-Derived Transforming Growth Factor-β1 Release and Activation in Animal Models of Aortic Valve Stenosis

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Objective—Aortic valve stenosis (AS) is characterized by fibrosis and calcification of valves leading to aortic valve narrowing, resulting in high wall shear stress across the valves. We previously demonstrated that high shear stress can activate platelet-derived transforming growth factor-β1 (TGF-β1), a cytokine that induces fibrosis and calcification. The aim of this study was to investigate the role of shear-induced platelet release of TGF-β1 and its activation in AS.

Approach and Results—We studied hypercholesterolemic Ldlr−/−ApoB100/100/Mttp−/−Mx1Cre+×+ (Reversa) mice that develop AS on Western diet and a surgical ascending aortic constriction mouse model that acutely simulates the hemodynamics of AS to study shear-induced platelet TGF-β1 release and activation. Reversa mice on Western diet for 6 months had thickening of the aortic valves, increased wall shear stress, and increased plasma TGF-β1 levels. There were weak and moderate correlations between wall shear stress and TGF-β1 levels in the progression and reversed Reversa groups and a stronger correlation in the ascending aortic constriction model in wild-type mice but not in mice with a targeted deletion of megakaryocyte and platelet TGF-β1 (Tgfβ1−/−). Plasma total TGF-β1 levels correlated with collagen deposition in the stenotic valves in Reversa mice. Although active TGF-β1 levels were too low to be measured directly, we found (1) canonical TGF-β1 (phosphorylated small mothers against decapentaplegic 2/3) signaling in the leukocytes and canonical and noncanonical (phosphorylated extracellular signal-regulated kinases 1/2) TGF-β1 signaling in aortic valves of Reversa mice on a Western diet, and (2) TGF-β1 signaling of both pathways in the ascending aortic constriction stenotic area in wild-type but not Tgfβ1−/− mice.

Conclusions—Shear-induced, platelet-derived TGF-β1 activation may contribute to AS. (Arterioscler Thromb Vasc Biol. 2014;34:1924-1932.)

Key Words: aortic valve stenosis ▪ blood platelets ▪ transforming growth factor beta
the released latent TGF-β1. This mechanism may contribute to the progression of AS because AV narrowing increases shear stress, resulting in greater release of platelet TGF-β1 and TGF-β1 activation, which in turn may lead to progressive valve narrowing and fibrosis and thus even greater shear stress.

To test our hypothesis, we studied plasma TGF-β1 levels longitudinally in Ldlr<sup>−/−</sup>ApoB<sup>100/100</sup>/Mttp<sup>−/−</sup>Mx1Cre<sup>+/−</sup> (Reversa mice) that develop both severe hypercholesterolemia and AS on a Western diet. To further test the role of platelet-derived TGF-β1 release under high shear stress, we compared the responses of wild-type (WT) mice and mice with a targeted deletion in megakaryocyte and platelet TGF-β1 (Tgfb1<sup>−/−</sup>) to acute surgical constriction of the ascending aorta. This model more closely simulates the hemodynamics of advanced human AS than the transverse aortic constriction model that we previously studied because the stenosis is proximal to the innominate artery. To complement these in vivo models, we also assessed in vitro the response of human AV cells to TGF-β1 and the effect of platelet-derived TGF-β1 on leukocyte phosphorylation of the protein small mothers against decapentaplegic 2/3 (Smad2/3), an indicator of active TGF-β1 signaling in the circulation.

**Material and Methods**

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

**Diet-Induced AV Stenosis**

A total of 74 Reversa mice (9–12 weeks of age) were placed on either a chow diet or a WD (TD 88137; Harlan Teklad) and divided into the groups illustrated in the online-only Data Supplement Figure I: (1) progression group: 28 mice on a WD for ≤12 months; (2) reversed group: 27 mice fed a WD for 6 months that then received 4 injections of polyinosinic-polycytidylic acid (pI-pC, 225 μg, IP) at 2-day intervals to inactivate the Mttp gene and switched to a chow diet for another 6 months; and (3) control group: 19 Reversa mice maintained on a chow diet for 12 months and treated with pI-pC at the age of 6 weeks.

**Figure 1.** Mice in the progression group have higher wall shear stress (WSS) and increased plasma transforming growth factor-β1 (TGF-β1) levels. A, Aortic valve (AV) cusp separation distance in the 3 groups. B, WSS across the AV in the 3 groups. C, Transvalvular peak velocity in the 3 groups. D, Plasma total TGF-β1 levels of mice in the 3 groups. E and F, Correlation between the changes in TGF-β1 levels and WSS from baseline to 6 months (E) and from 9 to 12 months (F) in the 3 groups. For A and B, *P<0.01 compared with control group at the same time point and #P<0.01 compared with the group’s own baseline value.
WSS Across the AV in Reversa Mice and the Stenosis Created in AAC Model

Flow measurements were obtained using the pulse-wave Doppler mode. WSS was calculated by the equation WSS=(4π×V_/r), where μ is the blood viscosity (estimated at 0.035 poise), V_ is the mean trans-stenotic velocity, and r is the radius (equal to half of the AV cusp separation distance in Reversa mice and the stenosis in the ascending aortic constriction [AAC] model).

**Results**

**Reversa Mice Are Hypercholesterolemic; a WD Exacerbates Hypercholesterolemia and Leads to AS After 6 Months of a WD; Activating the Cre Recombinase With pI-pC Reversers Hypercholesterolemia and Attenuates, But Does Not Stop AS Progression**

The experimental design is shown in Figure I in the online-only Data Supplement. The cholesterol levels in the control group on a chow diet remained at approximately the same level throughout the 12 months of the study, whereas those on a WD (progression group) developed significantly increased plasma cholesterol levels (Figure II in the online-only Data Supplement; all P<0.01). Injection of pI-pC to Reversa mice at 6 months, coupled with switching the mice from a WD to a chow diet (reversed group), resulted in a rapid decrease in cholesterol levels to approximately their initial values (Figure II in the online-only Data Supplement).

The mean AV cusp separation distance, a measure of AS, was within the range of 0.85 to 0.95 mm in the control mice before and throughout the study (Figure 1A). In sharp contrast, AV cusp distance steadily decreased in the progression group during the 12 months of the experiment, going from 0.86±0.03 to 0.31±0.03 mm (P<0.01). Reversal of hypercholesterolemia slowed the rate of reduction in AV cusp distance, such that it was 0.46±0.03 mm at 12 months (P<0.01 compared with the progression group value; Figure 1A). Feeding Reversa mice a WD for 6 months led to AV thickening and increased AV collagen deposition, and the abnormalities were more severe after 12 months (Figure III progression panels in the online-only Data Supplement). Reversal of hypercholesterolemia at 6 months slowed the progression of AV thickening from 6 to 12 months but had no effect on collagen deposition (Figure III reversed panels in the online-only Data Supplement).

