Targeted Disruption of Smad4 in Cardiomyocytes Results in Cardiac Hypertrophy and Heart Failure

Jian Wang,* Ning Xu,* Xinheng Feng, Ning Hou, JiShuai Zhang, Xuan Cheng, Yeguang Chen, Youyi Zhang, Xiao Yang

Abstract—Transforming growth factor-βs (TGF-βs) are pleiotropic cytokines involved in many physiological and pathological processes, including heart development and heart disease. Smad4 is the central intracellular mediator of TGF-β signaling. To investigate the function of Smad4 in heart development further, we generated a strain of cardiomyocyte-specific Smad4 knockout mice using the Cre–loxP system. Unexpectedly, the deletion of Smad4 in cardiomyocytes resulted in cardiac hypertrophy characterized by an increase in the size of cardiac myocytes, age-associated fibrosis, and reexpression of certain fetal genes. Approximately 70% of the Smad4 mutant mice died spontaneously between 5 and 12 months of age. Echocardiography and an invasive hemodynamic study of the left ventricle revealed markedly decreased cardiac contractility in Smad4 mutant mice compared with littermate controls. Moreover, phosphorylated extracellular signal–regulated kinase (ERK) 1/2 and mitogen-activated protein kinase–ERK (MEK) 1 were increased in the Smad4 mutants, suggesting that an upregulation of MEK1–ERK1/2 signaling as a consequence of deletion of Smad4 underlies the impaired cardiac function. These results reveal an important function of Smad4 in cardiac remodeling and suggest that an altered cellular response to TGF-β could be a mechanism by which cardiac myocytes undergo hypertrophy. (Circ Res. 2005;97:821-828.)

Key Words: Smad4 ■ cardiac hypertrophy ■ heart failure ■ mitogen-activated protein kinase ■ Cre–LoxP system

Heart failure (HF), the final common outcome of most primary cardiovascular diseases, is one of the most frequent causes of death worldwide. The reasons underlying HF are diverse but often relate to hypertrophy of cardiac myocytes and fibrosis. Cardiac hypertrophy is defined as an increase in ventricular wall thickness accompanied by an increase in cardiomyocyte cell volume and the reexpression of certain fetal genes. Hypertrophy of the adult myocardium can occur in response to diverse pathophysiological stimuli such as ischemic heart diseases, hypertension, and valvular insufficiency. Although initial cardiac hypertrophy is likely an adaptive mechanism, prolonged hypertrophy predisposes an individual to arrhythmias, sudden death, and HF. Greater knowledge of the molecular pathways that lead to cardiac hypertrophy may increase our understanding of the mechanisms underlying the development of HF.

Numerous regulatory pathways are implicated in the transcription of hypertrophic signaling, including mitogen-activated protein kinase (MAPK), calcineurin, and growth factor signaling pathways. One pathway that has received attention is mediated by the transforming growth factor (TGF)-β family. TGF-βs are produced by cardiomyocytes and non-myocytes within the heart and act as autocrine or paracrine growth factors. TGF-β is particularly elevated in hypertrophic myocardium during the transition from stable hypertrophy to HF in experimental models and human HF. Transgenic mice overexpressing TGF-β exhibit significant cardiac hypertrophy, which is characterized by hypertrophic growth of cardiac myocytes and interstitial fibrosis. Functional in vivo blocking of TGF-β, with neutralizing antibodies or by disruption of 1 allele of TGF-β, consistently prevents myocardial fibrosis.

