Endogenous Thrombospondin 1 Protects the Pressure-Overloaded Myocardium by Modulating Fibroblast Phenotype and Matrix Metabolism

Ying Xia, Marcin Dobaczewski, Carlos Gonzalez-Quesada, Wei Chen, Anna Biernacka, Na Li, Dong-Wook Lee, Nikolaos G. Frangogiannis

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Abstract—The matricellular protein thrombospondin (TSP) 1 is induced after tissue injury and may regulate reparative responses by activating transforming growth factor-β, by suppressing angiogenesis and by modulating matrix metabolism. We hypothesized that endogenous TSP-1 may be involved in the pathogenesis of cardiac remodeling in the pressure-overloaded heart. Myocardial TSP-1 expression was increased in a mouse model of pressure overload because of transverse aortic constriction. TSP-1−/− mice exhibited increased early hypertrophy and enhanced late dilation in response to pressure overload. Pressure-overloaded TSP-1 null mice had intense degenerative cardiomyocyte changes, exhibiting more extensive sarcomeric loss and sarcolemmal disruption when compared with wild-type hearts. Accentuated hypertrophy and cardiomyocyte injury in TSP-1−/− hearts was accompanied by increased myofibroblast density. However, despite a 2-fold higher infiltration of the cardiac interstitium with myofibroblasts, pressure-overloaded TSP-1 null hearts did not exhibit significantly increased collagen content when compared with wild-type hearts. The disproportionately low collagen content in TSP-1 null hearts was attributed to infiltration with abundant, but functionally defective, fibroblasts that exhibited impaired myofibroblast differentiation and reduced collagen expression in comparison with wild-type fibroblasts. Impaired myofibroblast activation in TSP-1 null hearts was associated with reduced Smad2 phosphorylation reflecting defective transforming growth factor-β signaling. Moreover, TSP-1 null hearts had increased myocardial matrix metalloproteinase 3 expression and enhanced matrix metalloproteinase 9 activation after pressure overload. TSP-1 upregulation in the pressure-overloaded heart critically regulates fibroblast phenotype and matrix remodeling by activating transforming growth factor-β signaling and by promoting matrix preservation, thus preventing chamber dilation. (Hypertension. 2011;58:902-911.) • Online Data Supplement

Key Words: fibrosis ■ matricellular proteins ■ cardiac remodeling ■ thrombospondin 1 ■ myofibroblast ■ transforming growth factor-β1 ■ collagen

Pressure overload induces cardiac remodeling through effects on both cardiomyocytes and cardiac interstitial cells. Hypertrophy and apoptosis of cardiac myocytes play an important role in the development of heart failure in the pressure-overloaded heart.1,2 On the other hand, fibroblast activation is a hallmark of the cardiomyopathic process after pressure overload because of transverse aortic constriction. TSP-1−/− mice exhibited increased early hypertrophy and enhanced late dilation in response to pressure overload. Pressure-overloaded TSP-1 null mice had intense degenerative cardiomyocyte changes, exhibiting more extensive sarcomeric loss and sarcolemmal disruption when compared with wild-type hearts. Accentuated hypertrophy and cardiomyocyte injury in TSP-1−/− hearts was accompanied by increased myofibroblast density. However, despite a 2-fold higher infiltration of the cardiac interstitium with myofibroblasts, pressure-overloaded TSP-1 null hearts did not exhibit significantly increased collagen content when compared with wild-type hearts. The disproportionately low collagen content in TSP-1 null hearts was attributed to infiltration with abundant, but functionally defective, fibroblasts that exhibited impaired myofibroblast differentiation and reduced collagen expression in comparison with wild-type fibroblasts. Impaired myofibroblast activation in TSP-1 null hearts was associated with reduced Smad2 phosphorylation reflecting defective transforming growth factor-β signaling. Moreover, TSP-1 null hearts had increased myocardial matrix metalloproteinase 3 expression and enhanced matrix metalloproteinase 9 activation after pressure overload. TSP-1 upregulation in the pressure-overloaded heart critically regulates fibroblast phenotype and matrix remodeling by activating transforming growth factor-β signaling and by promoting matrix preservation, thus preventing chamber dilation. (Hypertension. 2011;58:902-911.) • Online Data Supplement

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Thrombospondin (TSP) 1 is the archetypal matricellular protein, a 450-kD homotrimeric protein with multiple functional domains. TSP-1 is upregulated in injured tissues and is capable of regulating wound healing and tissue remodeling by modulating a variety of cellular functions essential to the reparative process. First, TSP-1 functions as an essential activator of transforming growth factor (TGF-β) signaling through an association with the latent complex that results in a conformational rearrangement in the latency-associated peptide (LAP) and renders the TGF-β dimer biologically active. Second, TSP-1 exerts potent angiostatic actions through direct effects on endothelial cells that result in inhibition of proliferation and increased apoptosis. Third, TSP-1 directly inhibits inflammation through CD47-mediated interactions. Fourth, TSP-1 modulates matrix metabolism by inhibiting matrix metalloproteinase (MMP) activation. We, and others, have reported important effects of TSP-1 in cardiac injury and repair. In healing myocardial infarction, TSP-1 is selectively deposited in the border zone and may serve as a protective “barrier” that protects the noninfarcted myocardium from extension of the inflammatory infiltrate by locally activating anti-inflammatory signals. Moreover, in a rat model of diabetic cardiomyopathy exacerbated by abdominal aortic coarctation, TSP-1 expression was upregulated, and administration of a peptide antagonist of TSP-1-dependent TGF-β activation prevented the development of cardiac fibrosis. Although these findings suggest that TSP-1 may modulate myocardial inflammation and repair, its role in cardiac remodeling because of pressure overload has not been investigated.

