Smad3 Signaling Promotes Fibrosis, While Preserving Cardiac and Aortic Geometry in Obese Diabetic Mice

Biernacka et al: Smad3 in Diabetic Cardiac Fibrosis

Anna Biernacka1*, MD; Michele Cavalera1*, PhD; Junhong Wang1, MD; Ilaria Russo, MBiol1; Arti Shinde1, PhD; Ping Kong1, PhD; Carlos Gonzalez-Quesada1,2, MD; Vikrant Rai1, MD; Marcin Dobaczewski1,2, MD; Dong-Wook Lee1, MD; Xiao-Fan Wang3, PhD; Nikolaos G. Frangogiannis1,2, MD

1The Wilf Family Cardiovascular Research Institute, Department of Medicine (Cardiology), Albert Einstein College of Medicine, Bronx NY; 2Department of Medicine, Baylor College of Medicine, Houston, TX; 3Department of Pharmacology and Cancer Biology, Duke University School of Medicine, Durham NC.

*These authors contributed equally

Correspondence to
Nikolaos Frangogiannis, MD
The Wilf Family Cardiovascular Research Institute, Albert Einstein College of Medicine
1300 Morris Park Avenue Forchheimer G46B, Bronx NY 10461.
Tel: 718-430-3546
Fax: 718-430-8989
Email: nikolaos.frangogiannis@einstein.yu.edu

DOI: 10.1161/CIRCHEARTFAILURE.114.001963

Journal Subject Codes: Subject codes: [113] Obesity; [115] Remodeling; [147] Growth factors/cytokines; [148] Heart failure - basic studies
Abstract

Background—Heart failure in diabetics is associated with cardiac hypertrophy, fibrosis and diastolic dysfunction. Activation of transforming growth factor (TGF)-β/Smad3 signaling in the diabetic myocardium may mediate fibrosis and diastolic heart failure, while preserving matrix homeostasis. We hypothesized that Smad3 may play a key role in the pathogenesis of cardiovascular remodeling associated with diabetes and obesity.

Methods and Results—We generated leptin-resistant db/db Smad3 null mice (dbSKO) and db/db Smad3 +/- animals (dbShet). Smad3 haploinsufficiency did not affect metabolic function in db/db mice, but protected from myocardial diastolic dysfunction, while causing left ventricular chamber dilation. Improved cardiac compliance and chamber dilation in dbShet animals was associated with decreased cardiomyocyte hypertrophy, reduced collagen deposition and accentuated matrix metalloproteinase (MMP) activity. Attenuation of hypertrophy and fibrosis in dbShet hearts was associated with reduced myocardial oxidative and nitrosative stress. dbSKO mice had reduced weight gain and decreased adiposity associated with attenuated insulin resistance, but also exhibited high early mortality, in part due to spontaneous rupture of the ascending aorta. Ultrasound studies showed that both lean and obese Smad3 null animals had significant aortic dilation. Aortic dilation in dbSKO mice occurred despite reduced hypertension, and was associated with perturbed matrix balance in the vascular wall.

Conclusions—Smad3 mediates diabetic cardiac hypertrophy, fibrosis and diastolic dysfunction, while preserving normal cardiac geometry and maintaining the integrity of the vascular wall.

Key Words: obesity, diabetic cardiomyopathy, fibrosis, cardiac remodeling, TGF-β
Diabetes and obesity are associated with an increased incidence of cardiovascular disease\textsuperscript{1,2} and are strong independent predictors of heart failure\textsuperscript{3}. Development of heart failure in diabetics is not only due to an increased incidence of ischemic heart disease, but is also associated with a cardiomyopathy independent of coronary disease, termed diabetic cardiomyopathy. Diabetic cardiomyopathy is characterized by extensive fibrotic changes and cardiomyocyte hypertrophy leading to increased myocardial stiffness and diastolic dysfunction\textsuperscript{4,5}. Despite its potential significance in diabetes-associated heart disease, the pathophysiologic basis of diabetic fibrosis remains poorly understood.

Extensive evidence suggests that transforming growth factor (TGF)-β is an essential fibrogenic mediator\textsuperscript{6}; however, the relative role of TGF-β-activated pro-fibrotic pathways remains unknown. In some studies, pro-fibrotic actions of TGF-β were attributed to downstream activation of the Smad3 pathway\textsuperscript{7,8}, a key intracellular effector signal in TGF-β-mediated fibrosis\textsuperscript{9}. Other investigations using models of cutaneous, pulmonary, and renal fibrosis suggested crucial fibrogenic actions of Smad-independent cascades\textsuperscript{10,11}. In addition to its pro-fibrotic actions, TGF-β may also contribute to the development of cardiac hypertrophy, acting downstream of angiotensin II\textsuperscript{12}. Induction and activation of TGF-β is consistently found in diabetic tissues and has been implicated in the pathogenesis of organ dysfunction; stimulation of TGF-β signaling pathways is associated with cardiac fibrosis in experimental models of type 1\textsuperscript{13} and type 2 diabetes\textsuperscript{14}. Accordingly, we hypothesized that activation of the Smad3 cascade in diabetic tissues, may play a critical role in the pathogenesis of hypertrophic and fibrotic remodeling in the diabetic heart.

In order to test this hypothesis, we generated obese diabetic leptin-resistant db/db mice that lack Smad3. Our findings show for the first time that Smad3 is implicated in the
pathogenesis of fibrosis, cardiomyocyte hypertrophy and diastolic dysfunction in obese diabetic mice, but also exerts important homeostatic functions, preserving left ventricular and aortic geometry. Smad3 disruption resulted in chamber dilation, associated with enhanced myocardial matrix metalloproteinase (MMP) activity, suggesting that myocardial Smad3 signaling is important for preservation of the interstitial matrix and maintains chamber geometry. Moreover, complete loss of Smad3 was associated with aortic dilation and an increased incidence of spontaneous aneurysmal rupture in both lean and db/db animals suggesting an important role for Smad3 in preserving integrity of the vascular wall.

Methods
(Details are provided in the supplement)

Generation of db/db mice lacking Smad3. Animal studies were approved by the Institutional Animal Care and Use Committee at Albert Einstein College of Medicine and the Animal Protocol Review Committee at Baylor College of Medicine. Because homozygous db/db mice are sterile, we crossed heterozygous db/+ with heterozygous Smad3 +/- (both from our own colonies on a C57BL6J background). We genotyped (using PCR) the first generation of offspring (F1) for the db and Smad3 allele and subsequently identified male and female double heterozygous mice, using them to breed the second generation of offspring (F2). For survival analysis male and female db/db mice, Smad3 -/- (Shet) mice, Smad3 +/- mice (SKO), db/db Smad3 +/- (dbShet) mice and db/db Smad3 -/- mice (dbSKO) were followed for 6 months and deaths were recorded.

Echocardiography and aortic ultrasound: WT, Shet, SKO, db/db, dbShet and dbSKO mice were imaged using a Vevo770 ultrasound system (VisualSonics, Toronto, Canada).
Assessment of body fat content: Body composition was quantitatively assessed using nuclear magnetic resonance.

Immunohistochemistry and histology: Histopathological analysis was performed using zinc formalin-fixed, paraffin-embedded hearts from 6 month-old mice.

RNA extraction and qPCR analysis: Quantitative PCR was performed using the SYBR green method on the iQ™5 Real-Time PCR Detection System. PCR primers are listed in Supplemental Table 1.

Protein extraction and western blotting: Protein was isolated from WT, db/db and dbShet hearts and Western blotting was performed.

Zymography: Matrix metalloproteinase (MMP) activity in murine WT, db/db and dbShet hearts was assessed by gelatin zymography.

Biochemical assessment of collagen content: Myocardial collagen content was measured using a hydroxyproline assay.

Assessment of plasma glucose, insulin and HOMA IR: Serum insulin levels were measured using the mouse enzyme-linked immunosorbent assay kits. Glucose levels were measured by the glucose oxidase method.

Arterial catheterization and blood pressure measurements: Invasive blood pressure measurements were performed with a Pressure:Volume analysis system.

Pressure-Volume analysis in isolated perfused hearts: Left ventricular pressure-volume analysis was performed using progressive isovolumic Langendorff retrograde perfusion of isolated murine hearts.
**Assessment of oxidative stress:** Myocardial reactive oxygen species (ROS) levels were measured using a fluorometric assay based on the conversion of 2', 7'-dicholrofluorescein-diacetate (DCFDA) to the highly fluorescent DCF in the presence of ROS\(^\text{15}\).