**Both WSS Across the AV and Plasma Total TGF-β1 Levels Increase After 6 Months in Reversa Mice on a WD**

Transvalvular peak velocity and WSS on the ventricular side of the AV, which is ≈3x to 4x higher than on the aortic side under normal circumstance, remained unchanged in the control group throughout the experiment (Figure 1B and 1C). In sharp contrast, ventricular WSS increased from 191±20 to 412±32 dyn/cm² after 6 months of WD in the progression group (P<0.01 versus control group at 6 months). It continued to increase during the next 6 months in the progression group, reaching a value of 1053±189 dyn/cm². WSS was lower in the reversed group at the 9-month time point than in the progression group (P<0.01). Between 9 and 12 months, WSS in the reversed group increased 38% but still remained significantly lower than in the progression group (P=0.04; Figure 1B).

Reversa mice on a WD developed systolic dysfunction after 12 months (Figure IV in the online-only Data Supplement).

Plasma total TGF-β1 levels in the control group were 0.87±0.05 ng/mL at baseline and remained ≤1.00±0.02 ng/mL throughout the 12 months of the experiment (Figure 1D). In contrast, in the progression group, the plasma total TGF-β1 increased from 1.02±0.02 ng/mL to 1.67±0.10, 1.94±0.18, and 1.79±0.11 ng/mL after 3, 6, and 9 months of WD, respectively; the value remained elevated at 1.90±0.16 ng/mL after 12 months (all P<0.01 versus baseline value and P<0.05 versus control group at 0, 3, 6, 9, and 12 months). TGF-β1 levels were lower in mice in the reversed group than in the progression group at the 9-month time point (1.11±0.05 ng/mL; P<0.001) but still higher than in the control group (P<0.05). Plasma TGF-β1 levels increased to 1.54±0.10 ng/mL at 12 months in the reversed group (39% increase from 9 months), a value significantly higher than that of the control group (P=0.01) but lower than that of the progression group (P=0.03; Figure 1D).

There was considerable variability between animals, but there was a weak correlation between the change in WSS from baseline to 6 months and the change in plasma TGF-β1 levels during the same time period in the progression group (r=0.37; P=0.03) but not in the control group (r=−0.38; P=0.08; Figure 1E). There was a trend toward a correlation between the change in plasma total TGF-β1 from 9 months to 12 months and the change in WSS during the same period in the reversed group (r=0.50; P=0.06; Figure 1F). The changes in plasma TGF-β1 levels from baseline to 6 months and from 9 months to 12 months did not correlate with changes in cholesterol levels in either group during the same time periods (data not shown).

**Association Among Plasma TGF-β1 Levels, AV Collagen Deposition, and Systolic Cardiac Function**

The association between plasma TGF-β1 and the area of the AV staining positive for collagen, a measure of valve fibrosis, was measured at 6 and 12 months (Figure 2A and 2B). There was no association in control animals at either time point (r=0.01 and 0.24, respectively; P=0.97 and 0.45, respectively; Figure 2A and 2B, control group). In the progression group, the correlation coefficients at 6 and 12 months were 0.47 and 0.78, but the associations were not statistically significant (P=0.19, n=9 and P=0.08, n=8; Figure 2A). The correlation in the regression group at 12 months (n=15) was statistically significant (r=0.77; P<0.01; n=15; Figure 2B). Plasma TGF-β1 levels showed a trend toward a negative association with ejection fraction in both the reversed and progression groups at 12 months (Figure 2D; r=−0.50 and −0.63, respectively; P=0.06 and P=0.09, respectively) but not at 6 months (Figure 2C).

**Association Among Vascular Calcification, Plasma TGF-β1 Levels, and WSS**

Reversa mice on a chow diet did not develop vascular calcification (n=8) as judged by alizarin red staining in either their AVs or ascending aortas (Figure 2E; Figure V in the online-only Data Supplement). In contrast, 8 of 8 mice on a WD in the progression group and 12 of 15 mice in the reversed group developed calcifications (P<0.01 for progression group and P<0.01 for reversed group versus control group; P=0.03...
Almost all of the calcifications were found in the ascending aorta above the AVs. The calcification was, for the most part, localized to relatively small areas, raising the possibility that they were in reaction to a jet of blood hitting the wall as it exited from the narrowed valve. There was no correlation between the extent of calcification and either the TGF-β1 level or WSS ($r=0.30$, $P=0.47$ and $r=0.24$, $P=0.26$, respectively).

**Both the Canonical and Noncanonical TGF-β1 Signaling Pathways Were Activated in the AVs of Reversa Mice in Both Progression and Reversed Groups**

Figure 3A and 3B shows representative sections of AVs stained for phosphorylated Smad2/3 (p-Smad2/3), a canonical TGF-β1 pathway intermediate, and phosphorylated extracellular signal–regulated kinases 1/2 (p-Erk1/2), a noncanonical TGF-β1 pathway intermediate, respectively, in the 3 groups at different time points. The AVs stained more intensely for p-Smad2/3 in the progression group than in the control group at 6 and 12 months, and most of these p-Smad2/3–positive cells expressed vimentin, indicating that they were valvular interstitial cells (VICs) rather than endothelial cells (Figure 3A). There was baseline p-Erk1/2 staining in endothelial cells (identified by von Willebrand factor staining) in mice on a chow diet. The percentage of cells staining for p-Erk1/2 dramatically increased after the development of AS in the progression group at both 6 and 12 months. Most p-Erk1/2–positive cells did not express von Willebrand factor, indicating that they were VICs rather than endothelial cells (Figure 3B). p-Smad2/3 and p-Erk1/2 staining of the valve leaflets of mice in both the reversed and the progression groups after 12 months was more intense than in the control group ($P<0.05$ for both; Figure 3). There was no significant association between either p-Smad2/3 or p-Erk1/2 staining and collagen deposition in either the reversed or the progression group at 12 months (Figure VI in the online-only Data Supplement).