Our study examines for the first time the role of TSP-1 in the development of the fibrotic cardiomyopathy associated with pressure overload. We report that TSP-1 is markedly upregulated in the myocardial interstitial space after experimental transverse aortic constriction (TAC). TSP-1 gene disruption resulted in adverse cardiac remodeling of the pressure-overloaded heart, associated with infiltration of the myocardium with abundant, but dysfunctional, fibroblasts that exhibited defective myofibroblast transdifferentiation and decreased matrix-synthetic capacity. Accumulation of functionally impaired fibroblasts in the remodeling myocardium was associated with defective activation of TGF-β/Smad3 signaling and enhanced MMP activity. Our findings suggest that TSP-1 deposition in the extracellular matrix of the pressure-overloaded heart activates TGF-β-mediated signaling in reparative cardiac fibroblasts and preserves the extracellular matrix, affording protection from adverse remodeling.

Methods
A detailed description of the Methods is provided in the online Data Supplement (please see http://hyper.ahajournals.org). Animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Baylor College of Medicine and Albert Einstein College of Medicine institutional review boards. Male and female, wild-type (WT) and TSP-1 null C57/BL6 mice from our colony were genotyped using established protocols and underwent TAC as described previously. Functional assessment of the pressure-overloaded mouse heart was performed using echocardiography. At the end of the experiment, the heart was excised, fixed in zinc formalin, and embedded in paraffin for histological studies or frozen for RNA or protein isolation. Animals used for histology underwent 3, 7, and 28 days of banding (n=8 per group). Additional groups of mice were used for RNA and protein extraction after 3 (n=7) or 7 days (n=7) of banding, for isolation and flow cytometric analysis of cardiac fibroblasts (WT: n=10; TSP-1−/−: n=6), and for assessment of collagen content using a biochemical hydroxyproline assay (WT: n=9; knockout: n=12). As a control, a “sham” operation without aortic constriction was performed on age-matched mice (WT: n=6; RNA: n=6; protein: n=6). The effects of TSP-1 loss on the inflammatory and fibrotic responses were studied using immunohistochemistry, quantitative histology, quantitative PCR, ribonuclease protection assays, and Western blotting. Effects on apoptosis of cardiomyocytes and interstitial cells were investigated using TUNEL staining combined with wheat germ agglutinin lectin fluorescence to outline the surface of cardiomyocytes. The consequences of TSP-1 deficiency on TGF-β signaling were studied through assessment of Smad2 phosphorylation using Western blotting. Effects on matrix metabolism were assessed using zymography, whereas collagen content was measured with a hydroxyproline biochemical assay. The role of TSP-1 in modulating fibroblast phenotype and function in the pressure-overloaded myocardium was studied using isolated cardiac fibroblasts and flow cytometry on cells harvested from the myocardium.

Results

TSP-1 Is Upregulated in the Pressure-Overloaded Myocardium
Using quantitative PCR we demonstrated that TSP-1 mRNA expression was markedly induced in the pressure-overloaded heart after 3 to 7 days of TAC (Figure 1A). Immunohistochemical staining demonstrated that TSP-1 protein was primarily localized in the cardiac interstitial and perivascular space after 7 to 28 days of TAC (Figure 1B and 1C). TSP-1 immunoreactivity was absent in control mouse myocardium.

TSP-1 Null Mice Exhibit Increased Early Mortality After TAC
TSP-1 null and WT mice exhibited comparable mortality after TAC (survival after 28 days of TSP-1−/− 57.9% versus WT 55.3%; P value not significant; Figure 1D). However, TSP-1 null mice had significantly increased early mortality over the first 3 days after TAC (percentage of survival after 3 days of TSP-1−/− 65.7% versus WT 80.3%; P=0.05).

TSP-1 Null Mice Have Increased Hypertrophy and Dilatation After Pressure Overload
In the absence of injury, WT and TSP-1 null mice had comparable cardiac dimensions, function, and left ventricular (LV) mass (Table 1). After 7 days of TAC, WT mouse hearts exhibited a marked increase in LV mass, associated with reduced LV end-diastolic diameter, indicating the development of concentric LV hypertrophy. At the same time point, TSP-1 null mice had accentuated hypertrophic remodeling exhibiting significantly higher LV mass and a trend for a higher LV end-diastolic diameter in comparison with WT animals (Table 1). After 28 days of TAC, LV mass was comparable between TSP-1−/− and WT mice; however, TSP-1 null animals exhibited significantly higher LV end-diastolic diameter when compared with WT mice (P<0.05; Table 1). Thus, TSP-1 deficiency was associated with increased early hypertrophy and enhanced late dila-
tion in response to pressure overload. LV fractional shortening was comparable between WT and TSP-1−/− mice at all of the time points examined, suggesting that the absence of TSP-1 did not affect the development of systolic dysfunction in the pressure-overloaded heart.