Recent studies indicate that angiotensin II (Ang II) and TGF-β interact to modulate cardiac remodeling. Ang II–induced increases in Tgb1 expression correlate with cardiac hypertrophy, fibrosis, and expression of fetal isoforms of cardiac myofibrillar proteins. Targeted disruption of TGF-β attenuates Ang II–mediated cardiac hypertrophy, indicating that TGF-β is an important mediator of the hypertrophic growth response of the heart to Ang II. However, the molecular mechanism of TGF-β signaling during the process of cardiac hypertrophy and HF remains poorly understood.
TGF-βs signal through cell surface serine/threonine kinase receptors, intracellular Smad and non-Smad mediators, MAPKs, and phosphatidylinositol 3-kinase. The activated MAPK pathway appears to be involved in the pathogenesis of cardiac hypertrophy. There are 8 distinct Smad proteins that can be divided into three functional classes: the receptor-regulated Smads (Smad1, 2, 3, 5, and 8), the comediator Smads (Smad4), and the inhibitory Smads (Smad6 and Smad7). Previous studies suggest that increases in Smad2 and Smad4 protein expression correlates with elevated cardiac collagen turnover in failing cardiomyopathic hearts. However, it is not known whether there is a causal relationship between increased Smad4 and cardiomyocyte hypertrophy.

Targeted disruption of Smad4, a central cytoplasmic mediator of TGF-β signaling, results in early embryonic lethality in mice, precluding assessment of the function of Smad4 in cardiac development. Therefore, we deleted the Smad4 gene specifically in cardiac myocytes using the Cre–loxP system. Unexpectedly, mice with cardiac-selective deletion of Smad4 developed significant cardiomyocyte hypertrophy and cardiac fibrosis leading to HF. These data indicate that the blockade of the cellular response of cardiac myocytes to TGF-β is sufficient to trigger hypertrophy and remodeling of the heart, suggesting an important role for Smad4 in the pathogenesis of cardiac hypertrophy and failure.

Materials and Methods

Mouse Strains

Mice that were homozygous for the floxed Smad4 allele (Smad4lox/lox) were bred with transgenic mice in which α-myosin heavy chain (α-MHC) regulatory element controls Cre recombinase expression (α-MHC-Cre). To examine the spatial distribution of Cre-mediated recombination, the α-MHC-Cre transgenic mice were bred with ROSA26 reporter mice. Animals were handled in accordance with institutional guidelines.

X-Gal Staining Procedures

X-Gal staining of postnatal day 20 (P20) hearts was performed using a procedure described. Stained heart tissues were postfixed in 4% paraformaldehyde at 4°C overnight and paraffin embedded for histological analysis.

Southern Blot and Northern Blot

Southern and Northern blot were performed as described. Probes for atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC), were cDNA fragments generated by RT-PCR using the following primers: β-MHC, 5’-TCGAGGCTCCAGGTCTTACACCC-3’ and 5’-GCGTAACACCCCTGTCAAAGTTCC-3’; ANP, 5’-CTGCCAGACACCCAGTAAGGAG-3’ and 5’-TGCCAAGGAGTATGCTCAGACACCC-3’; BNP, 5’-CAGCATCCTTGGATGTTTGCGGG-3’ and 5’-GTGGTGCTCCAGAGCCTGGGGGAAAGAAGAG-3’.

Histology and Immunohistochemistry

Heart tissues were fixed in 4% paraformaldehyde at 4°C overnight, embedded in paraffin, and sectioned at 5 μm. Sections were stained with hematoxylin and eosin (H&E), Masson trichrome (to examine interstitial fibrosis), periodic acid Schiff (PAS) (to visualize the cardiomyocyte border), or laminin antibody (Boster); Smad4, P-Smad2 (Cell Signaling), and BrdU (bromodeoxyuridine) staining and electron microscopic analysis were performed as described.

Morphometric Analysis of Isolated Cardiac Myocytes

Ca2+-tolerant cardiac myocytes were obtained from the left ventricle using a protocol described. The hearts from 3-month-old mice were retrogradely perfused and enzymatically dissociated with 0.3% collagenase. The dissociated cells were plated on laminin-coated dishes. Unattached cells were removed by changing the medium 1 hour after plating. Morphometric analysis was performed with IPLab software (Scanalytics).

Echocardiography

Mice were anesthetized with tribromoethanol and analyzed for anatomy and function on a Vivid 7 Dimension cardiovascular ultrasound system with a 12-MHz microprobe (GE Healthcare). Echocardiographic measurements were taken on M-mode in triplicate from more than 4 separate mice per group.