**Statistical analysis:** Data are expressed as mean ± SEM. For comparisons of two groups unpaired, 2-tailed Student’s t-test using (when appropriate) Welch’s correction for unequal variances was performed. The Mann-Whitney test was used for comparisons between 2 groups that did not show Gaussian distribution. For comparisons of multiple groups, 1-way ANOVA was performed followed by Tukey’s multiple comparison test. The Kruskall-Wallis test, followed by Dunn’s multiple comparison post-test was used when one or more groups did not show Gaussian distribution. Although adjustments for multiple comparisons in ANOVA were implemented, no additional adjustment for multiple testing was performed. Survival analysis was performed using the Kaplan-Meier method. Mortality was compared using the log rank test.

**Results**

1. **Cardiac hypertrophy, fibrosis, and TGF-β/Smad activation in db/db hearts.**

db/db mice had a marked and progressive increase in left ventricular (LV) mass (Figure 1A-C) reflecting predominant hypertrophic remodeling. Female db/db mice had earlier development of cardiac hypertrophy (Supplemental Figure I). db/db hearts showed a marked increase in collagen content, a trend towards increased myocardial TGF-β1 expression and markedly higher p-Smad2 levels (Figure 1D-F).
2. Smad3 loss is associated with increased mortality in lean and in db/db mice.

Follow-up to the age of 6 months showed that db/db and WT animals have comparable survival curves. Smad3 heterozygosity did not affect survival in db/db and WT animals (Figure 2A). However, both lean SKO and dbSKO animals had significantly increased mortality when compared to their corresponding Smad3+/+ littermates. Survival of dbSKO mice after 6 months of age was very rare (Figure 2B). In contrast, about 40% of SKO mice survived up to 6 months of age. Complete loss of Smad3 in db/db mice was associated with attenuated weight gain due to significant reductions in both lean and percent fat (Figure 2C-E). Although WT and db/db mice had comparable lean weight (Figure 2H), fat weight and percent fat were significantly higher in db/db animals (Figure 2I-J). Complete Smad3 loss in db/db mice was associated with reduced weight gain, associated with reductions in both lean weight and fat weight (Figure 2G). db/db mice had markedly increased body weight (BW) (Figure 2G). Although WT and db/db mice had comparable lean weight (Figure 2H), fat weight and percent fat were significantly higher in db/db animals (Figure 2I-J). Complete Smad3 loss in db/db mice was associated with attenuated weight gain due to significant reductions in both lean weight and fat weight (Figure 2G-I). In both lean and db/db animals, Smad3 haploinsufficiency had no effects on weight, lean weight and fat weight at 2-4 months of age. However, at 6 months, dbShet animals and Shet mice had increased adiposity. 6 month-old Shet mice had higher fat weight and fat percent content than age-matched WT mice (Figure 2E-F). Moreover, 6 month-
old dbShet mice had significantly higher fat weight than corresponding db/db animals (Figure 2I). Fat percent content was comparable between db/db abd dbShet mice at all timepoints studied (Figure 2J).

Smad3 loss did not affect fasting plasma glucose, insulin levels and HOMA IR in lean animals. db/db mice exhibited hyperglycemia, hyperinsulinemia, and markedly increased HOMA IR. Complete loss of Smad3 in db/db mice was associated with significantly attenuated hyperglycemia and a trend towards reduced insulin levels and decreased HOMA IR (Figure 2K-M). In contrast, Smad3 haploinsufficiency did not affect metabolic parameters in db/db animals.

4. dbSKO mice exhibit cardiac dilation and systolic dysfunction.

In lean mice, global Smad3 loss did not affect chamber dimensions and systolic function. SKO mice and WT controls had comparable LVEDD (Figure 3A, Supplemental Table 2) and LVEDV (Figure 3B); however, because of their smaller size, Smad3 null animals had a significantly higher LVEDD:BW ratio (Figure 3C). Smad3 loss in lean mice did not affect ejection fraction and LV mass (Figure 3D-E).

In db/db mice, both partial and complete Smad3 loss was associated with chamber dilation and a modest but significant reduction in ejection fraction. dbShet mice had significantly higher LVEDD and LVEDV (Figure 3F-H, Supplemental Figure III) than db/db animals. Female dbShet mice exhibited more severe chamber dilation than male animals (Supplemental Table 3). Although complete loss of Smad3 did not affect absolute chamber dimensions in db/db mice, dbSKO animals exhibited a much higher LVEDD:BW ratio (Figure 3G). Chamber dilation in dbShet and dbSKO mice was associated with mildly reduced ejection fraction (Figure 3I). Moreover, LV mass and cardiomyocyte size was significantly lower in dbSKO animals (Figure
3J-K, Supplemental Figure IV). Because dbSKO mice did not survive past the age of 6 months, comparison of ventricular dimensions and function in older animals (6-12 months of age) was limited to db/db and dbShet animals. When compared to db/db animals, dbShet had significantly increased chamber dimensions and volumes (Figure 3L-N), and exhibited a mild reduction in ejection fraction (Figure 3O), but comparable LV mass (Figure 3P). Despite reduction in ejection fraction, stroke volume and cardiac output were preserved in dbShet mice. When compared with WT animals, db/db mice had increased stroke volume and cardiac output. At 4-8 months of age, dbShet animals exhibited significantly higher cardiac output than db/db animals, likely reflecting the increased chamber dimensions (Supplemental Figure III).

5. Smad3 haploinsufficiency attenuates diastolic dysfunction in db/db mice.

The effects of the db mutation on fat content, metabolic function and cardiac geometry in the absence of Smad3 are summarized in Supplemental Figure V. Because dbSKO mice died during the first 6 months of their life, we could only study the effects of partial loss of Smad3 on diastolic function by comparing pressure:volume relations in isolated perfused db/db and dbShet hearts. At 12 months of age, db/db mice had a shift of the end-diastolic pressure:volume curve to the left and exhibited an increased chamber stiffness constant. When compared with age-matched db/db animals, dbShet mice had a shift of the end-diastolic pressure:volume curve to the right and a marked reduction in chamber stiffness constant reflecting improved ventricular compliance (Figure 4A-B).
6. *dbShet* mice have reduced cardiac fibrosis and enhanced MMP activity.

Because Smad3 may affect cardiac geometry and function by regulating matrix metabolism, we studied the effects of Smad3 disruption on myocardial collagen deposition, MMP expression and activity. Histochemistry and a hydroxyproline assay showed that *dbShet* mice had markedly lower myocardial collagen content than corresponding *db/db* animals; however, collagen mRNA levels were not different between groups (Figure 4C-F). When compared with *db/db* mice, *dbShet* animals had increased myocardial MMP-2 and MMP-9 activity, despite exhibiting comparable MMP-2 and lower MMP-9 mRNA levels (Figure 4G-K).

7. Reduced fibrosis in *dbShet* hearts is associated with attenuated oxidative and nitrosative stress

When compared with WT animals, *db/db* mice had higher myocardial ROS levels and increased expression of 3-nitrotyrosine (Figure 5). Both partial and complete Smad3 loss attenuated myocardial oxidative stress in WT and in *db/db* mice (Figure 5A). Moreover, in *db/db* hearts, Smad3 deficiency attenuated the increase in 3-nitrotyrosine levels (Figure 5B-C).

8. Attenuated oxidative stress in *dbShet* myocardium is associated with reduced inflammation.

When compared with *db/db* animals, *dbShet* mice had comparable myocardial Interleukin IL-1β mRNA expression, but lower levels of tumor necrosis factor-α and monocyte chemoattractant protein-1, and attenuated macrophage infiltration (Supplemental Figure VI).

9. Increased mortality in Smad3 KO is due, in part, to aortic rupture.

Autopsies demonstrated that some SKO and *dbSKO* mice died with hemothorax, resulting from aortic rupture; aortic casts showed aneurysmal dilation (Figure 6A-C). Mortality due to
aneurysmal rupture was significantly increased in dbSKO mice (44.4% of autopsied deaths) and in SKO animals (20% of deaths). Only one dbShet mouse died of aortic aneurysmal rupture. No rupture deaths were noted in Shet, db/db and WT animals.

10. Smad3 loss causes progressive aortic dilation.

Aortic ultrasound showed that SKO mice had significantly increased diameter of the ascending aorta. dbSKO mice had earlier and accentuated aortic dilation. Smad3 haploinsufficiency was associated with increased aortic diameter in db/db, but not in WT animals (Figure 6D-E).