Effects of TSP-1 Absence on Cardiomyocyte Alterations and Apoptosis in the Pressure-Overloaded Heart
To examine the basis for enhanced cardiac remodeling after TAC in the absence of TSP-1, we compared the pathological alterations in WT and TSP-1 null pressure-overloaded hearts. In both WT and TSP-1 null mice, TAC resulted in widening of the cardiac interstitium and focal cardiomyocyte degeneration. TSP-1 null animals had more intense cardiomyocyte changes after 3 to 7 days of TAC in comparison with WT animals, exhibiting more extensive sarcomeric loss and sarcolemmal disruption (Figure 2A through 2D). Combined TUNEL/wheat germ agglutinin lectin staining (Figure 2E through 2H) was used to identify apoptotic cardiomyocytes in the pressure-overloaded heart.

In both WT and TSP-1 null mice, the majority of apoptotic cells in the pressure-overloaded myocardium were noncardiomyocytes; apoptotic cardiomyocytes were rare. In WT hearts, the density of apoptotic cardiomyocytes peaked after 3 days of TAC; in contrast, TSP-1 null animals had a delayed peak in the number of apoptotic cells after 7 days of TAC (Figure 2I). The number of apoptotic interstitial cells was comparable between WT and TSP-1 null hearts at all of the time points examined (Figure 2J).

Despite a Marked Increase in Myofibroblast Density, Pressure-Overloaded TSP-1−/− Hearts Have No Significant Increase in Collagen Content
TSP-1 absence was associated with markedly increased myofibroblast density after 7 to 28 days of TAC (Figure 3A through 3C). Despite showing a 2-fold higher myofibroblast infiltration, TSP-1 null animals had no significant increase in total, soluble, and insoluble collagen content after 7 days of TAC when compared with WT hearts (Figure 3).

### Table 1. Echocardiographic Analysis of Systolic Function and Chamber Dimensions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre</th>
<th>7 d</th>
<th>Pre</th>
<th>28 d</th>
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<tr>
<td></td>
<td>WT</td>
<td>−/−</td>
<td>WT</td>
<td>−/−</td>
</tr>
<tr>
<td>LVESD, mm</td>
<td>3.85±0.12</td>
<td>3.75±0.09</td>
<td>3.23±0.19*</td>
<td>3.60±0.15</td>
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<tr>
<td></td>
<td>(P=0.12 vs WT)</td>
<td></td>
<td></td>
<td>(P&lt;0.05 vs WT)</td>
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<tr>
<td>LVESD, mm</td>
<td>2.49±0.27</td>
<td>2.30±0.11</td>
<td>2.36±0.23</td>
<td>2.49±0.30</td>
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<tr>
<td></td>
<td>(P&lt;0.14 vs WT)</td>
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<td></td>
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<tr>
<td>LVFS, mg</td>
<td>0.36±0.05</td>
<td>0.39±0.02</td>
<td>0.27±0.04</td>
<td>0.32±0.06</td>
</tr>
<tr>
<td></td>
<td>(P&lt;0.05 vs WT)</td>
<td></td>
<td></td>
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<tr>
<td>LVM, mg</td>
<td>68±2.79</td>
<td>79.7±3.7</td>
<td>104.9±6.7*</td>
<td>134.1±10.8</td>
</tr>
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</table>

LVESD indicates left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVFS, left ventricular fractional shortening; LVM, left ventricular mass; WT, wild-type; pre, before.

*P<0.01 vs corresponding pre.
examined whether the altered response of TSP-1 absence does not affect the angiogenic response in the pressure-overloaded myocardium. Because TSP-1 is a potent regulator of angiogenesis, we assessed whether increased cardiac remodeling in pressure-overloaded TSP-1 null mice is associated with increased expression of inflammatory mediators. TSP-1 null and WT animals showed no significant differences in the intensity and time course of the myocardial inflammatory response after TAC. RPA (RNAse protection assay) analysis demonstrated that expression of the proinflammatory cytokines tumor necrosis factor-α, interleukin 1β, and interleukin 6 was comparable between TSP-1−/− and WT hearts after 3 to 7 days of TAC (Table 2). Mac-2 immunohistochemistry showed that macrophage density was comparable between WT and TSP-1 null mice after 3 to 28 days of TAC (Figure 4A through 4C). In addition, expression of the CC chemokine monocyte chemoattractant protein (MCP) 1, a critical regulator of mononuclear cell recruitment, was comparable between groups (Figure 4D).

**TSP-1 Absence Does Not Affect the Inflammatory Response in the Pressure-Overloaded Heart**

Because TSP-1 is an important modulator of the inflammatory response, we examined whether increased cardiac remodeling in pressure-overloaded TSP-1 null mice is associated with increased expression of inflammatory mediators. TSP-1 null and WT animals showed no significant differences in the intensity and time course of the myocardial inflammatory response after TAC. RPA (RNAse protection assay) analysis demonstrated that expression of the proinflammatory cytokines tumor necrosis factor-α, interleukin 1β, and interleukin 6 was comparable between TSP-1−/− and WT hearts after 3 to 7 days of TAC (Table 2). Mac-2 immunohistochemistry showed that macrophage density was comparable between WT and TSP-1 null mice after 3 to 28 days of TAC (Figure 4A through 4C). In addition, expression of the CC chemokine monocyte chemoattractant protein (MCP) 1, a critical regulator of mononuclear cell recruitment, was comparable between groups (Figure 4D).