In Vivo Blood Pressure Measurements

Blood pressure measurements were performed as described. Left ventricular (LV) hemodynamics were measured with a 1.4-F micro-manometer conductance catheter (Millar Instruments). Briefly, mice were anesthetized with intraperitoneal injections of tribromoethanol and atropine; the catheter was placed in the right carotid artery and advanced into the LV for the measurement of left intraventricular pressure.

Western Blot

Western blots were performed on myocardial extracts as described. Fifty micrograms of proteins were electrophoresed on 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Immunoblotting was performed according to the instructions of the manufacturer using the following antibodies: extracellular signal–regulated kinase (ERK), phosphorylated ERK and p38, eIF5 (Santa Cruz), phosphorylated MAPK–ERK (MEK) 1, phosphorylated p38, Akt, and phosphorylated Akt, p70s6k, phosphorylated p70s6k (Cell Signaling).

Statistical Analysis of Data

All results were presented as mean±SEM. All statistical analyses were performed using SPSS software. Statistical differences were determined by Student’s t test. Probability values of P<0.05 were considered significant.

Results

Targeted Ablation of Smad4 Gene in Cardiomyocytes

To develop a cardiomyocyte-specific Smad4 knockout mouse, we used the Cre–loxP strategy. We generated a mouse strain (α-MHC-Cre) that specifically expressed Cre recombinase in cardiomyocytes using the α-MHC promoter. Northern blot analysis showed that Cre transcripts were detected in only heart tissue of the α-MHC-Cre transgenic mouse (data not shown). We further analyzed the expression pattern of Cre recombinase in this transgenic mouse using the ROSA26 reporter mouse strain. Whole-mount LacZ staining for multiple tissues of the α-MHC-Cre and ROSA26 double transgenic mice revealed that only hearts were stained blue (Figure 1A and data not shown). Analysis of heart sections from the P20 mouse revealed LacZ staining in >90% of cardiomyocytes (Figure 1B and 1C). These data demonstrated that the Cre recombinase was expressed exclusively in the cardiac tissues of the α-MHC-Cre transgenic mice, indicating that the α-MHC-Cre transgenic mice we generated could be used to achieve Cre-mediated recombination in cardiomyocytes.

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used as controls because there were no apparent differences in the structures observed among Smad4<sub>Co/Co</sub>:α-MHC-Cre and Smad4<sub>Co/Co</sub> or Smad4<sub>Co/+</sub> or wild-type mice (supplemental Figure 1A, available online at http://circres.ahajournals.org and data not shown). Cre-mediated excision of exon 8 in various tissues isolated from Smad4<sub>Co/Co</sub>:α-MHC-Cre mice was evaluated by PCR and Southern blot analysis. PCR analysis of heart, liver, spleen, lung, kidney, intestine, brain, and muscle revealed that the Cre-mediated recombination occurred exclusively in cardiac tissue (data not shown). This was confirmed by Southern blotting (Figure 1D). Northern blot analysis revealed that Smad4 mRNAs were significantly decreased in hearts of Smad4<sub>Co/Co</sub>:α-MHC-Cre mice compared with control mice (Figure 1E). These data were consistent with the Western blot analysis data (Figure 1F) and the immunohistochemistry for SMAD4 (Figure 1G and 1H). In addition, less-phosphorylated Smad2 (P-Smad2) nuclei translocation was found in mutant hearts (Figure 1I and supplemental Figure IB and IC). These findings indicate that the Smad4 gene was efficiently disrupted in cardiomyocytes by α-MHC-Cre-mediated recombination.

**Targeted Disruption of Smad4 in Cardiomyocytes Caused Cardiac Hypertrophy and Shortened Lifespan**

The Smad4<sub>Co/Co</sub>:α-MHC-Cre mutant mice were born normally and had normal cardiac structure (data not shown). The mutant mice developed to adulthood and were fertile. However, a significantly shortened lifespan was observed in Smad4<sub>Co/Co</sub>:α-MHC-Cre mutant mice (Figure 1J). Seventy percent of Smad4 mutant mice died suddenly between 5 and 12 months of age.