**Treating AV Interstitial Cells (VICs) In Vitro With TGF-β1 Activates the Erk1/2 Noncanonical TGF-β1 Signaling Pathway Independent of Canonical Pathway Activation**

The increased WSS across the AVs, the intermediate plasma TGF-β1 levels, and the continued activation of noncanonical pathways in VICs of AVs in the reversed group at 12
months despite the reduction in cholesterol levels suggested that shear-induced latent TGF-β1 release and activation may have its effect on AVs through the noncanonical TGF-β1 pathway. Because the data from the in vivo mouse studies suggested that TGF-β1 might be able to stimulate both the canonical and noncanonical pathways of TGF-β1 signaling in VICs, we tested the effect of exogenous TGF-β1 on VICs. We found that TGF-β1 transiently activated both the canonical and noncanonical pathways (Figure 4A). To test whether Erk1/2 phosphorylation required p-Smad2/3 signaling, we reduced Smad2 levels by ≈80% using small interfering RNA and then activated the cells with TGF-β1; Erk1/2 phosphorylation still occurred in response to TGF-β1 although there was virtually no Smad2/3 phosphorylation (Figure 4B), suggesting that the noncanonical pathway activation in VICs was independent of the activation of the canonical pathway. To further determine the relative contribution of the canonical and noncanonical TGF-β1

Figure 3. Canonical and noncanonical transforming growth factor-β1 (TGF-β1) signaling pathways in aortic valves (AVs). A, Representative images of canonical TGF-β1 signaling judged by phosphorylated small mothers against decapentaplegic 2/3 (p-Smad2/3) in the AVs from mice in the 3 groups at 6 and 12 months. An antibody to vimentin was used to identify valvular interstitial cells. B, Representative images of noncanonical TGF-β1 signaling judged by phosphorylated extracellular signal–regulated kinases 1/2 (p-Erk1/2). An antibody to von Willebrand factor (vWF) was used to identify endothelial cells. Quantification of mean fluorescent intensity (MFI) of p-Smad2/3 and p-Erk1/2 (+) cells in the AVs is shown in upper right small panels in A and B. *P<0.05. ns indicates nonsignificant.

Figure 4. Activation of canonical and noncanonical transforming growth factor-β1 (TGF-β1) signaling pathways after exposure of cultured human aortic valvular interstitial cells (VICs) to TGF-β1. A, Immunoblot of VIC after treatment with 0.5 ng/mL TGF-β1. B, Effect of reducing small mothers against decapentaplegic (Smad) 2 in VIC with small interfering RNA (RNAi) on TGF-β1–induced extracellular signal–regulated kinase (Erk) 1/2 phosphorylation. C, Changes in mRNA levels of fibrosis-related and calcification-related genes after knocking down Smad2 or Erk2. *P<0.05 vs control, n=3. α-SMA indicates α-smooth muscle actin; ALP, alkaline phosphatase; BMP4, bone morphogenetic protein; Col1a2, collagen, type I, alpha 2; Col3a1, collagen, type III, alpha 1; CTGF, connective tissue growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MMP13, matrix metalloproteinase 13; p-Erk1/2, phosphorylated Erk1/2; p-Smad2/3, phosphorylated Smad2/3; RNAi, ribonucleic acid interference; Runx2, Runt-related transcription factor 2; and scramble, a negative control siRNA with no homology to any known mammalian gene.
pathways to fibrosis or calcification in VICs, Smad2 or Erk2 was knocked down separately and fibrosis-related and calcification-related gene expression levels were analyzed after 48 hours of TGF-β1 treatment. TGF-β1 treatment led to increased mRNA levels in both fibrosis-related and calcification-related genes in control cells treated with a scrambled small interfering RNA. In sharp contrast, knocking down either Smad2 or Erk2 significantly decreased the response to TGF-β1 (Figure 4C). These data suggest that both the canonical and noncanonical TGF-β1 pathways contribute to fibrosis and calcification.

Acute Surgical Constriction of the Ascending Aorta in WT and Tgfb1flox Mice Leads to Increases in Plasma Total TGF-β1 Levels and WSS; the TGF-β1 Levels Correlated With WSS in WT But Not Tgfb1flox Mice

To further assess the effect of increased WSS on TGF-β1 levels in a mouse model that simulates the hemodynamics of AS in the absence of hypercholesterolemia, we performed AAC surgery on WT mice and Tgfb1flox mice. The experimental scheme is shown in Figure 5A. In response to surgery, WSS at the site of stenosis increased 20-fold 1 week after the surgery and remained elevated at 4 weeks (Figure 5A, inset). Baseline TGF-β1 levels of Tgfb1flox mice were 30% lower (median, 0.72 ng/mL; interquartile range, 0.60–0.80 ng/mL) than those of WT mice. In WT mice, total TGF-β1 levels increased from a median of 1.02 ng/mL (interquartile range, 0.92–1.36 ng/mL; n=23) before surgery to 1.26 ng/mL (interquartile range, 1.06–1.58 ng/mL; 24% increase) and 1.35 (interquartile range, 1.08–1.54 ng/mL; 32% increase) at 1 and 4 weeks after AAC surgery, respectively (both P=0.01; Figure 5B). Tgfb1flox mice had 35% to 40% lower TGF-β1 levels than WT mice at 1 week and 4 weeks after surgery (0.82 ng/mL at both 1 and 4 weeks; n=20; P<0.01 at both time points versus WT mice; Figure 5B). WSS at the site of the stenosis correlated positively with plasma total TGF-β1 levels in WT but not Tgfb1flox mice after AAC surgery (Figure 5C and 5D). The left ventricular ejection fraction decreased in both groups by ≈27% at both 1 and 4 weeks after AAC surgery (data not shown). Ejection fraction correlated negatively with the plasma total TGF-β1 levels in WT mice 1 week and 4 weeks after surgery (both P<0.01) but not in Tgfb1flox mice (both P>0.05; Figure 5E and 5F). Plasma total TGF-β1 levels correlated positively with the left ventricular interstitial fibrosis score in the combined group of WT and Tgfb1flox mice (r=0.48; P=0.025; n=43). Left ventricular systolic function (ejection fraction) was negatively associated with the fibrosis score in the group composed of WT and Tgfb1flox mice (r=−0.87; P<0.001; n=43; Figure VII in the online-only Data Supplement). WT mice showed evidence of both canonical and noncanonical signaling in the ascending aorta near to where the stenosis was created, suggesting local TGF-β1 activation under high shear stress (Figure VIII in the online-only Data Supplement). In sharp contrast, the Tgfb1flox mice had much less evidence of TGF-β1 signaling, suggesting that platelet-derived TGF-β1 rather than tissue-derived TGF-β1 was the source of the active TGF-β1.