**TSP-1 Absence Does Not Affect the Angiogenic Response in the Pressure-Overloaded Myocardium**

Because TSP-1 is a potent regulator of angiogenesis, we examined whether the altered response of TSP-1−/− hearts to pressure overload may be attributed to changes in microvascular density. Cardiac microvessels were identified using staining with the endothelial marker CD31 (Figure 4E and 4F). TSP-1 null and WT animals showed no significant difference in microvascular density after 7 to 28 days of pressure overload (Figure 4G and 4H).

**Impaired Activation of TGF-β/Smad2/3 Signaling in the Pressure-Overloaded Heart**

The TGF-β/Smad2/3 pathway plays an important role in the pathogenesis of cardiac fibrosis. Because TSP-1 is an essential activator of TGF-β, we examined the effects of TSP-1 gene disruption on TGF-β signaling in the pressure-overloaded heart. WT and TSP-1 null hearts exhibited comparable levels of TGF-β1 mRNA and protein expression after 3 to 7 days of TAC (Figure 5A and 5B). Despite showing comparable TGF-β1 levels, TSP-1−/− animals had significantly reduced expression of p-Smad2 in the pressure-overloaded heart after 7 days of TAC, suggesting an important role for TSP-1 in activation of profibrotic TGF-β signaling (Figure 5C and 5D). In the absence of pressure overload, sham WT and TSP-1 null hearts had comparable myocardial p-Smad2 expression (P value not significant).
TSP-1 Null Mice Show Increased MMP-3 Expression and Enhanced MMP-9 Activity in the Pressure-Overloaded Myocardium

MMPs play an important role in cardiac remodeling, and TSP-1 regulates MMP expression and activity both indirectly by activating TGF-β and directly through inhibition of MMP-9 activity. Accordingly, we examined whether TSP-1 absence affected MMP expression in the pressure-overloaded heart. After 7 days of TAC, TSP-1−/− animals exhibited markedly higher expression of MMP-3 protein in the pressure-overloaded myocardium in comparison with WT animals (Figure 6B). In contrast, MMP-2, MMP-9, and tissue inhibitor of metalloproteinase 1 protein levels were comparable among groups. In addition, zymographic assessment of MMP activity demonstrated that active MMP-9 levels were significantly higher in TSP-1−/− hearts (Figure 6F). Active MMP-2 levels were comparable among groups (Figure 6E).

TSP-1 Null Fibroblasts Harvested From the Pressure-Overloaded Heart Exhibit Impaired Myofibroblast Transdifferentiation and Reduced Collagen Synthesis

In the absence of TSP-1, cardiomyocyte injury in the pressure-overloaded heart is followed by replacement with abundant fibroblasts that produce disproportionately low amounts of collagen. We postulated that these findings may reflect functional impairment of TSP-1 null cardiac

Table 2. Cytokine mRNA Expression in the Pressure-Overloaded Heart

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>72 h</th>
<th>7 d</th>
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<tbody>
<tr>
<td>Ratio to L32</td>
<td>WT</td>
<td>−/−</td>
<td>WT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.0022±0.0005</td>
<td>0.0013±0.0002</td>
<td>0.0085±0.0001*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.003±0.0007</td>
<td>0.0018±0.0003</td>
<td>0.01±0.001*</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.007±0.001</td>
<td>0.004±0.003</td>
<td>0.023±0.008*</td>
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</table>

*P<0.01 vs corresponding sham.

TNF indicates tumor necrosis factor; IL, interleukin; WT, wild-type.
fibroblasts. To test this hypothesis we assessed type I collagen and α-smooth muscle actin (SMA) expression in myofibroblasts harvested from pressure-overloaded hearts after 7 days of TAC using 2 distinct methods. First, early passage myofibroblasts cultured from pressure-overloaded hearts underwent dual immunofluorescence for α-SMA and type I collagen. TSP-1 null myofibroblasts were smaller, exhibited a poorly developed network of α-SMA+ myofilaments, and had reduced collagen type I staining in comparison with myofibroblasts harvested from WT hearts (Figure 7F and 7G). Second, cardiac cell suspensions were harvested from TSP-1 null and WT animals immediately after completion of a 7-day TAC protocol and were used for quantitative flow cytometric analysis (Figure 7A through 7E). Myofibroblasts were identified as α-SMA+/collagen I+ cells. Myofibroblasts isolated from pressure-overloaded TSP-1 null hearts had a significantly lower cell volume than cells isolated from WT hearts (Figure 7A). In addition, TSP-1 null cells exhibited significantly reduced mean fluorescent intensity for α-SMA (Figure 7B and 7C) and collagen I (Figure 7D and 7E). Thus, both immunofluorescence and flow cytometry experiments suggested that TSP-1 absence results in impaired myofibroblast transdifferentiation and activation after pressure overload.