Mice were euthanized at different ages for cardiac anatomy and histological analysis. Hearts from the Smad4<sub>Co/Co</sub>:α-MHC-Cre mice were larger than those of their littermates at the age of 20 days. A typical example, at the age of 3 months, is shown in Figure 1K. The Smad4<sub>Co/Co</sub>:α-MHC-Cre mice had increased heart weight/body weight (HW/BW) and heart weight/tibia length (HW/TL) ratios compared with their littermates (Figure 1L and 1M and supplemental Figure IA). Sections revealed an increase in LV wall and intraventricular septal wall thickness in Smad4<sub>Co/Co</sub>:α-MHC-Cre mice at the ages of 1 and 3 months (Figure 2A through 2D). Cardiac hypertrophy is frequently associated with reexpression of genes expressed during fetal and perinatal development and with upregulation of some cardiac proteins, such as ANP, BNP, and β-MHC. Northern blot analysis of total RNA showed that these genes were significantly upregulated in the Smad4 mutant hearts compared with the controls (Figure 2E). These data demonstrated that Smad4 deficiency in the cardiac tissue led to cardiac hypertrophy.

**Loss of Smad4 Correlates With the Increased Size of Cardiomyocytes**

We further examined whether the increased heart size was attributable to cellular growth (increase in cell size) or proliferation (increase in cell number). Cardiomyocyte proliferation was assessed by BrdU staining. There was no significant difference in cardiomyocyte proliferation between

To disrupt the Smad4 gene in cardiomyocytes, we bred a mouse strain containing the Smad4 conditional alleles (Smad4<sub>Co/Co</sub>)<sup>20</sup> with the α-MHC-Cre transgenic mice. The acquired Smad4<sub>Co/Co</sub>:α-MHC-Cre offspring were further bred with Smad4<sub>Co/+</sub> mice to generate Smad4<sub>Co/Co</sub>:α-MHC-Cre mice. In this article, the Smad4<sub>Co/Co</sub>:α-MHC-Cre mice were
the Smad4<sup>Cre/Co</sup>;α-MHC-Cre mice and their littermates at embryonic day 18.5 (E18.5) and P3 (Figure 2F and supplemental Figure ID through IG). Cross-sections of hearts confirmed LV enlargement (Figure 3A and 3B) and showed a remarkable increase in myocyte cell size in Smad4 mutants compared with that found in control mice using H&E staining (Figure 3C and 3D), PAS staining (Figure 3E and 3F), and laminin immunohistochemistry staining (Figure 3G and 3H).

We also measured cell size of the individual cardiac myocytes in isolated adult myocyte preparations (Figure 3I and 3J). The cardiac myocytes (n=150) dissociated from the Smad4 mutant heart at 3 months of age (n=3) had a greater mean cell area than controls (1046.9±72.2 μm<sup>2</sup> in controls versus 2227.5±137.3 μm<sup>2</sup> in Smad4 mutants; P<0.01). Both the long axis length (71.2±1.6 μm in controls versus 89.5±0.9 μm in Smad4 mutants; P<0.05) and the short axis length (14.1±1.0 μm in controls versus 25.9±0.8 μm in Smad4 mutants; P<0.01) were significantly increased. These data indicate that the increase in ventricular thickness observed in Smad4<sup>Cre/Co</sup>;α-MHC-Cre mice resulted from the increased size of cardiomyocytes.

Mice Lacking Cardiac Smad4 Developed Cardiac Fibrosis and Ultrastructural Architectural Defects With Advanced Age

Fibrosis changes always accompany cardiac hypertrophy. Masson’s trichrome staining of heart sections at different stages was used to evaluate the degree of fibrosis in Smad4 mutants. No significant fibrosis was observed in Smad4 mutant mice less than 6 months of age (Figure 4A and 4B). At the age of 7 months, the Smad4 mutant hearts exhibited severe fibrosis (Figure 4D), whereas the controls had no significant fibrosis (Figure 4C). These results suggest that cardiac hypertrophy caused by the loss of Smad4 gradually led to maladaptive cardiac remodeling with age.