Platelet-Derived TGF-β1 Can Initiate Phosphorylation of Smad2/3 in CD45+ Leukocytes After Stirring Whole Blood

Because plasma levels of active TGF-β1 in both Reversa mice and mice that underwent AAC surgery were too low to be detected by ELISA, we tried to develop a surrogate indicator of circulating activated TGF-β1. The first step was to check the phosphorylation of Smad2/3 in leukocytes contained in whole blood with or without stirring to increase shear. We found that stirring the blood of WT mice for 40 minutes dramatically increased the percentage of p-Smad2/3–positive leukocytes from 1.6±0.4% to 30.7±8.9% (n=6; P=0.003; Figure 6B, with a representative experiment shown in Figure 6A). To assess the role of TGF-β1 in p-Smad2/3, we also stirred whole blood from Tgfb1flox mice and found a much lower p-Smad2/3 response, going from 1.2±0.3% to 7.2±2.3% (n=6; P=0.08 compared with unstirred value and P<0.001 compared with value in WT mice after stirring). Similar results were obtained by immunoblotting of lysed whole blood (Figure 6C). To further assess whether the increase in leukocyte p-Smad2/3 in WT mice was because of active TGF-β1, we repeated the experiments in the presence of an antibody to TGF-β1 and found that the antibody attenuated the stirring-induced increase in WT leukocyte p-Smad2/3 by 44% (13.5±7.3%; n=6; P=0.03; Figure 5).

Figure 5. Plasma total transforming growth factor-β1 (TGF-β1) levels increase after ascending aorta constriction (AAC) surgery; the increased TGF-β1 levels correlate with wall shear stress (WSS) at the site of the stenosis in wild-type (WT) but not Tgfb1flox mice. A, Experimental design and WSS (inset) before (pre) and 1 and 4 weeks after AAC surgery. B, Plasma total TGF-β1 levels before and 1 and 4 weeks after AAC surgery in WT and Tgfb1flox mice (median is shown on the plot; P<0.001 between the 2 groups). C and D, Correlations between plasma total TGF-β1 levels and WSS at the site of aortic stenosis in WT and Tgfb1flox mice at 1 week (C) and 4 weeks (D) after AAC surgery. E and F, Correlation between plasma total TGF-β1 levels and ejection fraction in WT and Tgfb1flox mice at 1 week (E) and 4 weeks (F) after AAC surgery. Tgfb1flox mouse is Tgfb1flox/Pf4Cre/−.
Reversa Mice on a WD for 3 Months Have More p-Smad2/3 in Their CD45\(^+\) Leukocytes Than Those on Chow Diet or WT Mice on Chow Diet, Suggesting the Presence of More Circulating Active TGF-\(\beta\)1

Based on the ex vivo assay described above, we tested whether there was more p-Smad2/3 in the leukocytes in the blood of Reversa mice on a WD for 3 months without stirring than in the leukocytes of age- and sex-matched Reversa mice on a chow diet or WT mice. We found that 5.8±1.0% of CD45\(^+\) leukocytes were p-Smad2/3 positive in Reversa mice on a WD compared with 1.2±0.3% of leukocytes in Reversa mice on chow diet and 2.1±0.5% in WT mice (\(P=0.04\) and \(P=0.01\), respectively, versus Reversa mice on a WD diet; Figure 6D). Similarly, we found higher leukocyte p-Smad2/3 mean fluorescent intensity in Reversa mice on a WD (302±23 arbitrary units [AU]) than Reversa mice on a chow diet and WT mice (236±17 AU and 244±11 AU, respectively; both \(P=0.04\); Figure 6E). A representative p-Smad2/3 flow cytometry histogram for 1 mouse in each group is shown in Figure 6F.

Discussion

AS, characterized by reduced AV area and increased shear stress, is estimated to affect >25% of people aged >65 years.\(^3\) Although inflammation, oxidative damage, and osteogenesis have been reported to contribute to the pathogenesis of AS, to date there is no effective medical therapy,\(^6,22\) and so AV replacement, either surgically or percutaneously, is the only effective treatment.\(^1,23\) Based on our previous observation that high shear stress can induce release and activation of platelet-derived latent TGF-\(\beta\)1,\(^11\) a cytokine that can induce fibrosis and calcification in explanted AV interstitial cells,\(^12,24\) we hypothesized that high shear stress across a stenotic AV could both induce release of platelet TGF-\(\beta\)1 and activate it in the circulation, leading to progressive AV fibrosis, calcification, and further stenosis. To test this hypothesis, we used a nonsurgical model of diet-induced AS and a surgical model of AS that closely simulates the hemodynamics of severe AS.

In the hypercholesterolemic mouse model, we found that the onset of AV abnormalities occurred within 3 months after starting the WD. Plasma TGF-\(\beta\)1 levels also increased within 3 months in these animals and remained elevated thereafter. Although there was a weak correlation between the increase in TGF-\(\beta\)1 and the increase in WSS from baseline to 6 months, the TGF-\(\beta\)1 increase reached near-maximum levels at 3 months, and degranulation.\(^25,26\) Additionally or alternatively, hypercholesterolemia may have increased release of TGF-\(\beta\)1 from sources other than platelets.\(^27,28\) Support for a role for hypercholesterolemia comes from the data in the reversed group of Reversa mice. Their hypercholesterolemia was nearly normalized at 9 months associated with a significant reduction in plasma TGF-\(\beta\)1 levels. Furthermore, Zhou et al\(^29\) reported a correlation between cholesterol and plasma TGF-\(\beta\)1 levels in

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Figure 6A–6C). To assess whether platelet granule release was required for the increase in leukocyte p-Smad2/3, we also tested the effect of adding the platelet activation inhibitor prostaglandin E\(_1\). It also attenuated the increase in leukocyte p-Smad2/3 as shown by immunoblotting (Figure 6C). We concluded that platelet-derived, shear (stirring)-activated TGF-\(\beta\)1 was responsible for the increase in leukocyte p-Smad2/3.