11. Aortic dilation in the absence of Smad3 is not due to increased systemic blood pressure.

Because an increased hemodynamic load induces aortic dilation and increases wall tension we examined whether the aortic pathology in the absence of Smad3 is due to systemic hypertension. SKO and dbSKO mice had lower systemic blood pressure than corresponding Smad3 +/+ animals (Figure 6F-I), suggesting that aortic dilation in the absence of Smad3 is not due to hemodynamic overload.

12. SKO mice have increased SMC apoptosis and enhanced MMP expression.

Because Smad3 loss may affect both matrix metabolism and SMC phenotype, we compared the morphology of the aortic matrix network and SMCs between WT and Smad3 null animals (Supplemental Figure VII). When compared with WT, Shet mice had increased aortic SMC density, while SKO mice had a trend towards increased density. Both partial and complete loss of Smad3 was associated with reduced aortic SMC size. TUNEL/α-SMA staining identified occasional apoptotic SMCs in the aortic media. Apoptotic SMCs were very rare in WT aortas;
but were significantly increased in SKO and Shet animals (Supplemental Figure VII). Smad3 loss significantly increased MMP-8 and MMP-9 mRNA expression in the aorta, but did not significantly affect collagen and TIMP-1 expression levels (Supplemental Table 4).

13. *dbSKO* mice have attenuated aortic matrix synthesis.

When compared with WT, *db/db* mice had comparable aortic SMC density (Supplemental Figure VIII), but exhibited significantly increased SMC size and had an increased number of apoptotic aortic SMCs. When compared with *db/db*, *dbSKO* mice had significantly reduced aortic SMC size, but comparable numbers of apoptotic SMCs. *db/db* mice exhibited significantly increased aortic extracellular matrix protein synthesis. When compared with *db/db* mice, *dbShet* and *dbSKO* had reduced collagen and tropoelastin synthesis. MMP and TIMP mRNA was also increased in *db/db* aortas; however the MMP-2:TIMP-1 ratio was significantly lower than in WT animals indicating predominance of matrix-preserving mediators (Supplemental Table 5).

**Discussion**

We demonstrate for the first time that Smad3 signaling mediates cardiac fibrosis and diastolic dysfunction in leptin-resistant diabetic mice, while preserving cardiac geometry and integrity of the vascular wall. In *db/db* mice, Smad3 disruption decreases fibrotic remodeling, reduces cardiac stiffness, and improves ventricular compliance; the effects of Smad3 loss are associated with decreased oxidative and nitrosative stress. Moreover, complete loss of Smad3 in both lean and obese mice is associated with aortic dilation and early death, due in part, to an increased incidence of aortic rupture.
Smad3 mediates diabetic diastolic dysfunction, while preserving chamber geometry.

Cell biological investigations, animal model experiments and studies in human subjects suggest that the metabolic alterations associated with diabetes and obesity activate TGF-β/Smad-dependent signaling. At the cellular level, high glucose potently stimulates TGF-β/Smad3, increasing cell size and enhancing protein synthesis. In experimental models of type 1 and type 2 diabetes, TGF-β/Smad3 activation is associated with tissue fibrosis. Rats with streptozotocin-induced diabetes and mice fed a high-fat diet exhibit myocardial activation of TGF-β/Smad3. Our findings show intense myocardial activation of the TGF-β/Smad2/3 axis in obese diabetic db/db mice; increased TGF-β/Smad signaling is associated with cardiac fibrosis and development of concentric ventricular hypertrophy.

Diastolic dysfunction is the hallmark of diabetic cardiomyopathy and may be due to cardiac hypertrophy, or to the development of interstitial fibrosis. Our findings demonstrate that in the db/db mouse model of fibrotic diabetic cardiomyopathy, partial loss of Smad3 improves ventricular compliance. However, attenuated diastolic dysfunction in dbShet mice comes at a cost: Smad3 disruption increases chamber dilation. Whether complete absence of Smad3 further accentuates dilative remodeling is unclear. Although the LVEDD:BW ratio is markedly increased in dbSKO animals, the smaller size of mice with complete loss of Smad3 makes direct comparisons of chamber dimensions difficult to interpret. Tibial length could have provided a more appropriate method for adjustment, but was not assessed in this study. The altered geometric and functional characteristics of the ventricle are associated with reduced collagen deposition and accentuated myocardial MMP activation. Thus, in the diabetic heart, activation of Smad3 signaling promotes fibrosis, while exerting matrix-preserving actions necessary for preservation of chamber geometry. The anti-fibrotic effects of Smad3 loss may
result from direct modulation of fibroblast function, or may reflect Smad-dependent actions in cardiomyocytes or vascular cells that may regulate fibroblast activation through paracrine or contact-dependent mechanisms.

*The link between Smad3 and oxidative stress*

Quantitative assessment of myocardial oxidative stress revealed that both partial and complete Smad3 loss markedly attenuated ROS levels and reduced 3-nitrotyrosine expression in db/db hearts (Figure 5). Oxidative stress may play an important role in the pathogenesis of the cardiomyopathy associated with diabetes and obesity acting, at least in part, through stimulation of pro-fibrotic signaling. Increased ROS levels may explain some of the fibrogenic effects of Smad3 in the diabetic myocardium. The molecular links between the Smad3 cascade and the ROS system remain unknown. *In vitro* experiments have implicated Smad3 signaling in activation of oxidative stress in epithelial cells, hepatocytes and SMCs and suggested that Smad3 signaling may mediate TGF-β-induced repression of antioxidant enzymes, such as manganese superoxide dismutase (MnSOD) and catalase.

*Smad3 loss results in aortic dilation and rupture*

Autopsy showed that, in many cases, early death of dbSKO mice was due to spontaneous aortic rupture. Aortic ultrasound demonstrated that Smad3 loss caused significant dilation of the ascending aorta in both obese and lean animals (Figure 6). Perturbations of TGF-β signaling have been associated with aortic aneurysm formation. Overactive canonical and non-canonical TGF-β responses play a key role in aortic dilation in Marfan’s syndrome. On the other hand, disrupted TGF-β signaling has also been associated with aneurysm formation. Several
studies have identified aortic aneurysmal disease in patients with Smad3 mutations\textsuperscript{31}; however, whether the effects of the mutations are due to disrupted or overactive TGF-\(\beta\) responses remains unknown. In a Dutch family with syndromic aortic aneurysmal disease, a \textit{SMAD3} heterozygous mutation was identified and was associated with immunohistochemical evidence of increased expression of phosphorylated Smad3\textsuperscript{32, 33}. In mice, Smad3 loss impaired aortic biomechanics and resulted in accentuated aortic inflammation and enhanced aneurysm formation upon infusion of angiotensin II\textsuperscript{14}. Smad3 signaling may play an important role in preserving the integrity of the aortic wall by promoting matrix protein deposition and by modulating the balance between MMPs and their inhibitors\textsuperscript{9}.

\textit{What is the basis for the effects of Smad3 in the cardiovascular system?}

Our findings suggest that Smad3 exerts both detrimental and protective effects on the diabetic heart and vasculature. Smad3 mediates cardiac fibrosis and diastolic dysfunction in db/db hearts, but also plays an important homeostatic role, preserving cardiac geometry and maintaining the integrity of the aortic wall. The adverse consequences of Smad3 loss in db/db mice cannot be explained by worse obesity, accentuated metabolic dysfunction or hemodynamic changes. When compared with db/db animals, dbSKO mice had significantly reduced fat content and attenuated insulin resistance. Moreover, loss of Smad3 attenuated the hypertensive response observed in db/db mice and, according to Laplace’s law, would be expected to confer protection from aortic dilation and rupture by reducing wall tension. Thus, the detrimental effects of Smad3 loss on the geometry of the heart and vessels appear to involve structural perturbations of the cardiac and vascular extracellular matrix. Imbalance between matrix-preserving and matrix-degrading signals may play an important role in the pathogenesis of aortic dilation and rupture in the
absence of Smad3. Moreover, effects of Smad3 disruption on vascular SMC phenotype may also be implicated (Supplemental Figures VII-VIII).

The benefits and perils of Smad3 inhibition in diabetic cardiomyopathy

Smad3 signaling is critically involved in the pathogenesis of diabetic fibrosis and diastolic dysfunction. Considering the high incidence of diastolic dysfunction in aging diabetic patients, Smad3 inhibition may be a promising therapeutic strategy to attenuate myocardial fibrosis and to protect from heart failure. However, therapeutic approaches targeting the Smad3 cascade may carry significant risks. Overzealous Smad3 inhibition may promote cardiac dilation and may accelerate aortic aneurysm formation, or cause aortic rupture in susceptible individuals.