To examine whether the ultrastructural architecture was affected by disruption of the Smad4 gene, LV tissues from 3- and 7-month-old mutant and control mice were subjected to electron microscopy. Abnormal mitochondria were observed in cardiac myocytes from 3-month-old Smad4<sup>Cre/Co</sup>;α-MHC-Cre mice (Figure 4E and 4F). The myocytes from 7-month-old Smad4<sup>Cre/Co</sup>;α-MHC-Cre mice showed abnormal dispersion and disorganization of the Z-bands and mitochondria, whereas control myocytes exhibited typical registry of...
Z-bands and normal organization of mitochondria (Figure 4G and 4H). An increase in myofibrillar size was also evident in 7-month-old Smad4Co/Co;H9251-MHC-Cre mice, along with an increased cell size (Figure 4H).

Loss of Smad4 in the Heart Resulted in Decreased Cardiac Contractility

Because hemodynamic loading status is 1 of the determinants of heart size, we assessed the LV dimensions and systolic function of Smad4Co/Co;α-MHC-Cre mice by means of M-mode echocardiography (Figure 5A; supplemental Table I). At 1-month old, there was an increase in LV mass and wall thickness of Smad4Co/Co;α-MHC-Cre mice (Figure 5B and 5C). The LV diastolic and systolic diameters of Smad4Co/Co;α-MHC-Cre mice did not differ from those of their littermates (supplemental Table I). Fractional shortening (FS), an echocardiographic index of LV contractile function, of Smad4Co/Co;α-MHC-Cre mice was not altered (Figure 5D). An increase in HW in the absence of cardiac dysfunction suggests that the increase in heart size in Smad4Co/Co;α-MHC-Cre mice was most likely not a secondary response to the changes in hemodynamic loading status. For 3-month-old and 7-month-old mice, an increase in the wall thickness was also evident in Smad4 mutants (Figure 5C). Smad4 mutant mice had significantly depressed cardiac contractility, as indicated by decreased FS (Figure 5D; supplemental Table I). Moreover, valvular regurgitation was observed in some of the 7-month-old Smad4Co/Co;α-MHC-Cre mice (data not shown).

Functional invasive hemodynamic measurements were performed to confirm the occurrence echocardiographic alterations. After anesthesia and LV catheterization, the LV diastolic end pressure was increased (Figure 5E) and the maximum of change in pressure/change in time was decreased in the 3- and 7-month-old mice compared with those in control mice (Figure 5F). The mean arterial blood pressure, heart rates, and other hemodynamic variables were not significantly different (Figure 5G and supplemental Table II). The results reflected severe impairment of systolic function.
MAPK Activation in Smad4-Deficient Hearts

Previous studies indicate that the MAPK family is a downstream mediator of the biological effects of TGF-β.27 To examine whether the MAPK pathway is involved in the cardiac dysfunction observed in Smad4 mutant mice, the protein levels of phosphorylated MEK1, ERK1/2, p38, JNK, and Akt of cardiac extracts from 3 Smad4<sup>LoxP/cre</sup> α-MHC-Cre<sup>−/−</sup> and 3 control<sup>+/−</sup> mice at 1, 3, and 7 months of age were measured by Western blot analysis (Figure 6). The phosphorylation levels of ERK1/2 and MEK1 at 1 and 3 months of age were significantly enhanced in Smad4 mutants compared with those found in their littermate controls, whereas the phosphorylation levels of p38 and JNK were not different. Previous studies have suggested that the phosphatidylinositol 3-kinase–Akt signaling pathway is critical in cardiac hypertrophy. Thus, we also examined whether Akt activation is involved in cardiac hypertrophy in Smad4 mutant mice. There were no significant differences in the levels of phosphorylated Akt (Figure 6) or p70 S6 kinase (data not shown). Together, these results indicate that the activation of MEK1–ERK1/2 signaling pathway may be a possible mechanism underlying the cardiac hypertrophy in Smad4 mutants.