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Figure 6. Effect of stirring blood from wild-type (WT) and Tgfb1\(^{flox}\) mice on platelet-derived transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1) release and activation; phosphorlated small mothers against decapentaplegic 2/3 (p-Smad2/3) levels in leukocytes of Reversa mice. A, A representative experiment to detect p-Smad2/3 in CD45\(^+\) leukocytes in WT and Tgfb1\(^{flox}\) in untreated blood (top) and blood stirred for 40 minutes at 37°C (middle). The inhibitory effect of adding a TGF-\(\beta\)1 neutralizing antibody to CD45\(^+\) leukocytes before and after stirring, with or without the antibody to TGF-\(\beta\)1 (n=6). \(*P<0.01, **P<0.001. C, Immunoblots of unstirred or stirred whole blood in the absence or presence of the TGF-\(\beta\)1 antibody or prostaglandin E\(_1\) (PGE\(_1\); 4 μmol/L) after lysing red blood cells. Tgfb1\(^{flox}\) mouse is Tgfb1\(^{flox}\)PF4-Cre\(^{-}\). D, Percentage of p-Smad2/3(+) leukocytes in each group. E, Geometric mean fluorescence intensity (MFI) of p-Smad2/3 in leukocytes in each group after 3 months. F, Representative histograms for p-Smad2/3 in leukocytes. Leukocytes were identified with an antibody to CD45. n=5 in WT and Reversa chow group and n=6 in WT and Reversa WD groups. AU indicates arbitrary unit; and WD, Western diet.
apolipoprotein E–deficient mice placed on a high-fat diet. The increase in plasma TGF-β1 levels between 9 and 12 months in the reversed group was associated with an increase in WSS but these mice had nearly normal cholesterol levels, indicating a potential role of WSS in increasing plasma TGF-β1 levels in the absence of hypercholesterolemia. Collectively, these data suggest a complex relationship between hypercholesterolemia and WSS as factors in increased TGF-β1 levels. To further assess the impact of increased aortic WSS on TGF-β1 levels in the absence of hypercholesterolemia and assess the contribution of platelet TGF-β1 to any change in plasma TGF-β1, we surgically constricted the ascending aorta of WT mice and Tgfb1<sup>fl<sup>x</sup> mice whose platelet TGF-β1 levels are only ≈15% of those of WT mice. Plasma TGF-β1 levels increased in both WT mice and Tgfb1<sup>fl<sup>x</sup> mice but only correlated with increased WSS in WT mice but not Tgfb1<sup>fl<sup>x</sup> mice. These data further support the hypothesis that high shear stress can lead to release of platelet-derived TGF-β1.

Although we could not detect active TGF-β1 in plasma of Reversa mice, probably because the current assays are not sensitive enough to detect the picomolar levels expected based on total TGF-β1 levels, the relatively low percentage of total TGF-β1 that becomes activated by shear,<sup>31,32</sup> and the reported short survival of active TGF-β1 in the circulation,<sup>33,34</sup> we found activation of both canonical (p-Smad2/3) and noncanonical (p-Erk1/2) pathways in VICs of the stenotic AVs, indicating the possibility of local TGF-β1 activation under high shear stress. We also found evidence of both canonical and noncanonical TGF-β1 signaling in the local area of high shear in the ascending aorta of WT mice in the AAC model. There was much less TGF-β1 signaling in the Tgfb1<sup>fl<sup>x</sup> mice, suggesting that platelet-derived TGF-β1 is the likely source of the active TGF-β1. In vitro studies of cultured human AV interstitial cells with exogenous TGF-β1 supported these in vivo findings and indicated that activation of the noncanonical pathway is independent of the activation of the canonical pathway on VICs. These data are in accordance with previous studies indicating that TGF-β1 independently activates both the canonical and the noncanonical pathways.<sup>12</sup> The identification of a potential role of the noncanonical pathway in AS in this model is of interest because Erk1/2 is important in epithelial mesenchymal transition,<sup>35</sup> regulating cell proliferation and apoptosis<sup>36</sup> and calcification of VICs.<sup>37</sup> We found that increased Smad and Erk signaling persisted in the regression group at 12 months despite the reduction in cholesterol and the partial reduction in plasma TGF-β1 levels, raising the possibility that even a modest elevation in plasma TGF-β1 may be sufficient to induce signaling.

Because active TGF-β1 could not be directly detected in the plasma of mice, we developed an indicator of platelet-derived circulating active TGF-β1 based on our in vitro findings that stirring mouse whole blood produced an increased p-Smad2/3 in leukocytes. Support for the increase in p-Smad2/3 being because of stimulation by TGF-β1 came from data showing that an antibody to TGF-β1 inhibited the Smad2/3 phosphorylation, as did the platelet granule release inhibitor prostaglandin E<sub>2</sub>. Further support for the platelet origin of the TGF-β1 in these studies came from parallel studies using Tgfb1<sup>fl<sup>x</sup> mice because the p-Smad2/3 signal was dramatically reduced with the blood from these animals. By using this assay, we found a significant increase in leukocyte p-Smad2/3 in the Reversa mice on the WD but not on the Chow diet. Thus, although it has been postulated that shear force may activate TGF-β1 in a paracrine fashion through valve tissue stretching and mechanical, traction-based cellular activation,<sup>4</sup> our results suggest that additionally or alternatively shear force may lead to activation of circulating TGF-β1.

In summary, our in vivo data provide direct or indirect evidence for (1) a complex relationship between hypercholesterolemia and shear in increasing plasma TGF-β1 levels in a diet-induced mouse model of AS; (2) a role for TGF-β1 signaling through both its canonical and noncanonical pathways in the AV abnormalities in this model; and (3) the presence of active TGF-β1 in the circulation in this model. Complementary in vitro data demonstrate that shear stress can release TGF-β1 from platelets and activate it to levels that can induce a biological response in leukocytes. These data raise the possibility that interfering with TGF-β1 activation or its effects on the canonical and noncanonical pathways may slow the progression of AS. It will, however, require direct demonstration that preventing TGF-β1 release or activation, or neutralizing its activity, prevents pathological AV changes to establish a causal relationship.

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Disclosures

Dr Coller has royalty interests in abxiximab (Centocor) and the VerifyNow assays (Accumetrics) and serves as a consultant to Chugai Pharmabody. The other authors report no conflicts.

References

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Significance

Aortic valve stenosis is a disorder of major medical importance with no effective medical therapy. Transforming growth factor-beta1 has been implicated in the pathogenesis, but its precise role has not been defined. Although it has been postulated that shear force may activate transforming growth factor-beta1 in a paracrine fashion through valve tissue stretching and mechanical, traction-based cellular activation, our results suggest that additionally, shear force may lead to release and activation of platelet-derived transforming growth factor-beta1. They also indicate that transforming growth factor-beta1 may affect aortic valves through both canonical and noncanonical signaling pathways. These data provide a modified paradigm for the pathogenesis of aortic valve stenosis and identify potential new therapeutic targets.
Association Between Shear Stress and Platelet-Derived Transforming Growth Factor-β1 Release and Activation in Animal Models of Aortic Valve Stenosis
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Association Between Shear Stress and Platelet-derived TGF-β1 Release and Activation in Animal Models of Aortic Valve Stenosis

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This online supplement consists of eight supplementary figures.

FIGURE LEGENDS:

Supplementary Figure I: Experimental scheme. Reversa mice were divided into control, progression, and reversed groups.

Supplementary Figure II. Plasma cholesterol levels in control, reversed, and progression groups. Data reported as mean ± SEM. n=19 in control group, n=28 progression group and n=27 in reversed group at 0 and 6 month, and n=12 in control group, n=8 progression group and n=15 in reversed group at 12 months. * p<0.01 compared with control group at the same time point, # p<0.01 compared with baseline values (0 time).