Sources of Funding

Supported by NIH grants R01 HL76246, R01 HL85440.

Disclosures

None.

References


Figure Legends

**Figure 1.** db/db mice exhibit cardiac hypertrophy and fibrosis associated with activation of TGF-β/Smad. A. db/db mice had significantly higher LV mass than corresponding lean WT animals. B. db/db and WT animals had comparable LVEDD between 1-2 months of age and exhibited only subtle increases in chamber dimensions at 4-6 months. C. LVEDD:LVmass ratio was markedly lower in db/db animals reflecting predominant hypertrophic remodeling. D. 6 month-old db/db hearts were fibrotic showing a marked increase in collagen content (measured with a hydroxyproline assay). E. There was a trend towards increased TGF-β1 mRNA expression in db/db hearts. F. Expression of p-Smad2 was increased in db/db myocardium (*p<0.05, **p<0.01 vs. WT). (echocardiography WT n=25-73/group, db/db n=14-44/group; collagen n=8/group; mRNA n=15/group; western blotting n=8/group).

**Figure 2.** Smad3 loss increases mortality in both lean and db/db mice and is associated with attenuated weight gain and reduced adiposity. A. WT mice, db/db mice, Shet and dbShet mice had comparable survival curves. B. When compared with WT animals, Smad3 knockout mice (SKO) had higher mortality (**p<0.01). In comparison to db/db, db/db Smad3 -/- (dbSKO) animals had higher mortality (**p<0.01). All dbSKO mice died before 6 months of age. C. SKO mice had significantly lower body weight than corresponding lean WT animals (**p<0.01). D-E: Reduced weight in SKO mice was due to reductions in both lean weight (D) and fat content (E). F. 2 month-old SKO mice had lower fat % content than WT animals (**p<0.01); however at 4 and 6 months, fat % was not significantly different between WT and SKO. Shet animals had increased fat % content at 6 months of age (^p<0.01 vs. WT). G. db/db mice had a marked increase in body weight at 2-6 months of age (^^p<0.01 vs. age-matched WT mice).
comparison to db/db, dbSKO mice had markedly lower body weight (**p<0.01). H-I. Reduced weight gain in dbSKO mice was due to reductions in both lean weight and fat content (*p<0.05, **p<0.01 vs. db/db). At 6 months of age, dbShet mice had a statistically significant increase in fat content when compared with db/db animals (^p<0.01 vs. db/db). J. Percent fat content was markedly increased in db/db animals (^p<0.01 vs. corresponding WT). However, Smad3 loss in dbSKO animals was associated with reduced percent fat content only at 4 months of age, suggesting that lower body weight in the absence of Smad3 was predominantly related to stunted growth. K. db/db and dbShet mice had significantly increased plasma glucose levels when compared with WT animals (^p<0.01 vs. WT); dbSKO mice had attenuated hyperglycemia. L. Insulin levels were also markedly increased in db/db and dbShet animals (^p<0.05 vs. WT); however dbSKO mice had comparable insulin levels with lean WT controls. M. HOMA-IR was also significantly higher in db/db and in dbShet mice when compared with corresponding WT controls (^p<0.05, ^p<0.01 vs. WT); in contrast, in dbSKO mice, HOMA IR was comparable with WT animals suggesting attenuated insulin resistance. Survival: WT n=86, Shet n=175, SKO n=70, db/db n=32, dbShet n=55, dbSKO n=17; weight and adiposity: WT n=14, db/db n=16, dbShet n=25, dbSKO n=19 (2mo) n=9 (4mo), Shet n=16, SKO n=19 (2mo) n=17 (4mo); metabolic analysis: n=6.

Figure 3. Smad3 heterozygosity in db/db mice is associated with increased chamber dilation and a modest reduction in ejection fraction. Systolic function, dilative and hypertrophic remodeling were compared between lean WT mice and corresponding Shet and SKO animals (A-E), and between db/db, dbShet and dbSKO mice (F-J) using echocardiography. Panels K-O show comparison of echocardiographic endpoints between WT, db and dbShet animals at 6 and 12
months of age (at these timepoints comparison with SKO and dbSKO mice is not possible due to their early mortality)  

**A-E**: Shet and SKO mice had modest chamber dilation with preserved systolic function.  

**A**: At 4 months of age, Shet mice had higher LVEDD than WT animals (**p<0.01**).  

**B**: LVEDV was significantly higher in Shet animals at 4 months of age.  

**C**: When adjusted to body weight, SKO animals also exhibited an increase in LVEDD:BW ratio.  

**D**: Ejection fraction was comparable between groups at 2 and 4 months of age.  

**E**: No statistically significant differences in LV mass were noted.  

**F**: dbShet mice had a significantly higher LVEDD than db/db animals.  

**G**: dbSKO mice had significantly increased LVEDD:body weight ratio.  

**H**: LVEDV was significantly higher in dbShet mice at 4 months of age.  

**I**: When compared with age-matched WT animals, db/db mice had increased ejection fraction (^p<0.01 vs WT).  

When compared with db/db animals, dbShet mice and dbSKO animals had a modest, but significant reduction in ejection fraction (**p<0.01 vs db/db).  

**J-K**: db/db mice had increased LV mass and higher cardiomyocyte size than age-matched lean controls (*p<0.05, **p<0.01 vs. WT). dbSKO mice had attenuated LV hypertrophy, suggested by reduced LV mass (J) and decreased cardiomyocyte size (K) (*p<0.05, **p<0.01 vs. db/db).  

**L-N**: At 6-12 months of age, dbShet mice had persistent chamber dilation (*p<0.05, **p<0.01 vs. age-matched db/db) without significant progression of dilative remodeling.  

**O**: Ejection fraction was significantly lower in dbShet mice at 6 and 8 months of age (**p<0.01 vs. db/db); however progressive systolic dysfunction was not observed.  

**P**: In comparison to WT animals, db/db mice had significantly higher LV mass at 6-8 months of age (^p<0.01 vs. WT). dbShet mice and db/db mice had comparable LV mass at 6 and 12 months of age (2 mo of age: WT n=14, Shet=26, SKO n=10, db/db n=16, dbShet n=15, dbSKO n=19; 4mo of age: WT n=45, Shet n=27, SKO n=6, db/db n=42, dbShet n=16, dbSKO n=10, Shet n=16) (^p<0.05, ^p<0.01 vs. age-matched WT).
**Figure 4.** dbShet mice exhibit attenuated diastolic dysfunction associated with reduced matrix deposition and increased MMP activity.  
A. Pressure:volume relations in 12 month-old animals demonstrated that db/db mice exhibit a shift of the curve to the left and that dbShet hearts had improved compliance. B. Left ventricular chamber stiffness constant was increased in db/db mice. Partial loss of Smad3 reduced the chamber stiffness constant in db/db animals (*p<0.05, **p<0.01). C-D. Improved diastolic function in dbShet hearts was associated with reduced collagen content, assessed through Sirius red staining (C) and through a hydroxyproline assay (D). E-F. Reduced collagen in dbShet hearts was not due to differences in collagen transcription. G-I. dbShet animals had increased myocardial MMP9 (H) and MMP2 (I) activity, assessed with zymography (G). Accentuated MMP activity in dbShet hearts was not due to increased MMP2 (J) or MMP9 (K) transcription.

**Figure 5.**  
A. Smad3 loss attenuates oxidative and nitrosative stress in WT and db/db hearts. DCFDA assay showed that db/db hearts had increased ROS levels when compared with age-matched WT hearts (*p<0.05 vs WT). Partial or complete loss of Smad3 markedly reduced myocardial ROS levels in WT and in db/db animals (^p<0.05, ^^p<0.01 vs. WT; **p<0.01 vs. db/db, n=6/group). B-C. 3-nitrotyrosine expression was markedly increased in db/db hearts (**p<0.01 vs WT). Smad3 loss in db/db mice resulted in attenuated 3-nitrotyrosine expression (***p<0.01 vs. db/db).