Discussion

The cardiomyocyte-specific knockout of Smad4 induced cardiac hypertrophy, as indicated by a significant increase in absolute HW/BW and HW/TL ratios. Myocardial hypertrophy in this model was characterized by the increased size of cardiomyocytes, cardiac fibrosis with advanced age, and reexpression of fetal genes. The Smad4 mutant mice experienced sudden death caused by heart dysfunction. Furthermore, the present findings indicate that the expression levels of phosphorylated MEK1 and ERK1/2 were elevated in Smad4 mutant hearts. Thus, the current study is the first direct evidence of an important role for Smad4 in the hypertrophic response of the heart.

Given the well-identified roles of TGF-β ligands and receptors in heart development, and the frequently cited role of Smad4 as a common mediator of TGF-β superfamily signaling,28 it is surprising that the cardiac myocyte–specific Smad4 mutant mice did not exhibit more severe phenotypes. Previous studies indicate that several genes encoding bone morphogenetic proteins (BMPs), TGF-βs, and their signal transducers are expressed in the developing and adult heart and that these molecules exhibit overlapping and distinct spatial and temporal patterns.29,30 Heart formation in Bmp2 mutants is grossly abnormal,31 whereas targeted disruption of TGF-β1 and TGF-β2 genes results in various degrees of heart defects, including the failure of myocardialization of the mesenchyme of the atrial septum and the ventricular outflow tract as well as deficient valve differentiation in TGF-β2 mutant embryos.32–34 Failed chamber septation and reduced trabeculation are present in Bmp5;Bmp7 double mutant mice.35 When ALK3, a type IA BMP receptor, is knocked out in cardiomyocytes using the Cre–LoxP system, mice die before E18.5 because of defects in cardiac cushion and myocardium.36 Recent studies also suggest that the loss of Smad5 results in abnormal development of heart and apoptosis of cardiac myocytes.24,37 Unexpectedly, heart formation and morphogenesis were not apparently affected in the cardiomyocyte-specific Smad4 knockout mice. This finding suggests that the Smad4 might not be the requisite mediator of all TGF-β signals in cardiac myocytes. Indeed, recent studies indicate that several TGF-β-regulated and BMP-regulated processes still occur in the gastrulating mouse embryo in the absence of Smad4, raising questions regarding the central role of Smad4 in TGF-β signaling.38

Numerous studies indicate that TGF-β1 plays a pivotal role in the development of cardiac hypertrophy and heart failure.39 Clinical evidence suggests that patients with idiopathic hypertrophic obstructive cardiomyopathy have elevated expression of TGF-β1, and its receptor in both cardiomyocytes and fibroblasts.40 Transgenic mice that overexpress TGF-β1 consistently exhibit cardiac hypertrophy characterized by hypertrophic growth of cardiac myocytes and interstitial fibrosis,41 whereas targeted disruption of TGF-β1 or blocking of TGF-β1 using neutralizing antibodies inhibits cardiomyocyte hypertrophy.

Originally, we hypothesized that targeted disruption of Smad4 in cardiac myocytes would block hypertrophic responsiveness. In fact, the opposite occurred. The cardiomyocyte-restricted knockout of Smad4 resulted in an early onset of cardiac myocyte hypertrophic growth, characterized by an increase in the size of cardiac myocytes and increased expression of molecular markers for cardiomyocyte hypertrophy (β-MHC, ANP, and BNP). This was followed by progressive cardiac fibrosis with advanced age. A significant incidence of sudden death was observed in Smad4 mutant mice. The mutant mice showed no changes of appearance or behavior before death. Therefore, it appears that the intracellular signaling pathway that mediates the responsiveness of cardiomyocyte to TGF-β is required for maintaining the homeostasis of heart, suggesting that a lack of responsiveness to TGF-β is one of the molecular bases of myocardial hypertrophy and heart failure.
The MAPKs represent another major type of signaling intermediate for TGF-β. The 3 major members, ERKs, JNK, and p38–MAPK, are implicated in the development of cardiac hypertrophy and heart failure. The current study found a transient activation of ERK1/2 in Smad4 mutant mice and no activation of JNK and p38 in Smad4 mutants. These results provide evidence that elevated ERK1/2 signaling is one of the molecular mechanisms underlying the cardiac hypertrophy of Smad4 mutants. In vitro studies indicate that ERK1/2 becomes activated in cardiac myocytes in response to agonist stimulation or cell stretching. MEK1 transgenic mice that express the activated ERK1/2 in the heart exhibit concentric hypertrophy. Recent studies suggest that increased ERK is associated with the enhanced concentric cardiac hypertrophy and increased mortality in pressure-overloaded adiponectin-deficient mice. MEK1–ERK1/2 signaling also appears to interact with calcineurin to regulate the cardiac cellular growth. We found that blocking the signaling also appears to interact with calcineurin to regulate responsiveness of cardiac myocytes to TGF-β. MEK1–ERK1/2 pathway and induce phenotypes other than lethality up to 12 months of age. Thus, the loss of Smad4 mutant mice play a direct role in the pathogenesis of cardiac hypertrophy and HF.

Acknowledgments
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References


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Online Supplemental Data

ONLINE TABLE 1.
Echocardiographic Characterization of Cardiomyocyte Specific Smad4 Knockout Mice

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<th>1 Month Cre/Co/+ N=6</th>
<th>1 Month Cre/Co/Co N=7</th>
<th>3 Months Cre/Co/+ N=7</th>
<th>3 Months Cre/Co/Co N=8</th>
<th>7 Months Cre/Co/+ N=5</th>
<th>7 Months Cre/Co/Co N=8</th>
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<tr>
<td>Heart rate (bpm)</td>
<td>380±28.66</td>
<td>394.5±15.32</td>
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<td>IVSs (mm)</td>
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<td>LVIDs (mm)</td>
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<td>LVPWd (mm)</td>
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<td>LVPWs (mm)</td>
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<td>LVM (mg)</td>
<td>78.13±5.26</td>
<td>104.68±8.48*</td>
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<td>139.73±7.76*</td>
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<td>%FS</td>
<td>39.79±2.24</td>
<td>35.84±2.67</td>
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<td>30.09±0.76*</td>
<td>41.14±1.29</td>
<td>29.48±2.48*</td>
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The results presented are means ± SEM *P < 0.05 versus control mice.
Bpm = heart beats per minute; IVSd = septum in diastole; IVSs = septum in systole; LVIDd = left ventricular end-diastolic dimension; LVIDs = left ventricular end-systolic dimension; LVM = left ventricular mass; %FS = percent fractional shortening.
## ONLINE TABLE 2.  Hemodynamic Characterization of Cardiomyocyte Specific Smad4 Knockout Mice

<table>
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<th>1 Month</th>
<th>3 Months</th>
<th>7 Months</th>
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<td>N=6</td>
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<td>MAP (mmHg)</td>
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<td>dP/dt_{min} (mmHg/s)</td>
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<td>81.83 ± 6.77</td>
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<td>Heart rate (beats/min)</td>
<td>388.79 ± 8.03</td>
<td>368.12 ± 19.57</td>
<td>354.19 ± 22.82</td>
</tr>
</tbody>
</table>

The results presented are means ± SEM  ^{*} P < 0.05 versus control mice.
Figure legend for supplemental Figure 1. (A) HW/BW (mg/g) ratios of Co/+ (n=9), Co/Co (n=11), Cre/Co/+ (n=10), Cre/Co/Co (n=10) mice at 1 month of age. * $P < 0.05$, significant different from age-matched controls. (B-C) Immunohistochemistry for P-Smad2 in control (A) and Smad4 mutant mice (B). (D-G) BrdU labeling was performed on heart sections from control (D and F) and Smad4 mutant (E and G) mice at E18.5 (D and E) and P3 (F and G) of age. Scale bar: (A-B) 10 $\mu$m; (D-E) 10 $\mu$m; (F-G) 20 $\mu$m.