Supplementary Figure III: Histology of AV in control, reversed, and progression groups at 6 and 12 months. (A) Representative images of H&E-stained AVs and corresponding AV thickness data for all animals. (B) Representative images of trichrome-stained AVs and corresponding analysis of the percentage of the area of the AV sections that stained positive for collagen (blue). Numbers of mice in each group at different time points are the same as reported in supplementary figure I. * p<0.05; # p<0.05 progression group at 6 months vs. reversed group at 12 months; ns: no significance.
Supplementary Figure IV. Systolic cardiac function in control, reversed, and progression groups. (A) Ejection fraction and (B) Fractional shortening. Data reported as mean ± SEM. Number of mice in each group at different time points are the same as reported in supplementary figure I. * p<0.01 compared with control group at the same time point, # p<0.01 compared with baseline (0 time).

Supplementary Figure V: Calcification of aortic arch and aortic root of Reversa mice in the three groups using Alizarin red staining. Representative images were shown in each group.

Supplementary Figure VI. Association between p-Smad2/3 or p-Erk1/2 staining on AVs of Reversa mice and collagen deposition on AVs. (A) p-Smad2/3 (B) p-Erk1/2.

Supplementary Figure VII. Association between interstitial fibrosis score and total TGF-β1 level (A) or EF (B). EF: ejection fraction.

Supplementary Figure VIII. Canonical and non-canonical TGF-β1 signaling pathways in the ascending aorta where the stenosis was created in the AAC model. (A) Representative images of canonical TGF-β1 signaling as judged by p-Smad2/3 staining in the ascending aorta of WT and Tgfb1floxflox mice 4 weeks after AAC surgery. Quantification of mean fluorescence intensity (MFI) is shown in (B) (n=6). (C) Representative images of non-canonical TGF-β1 signaling as judged by p-Erk1/2 staining in the ascending aorta of WT and Tgfb1floxflox mice 4 weeks after AAC surgery. Quantification of the percentage of cells staining positive is shown in (D) (n=6). An antibody to vimentin was used to identify valvular interstitial cells. * p<0.05.
Supplementary Figure I

The diagram illustrates the progression and reversal of conditions over time, with different colors indicating different stages. The timeline is divided into segments labeled 'chow diet', 'Western diet', and 'Progression'.

- **Left Side**:
  - 'Reversa' followed by 'pl-pC' indicating a reversal point.
  - A time line from -1 to 12 months, with '0 (pre)' indicating the baseline.

- **Right Side**:
  - 'Reversed' and 'Control' labels, indicating different outcomes.
  - The timeline includes the progression of 'chow diet' and 'Western diet' periods, with 'pl-pC' as a transition point.

- **Activities**:
  - Echocardiography, and blood sampling at 3, 6, 9, and 12 months.
  - Histology/immunohistochemistry at 12 months.
Supplementary Figure II

The graph illustrates the changes in cholesterol levels (mg/dl) over time (months) for three different groups: Control (circles), Reversed (upward-pointing triangles), and Progression (downward-pointing triangles).

- Control group shows a steady increase in cholesterol levels from 0 to 9 months, followed by a slight decrease in the last month.
- Reversed group experiences a sharp increase from 0 to 3 months, followed by a decrease to a lower level than the Control group by 9 months.
- Progression group starts with the highest cholesterol levels, which decrease sharply by 3 months, followed by a slight increase near the end of the study period.

Statistical significance is indicated by asterisks and hash symbols:
- The differences between Control and Reversed groups at 3 months are statistically significant (p<0.001).
- The differences between Reversed and Progression groups at 3 months are also statistically significant (p=0.02).
- The differences between Progression and Control groups at 9 months are statistically significant (p=0.06).

The graph shows a clear trend of increasing cholesterol levels followed by a decrease, with the Progression group demonstrating the most pronounced changes.
Supplementary Figure III

A

6 mon

Control  Progression  Reversed

12 mon

B

6 mon

12 mon
Supplementary Figure IV

(A) Ejection fraction (%) over time (months) for Control, Reversed, and Progression groups. Significant differences are indicated by * (p=0.06) and # (p=0.07).

(B) Fractional shortening (%) over time (months) for Control, Reversed, and Progression groups. Significant differences are indicated by * (p=0.06) and # (p=0.07).
Supplementary Figure VII

A

Total TGF-β1 (ng/ml) vs. Fibrosis score

$r = 0.48$, $p = 0.025$

B

EF (%) vs. Fibrosis score

$r = -0.87$, $p < 0.001$
Supplemental Methods

Mice

Wild-type (C57Bl/6, WT) mice were purchased from Jackson Laboratory. Reversa mice (Ldl<sup>-/-</sup>Apo<sup>100/100</sup>/Mttp<sup>fl/fl</sup>/Mx1Cre<sup>+/+</sup>) are Ldl<sup>-/-</sup> mice homozygous for an apo-B100-only allele, a conditional (“floxed”) Mttp allele and a Mx1-Cre transgene that causes near complete recombination in liver and spleen (kindly provided by Dr. Donald Heistad from University of Iowa).<sup>1</sup>-<sup>3</sup> Mice with a targeted deletion of Tgfb1 in megakaryocytes and platelets (Tgfb1<sup>flox/flox</sup>PF<sub>4</sub>-Cre<sup>+/+</sup>, abbreviated as Tgfb1<sup>flox</sup>; a kind gift of Dr. Jay Degen of the University of Cincinnati) was generated by interbreeding mice carrying a “floxed” TGF-β1 allele (Tgfb1<sup>flox</sup>, provided by Dr. Tom Deotschman, University of Arizona, Tucson, AZ)<sup>4</sup> and C57Bl/6 transgenic mice expressing Cre recombinase under the control of the megakaryocyte-specific platelet factor 4 (PF4) promoter (provided by Dr. Radek Skoda, University Hospital Basel, Basel, Switzerland).<sup>5</sup> All mice were housed in a controlled environment (23 ± 2°C; 12 hours light/dark cycles). All experimental procedures were approved by the Rockefeller University Institutional Animal Care and Use Committee (IACUC).

Diet-induced aortic valve stenosis

A total of 74 Reversa mice (8-12 weeks of age) were placed on either a chow diet or a western diet (WD, TD 88137; Harlan Tekland) and divided into 1) progression group: 28 mice on a WD for up to 12 months; 2) reversed group: 27 mice fed a WD for 6 months that then received 4 injections of pl-pC (225 µg, i.p.) at two-day intervals to inactivate the Mttp gene (and thus reduce their cholesterol levels) and switched to a chow diet for another 6 months; and 3) control group: 19 Reversa mice maintained on a chow diet for 12 months and treated with pl-pC at the age of 6 weeks. Echocardiography (see below) was performed before starting the special diets and every three months thereafter. Blood samples were obtained at the same time heart and aortic valve functions were evaluated under ultrasound guidance from left ventricle (LV) inflow tract.