**Figure 6.** SKO and dbSKO mice had aortic dilation and a high incidence of spontaneous rupture.  
A. Hemothorax in a 2 month-old SKO mouse that died spontaneously.  
B. Dilated ascending aorta in a dbSKO animal.  
C. Casts of the aorta show dilation and aneurysm formation (arrows) in
a 4 month-old dbSKO and a 2 month-old SKO mouse. D. Ultrasound showed that at 2 months of age, the ascending aorta was significantly dilated in dbShet and in dbSKO mice (*p<0.05, **p<0.01 vs. db/db). E. At 4 months of age, lean Shet and SKO mice had increased aortic diameter (*p<0.05, **p<0.01 vs. WT). dbShet and dbSKO exhibited increased aortic dimensions in comparison to db/db animals (**p<0.01 vs. db/db). (WT n=10, Shet n=11, SKO n=16; db/db n=14, dbShet n=13, dbSKO n=9). F-I. Aortic dilation in SKO and dbSKO mice was not due to increased systemic blood pressure. When compared with 4-month old WT animals, age-matched Shet and SKO mice had lower systolic (F) and diastolic (G) blood pressure (*p<0.05, **p<0.01 vs. WT). 4 month-old db/db mice had higher systolic (H) and diastolic (I) blood pressure than lean WT animals (^\^p<0.01 vs. WT). Smad3 loss in db/db mice protected from increase in systemic blood pressure (H, I) (**p<0.01). (WT n=8, db/db n=6; dbShet, dbSKO, SKO, Shet n=5).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Smad3 Signaling Promotes Fibrosis, While Preserving Cardiac and Aortic Geometry in Obese Diabetic Mice

Anna Biernacka, Michele Cavalera, Junhong Wang, Ilaria Russo, Arti Shinde, Ping Kong, Carlos Gonzalez-Quesada, Vikrant Rai, Marcin Dobaczewski, Dong-Wook Lee, Xiao-Fan Wang and Nikolaos G. Frangogiannis

_Circ Heart Fail._ published online May 18, 2015; Circulation: Heart Failure is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2015 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-3289. Online ISSN: 1941-3297

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circheartfailure.ahajournals.org/content/early/2015/05/18/CIRCHEARTFAILURE.114.001963

Data Supplement (unedited) at:
http://circheartfailure.ahajournals.org/content/suppl/2015/05/20/CIRCHEARTFAILURE.114.001963.DC1
http://circheartfailure.ahajournals.org/content/suppl/2015/05/20/CIRCHEARTFAILURE.114.001963.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation: Heart Failure can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation: Heart Failure is online at:
http://circheartfailure.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL:

SUPPLEMENTAL METHODS:

**Generation of db/db Smad3 null mice:** Animal studies were approved by the Institutional Animal Care and Use Committee at Albert Einstein College of Medicine and the Animal Protocol Review Committee at Baylor College of Medicine. Lepr<sup>dm/+</sup> on a C57BL6 background (db/+) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The Smad3 +/- mice (in a C57BL6J background) were obtained from our own colony. Genotyping was performed through established PCR protocols. Because homozygous db/db mice are sterile, we crossed heterozygous db/+ with heterozygous Smad3 +/- (both on a C57BL6 background). We genotyped the first generation of offspring (F1) for the db and Smad3 allele and subsequently identified male and female double heterozygous mice, using them to breed the second generation of offspring (F2). For survival analysis male and female db/db mice, Smad3 +/- (Shet) mice, Smad3 -/- mice (SKO), db/db Smad3 +/- (dbShet) mice and db/db Smad3 -/- mice (dbSKO) were followed for 6 months and deaths were recorded.

**Echocardiography and aortic ultrasound:** For echocardiographic and aortic ultrasound analysis WT, Shet, SKO, db/db, dbShet and dbSKO mice were imaged at 2, 4, and 6 months of age; WT, Shet, db/db and dbShet mice were also imaged at 8, 10 and 12 months of age. Mice were initially anesthetized in a chamber (5% isoflurane and 95% oxygen) and afterwards were supinely placed on a heating pad at 37°C on maintenance anesthesia (5% isoflurane, 95% oxygen at 4 L/min). Echocardiographic assessment was performed using a Vevo770 ultrasound (Visualsonics, Toronto, Canada) with a real time micro-visualization transducer (RMVB710B, 12-38 MHz at a frame rate of 110-120 frames per second) applied parasternally to the shaved chest wall. Images were taken in the parasternal long axis, parasternal short axis and short axis M-mode (SAMM). SAMM images were acquired by vertically placing the M-mode cursor at the parasternal short axis view when both papillary muscles were visualized. Views and data were exported for offline calculation using dedicated Vevo 770 quantification software (Vevo 770 v. 3.0.0). Images from the SAMM were used to measure the left ventricular end-diastolic diameter (LVEDD), Left ventricular end-systolic diameter (LVESD), left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), left ventricular mass, the ejection fraction and fractional shortening. Images from the parasternal long axis were used to assess stroke volume and cardiac output.

**Assessment of body fat content:** Body composition (body fat mass and lean mass) was quantitatively assessed using nuclear magnetic resonance (EchoMRI Whole Body Composition Analyzer, Echo Medical System – Houston, TX) in living, non-anesthetized mice at the Albert Einstein College of Medicine Diabetes Research Center, Animal Physiology Core.

**Immunohistochemistry and histology:** For histopathological analysis mice were sacrificed at 6 months of age; the hearts were fixed in zinc-formalin and embedded in paraffin. Sequential 5 μm sections were cut by microtomy. Collagen fibers were identified by Picrosirius Red staining using protocols established in our laboratory. For macrophage identification, immunohistochemical staining was performed using the anti-mouse Mac2 antibody as previously described. Stained sections were scanned using a Zeiss Axio Imager M2 microscope equipped with a Zeiss Axiocam MRm digital camera. Assessment of macrophage density was performed for each heart by counting the number of Mac2-positive profiles in 10 high-power fields from two sections. Samples from the ascending aorta were obtained from 4 month old animals and were also fixed in zinc formalin and embedded in paraffin. Identification of the elastin network...
was performed using Verhoeff-Van Gieson staining as previously described by our group. Immunofluorescent staining with an anti-α-SMA antibody (1:100, Sigma) was used to identify vascular smooth muscle cells in the aortic media as previously described. Smooth muscle cell density in the aortic media was measured by quantitating the number of α-SMA+ profiles in high power fields spanning the entire aortic cross-section. Smooth muscle cell size was measured for each animal by quantitating the mean cross-sectional area of 50 α-SMA+ cells. Only cells with a central nucleus were used for quantitation. Identification of apoptotic smooth muscle cells was performed by combining fluorescent TUNEL staining with α-SMA immunofluorescence as previously described.

**RNA extraction and qPCR analysis:** Isolated total RNA from the hearts and aortas 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). The sequences of the primers used in the study are listed in Table 4. The following genes were assessed: type I collagen, type III collagen, tropoelastin, MMP-2, MMP-3, MMP-8, MMP-9, TIMP-1, TIMP-2, TGF-β1, TNF-α, IL-1β, MCP-1, and 18S. Each sample was run in triplicate.

**Protein extraction and western blotting:** Protein was isolated from whole WT, db/db and dbShet hearts at 6 months of age (n=8/group) and Western blotting with rabbit anti-Smad2, anti-phosphorylated-Smad2 (from Cell Signaling), anti-nitrotyrosine, and anti-GAPDH antibodies (Santa Cruz) was performed from Cell Signaling) was performed as described previously. The gels were imaged by ChemiDoc™ MP System (Bio Rad) and analyzed by Image Lab 3.0 software (Bio Rad).

**Zymography:** MMP activity in murine WT, db/db and dbShet hearts (n=8 per group) was assessed by gelatin zymography as previously described. Whole hearts 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 minutes, 10,000 g), and 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 50 V/gel through stacking phase and 100 V/gel for the separating phase, maintaining a running buffer temperature of 4°C. Subsequently, the gels were renatured in 2.5% Triton X-100 for 30 minutes, rinsed in water, and incubated for 48 hours 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 Image Lab 3.0 software.

**Assessment of collagen content using a biochemical assay:** To assess collagen deposition in murine hearts we used previously described method that utilizes hydroxyproline content as surrogate measure of collagen. Briefly, WT, db/db and dbShet hearts (n=8 per group) were lyophilized and dry weights were recorded. Subsequently hearts were homogenized with 6N hydrochloric acid, transferred to 5 ml glass ampoules, purged with nitrogen gas and sealed using propane torch. Samples were allowed to hydrolyze at 110 °C for 16h. Subsequently, samples were evaporated in a vacuum centrifuge (Labconco) 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 collagen was expressed as μg of collagen/mg of dry tissue.

**Assessment of plasma glucose, insulin and HOMA IR:** Animals used for assessment of metabolic parameters in the serum were euthanized at 4 months of age and blood was collected by aortic puncture into EDTA anticoagulant-coated tubes. Plasma was extracted by centrifugation at 850 x g for 15 minutes at 4°C and stored at -80 °C. Mice were fasted 12 hours before collection of the blood. Serum insulin levels were measured using the mouse enzyme-linked immunosorbent assay kits as previously described (Crystal Chem). Glucose levels were measured by the glucose oxidase method (GM7; Analox, London,UK). HOMA-IR was calculated as fasting insulin (expressed as microunits/ml) × fasting glucose (expressed as millimoles/l)/22.5.

**Arterial catheterization and blood pressure measurements:**
Invasive blood pressure measurements were performed with a commercial Pressure Volume analysis system (Scisense). Mice undergoing blood pressure measurement were anesthetized with isoflurane and allowed to rest for 10 min to stabilize BP. The right carotid artery was exposed by blunt dissection and tied in three spots 3 mm apart with 6-0 silk suture. Distal suture was tied permanently with two knots whereas mid and proximal sutures were tied with one knot. Using fine scissors small incision was made between distal and mid suture. 1.2F pressure-volume catheter (Scisense) was inserted and advanced until the tip of the catheter passed mid suture which was tightened securing the catheter. Subsequently, the proximal suture was removed and the catheter was advanced to the aortic arch and secured with a suture. The catheter was left in place for 10 min to allow blood pressure equilibration. Pressure tracings were recorded for 5 min and analyzed to derive average systolic and diastolic blood pressure.

**Pressure:Volume analysis in isolated perfused hearts:**
Left ventricular pressure-volume analysis was performed using progressive isovolumic Langendorff retrograde perfusion of isolated murine hearts. Briefly, mice were injected intraperitoneally with heparin 10,000U/Kg; Sigma, St Louis, MO, USA) and anesthetized with Isoflurane as previously described. The hearts were rapidly excised and perfused in a retrograde manner at a constant perfusion pressure of 80 mmHg with modified Krebs- Henseileit buffer containing (mmol): NaCl 118, NaHCO3 24, KCl 4.7, KH2PO4 1.2, MgSO4 1.2, CaCl2 1.8, Glucose 11, Pyruvate 2. Buffer was oxygenated with 95% O2 and 5% CO2 at 37°C, to yield a pH of 7.4. A hand-made balloon (polyvinyl chloride film) connected to a polyethylene tube (PE50), was inserted into the LV through the mitral valve via an incision of the left atrium and was connected to a pressure transducer. The hearts were paced from the right ventricle using a stimulator at 420 beats per minute. Functional data were recorded at 1 KHz on a data acquisition system. After 20 min of stabilization, the balloon was progressively inflated in 2-μL increments until LV developed pressure (LVDP) became asymptotic and then began to decline. LVDP was calculated as the difference between peak-systolic pressure and LV end-diastolic pressure (LVEDP). Three independent runs for each animal were collected and averaged for purpose of calculation. A final pressure-volume exponential relationship obtained as described. The data for LVEDP were plotted as a function of increasing balloon volumes, and were plotted to a best-fit exponential curve (P = b·exp(KV)), where V is volume, and P is LV pressure generated for each
mouse. The volumes at a given pressure were averaged for animals in each group. The pressure-volume curves were standardized for pressures while volumes were used to calculate variability. The chamber stiffness constant (K) was determined by fitting the end-diastolic pressure-volume curves from individual hearts to an exponential function.\textsuperscript{10, 11}

**Generation of aortic casts:**

Aortic tree casting was performed in selected animals using Batson’s No. 17 Plastic Replica and Corrosion Kit (Polysciences, Inc. catalog #07349). The red pigment in the amount of 10 ml was added to 200 ml of Base Solution A, stir vigorously until mixed and divided in two equal parts to allow more control of the polymerization steps. Carefully, 40 ml of the Catalyst was added to 100 ml of Base Solution and set aside until the second half is mixed. Subsequently, 24 drops of Promoter C was added to the second half of the Base Solution A and mixed slowly on a magnetic stirrer. The two solutions were then added together and stirred to mix. The chest and abdominal cavity of previously deceased or euthanized animal was opened by a midline thoracotomy and laparotomy and the heart and aorta were exposed under the microscope. The cast solution was injected directly to the left ventricle with disposable polyethylene syringe and large needle. The casting material was then pumped with the slow flow rate into the aortic circulation with microscopic inspection of filling up the major arteries, until definitive resistance was achieved. After the procedure the animal was kept for 2 to 3 hours in 4°C to aid in the dissipation of the exothermic reaction caused by polymerization and to prevent expansion and distortion of the cast. Subsequently, the mouse was placed in the Maceration Solution (Polysciences, Inc catalog #07359) at 50°C overnight to corrode. The amount of solution was at least 2 to 3 times the volume of the mouse to be macerated. The following day the specimen was removed and rinsed in water to remove the excess material and immersed again in the maceration solution to allow the extra fluid to penetrate additional tissue. The procedure was repeated up to several days, until all tissues were removed from the cast. The cleaned specimen was immobilized to protect a delicate structure and photographed with the digital camera.

**Assessment of oxidative stress:** Myocardial reactive oxygen species (ROS) levels were measured using a fluorometric assay based on the conversion of 2', 7'-dicholrofluorescein-diacetate (DCFDA) to the highly fluorescent DCF in the presence of ROS.\textsuperscript{12}

**Statistical analysis:** For comparisons of two groups unpaired, 2-tailed Student’s t-test using (when appropriate) Welch’s correction for unequal variances was performed. The Mann-Whitney test was used for comparisons between 2 groups that did not show Gaussian distribution. For comparisons of multiple groups, 1-way ANOVA was performed followed by Tukey’s multiple comparison test. The Kruskall-Wallis test, followed by Dunn’s multiple comparison post-test was used when one or more groups did not show Gaussian distribution. Although adjustments for multiple comparisons in ANOVA were implemented, no additional adjustment for multiple testing was performed. Survival analysis was performed using the Kaplan-Meier method. Mortality was compared using the log rank test.
SUPPLEMENTAL RESULTS:

1. Female db/db mice exhibit accelerated cardiac hypertrophy and increased chamber dimensions.

When compared with corresponding WT animals, female db/db mice exhibited increased LV mass at a younger age than male animals, suggesting an accelerated hypertrophic response (Supplemental Figure IA-B). Moreover, female, but not male db/db mice exhibited modest chamber dilation at 2-6 months of age (SFIC-D).

2. Smad3 loss is associated with skeletal abnormalities in both lean and obese animals.

In addition to the significant reductions in weight, Smad3 loss in both lean and leptin-resistant mice was associated with skeletal abnormalities. Lean Smad3 null mice often developed kyphosis and displayed rib cage malformations, sometimes resulting in a concave groove at the base of the sternum (Supplemental Figure IIA-E). The presence of medially torqued forepaws was noted in approximately 30% of lean Smad3 null mice (SFIC). Lean Smad3 null mice also had frequent hair loss and rectal prolapse (Figure SFIID, occurring in up to 70% of mice by the age of 6 months) and a tendency to develop tumors and dermatitis. Leptin resistant Smad3 null mice (dbSKO) showed a characteristic dome-shaped body (Figure SFIIF) and displayed similar skeletal deformities (Figure SFIIG), but did not exhibit rectal prolapse, or hair loss.

3. Effects of the db mutation on the phenotype of Smad3 null mice.

db/db mice have a mutation in the leptin receptor gene and develop leptin resistance. Supplemental figure V summarizes the effects of the db mutation on the phenotype of Smad3 null animals. The db mutation increased fat weight and percent fat content but did not affect lean weight in Smad3 KO mice (SFVA-C). Metabolic consequences were relatively modest: glucose levels and HOMA IR were comparable between SKO and dbSKO animals, while dbSKO mice had a trend towards higher insulin levels (SFVD-F). The db mutation did not affect cardiac geometry and function in Smad3 null mice (SFVG-I). In contrast to the marked effects of the db mutation on myocardial oxidative stress in Smad3 +/+ animals (Figure 5A), no significant difference was observed in cardiac oxidative stress between SKO and dbSKO mice (SFVJ). However, much like in lean Smad3 +/+ mice, the db mutation was associated with modest, but significant increases in systolic and diastolic blood pressure in Smad3 KO mice (SFVK-L).

4. Enhanced matrix degradation in dbShet myocardium is not due to accentuated inflammation.

Inflammatory cytokines stimulate MMP expression and activation inducing matrix degradation. Accordingly, we examined whether enhanced matrix remodeling upon Smad3 disruption in db/db animals is due to accentuated myocardial inflammation. When compared with 2 month-old db/db animals, age-matched dbShet mice had comparable myocardial IL-1β mRNA expression and decreased levels of TNF-α and MCP-1 mRNA (Supplemental Figure VIA-C). Moreover, at 12 months of age, dbShet mice had attenuated macrophage infiltration (SFVID-E). Taken together the findings demonstrate that Smad3 haploinsufficiency is associated with reduced inflammatory activity in db/db hearts, suggesting that increased MMP activity in these animals is not due to accentuation of the myocardial inflammatory response.
**SUPPLEMENTAL TABLES:**

Supplemental Table 1:

**Primers used for qPCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tropoelastin</td>
<td>GAGGTCCAGGCGATTTGAGCTGC</td>
<td>ATATGTCCGGGATGCCAACTC</td>
</tr>
<tr>
<td>Collagen I</td>
<td>GCTGCATAACACCTTCGCTCA</td>
<td>GTCCCTCGACTCCATCACCTCA</td>
</tr>
<tr>
<td>Collagen III</td>
<td>GCACAGGAGTCCACACCTAGA</td>
<td>CAGGAAGCAGGAGGAGCAGG</td>
</tr>
<tr>
<td>MMP-2</td>
<td>TTCAACCGTGGGAAGAACAGAAT</td>
<td>AACTTGCCGGTGCATTCCATC</td>
</tr>
<tr>
<td>MMP-3</td>
<td>TCAGTGACATTCTCCAGTTTAC</td>
<td>AGGATGCGCTTCTCCATATCT</td>
</tr>
<tr>
<td>MMP-8</td>
<td>TTTGATGGACCGAAATGGAAT</td>
<td>GAGCGACCCAGAGAAATAGG</td>
</tr>
<tr>
<td>MMP-9</td>
<td>CCGACTCCAGGCTTATTTATT</td>
<td>GAGTTGATAGGCGCTGGTGGT</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>ATTCAAGGCTGTGGGAAATG</td>
<td>CTCAGGATAGGCGGAGGAC</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>CACAGACTTCAGCAGATGGGA</td>
<td>CTTGGAAGCAGATGGTGG</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>AATCAAGTGGAGAGCAACATG</td>
<td>AGCCCTGTATTCCGATCCT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>GATTATGGCTCCAGGCTCAA</td>
<td>CTCCCCTTGCCAGAACTCAGG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>CAGGCGAGCAGATCCACTCA</td>
<td>AGCTCATATGGGCTCGACAG</td>
</tr>
<tr>
<td>MCP-1</td>
<td>CCGATGGATGGGCTGAGA</td>
<td>TCTGACCCCCATCTCCTTG</td>
</tr>
<tr>
<td>18S</td>
<td>TCAGATACCGTGAGTTT</td>
<td>CTTTAAAGTTCAGGATTTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AAGCAGGGCTTCCATTTGAC</td>
<td>CACCAGTGAATCCAGGACA</td>
</tr>
</tbody>
</table>


Supplemental Table 2: Effects of Smad3 loss on cardiac geometry and function in male and female lean mice (4mo of age)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Shet</th>
<th>SKO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M (n=24)</td>
<td>F (n=21)</td>
<td>M (n=17)</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.9±0.08</td>
<td>3.4±0.09</td>
<td>4.5±0.04**</td>
</tr>
<tr>
<td>LVEDV (mm³)</td>
<td>67.4±2.6</td>
<td>48.8±3.1</td>
<td>91.2±1.6**</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>115.7±4.8</td>
<td>97.7±4.9</td>
<td>124.9±5.8</td>
</tr>
<tr>
<td>Éjection ration (%)</td>
<td>64.6±1.9</td>
<td>63.2±2.2</td>
<td>56.4±2.8*</td>
</tr>
</tbody>
</table>

**p<0.01 vs. corresponding WT.

Supplemental Table 3: Effects of Smad3 loss on cardiac geometry and function in male and female db/db mice (4 mo of age)

<table>
<thead>
<tr>
<th></th>
<th>db/db</th>
<th>dbShet</th>
<th>dbSKO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M (n=27)</td>
<td>F (n=15)</td>
<td>M (n=7)</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>4.1±0.06</td>
<td>4.0±0.08^^</td>
<td>4.5±0.09*</td>
</tr>
<tr>
<td>LVEDV (mm³)</td>
<td>74±2.7</td>
<td>72.1±3.4^^</td>
<td>87.1±8.4</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>150.6±9.5^^</td>
<td>157.5±9.0^^</td>
<td>133.2±10.6</td>
</tr>
<tr>
<td>Éjection ration (%)</td>
<td>71.1±1.4^^</td>
<td>75.0±2.2^^</td>
<td>65.6±4.2</td>
</tr>
</tbody>
</table>

^^p<0.01 vs. corresponding WT; *p<0.05, **p<0.01 vs. corresponding db/db

Supplemental Table 4:
Effects of Smad3 loss on aortic levels of genes associated with matrix production and metabolism in lean mice

<table>
<thead>
<tr>
<th>Gene:18S</th>
<th>WT</th>
<th>Shet</th>
<th>SKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen 1</td>
<td>1.0±0.2</td>
<td>1.8±0.7</td>
<td>4±2.0 (p=NS)</td>
</tr>
<tr>
<td>Collagen 3</td>
<td>1.0±0.3</td>
<td>1.3±0.5</td>
<td>1.8±0.7 (p=NS)</td>
</tr>
<tr>
<td>Tropoelastin</td>
<td>1.0±0.2</td>
<td>1.8±0.5</td>
<td>8.2±3.8*</td>
</tr>
<tr>
<td>MMP2</td>
<td>1.0±0.1</td>
<td>1.6±0.3</td>
<td>2.8±0.7 (p=0.11)</td>
</tr>
<tr>
<td>MMP3</td>
<td>1.0±0.2</td>
<td>1.8±0.5</td>
<td>6.9±2.5 (p=0.11)</td>
</tr>
<tr>
<td>MMP8</td>
<td>1.0±0.1</td>
<td>0.8±0.2</td>
<td>5.4±2.3*</td>
</tr>
<tr>
<td>MMP9</td>
<td>1.0±0.2</td>
<td>1.8±0.5</td>
<td>4±0.7**</td>
</tr>
<tr>
<td>TIMP1</td>
<td>1.0±0.3</td>
<td>1.7±0.6</td>
<td>5.4±2.9 (p=NS)</td>
</tr>
<tr>
<td>TIMP2</td>
<td>1.0±0.2</td>
<td>1.0±0.3</td>
<td>1.7±0.5 (p=NS)</td>
</tr>
</tbody>
</table>
Supplemental Table 5:
Effects of Smad3 loss on aortic levels of genes associated with matrix production and metabolism in leptin-resistant db/db mice

<table>
<thead>
<tr>
<th>Gene expression (normalized WT=1)</th>
<th>WT</th>
<th>db/db</th>
<th>dbShet</th>
<th>dbSKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>1.0±0.1</td>
<td>21.2±5.7^^</td>
<td>9.0±2.8*</td>
<td>8.5±2.1*</td>
</tr>
<tr>
<td>Collagen III</td>
<td>1.0±0.2</td>
<td>7±2^^</td>
<td>5.7±1.5</td>
<td>3±0.7 (p=0.09)</td>
</tr>
<tr>
<td>Tropoelastin</td>
<td>1.0±0.2</td>
<td>31.8±7.9^^</td>
<td>7.8±4.4**</td>
<td>7.1±1.6**</td>
</tr>
<tr>
<td>MMP2</td>
<td>1.0±0.1</td>
<td>5.8±1.4^^</td>
<td>5±0.9</td>
<td>2.5±0.4 (p=0.15)</td>
</tr>
<tr>
<td>MMP3</td>
<td>1±0.2</td>
<td>27.5±7.1^^</td>
<td>17.3±6.9</td>
<td>5.0±1.1 (p=0.06)</td>
</tr>
<tr>
<td>MMP8</td>
<td>1±0.4</td>
<td>2.6±0.7</td>
<td>1.8±0.6</td>
<td>1.3±0.5 (p=0.15)</td>
</tr>
<tr>
<td>MMP9</td>
<td>1±0.2</td>
<td>3.1±0.8^</td>
<td>1.6±0.3</td>
<td>0.9±0.3*</td>
</tr>
<tr>
<td>TIMP1</td>
<td>1±0.1</td>
<td>24.6±6.7^^</td>
<td>15.2±2.8</td>
<td>10.3±3.1*</td>
</tr>
<tr>
<td>TIMP2</td>
<td>1±0.2</td>
<td>6.3±1.6^^</td>
<td>3.5±0.7</td>
<td>2.4±0.5 (p=0.15)</td>
</tr>
<tr>
<td>MMP2:TIMP1</td>
<td>1±0.1</td>
<td>0.3±0.01^^</td>
<td>0.4±0.1</td>
<td>0.3±0.1 (p=NS)</td>
</tr>
</tbody>
</table>
**Supplemental figure I:** Female db/db mice exhibit accelerated cardiac hypertrophy and increased chamber dimensions. A-B. Male db/db (db) mice had increased LV mass at 4-8 months of age (A), whereas female animals developed hypertrophy at 2 months of age (B). Male db/db mice and corresponding WT controls had comparable LVEDD; however, female db/db animals exhibited modest chamber dilation at 2-6 months of age (Male mice, WT 1mo: n=21, WT 2mo: n=7, WT 4mo: n=8, db 1mo: n=11, db 4mo: n=27, db 6 mo n=26, db 8 mo n=12; Female mice, WT 1mo n=52, WT 2mo n=18, WT 4mo n=21, WT 6 mo n=21, WT 8 mo n=12, db 1mo n=4, db 4mo n=6, db 6 mo n=15, db 8 mo n=7; *p<0.05, **p<0.01 vs. age-matched WT)
Supplemental figure II: Smad3 loss is associated with skeletal abnormalities in both lean and db/db mice. As previously reported, Smad3 KO mice (SKO) have a high incidence of skeletal malformations, including kyphosis (A - arrow), rib cage malformations (B- arrow) and medially toqued forepaws (C-arrow). In some cases, SKO mice developed rectal prolapse (D- arrow), tumors (E- arrow) hair loss and dermatitis (not shown). dbSKO mice had a domed appearance (F) and exhibited many of the skeletal abnormalities noted in SKO mice (G – arrow shows kyphosis).
Supplemental figure III: A-D. Representative echocardiograms of a WT (A), a db/db (B), a dbShet (C) and a dbSKO (D) mouse at 4 months of age illustrate dilative remodeling of the ventricle in the absence of Smad3 (quantitative analysis shown in figure 3). E-H. Effects of Smad3 loss on stroke volume (E, G) and on cardiac output (F, H) in db/db mice. When compared with WT animals, db/db mice exhibited increased stroke volume and cardiac output (^p<0.01 vs. WT). At 4, 6 and 8 months of age, dbShet mice exhibited higher cardiac output than corresponding db/db animals (**p<0.01 vs. age-matched db/db).
Supplemental figure IV: Cardiomyocyte size was assessed using WGA lectin. Images show representative sections from WT (A), Shet (B), SKO (C), db/db (D), dbShet (E) and dbSKO (F) mice. Counterstained with DAPI. Scalebar=25μm
Supplemental figure V: Direct comparison of metabolic function, cardiac geometry and function and systemic blood pressure between SKO and dbSKO mice illustrates the effects of the db mutation on the phenotype of Smad3 null animals. Central leptin resistance increases fat weight (A) and fat percent content (C) in 2 month old Smad3 null mice, without affecting lean weight. The metabolic effects of the db mutation are modest, as dbSKO mice and SKO animals have comparable blood glucose levels at 4 months of age (D), while exhibiting a trend towards higher insulin levels (E) and comparable HOMA IR. LVEDV (G), ejection fraction (H) and LV mass (I) are comparable between SKO and dbSKO mice at 2 and 4 months of age. SKO and dbSKO animals have comparable myocardial oxidative stress (J). Much like in Smad3 +/+ mice,
central leptin resistance in Smad3 null animals is associated with modest but significant elevations in systolic and diastolic blood pressure (K-L).

Supplemental figure VI: Accentuated myocardial MMP activity and chamber dilation in dbShet mice is not due to increased inflammation. A-C: In comparison to db/db animals, dbShet mice had comparable myocardial IL-1β mRNA expression (A) and lower TNF-α (B) and MCP-1 (C) levels. D-E: Macrophages were identified in the myocardium using Mac2 staining. db/db mice had higher macrophage density than WT animals; however dbShet animals exhibited significantly lower macrophage infiltration that db/db mice (sections were counterstained with eosin) (n=8 per group).
Supplemental Figure VII: Smad3 loss in lean animals is associated with reduced SMC size, increased SMC apoptosis and increased aortic MMP expression. A-C: Immunofluorescence for α-SMA identified SMCs in the aortic media of WT (A), Shet (B) and SKO (C) mice. D: SMC
density.

**Figure Details:**
- **Supplemental Figure VII**: Smad3 loss in lean animals is associated with reduced SMC size, increased SMC apoptosis and increased aortic MMP expression. 
- **A-C**: Immunofluorescence for α-SMA identified SMCs in the aortic media of WT (A), Shet (B) and SKO (C) mice. 
- **D**: SMC density.

**Legend:**
- **SMC density**: 
  - WT
  - Shet
  - SKO
  - *p<0.19
- **SMC size**: 
  - WT
  - Shet
  - SKO
  - *p<0.19
- **MMP2**: 
  - WT
  - Shet
  - SKO
  - *p<0.11
- **MMP3**: 
  - WT
  - Shet
  - SKO
  - *p<0.11
- **MMP9**: 
  - WT
  - Shet
  - SKO
  - **p<0.05

**Images:**
- **A**: Immunofluorescence for α-SMA in WT mouse.
- **B**: Immunofluorescence for α-SMA in Shet mouse.
- **C**: Immunofluorescence for α-SMA in SKO mouse.
- **D**: Bar graph showing SMC density.
- **E**: Bar graph showing SMC size.
- **F**: Bar graph showing apoptotic cells.
- **G**: Bar graph showing MMP2 levels.
- **H**: Bar graph showing MMP3 levels.
- **I**: Bar graph showing MMP9 levels.
- **J**: Histological section of WT aorta.
- **K**: Histological section of Shet aorta.
- **L**: Histological section of SKO aorta.
- **M**: Graph showing elastin staining.
- **N**: Graph showing collagen staining.
density was significantly higher in Shet mice; SKO mice had a trend towards a higher SMC density. **E.** In comparison to WT animals, Shet and SKO mice had significantly lower aortic SMC size. **F.** The number of apoptotic SMCs detected through TUNEL staining was significantly higher in Shet and SKO mice. In contrast, apoptotic SMCs were extremely rare in WT aortas. However, SKO aortas had significantly increased expression of MMP-2 (**G**), -3 (**H**) and -9 (**I**). **J-L.** The morphology of the aortic matrix was studied in WT (**J**), Shet (**K**) and SKO animals (**L**) using VVG staining. Microscopically, both Shet and SKO mice had a well-developed elastin network in the aortic media; elastin content was comparable between groups (**M**). **N.** Collagen-stained area assessed through Sirius red histochemistry was decreased in Shet and SKO animals (*p<0.05, **p<0.01* vs. WT; WT n=8, Shet n=10, SKO n=11) (Scalebar=100μm).
Supplemental Figure VIII: Smad3 loss in db/db mice is associated with reduced SMC size and decreased matrix protein synthesis in the aortic wall. A-D: SMCs were identified in WT (A), db/db (B), dbShet (C) and dbSKO (D) animals. SMC density was comparable between groups (E). F: SMC size was increased in db/db mice (^p<0.01 vs. WT); however, Smad3 loss was associated with a significant decrease in SMC size in db/db animals (**p<0.01). G: db/db mice had an increased incidence of apoptotic SMCs in the aortic wall; however, Smad3 absence did not significantly affect the number of apoptotic cells. H-J: VVG staining in WT, db/db (H), dbShet (I) and dbSKO (J) animals shows no significant microscopic abnormalities. However, db/db aortas had markedly increased expression of matrix protein genes, including collagen I (K), collagen III (L) and tropoelastin (M). Smad3 loss markedly attenuated aortic matrix protein...
Moreover, TIMP-1 expression was increased in db/db aortas and was significantly suppressed in the absence of Smad3 (\(^*\)p<0.01 vs. WT; \(^*\)*p<0.05, \(^**\)*p<0.01 vs. db/db). Quantitative analysis of elastin-stained area (using VVG staining) suggested that Smad3 loss did not affect the relative elastin content of the aortic media (O). Sirius red staining showed no significant effects of Smad3 loss on collagen content in db/db mice (P). Despite increased levels of collagen mRNA expression, the ratio of collagen stained area to the medial area was lower in db/db mice when compared with WT controls (\(^*\)p<0.05 vs. WT), possibly reflecting (at least in part) the larger size of smooth muscle cells (F) in these animals (WT n=7, db/db n=5, dbShet n=6, dbSKO n=5) (Scalebar=100\(\mu m\)).

SUPPLEMENTAL REFERENCES