**Discussion**

Matricellular proteins are a family of extracellular matrix proteins that do not provide structural support but serve as modulators of cell:matrix interactions, transducing signals during development and after tissue injury. Increased expression of matricellular proteins is a hallmark of cardiac remodeling. Studies using genetically targeted

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**Figure 4.** Thrombospondin (TSP) 1−/− and wild-type (WT) mice show comparable infiltration of the pressure-overloaded heart with macrophages and exhibit no significant difference in microvascular density. A and B, Mac2 immunohistochemistry was used to identify macrophages in WT (A) and TSP-1 null (B) pressure-overloaded hearts. C, Quantitative analysis showed infiltration of the pressure-overloaded myocardium with Mac2+ cells after 3 to 7 days of transverse aortic constriction (TAC; \(P < 0.01\) vs sham). The density of Mac2+ macrophages was comparable between WT and TSP-1 null animals at all of the time points examined. D, Quantitative PCR demonstrated that cardiac mRNA expression of the CC chemokine monocyte chemoattractant protein (MCP) 1, a potent mononuclear/macrophage chemoattractant, was comparable between TSP-1 null and WT hearts. E and F, Vascular endothelial cells were identified in the mouse myocardium using staining for CD31. Microvascular density was compared between WT (B) and TSP-1−/− (F) animals. No statistically significant difference was observed in subendocardial (G, endo) and subepicardial (H, epi) microvascular density between WT and knockout hearts after 7 to 28 days of TAC (\(P\) value not significant).

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**Figure 5.** Thrombospondin (TSP) 1 null mice exhibit impaired activation of transforming growth factor (TGF)-β/Smad2 signaling. A, Quantitative PCR demonstrated that TGF-β1 mRNA was upregulated in pressure-overloaded hearts after 3 days of transverse aortic constriction (TAC; \(P < 0.05\) vs sham). However, TGF-β1 mRNA (A) and protein (B) levels were comparable between TSP-1 null and wild-type (WT) animals. C, D, Activation of the Smad2/3 pathway was assessed using Western blotting for p-Smad2. TSP-1 null hearts exhibited significantly lower Smad2 phosphorylation after 7 days of transverse aortic constriction (TAC) than WT hearts \((P < 0.05)\).
mice have suggested that several members of the matricellular family play an important role in the pathogenesis of cardiac remodeling associated with pressure overload by modulating responses that may involve cardiomyocytes and fibroblasts. Osteopontin induction in the pressure-overloaded myocardium mediates cardiomyocyte hypertrophy through integrin-associated interactions without affecting development of fibrosis. Secreted protein acidic and rich in cysteine signaling is essential for postsynthetic collagen processing and cross-linking but does not affect cardiac hypertrophy; in its absence collagen deposition is reduced and myocardial diastolic stiffness is attenuated. Members of the TSP family have been suggested to play a role in cardiac remodeling. TSP-2 is induced in the pressure-overloaded myocardium associated with increased early hypertrophy and accentuated collagen synthesis. Alterations in 2 distinct pathways appear to be responsible for defective fibroblast activation and increased matrix remodeling in the pressure-overloaded TSP-1−/− heart. First, TSP-1 absence was associated with increased early hypertrophy and accentuated cardiac injury. In response to pressure overload, TSP-1 null hearts were infiltrated with abundant, but functionally impaired fibroblasts that exhibited defective myofibroblast transdifferentiation and reduced collagen synthesis. Second, TSP-1 was involved in regulation of matrix metabolism; TSP-1 loss resulted in enhanced MMP-9 activity and accentuated MMP-3 expression in the pressure-overloaded TSP-1−/− hearts (**P<0.01 vs corresponding wild-type [WT]); in contrast MMP-2, MMP-9, and tissue inhibitor of metalloproteinase (TIMP) 1 levels were comparable between groups (P<0.05 vs sham). E, F, Zymography showed significantly increased MMP-2 and MMP-9 activity in the pressure-overloaded myocardium after 7 days of TAC (**P<0.05 vs sham). Active MMP-9 levels (aMMP-9) were significantly higher in TSP-1 hearts (**P<0.05 vs WT), whereas MMP-2 activity (aMMP-2) was comparable between WT and −/− hearts (B). G, A representative image of a zymogram comparing MMP activity in the pressure-overloaded myocardium between WT (+/+), and TSP-1−/− mice after 7 days of TAC. Bands corresponding with active MMP-2 and active MMP-9 are identified.

Figure 6. Thrombospondin (TSP) 1 null hearts have increased matrix metalloproteinase (MMP) 3 expression and enhanced MMP-9 activity after 7 days of pressure overload. A through D, Quantitative analysis of Western blotting showed that MMP-3 protein levels were significantly higher in pressure-overloaded TSP-1−/− hearts (**P<0.01 vs corresponding wild-type [WT]); in contrast MMP-2, MMP-9, and tissue inhibitor of metalloproteinase (TIMP) 1 levels were comparable between groups (P<0.05 vs sham). E, F, Zymography showed significantly increased MMP-2 and MMP-9 activity in the pressure-overloaded myocardium after 7 days of TAC (**P<0.05 vs sham). Active MMP-9 levels (aMMP-9) were significantly higher in TSP-1 hearts (**P<0.05 vs WT), whereas MMP-2 activity (aMMP-2) was comparable between WT and −/− hearts (B). G, A representative image of a zymogram comparing MMP activity in the pressure-overloaded myocardium between WT (+/+), and TSP-1−/− mice after 7 days of TAC. Bands corresponding with active MMP-2 and active MMP-9 are identified.

unknown. Our study demonstrates for the first time that TSP-1 is induced in the pressure-overloaded myocardium and may serve as a protective signal that prevents development of cardiac remodeling through effects on fibroblast function and matrix metabolism. TSP-1 absence was associated with increased early hypertrophy and accentuated cardiac injury. In response to pressure overload, TSP-1 null hearts were infiltrated with abundant, but functionally impaired fibroblasts that exhibited defective myofibroblast transdifferentiation and reduced collagen synthesis. Alternations in 2 distinct pathways appear to be responsible for defective fibroblast activation and increased matrix remodeling in the pressure-overloaded TSP-1−/− heart. First, TSP-1 absence was associated with attenuated TGF-β/Smad2 signaling, leading to an impairment in myofibroblast transdifferentiation and to reduced fibroblast-derived matrix synthesis. Second, TSP-1 was involved in regulation of matrix metabolism; TSP-1 loss resulted in enhanced MMP-9 activity and accentuated MMP-3 expression in the pressure-overloaded heart.

In the cardiac interstitium, extracellular matrix proteins not only form a structural framework that provides me-
chanical support but also transduce signals that regulate cell survival, phenotype, and function. TSP-1 limits adverse remodeling of the pressure-overloaded heart through modulatory effects on fibroblast function. Progressive cardiomyocyte loss triggers inflammatory and profibrotic pathways leading to infiltration of the pressure-overloaded myocardium with activated myofibroblasts. Although TSP-1 is known to exert direct anti-inflammatory actions through CD47, TSP-1 null and WT mice exhibited comparable levels of inflammatory signals in the pressure-overloaded myocardium (Table 2). However, TSP-1 absence significantly affected maturation and activation of myofibroblasts in the pressure-overloaded heart. Because of increased cardiomyocyte injury, TSP-1 null hearts exhibited markedly accentuated myofibroblast infiltration and more extensive areas of reparative fibrosis. However, despite the increased number of fibroblasts, TSP-1 null hearts showed no significant increase in collagen content after pressure overload (Figure 3), reflecting the functional impairment observed in TSP-1 null fibroblasts. Flow cytometry and immunofluorescence on cells harvested from the remodeling pressure-overloaded myocardium demonstrated that, in the absence of TSP-1, cardiac fibroblasts exhibited defective myofibroblast transdifferentiation and significantly reduced collagen synthesis (Figure 7). These defects were associated with a significant decrease in Smad2 phosphorylation, suggesting that impaired fibroblast activation in the absence of TSP-1 may be...
because of defective TGF-β activation (Figure 5). TSP-1 is critically involved in TGF-β activation by binding to the LAP, inducing a conformational change in the latent complex that renders the TGF-β dimer biologically active. In addition, the LAP:TSP-1 interaction may occupy LAP molecules, preventing the generation of new inactive LAP:TGF-β small latent complexes, because TSP-associate LAP does not confer latency on active TGF-β.15

Why does impaired myofibroblast activation in TSP-1 null pressure-overloaded hearts contribute to adverse remodeling? The functional consequences of TSP-1 loss are probably attributed to the combination of defective myofibroblast function and accentuated matrix remodeling. In the absence of TSP-1, impaired TGF-β signaling in cardiac fibroblasts results in decreased collagen deposition, leading to formation of a matrix that provides inadequate mechanical support to the ventricle. In addition, accentuated MMP-3 expression and MMP-9 activity in TSP-1−/−-pressure-overloaded hearts (Figure 6) may also contribute to adverse remodeling by promoting matrix degradation. Because MMP-3 is the most efficient MMP-9 activator identified to date,27 the increased MMP-9 activity observed in TSP-1 null pressure-overloaded hearts may be attributed to accentuated MMP-3 expression. Inhibitory effects of TSP-1 on MMP-9 activity have been suggested previously to play an important role in suppression of tumor growth.28 It should be noted that, despite the increased matrix degradation and enhanced cardiomyocyte injury observed in TSP-1 null hearts after pressure overload, cardiac systolic function was preserved. This finding suggests that TSP-1 absence does not affect cardiomyocyte contractility but may also reflect the compensatory effects of the increased LV mass in TSP-1−/− hearts.

Perspectives
The effects of TSP-1 in the remodeling heart are attributed to a combination of protective actions involving cardiac fibroblasts and the extracellular matrix. Pressure overload results in early MMP activation and matrix degradation; stimulation of a matrix-preserving fibroblast phenotype provides mechanical support to the myocardium preventing chamber dilation and adverse remodeling. TSP-1 induction in the cardiac interstitium may serve as a protective mechanism against cardiac remodeling that regulates the reparative properties of cardiac fibroblasts activating TGF-β signaling and inhibiting MMP activity, thus promoting matrix preservation.

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Disclosures
None.

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ENDOGENOUS THROMBOSPONDIN-1 PROTECTS THE PRESSURE-OVERLOADED MYOCARDIUM BY MODULATING FIBROBLAST PHENOTYPE AND MATRIX METABOLISM

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ONLINE SUPPLEMENT:
SUPPLEMENTAL METHODS:

1. Animal protocols.

Animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Baylor College of Medicine and Albert Einstein College of Medicine Institutional Review Boards. Male and female, 6-12 month-old WT and TSP-1 null C57/BL6 mice from our colony were genotyped using established protocols. Animals were anesthetized with inhaled isoflurane. Aortic banding was achieved by creating a constriction between the right innominate and left carotid arteries as previously described. The degree of pressure overload was assessed by measuring right-to-left carotid artery flow velocity ratio after constricting the transverse aorta. Only mice with a flow ratio from 5:1 to 10:1 were used for analysis. At the end of the experiment, the heart was excised, fixed in zinc-formalin, and embedded in paraffin for histological studies, or frozen for RNA/protein isolation. Animals used for histology underwent 3, 7, and 28 days of banding (n=8/group). Additional groups of mice were used for RNA and protein extraction after 3 (n=7) or 7 days (n=7) of banding, and for isolation and flow cytometric analysis of cardiac fibroblasts (WT, n=10; TSP-1 -/-, n=6). As a control, a "sham" operation without aortic constriction was performed on age-matched mice (histology n=6, RNA n=6, protein n=6).

2. Echocardiographic analysis

Short axis M-mode echocardiography was performed prior to instrumentation and before the end of each experiment (3, 7 or 28 days of TAC) using a Sequoia C256 system (Acuson, Mountain View, CA) as previously described. The following parameters were measured as indicators of function and remodeling: left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), fractional shortening (FS=[LVEDD-LVESD]x100/LVEDD), interventricular septal (IVS) thickness, posterior wall (PW) thickness and left ventricular mass (LV mass).

3. Immunohistochemistry and quantitative histology.

Sections were cut at 5 μm and stained immunohistochemically as previously described. The collagen network was labeled using picrosirius red staining. Myofibroblasts were identified using staining with an antibody to α-smooth muscle actin (α-SMA) (Sigma, St. Louis, MO) as spindle-shaped cells located outside the vascular media. Macrophages were labeled using a rat anti-mouse Mac-2 antibody (Cedarlane, Burlington NC). Endothelial cells were identified using staining with a rat anti-mouse CD31 antibody (Pharmingen, San Diego CA). Staining was performed with a peroxidase-based technique using the Vectastain ELITE kit (Vector, Burlingame CA) and developed with diaminobenzidine and nickel. The Mouse to Mouse (MOM) kit was used for α-SMA staining. For CD31 staining the Tyramide Signal Amplification (TSA) kit (Perkin Elmer, Boston MA) was used on sections pre-treated with trypsin. Quantitative assessment of myofibroblast and macrophage density was performed by counting the number of cells/myocardial area using Image Pro software. Microvascular density was assessed by counting the number of vascular profiles in the subendocardial and subepicardial area. Eight fields from three different stained sections were used for analysis.

4. Assessment of apoptosis using TUNEL staining and WGA lectin fluorescence.
Identification of apoptotic cardiomyocytes and interstitial cells in pressure-overloaded hearts was performed using fluorescent \textit{In situ} Cell Death Detection Kit (Roche) and WGA staining of cell membranes. Briefly, paraffin sections were incubated at 55°C for 4h and allowed to cool down for 2h. Subsequently, slides were deparaffinized, rehydrated in graded alcohols and subjected to antigen retrieval with 20 µg/ml Proteinase K (Promega) in modified TE buffer (50mM Tris Base, 1mM EDTA, 0.5% Triton X-100, pH 8.0) for 30 min at 37°C. Slides were washed several times in PBS and stained with 10 µg/ml biotynylated WGA lectin (Vector Labs) in PBS with Ca^{2+} and Mg^{2+} for 1h at RT. After several washes in PBS slides were stained steptavidin-Texas Red X complex (Invitrogen). Nuclei were stained with DAPI (Invitrogen). Finally, slides were incubated with TUNEL reaction mixture for 1h at 37°C. The densities (cells/mm²) of TUNEL+ cardiomyocytes and TUNEL+ noncardiomyocytes were quantitatively assessed in entire paraffin cross-section of myocardium and averaged using 3 nonadjacent sections from base, mid-myocardium and apex of each heart.

5. Collagen crosslinking assay.
To assess crosslinking of collagen in pressure overloaded hearts we adopted a method described by Mukherjee et al. In this method, PBS and sodium dodecyl sulfate (SDS) are used to remove the bulk of noncollagen proteins and freshly deposited collagen, leaving insoluble residue of crosslinked collagen. Incubation with cyanogen bromide (CNBr) allows determining the degree of collagen cross-linking based on solubility to CNBr. Briefly, hearts were lyophilized and dry weights were recorded. Subsequently hearts were homogenized with PBS and homogenate was centrifuged at 4,000 g for 10 min. Supernatant was retained and remaining pellet was rehomogenized with 10% SDS and incubated at 4°C for 2h. Homogenate was again centrifuged 4,000g for 10 minutes and supernatant was retained. The remaining residue was further extracted three times with PBS to remove the excess SDS. The residue then was rehomogenized in acetone and centrifuged at 4,000g for 10 minutes; the supernatant was discarded. This step was repeated, and the pellet was dried under vacuum. The acetone-dried powder was homogenized with solution of 20 mg/ml cyanogen bromide (Sigma) in 70% vol/vol formic acid. The homogenate was transferred to 5 ml glass ampoules, purged with nitrogen gas and sealed using propane torch. The reaction was allowed to proceed for 18 hours at 25°C. At the completion of the reaction, the digest was centrifuged at 5,000g for 20 minutes and supernatant was retained. At this point all collected supernatants and CNBr insoluble pellet were dried in vacuum centrifuge. Pellets were rehomogenized with 6N HCl, transferred 5 ml glass ampoules, sealed under vacuum and allowed to hydrolyze at 110°C for 16h. Subsequently, samples were evaporated in vacuum centrifuge and were oxidized using 1.27% chloramine T (Sigma, St. Louis, MO), 10% n-propanol, 0.2 M sodium citrate, and 0.5 M sodium acetate, 0.7 M sodium hydroxide at pH 6.5. After 20 min of incubation at room temperature, Erlich's solution (1 M p-dimethylaminobenzaldehyde [Sigma] in 70% n-propanol, 20% perchloric acid) was added and a 15 min incubation at 65°C performed. Absorbance was measured at 550 nm and the amount of hydroxyproline was determined against a standard curve. Total and insoluble collagen was expressed as µg of collagen/mg of dry tissue. The index of collagen crosslinking was defined as ratio of insoluble collagen to total collagen.

6. Ribonuclease protection assay (RPA)
mRNA expression levels of Tumor Necrosis Factor (TNF)-a, Interleukin (IL)-1b and IL-6 was determined using an RPA (RiboQuant; Pharmingen) as previously described.

7. Quantitative PCR

Isolated total RNA from mouse hearts was reverse transcribed to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad) following the manufacturer’s guidelines. Quantitative PCR was performed using the SYBR green (Bio-Rad) method on the iQ™5 Real-Time PCR Detection System (Bio-Rad). Primers were synthesized at the Baylor College of Medicine Child Health Research Center core facility. The following sets of primers were used in the study: TGF-β1 (forward), GACGAGCTGGTTGAGAGAAG, (reverse) CGCAGTGCCAAGAAGTCC; MCP-1 (forward) TGAAGTTGACCCGTAAATCTGAAG, (reverse) AAGGCATCACAGTCCGAGTC; TSP-1 (forward) AAGACATTCTCAGGAACAAAAGG, (reverse) TGTTGGAACGATAGC.

8. Protein extraction and western blotting

Protein was isolated from whole hearts. Western blotting was performed as previously described using the following antibodies: rabbit anti-mouse p-Smad2(ser465/467) (Cell Signaling), rabbit monoclonal anti-TGF-β antibody (Cell Signaling), goat anti-MMP-2, rat anti-MMP-3, goat anti-MMP-9 and rat anti-TIMP-1 (all from R&D).

9. Zymography

MMP activity in the pressure overloaded myocardium was examined by gelatin zymography as previously described. Myocardial samples were homogenized in 300 μl of an ice-cold extraction buffer containing cacodylic acid (10 mmol/L), NaCl (150 mmol/L), ZnCl₂ (20 mmol/L), NaN₃ (1.5 mmol/L) and 0.01% Triton X-100 (pH 5.0). Subsequently, the homogenate was centrifuged (4°C, 10 min, 10,000g), the supernatant decanted and saved on ice. The protein concentration in tissue extracts was measured using BCA Protein Assay Kit (Thermo Scientific). The myocardial extracts at final protein concentration of 20 μg were mixed in a ratio 1:2 with Zymogram Sample Buffer (Bio Rad) and loaded onto 10% polyacrylamide electrophoretic precast gels (Bio Rad) containing 1 mg/ml of gelatin under non-reducing conditions. The gels were run at 50V/gel through stacking phase and 100v/gel for the separating phase, maintaining a running buffer temperature of 4°C. Subsequently, the gels were renaturated in 2.5% Triton X-100 for 30 min, rinsed in water and incubated for 48h in Zymogram Development Buffer (Bio Rad) at 37°C. After incubation the gels were stained with Coomassie brilliant blue R-250 (Bio Rad) and subsequently destained until clear bands appeared against blue background. Digital images were scanned and the optical density of the bands was measured using ImageJ software.

10. Isolation of cardiac myofibroblasts

Cardiac fibroblasts were isolated from WT and TSP-1 null hearts after 7 days of TAC as previously described, fixed for 10 min in 2% paraformaldehyde (Sigma), permeabilized using 0.1% Triton-X (Sigma) and stained using dual immunofluorescence. Slides were stained with an anti-α-SMA antibody (Sigma), followed by incubation with a biotinylated secondary and amplification with Streptavidin Alexa Fluor 488 (Invitrogen). After brief treatment with avidin/biotin blocking kit (Vector) staining with a rabbit anti-type I collagen antibody was performed (Rockland Inc), followed by incubation with a biotinylated secondary antibody and amplification with Streptavidin Alexa Fluor 594 (Invitrogen).
11. Flow cytometry on cells harvesting of cells from the pressure-overloaded myocardium.

Single cell suspensions were prepared from WT (n=10) and KO (n=6) hearts after 7 days of TAC and underwent flow cytometric analysis as previously described 4. Flow cytometry was performed using the FITC-conjugated anti-α-SMA (Sigma, Clone 1A4) and purified rabbit anti-collagen I (Rockland Inc.) antibodies. Cell permeable DRAQ5 dye (Alexis Biochemicals) was used to define the gate for nucleated cells. Data analysis was performed using FlowJo (Tree Star, Inc).

12. Statistical analysis

Statistical analysis was performed using ANOVA followed by t-test corrected for multiple comparisons (Student-Newman-Keuls). Paired t-test was used to compare echocardiographic endpoints before instrumentation and after TAC. Data were expressed as mean±SEM. Statistical significance was set at 0.05.

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