Ascending aortic constriction (AAC)

Ascending aortic constriction was induced by controlled constriction of the ascending aorta as described by Hamawaki et al.<sup>6</sup>,<sup>7</sup> Briefly, mice were anesthetized with 1.5% isoflurane and the ascending aorta was surgically exposed by a mid-thorax incision. A
7-0 suture was placed around both the aorta and an adjacent 27-gauge (0.41 mm OD) needle proximal to the origin of the innominate artery. This temporarily caused complete occlusion of the aorta, but when the needle was removed the lumen was restored with an >80% reduction in diameter. Blood samples were obtained before surgery and 1 week and 4 weeks after surgery using the same technique described for the Reversa mice. Cardiac function was evaluated at the same time point using echocardiography.

**Echocardiography and systolic cardiac and aortic valve function**

Echocardiographic evaluation (Vevo770 system; VisualSonics, Toronto) of the heart and aortic valve function was performed in mice secured on a heating stage to maintain a body temperature between 36-37°C (monitored with a rectal temperature probe) and anesthetized with inhaled isoflurane (1.5%-2.0%; Aerrane, Baxter) at 1 L/min, 100% O2. Heart rate and ECG were continuously monitored (THM100, Indus Instruments). Using B-mode imaging, the transducer (30-MHz RMV707B) was positioned to image the longitudinal or short axis of the heart. Subsequently, the regions of interest were focused and cine loops were recorded for analysis. All echocardiographic measurements were made 3 times and the average values are reported.

**Aortic valve (AV) function assessment**

In brief, the AV of Reversa mice was located using B-mode in a modified left parasternal longitudinal axis window showing the left ventricular outflow tract, aortic root, and ascending aorta as described by Zhou et al. AV cusp movement was recorded in both M-mode (modified left parasternal longitudinal axis) and EKV-mode (modified left parasternal short axis). Briefly, the AV region in a modified left parasternal longitudinal axis was imaged by zooming at the level of the aortic root, showing the movement of the aortic cusps, and then AV cusp separation distance by M-mode was recorded.

**Systolic cardiac function**

Systolic cardiac function was evaluated by measuring the left ventricle ejection fraction (EF) and fractional shortening (FS) as described previously. Based on the B-mode imaging of the left ventricle long axis, EKV™ (ECG-Gated Kilohertz Visualization) mode was used to capture the rapid heart rate (500-650 beats per minute) and reconstruct the images. EF was calculated using parameters obtained by EKV mode, including
endocardial and epicardial areas and LV base-to-apex distance at end-systole and end-dialedstole.9

Wall shear stress (WSS) across the aortic valve in Reversa mice and the stenosis created in AAC model

Flow measurements were obtained using the pulse-wave Doppler mode after adjusting the position of transducer to keep the Doppler line parallel to the direction of flow so as to avoid the need for angle correction.10 In Reversa mice, the transducer was put at the position of the AV to measure the velocity across the valve. In the AAC model, the transducer was positioned over the ligated aortic region to measure the velocity across the stenosis. Since the WSS on the ventricular side of the AV has been shown to be higher than the WSS on the aortic side (64-71 dynes/cm² on the ventricular side vs. 15-25 dynes/cm² on the aortic side),12 we measured the WSS on the ventricular side. Wall shear stress was calculated by the equation \( \text{WSS} = \frac{4\mu V_m}{r} \), where \( \mu \) is the blood viscosity (estimated at 0.035 poise), \( V_m \) is the mean trans-stenotic velocity, and \( r \) is the radius (equal to half of the AV cusp separation distance in Reversa mice and the stenosis in AAC model).13,14

Plasma TGF-β1 and cholesterol

Plasma was prepared from blood obtained under ultrasound guidance from the LV inflow tract using a 30-gauge needle and 1 mL syringe containing 0.1 volume of 3.8% sodium citrate, pH 7.4 as described previously.9 Whole blood was centrifuged immediately at 12,000 g for 5 minutes at room temperature (RT) and aliquoted and stored at -80°C until assayed. Total TGF-β1 in plasma was measured with a 2-antibody ELISA assay specific for the activated form of TGF-β1 (R&D Systems) after converting latent TGF-β1 to active TGF-β1 by acidification (20 minute incubation at RT with 0.5 volume of 1 N HCl, followed by neutralization by adding the same volume of 1.2 N NaOH in 0.5 M HEPES). Plasma cholesterol levels were measured using an enzymatic colorimetric assay (439-17501, Wako Diagnostics).

Human valvular interstitial cell culture and Smad2 or Erk2 siRNA knock down

Human aortic valvular interstitial cells (VICs) were isolated from AVs purchased from the National Disease Research Interchange (DNRI, Philadelphia, PA).15 Briefly, the AV
leaflets were digested with collagenase II (600 U/ml) at 37°C for 10 minutes. The endothelial layer was gently removed by rotating a dry sterile swab on the surface of the leaflet. The remaining AV leaflets were further digested with collagenase II for 16-18 hours at 37°C and a suspension of single VICs obtained by passing the tissue through a 27g needle. VICs were pelleted, washed, and then cultured in M199 medium (11043-023, Life Technologies Inc.) with 10% FBS, 100 mg/L L-glutamine, 1% penicillin, streptomycin, and 1% amphotericin B (Fungizone, Life Technologies Inc.) at 37°C, 5% CO₂ for two to four passages. VICs were plated at 100,000 cells per well in 24-well or 6-well plates. After achieving ~80% confluence, VICs were treated with human platelet-derived TGF-β1 (101-B1-001, R&D) at 0.5 ng/ml for periods of time from 15 minutes to 24 hours. To reduce Smad2 levels, VICs were plated in 24 well plates for 24 hours and then transfected with siRNA specific for Smad2 (SI02757496, 5’-CAGGTAATGTATCATGATCCA-3’ and SI02636263, 5’-CTAGAAATACTCTCCATTAA-3’, Qiagen) using Lipofectamine RNAi MAX according to the manufacturer’s instructions. 6 hours after transfection, the transfection solution was removed and cells were cultured for 72 hours at 37°C before being stimulated with human platelet-derived TGF-β1 (0.5 ng/ml,100-B-001,R&D) for 1 hour at 37°C. For the gene expression experiment, cells were transfected with siRNA specific for either Smad2 or Erk2 (1022564, Qiagen) using the same protocol mentioned above. After removing the transfection solution from the wells, cells were cultured in medium supplied with 1% FBS for 72 hours. Cells were then stimulated with TGF-β1 at concentration of 5 ng/ml for 48 hours before being harvested for the gene expression studies. The use of human tissue was approved by the Rockefeller University Institutional Review Board (IRB).

Reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) of gene expression levels in VICs

After the treatment, cells were harvested and RNAs were isolated using RNasey Mini kit (74104, Qiagen) according to the manufacturer’s protocol. RNA with an A₂₆₀/₂₈₀ of >1.8 was used for cDNA synthesis with M-MLV reverse transcriptase (28025-013, Life Technology) and qPCR was performed in a 7500 Real-Time PCR system (Applied Biosystem) using SYBR green chemistry. Human Gapdh, Col1a2, Col3a1, CTGF, Vimentin, α-SMA, BMP4, Runx2, MMP13 and ALP primers were purchased from Integrated DNA Technologies.
Histology and immunofluorescent staining of mouse heart tissue and ascending aorta sections

Hearts with their attached aortic arches or ascending aorta with a knot placed around the area of constriction were excised, fixed in 10% neutral-buffered formalin for 24 hours, and processed for routine paraffin embedding. Tissues were cut in 5 μm thick sections and stained with hematoxylin and eosin (H&E) for general histology and Masson’s trichrome for collagen. For immunofluorescent staining, tissue sections were deparaffinized and rehydrated by immersing in xylene and a series of graded alcohols, followed by heat (97°C)-induced epitope retrieval using 10 mM citrate buffer, pH 7.0 for 30 minutes. Slides were first incubated for 1 hour at RT with 10% goat serum and then incubated overnight at 4°C with primary antibodies at final concentrations of 5 μg/ml. After washing in PBS and incubation with a fluorescent secondary antibody at RT for 1 hour, slides were washed with PBS three times and mounted with a fluorescence mounting medium containing diamidino-2-phenylindole (DAPI) to stain nuclei (H-1200, Vector). Images were photographed using an Olympus microscope equipped with a digital camera (BX60, Olympus). Primary antibodies included anti-phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (AB3849, Millipore), anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (9101S, Cell signaling), anti-vWf (LS-132638, LifeSpan Bioscience, Inc.) and anti-vimentin (ab20346, Abcam). The secondary antibodies were Alexa-488 or Alexa-594 conjugated goat anti-rabbit or goat anti-mouse (Life Technologies).

Alizarin Red staining for calcium on aorta of Reversa mice

The staining was performed according to the manufacturer’s protocol which is also reported by others. Briefly, tissue sections were deparaffinized, rinsed in distilled water and stained for 10 min in 2% (w/v) Alizarin Red solution (IW-3001, IHC world) in distilled water. The degree of red-orange staining was observed under the microscopy. Sections were then rinsed again, dehydrated in acetone and then in acetone-xylene (1:1) solution. Finally, sections were cleared in xylene and mounted using a synthetic mounting medium.

Immunoblot
Whole cell protein extracts were obtained by lysing VICs in Laemmli sample buffer (Bio-Rad) and heating to 100°C for 5 minutes. Samples were electrophoresed in 8% to 16% gradient Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes, which were then blocked for 1 hour with blocking buffer (927-40000, Odyssey). Membranes were incubated with the appropriate primary antibody in Odyssey blocking buffer with 0.1% Tween 20 (16 hours, 4°C), washed with Tris-buffered saline-Tween 20 (TBST), and incubated with the appropriate secondary antibody conjugated with an infrared fluorophore (LI-COR) for 1 hour at 4°C. Band intensities were visualized and quantified using the Odyssey system. Primary antibodies used included anti-phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (8828, Cell Signaling), anti-Smad2/3 (3102, Cell Signaling), anti-phospho-p44/42 MAPK (p-Erk1/2) (Thr202/Tyr204) (9101, Cell Signaling), anti-p44/42 MAPK (Erk1/2) (9107, Cell Signaling), anti-α-smooth muscle actin (α-SMA) (ab7817, Abcam), anti-α-smooth muscle actin (α-SMA) and anti-GAPDH (sc-365062, Santa Cruz).

To assess TGF-β1 signaling in whole blood after stirring, blood from WT and Tgfb1^flox^ mice was stirred for 40 minutes at 37°C, with or without PGE\textsubscript{1} (4µM) or an antibody that neutralizes active TGF-β1 (αTGF-β1, MAB240, R&D). Erythrocytes were then lysed (555899, BD) and the remaining cells were pelleted and lysed in Laemmli sample buffer. p-Smad2/3, total Smad2/3, and GAPDH were detected by immunoblotting and quantified as indicated above.

**Phosphorylation of Smad2/3 in leukocytes**

To assess TGF-β1 signaling before and after stirring, 200µl whole blood from WT and Tgfb1^flox^ mice was stirred for 40 minutes at 37°C with or without anti-TGF-β1 (αTGF-β1) antibody. Erythrocytes were lysed and cells were fixed using a lyse/fix buffer (555899, BD). To assess TGF-β1 signaling in circulating leukocytes of Reversa mice on either a chow diet or WD, 200µl whole blood was directly lysed and fixed without stirring. In both cases, cells were washed with phosphate buffered saline (PBS) once and then permeabilized with a proprietary buffer (BD™ Phosflow Perm Buffer III; 558050, BD) on ice for 30 minutes. After two washes with PBS/FBS buffer (554657, BD), cells were incubated with antibodies to CD45-Alexa 647 (103123, Biolegend) and p-Smad2/3-PE CF594 (562697, BD) and analyzed by flow cytometry using a BD LSR-II flow cytometer.

**Statistics**
Since the progression and reversed groups were treated the same for the first 6 months, data before the 6 month time point were pooled from these groups and reported as data from the progression group. Unless otherwise specified, continuous data are reported as mean ± standard error of measurement (SEM) if they were normally distributed and as median and interquartile range if they were not normally distributed. Data were tested for normality and treated accordingly. Differences in means between 2 independent groups were analyzed using a 2-sample Welch t test, whereas paired Student t test was used for paired samples. The Mann-Whitney U test was used for data that were not normally distributed and the Wilcoxon matched pairs test was used for paired samples. ANOVA or the Kruskal-Wallis test was used for comparisons involving more than two groups. Pearson product-moment correlation was used to correlate continuous values with underlying normality and sufficient sample size; otherwise, Spearman rank correlation ρ was used. Logrank test was used to test the difference of survival curves among groups. A 2-tailed p value of <0.05 was considered significant. Statistical analyses were performed using either Graphpad Prism 5.0 (La Jolla, CA) or R version 2.1.12. Histology images were analyzed using ImageJ (NIH) and immunoblots were analyzed using Odyssey system (LI-COR Bioscience, Lincoln, NE).

Reference:


