Cardiac-restricted Overexpression of TRAF3 Interacting Protein 2 (TRAF3IP2) Results in Spontaneous Development of Myocardial Hypertrophy, Fibrosis, and Dysfunction*

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TRAF3IP2 (TRAF3 interacting protein 2; previously known as CIK5 or Act1) is a key intermediate in the normal inflammatory response and the pathogenesis of various autoimmune and inflammatory diseases. Induction of TRAF3IP2 activates IκB kinase (IKK)/NF-κB, JNK/AP-1, and c/EBPβ and stimulates the expression of various inflammatory mediators with negative myocardial inotropic effects. To investigate the role of TRAF3IP2 in heart disease, we generated a transgenic mouse model with cardiomyocyte-specific TRAF3IP2 overexpression (TRAF3IP2-Tg). Echocardiography, magnetic resonance imaging, and pressure-volume conductance catheterization revealed impaired cardiac function in 2-month-old male transgenic (Tg) mice as evidenced by decreased ejection fraction, stroke volume, cardiac output, and peak ejection rate. Moreover, the male Tg mice spontaneously developed myocardial hypertrophy (increased heart/body weight ratio, cardiomyocyte cross-sectional area, GATA4 induction, and fetal gene re-expression). Furthermore, TRAF3IP2 overexpression resulted in the activation of IKK/NF-κB, JNK/AP-1, c/EBPβ, and p38 MAPK and induction of proinflammatory cytokines, chemokines, and extracellular matrix proteins in the heart. Although myocardial hypertrophy decreased with age, cardiac fibrosis (increased number of myofibroblasts and enhanced expression and deposition of fibrillar collagens) increased progressively. Despite these adverse changes, TRAF3IP2 overexpression did not result in cell death at any time period. Interestingly, despite increased mRNA expression, TRAF3IP2 protein levels and activation of its downstream signaling intermediates remained unchanged in the hearts of female Tg mice. The female Tg mice also failed to develop myocardial hypertrophy. In summary, these results demonstrate that overexpression of TRAF3IP2 in male mice is sufficient to induce myocardial hypertrophy, cardiac fibrosis, and contractile dysfunction.

Cardiac failure is a multifactorial terminal condition resulting from pathological events that include pressure/volume overload, myocardial ischemia/infarction and cardiomyopathies (1). A key component in these predisposing events is sustained inflammation, which contributes to the onset and progression of the critical determinants of cardiac failure: myocardial hypertrophy, cardiac fibrosis, and contractile dysfunction (2–4). Both nuclear factor κB (NF-κB) and activator protein 1 (AP-1)3 pathways have been shown to play critical roles in the development and progression of hypertrophy and fibrosis, with the activation of IκB kinase (IKK) and c-Jun N-terminal kinase (JNK) as the key intermediate steps (3). For example, mice with cardiac-restricted overexpression of constitutively active IKK spontaneously developed reversible inflammatory cardiomyopathy and heart failure (5). In concordance, overexpression of IκB-α super-repressor in the heart led to reduced hypertrophic response after angiotensin II/iso-proterenol infusion (6). Furthermore, sustained activation of p50 and p65, the major subunits of NF-κB, plays a causal role in the onset and progression of cytokine- and myocardial infarction-induced heart failure (7, 8), demonstrating a critical role

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3 The abbreviations used are: AP-1, activator protein 1; IKK, IκB kinase; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CTGF, connective tissue growth factor; LOX, lysyl oxidase; α-SMA, α-smooth muscle actin; WGA, wheat germ agglutinin; c/EBPβ; CCAAT/enhancer-binding protein β; TRAF3IP2, TRAF3 interacting protein 2; α-MyHC, α-myosin heavy chain; MMP, matrix metalloproteinase; qPCR, quantitative PCR; EF, ejection fraction; FS, fractional shortening; Tg, transgenic; NTg, nontransgenic; Coll, collagen type 1α1; M-mode, motion; B-mode, bidimensional mode; LV, left ventricular.
for sustained NF-κB activation in pathological cardiac remodeling and development of heart failure.

Contrary to IKK and NF-κB signaling, JNK and the components of its downstream target AP-1 (fra1 and JunD) exhibit differential effects in the heart. Although AP-1 is up-regulated during the early stages of the hypertrophic response, FOSL1 (fra-1) gene deletion failed to affect the hypertrophic response to chronic pressure overload or sustained β-adrenergic stimulation (9). In contrast, fra-1 overexpression resulted in premature decompensation. Similarly, transgenic mice with JunD overexpression in a cardiomyocyte-specific manner spontaneously developed ventricular dilation and contractile dysfunction (9). Notably, fra-1 transgenic mice simultaneously lacking JunD spontaneously developed dilated cardiomyopathy, characterized by increased cardiomyocyte death (9). These data suggest that chronic AP-1 activation can be either protective or detrimental to the heart. In addition to NF-κB and AP-1, the nuclear transcription factor CCAAT/enhancer-binding protein β (c/EBPβ) has been shown to contribute to physiological hypertrophy (10), indicating that activation of NF-κB, AP-1 and c/EBPβ contribute to development of myocardial hypertrophy and possible transition to failure.

One of the upstream regulators of these classic regulators of inflammation is the cytoplasmic adapter molecule TRAF3 interacting protein 2 (TRAF3IP2; previously known as CIKS or Act1) (11, 12). Using loss-of-function (knock-out) mouse models, a number of reports have described its causal role in the pathogenesis of various autoimmune and inflammatory diseases (13–17). However, no reports are available yet describing the consequences of TRAF3IP2 overexpression in vivo. In an in vitro study, Leonardi et al. (11) have demonstrated that ectopic overexpression of TRAF3IP2 by itself was sufficient to induce NF-κB activation through TRAF3IP2/IKK physical association. We previously demonstrated activation of JNK via TRAF3IP2/IKK/y interaction (18), implying that TRAF3IP2 overexpression could result in IKK, JNK, and c/EBPβ activation in vivo with the subsequent induction of inflammatory mediators, resulting in pathological changes. Therefore, we have generated a gain-of-function (transgenic) mouse model that overexpresses TRAF3IP2 in a cardiomyocyte-specific manner (TRAF3IP2-Tg) and investigated the consequences of its overexpression.

Results

Characterization of TRAF3IP2-Tg Mice—We have generated a transgenic mouse model that overexpresses TRAF3IP2 in a cardiomyocyte-specific manner using the α-myosin heavy chain (α-MyHC) promoter (TRAF3IP2-Tg, Fig. 1). RT-qPCR revealed markedly increased (∼200-fold) TRAF3IP2 mRNA expression in the hearts of male Tg mice as compared with control non-transgenic (NTg) littersmates (Fig. 2A). Its protein levels were also increased significantly in hearts by 3.48-fold, similar to those seen after angiotensin II infusion in wild type mice (18) (Fig. 2B). However, its protein levels were not altered in lung, liver, small intestine, and kidneys of the Tg mice (Fig. 2, C and D). Increased expression of TRAF3IP2 in the heart was detected specifically in cardiomyocytes (Fig. 2E) but not other myocardial constituent cells (fibroblasts, endothelial, and smooth muscle cells), confirming the generation of a Tg mouse that constitutively overexpresses TRAF3IP2 specifically in hearts (Fig. 2).

Cardiac-restricted TRAF3IP2 Overexpression Results in IKK/NF-κB, JNK/AP-1, c/EBPβ, and p38 MAPK Activation—Because TRAF3IP2 is an upstream regulator of IKK/NF-κB, JNK/AP-1, and c/EBPβ (11, 12, 19), we investigated whether TRAF3IP2 overexpression results in their spontaneous activation. The results show that phosphorylated levels of IKKβ and its downstream target p65, JNK and its downstream target c-Jun, and c/EBPβ were all increased significantly in hearts of
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p38 MAPK, a stress-regulated kinase (Fig. 2). In addition, TRAF3IP2 overexpression resulted in the activation of multiple transcription factors and stress-activated kinases that are known to induce inflammation and promote a hypertrophic response. However, the protein levels of TRAF3IP2, phospho-p65, and phospho-p38 MAPK remained unaltered in the hearts of female Tg mice (Fig. 2G).

Cardiac-restricted TRAF3IP2 Overexpression Results in Spontaneous Development of Myocardial Hypertrophy and Contractile Dysfunction—Because activation of NF-κB, AP-1, c/EBPβ, and p38 MAPK has been shown to contribute to hypertrophy development (5, 7–10, 20, 21), we next investigated whether TRAF3IP2 overexpression results in myocardial hypertrophy. At 2 months of age, both Tg and control NTg littermates underwent transthoracic echocardiography. Motion (M)-mode analysis revealed a hypertrophic response only in male Tg mice as evidenced by increased LV wall thickness (22%; 1.220 ± 0.037 versus 0.9983 ± 0.076 mm; p < 0.02) and decreased LV volume (19%; 17.61 ± 0.994 versus 21.89 ± 2.006 μl; p < 0.02; Fig. 3A, Table 1). Bidimensional (B)-mode analysis confirmed a decrease in chamber volume at both systole and diastole (Fig. 3B). The male Tg mice also exhibited cardiac dysfunction. The ejection fraction (EF) was decreased by 17% (51.32 ± 2.21 versus 61.64 ± 2.95%; p < 0.02), and fractional shortening (FS) was decreased by 21% (25.75 ± 1.37% versus 32.78 ± 2.18%; p < 0.03) (Fig. 3C; Table 1). However, these changes were not observed in the female Tg mice, which appeared to be normal (Table 2).

Supporting the echocardiographic data, cine-MRI revealed significantly thickened LV septal wall (S; mid-ventricular short axis at end-diastole) in the male Tg mice at 2 months of age (~27%; 1.18 ± 0.04 versus 0.928 ± 0.03 mm; p < 0.002; Fig. 3D). Furthermore, the EF was decreased by 16.5% (45.50 ± 0.7 versus 54.5 ± 2.6%; p < 0.02), the stroke volume was decreased by ~20% (21.7 ± 0.3 versus 27.0 ± 0.9 μl; p < 0.001), cardiac output was decreased by ~24% (8.0 ± 0.02 versus 10.5 ± 0.4 ml/min; p < 0.002), and peak ejection rate was decreased by ~18% (0.49 ± 0.02 versus 0.6 ± 0.03 μl/min; p < 0.03). However, end diastolic volumes were similar in both Tg and control NTg littermates. In addition, the diastolic function, peak filling rate, initial filling rate, and diastolic relaxation time were all similar in both groups (Table 1).

The findings from the pressure-volume conductance catheterization confirmed cardiac dysfunction in the male Tg mice as evidenced by decreased EF (10.5%; 56.17 ± 0.83 versus 62.73 ± 1.42%; p < 0.006), stroke volume (22%; 43.81 ± 2.314 versus
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56.15 ± 3.02 μl; \( p < 0.007 \), and cardiac output (−21%; 22.67 ± 0.67 versus 28.54 ± 1.35 ml/min; \( p < 0.0009 \); Table 1). However, end diastolic pressure, end systolic pressure, end diastolic volume, and end systolic volume did not vary between the two groups (Table 1). Together, these data demonstrate that male Tg mice with constitutive overexpression of TRAF3IP2 in a cardiomyocyte-specific manner spontaneously develop myocardial hypertrophy and contractile dysfunction.

Confirming echocardiographic and cine-MRI data, gross morphology showed increased heart size in the male Tg mice at 2 months of age (Fig. 4A, upper panel). Furthermore, the heart-to-body-weight ratios (−18%; 5.038 ± 0.09 versus 4.287 ± 0.06 mg/g; \( p < 0.001 \); Fig. 4A, lower panel), cardiomyocyte cross-sectional area (wheat germ agglutinin (WGA) staining; 451.1 ± 24.0 versus 303.0 ± 17.45 μm²; \( p < 0.0005 \); Fig. 4B), and expression of fetal genes ANP, BNP, and β-MyHC (Fig. 4C, mRNA, 16-, 9-, and 6-fold, respectively; protein, Fig. 4D), and GATA4 (Fig. 4D) were all increased in the Tg mice. However, the temporal studies showed that the hypertrophic response continued for up to 4 months of age but improved at later time periods (Fig. 4E).

In contrast to males, the female Tg mice failed to develop myocardial hypertrophy (heart weight to body weight ratios, 4%; Tg, 4.61 ± 0.04 versus NTg littermates, 4.43 ± 0.19 mg/g, \( n = 5–7 \)/group). Furthermore, ANP and GATA4 levels remained low, essentially at control levels (Fig. 4F), suggesting a gender-dependent effect of TRAF3IP2 in the hypertrophic response.

**TRAF3IP2 Overexpression Results in Cardiac Fibrosis**—Pathological hypertrophy is associated with a fibrotic response (22), with remodeling of the extracellular matrix playing a key role in its development and progression. The key structural proteins involved in cardiac fibrosis are collagens type Iα1 (ColIα1) and type IIIα1 (ColIIIα1). The expression levels of these matrix genes were increased markedly in the hearts of male Tg mice at 2 months of age (mRNA, Fig. 5A; protein, Fig. 5B). Furthermore, the mRNA expression of lysyl oxidase (LOX), a key regulator of collagen cross-linking and deposition of insoluble collagens (23), was also increased in the Tg heart, as was LOX enzyme activity (Fig. 5B). The mRNA levels of the growth factor CTGF, a positive regulator of collagen expression (24), were also increased in the Tg hearts (Figs. 5, A and B). Together, these data demonstrate that TRAF3IP2 overexpression results in increased expression of the fibrillar collagens (Iα1 and IIIα1) and their positive regulator CTGF, and activation of LOX in Tg hearts, changes that might have contributed to cardiac fibrosis.

Supporting the mRNA and protein data, immunofluorescence studies revealed increased deposition and co-localization of ColIα1 and IIIα1 in the Tg heart (Fig. 5C). However, unlike myocardial hypertrophy, which improved after 4 months of age (Fig. 5E), fibrosis progressed with age, with even higher collagen levels detectable at 8 months of age (Fig. 5D).

Activated fibroblasts are the predominant cell type responsible for increased expression and deposition of collagens (25, 26). Inflammatory and hypertrophic stimuli trigger the differentiation of resident cardiac fibroblasts into a more contractile and synthetic phenotype, called myofibroblasts (25). To determine whether TRAF3IP2 overexpression increases the number of myofibroblasts in the hypertrophied myocardium, we analyzed various markers of tissue components of the heart. The results show that whereas the expression of CD-31, a surface marker expressed predominantly on endothelial cells (27), remained unchanged (Fig. 6A). α-SMA, one of the markers of myofibroblasts (25, 28), was greatly increased in myofibroblasts.
TABLE 1
Anatomic and functional changes in 2-month-old TRAF3IP2-Tg and control NTg littermates

| Parameter                              | NTg                        | TRAF3IP2-Tg | p value |
|----------------------------------------|----------------------------|-------------|---------|
| n                                      | 6                          | 6           |         |
| Body weight (g)                        | 25.45 ± 0.75               | 26.10 ± 0.9 | 0.7     |
| Heart weight (mg)                      | 109.10 ± 3.56              | 131.5 ± 3.95| 0.001   |
| Heart weight/body weight (mg/g)        | 4.287 ± 0.06               | 5.038 ± 0.09| 0.003   |
| Cardiomyocyte cross-sectional area (μm²)| 303.0 ± 17.45              | 451.1 ± 24.0| 0.0005  |

Functional data

| Parameter                              | NTg                        | TRAF3IP2-Tg | p value |
|----------------------------------------|----------------------------|-------------|---------|
| n                                      | 6                          | 6           |         |
| Septal wall thickness (mm)             | 0.928 ± 0.03               | 1.18 ± 0.01 | 0.002   |
| LVMI (μl/mg)                           | 50.1 ± 2.6                 | 482.2 ± 1.4 | 0.001   |
| LVESV (μl)                             | 23.1 ± 2.4                 | 26.5 ± 1.1  | 0.31    |
| SV (%)                                 | 27.0 ± 0.9                 | 21.7 ± 0.3  | 0.001   |
| CO (ml/min)                            | 10.4 ± 0.5                 | 8.0 ± 0.2   | 0.002   |
| EF (%)                                 | 54.5 ± 2.6                 | 45.0 ± 0.7  | 0.002   |
| PER (%)                                | 6.0 ± 0.03                 | 4.99 ± 0.02 | 0.002   |
| PFR (%)                                | 0.79 ± 0.06                | 0.77 ± 0.08 | 0.009   |
| FFR (%)                                | 0.27 ± 0.05                | 0.19 ± 0.04 | 0.029   |
| Retention time (ms)                    | 28.15 ± 3.71               | 34.42 ± 2.32| 0.24    |
| Pressure volume conductance catheter   |                            |             |         |
| n                                      | 4                          | 4           |         |
| ESP (mmHg)                             | 55.37 ± 1.12               | 52.15 ± 1.08| 0.08    |
| EDP (mmHg)                             | 2.604 ± 0.19               | 1.939 ± 0.34| 0.14    |
| EDV (μl)                               | 25.56 ± 0.65               | 28.24 ± 1.22| 0.001   |
| SV (μl)                                | 40.67 ± 3.27               | 34.93 ± 2.90| 0.21    |
| CO (ml/min)                            | 56.15 ± 3.10               | 43.81 ± 2.31| 0.007   |
| EF (%)                                 | 62.73 ± 1.42               | 56.17 ± 0.83| 0.006   |
| Heart rate (beats/min)                 | 481.1 ± 14.77              | 573.0 ± 22.68| 0.008   |

TABLE 2
Cardiac functional changes in 2-month-old female TRAF3IP2-Tg and NTg mice

Results represent the mean ± S.E. (n = 5/group) and are considered statistically significant at p < 0.05. LVIDd, LV internal diameter in diastole; LVIDs, LV internal diameter in systole; LVAWd, LV anterior wall diameter in diastole; LVPWd, LV posterior wall diameter in diastole; LVIDv, LV end diastolic volume; LVAWv, LV anterior wall volume; LVPWv, LV posterior wall volume; EDV, end diastolic volume; ESV, end systolic volume; CO, cardiac output; FFR, Frank-Follmer; SI, stress index; EF, ejection fraction; FFR, filling fraction; SV, stroke volume. Results represent the mean ± S.E. and are considered statistically significant if p < 0.05.

| Parameter                              | Female NTg    | Female TRAF3IP2-Tg | p value |
|----------------------------------------|---------------|------------------|---------|
| LVIDd (mm)                             | 3.238 ± 0.068 | 3.14 ± 0.12     | 0.49    |
| LVIDs (mm)                             | 2.367 ± 0.109 | 2.243 ± 0.06    | 0.35    |
| LVAWd (mm)                             | 1.043 ± 0.017 | 1.178 ± 0.06    | 0.06    |
| LVPWd (mm)                             | 1.152 ± 0.035 | 1.250 ± 0.04    | 0.10    |
| LVAWv (mm)                             | 3.132 ± 0.034 | 1.338 ± 0.04    | 0.63    |
| EF (%)                                 | 56.51 ± 1.026 | 57.26 ± 4.6     | 0.87    |
| FFR (%)                                | 28.61 ± 0.644 | 29.41 ± 3.2     | 0.81    |

and vascular smooth muscle cells of Tg hearts (Fig. 6A). Furthermore, tensin, which is expressed in myofibroblasts (29), was detected only in the Tg hearts (Fig. 6B). Paxillin, expressed in both myofibroblasts and vasculature (29), also increased in the Tg hearts (Fig. 6C). Thus, TRAF3IP2 overexpression resulted in an increased number of myofibroblasts in the hypertrophied Tg heart. Supporting these observations, the Quantibody® Mouse Cytokine Antibody Array also showed a 2.4-fold increase in the expression of peristin (Table 3), another marker of myofibroblasts (29).

TRAF3IP2 Overexpression Results in Enhanced Expression of Proinflammatory Mediators in the Heart—Inflammation is a major risk factor associated with cardiac pathologies, including hypertrophy, fibrosis, dysfunction, and failure (2–4). Potent proinflammatory cytokines such as IL-6, IL-17, IL-18, and TNF-α mediate both hypertrophic and fibrotic responses in the heart and contribute to contractile dysfunction and heart failure development (30). The chemokine fractalkine also inhibits cardiomyocyte contractility (31). In the Tg mouse hearts there was an increase in the expression of IL-6 and IL-18 at both mRNA (Fig. 7A) and protein levels (Fig. 7B). Furthermore, antibody array analysis showed a significant increase in cytokines (IL-6, TWEAK (TNF-related weak inducer of apoptosis), TWEAKR, β-FG, IL-17, TNF-α, G-CSF and M-CSF), chemokines (fractalkine), and adhesion molecules (E-selectin, P-selectin, ICAM-1 (intercellular adhesion molecule 1), VCAM-1 (vascular cell adhesion molecule-1)) in Tg mouse hearts (Table 3). In addition, MMP-3 and MMP-10 levels were also higher in the Tg mice (Table 3). Interestingly, although expression levels of the pro-hypertensive mediators angiotensin-converting enzyme (ACE), angiotensin III, and renin-3 were elevated in Tg mice (Table 3), no significant changes in blood pressure were detected (tail cuff plethysmography; data not shown). Unlike in male Tg mice, the levels of most of these pro-inflammatory, pro-hypertrophic, and pro-fibrotic mediators remained low or unchanged in the hearts of female Tg mice (Table 3).

Macrophage infiltration is a common feature of cardiac inflammation (32), and Mac3 serves as a general marker for macrophages. Immunofluorescent studies showed an increased number of Mac3+ cells in the Tg hearts (Fig. 7C). Of note, macrophages contribute to adverse cardiac remodeling by expressing various pro-inflammatory, pro-hypertrophic, and pro-fibrotic mediators (32). Together, these results indicate that constitutive overexpression of TRAF3IP2 enhances the expression of various inflammatory mediators and the infiltration of inflammatory cells into the heart, possibly contributing to adverse cardiac remodeling in the male Tg mice.

Discussion

The results of this study show for the first time that cardiac-restricted TRAF3IP2 overexpression (TRAF3IP2-Tg) results in the spontaneous development of myocardial hypertrophy, cardiac fibrosis, and contractile dysfunction. Although hypertrophy resolved with age, fibrosis progressed in the Tg mice. These adverse cardiac changes occurred only in the male Tg mice. Overexpression of TRAF3IP2 did not appear to induce cell death despite progressive fibrosis, and the Tg mice failed to develop heart failure during the 8-month study period (data not shown). These results indicate that persistently elevated TRAF3IP2 levels play a causal role in adverse cardiac remodeling, at least in male mice.

The overexpression of TRAF3IP2 resulted in the activation of IKK/NF-κB, JNK/AP-1, c/EBPβ, and p38 MAPK, all of which contribute to the development of myocardial hypertrophy, fibrosis, contractile dysfunction, and heart failure (3, 5, 7–10, 20, 21), suggesting that TRAF3IP2 overexpression may result in
adverse cardiac remodeling. In fact, its overexpression resulted in myocardial hypertrophy, which was evident as early as 2 months of age, but this resolved by 6 months of age. In contrast, these mice showed progressive fibrosis, which did not resolve with age. Because cardiac fibrosis leads to diastolic dysfunction and heart failure development (33), we will continue to monitor these Tg mice until moribund.

In addition to activation of multiple transcription factors and p38 MAPK, TRAF3IP2 overexpression also increased the levels of GATA4, a member of the highly conserved zinc finger-containing transcription factors that regulates cardiomyocyte hypertrophy and induction of the fetal gene program (34). In fact, the Tg mice showed increased levels of ANP, BNP, and β-MyHC, all of which are GATA4-responsive genes (34). Sur-

FIGURE 4. Transgenic overexpression of TRAF3IP2 results in spontaneous development of myocardial hypertrophy. A, gross morphology of the heart (upper panel) and heart weight (HW) to body weight (BW) ratios (lower panel) in 2-month-old NTg and Tg mice. B, cardiomyocyte cross-sectional area was analyzed using WGA-stained heart sections. Scale: 50 μm. The mean area of cardiomyocytes was quantified and summarized on the right. C, mRNA expression of fetal genes (ANP, BNP, and β-MyHC) in LV tissues as analyzed by RT-qPCR. 18S served as an internal control. D, protein expression of fetal genes and GATA4 was analyzed by immunoblotting. Densitometric analysis of immunoreactive bands is summarized on the right. E, temporal changes in heart weight to body weight ratios. F, ANP and GATA4 expression in female Tg hearts (n = 4/group). Error bars represent S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus NTg littermates.
意外的是，在最近的一项研究中，GATA4 被证明以 TRAF3IP2 依赖的模式激活 NF-κB/H9260，表明 TRAF3IP2 可能通过多种途径激活 NF-κB/H9260，包括 IKK 和 GATA4，两者在 Tg 小鼠中增加。

不利/不适应性心脏重塑是心力衰竭发展的主要预测因子，并且与持续升高多种促炎细胞因子有关（30）。事实上，检测到增加的 IL-6，IL-17，IL-18，和 TNF-9251 水平。这些细胞因子各自通过激活胞内应激激活激酶，包括 p38 MAPK，TRAF6，与 TRAF3IP2 分子的物理关联（36），是 p38 MAPK 激活已知的诱导剂（37），并参与诱导多种促炎介质，以诱导心肌肥大和心脏纤维化。值得注意的是，p38 MAPK 也参与 GATA4 激活在心肌细胞（38）。

IL-6，一种促炎性和促心肌肥厚的细胞因子，通过刺激增加的胶原沉积诱导心脏纤维化，在部分通过 PI3K-Akt-GATA4-依赖的信号途径（40）。IL-18 也通过促进成纤维细胞纤维化和内皮细胞凋亡（41, 42）。增加的 TNF-9251 诱导心脏功能障碍，心肌病，不利 LV 重塑，凋亡，和 cachexia（43）。除了增加的 IL-6，IL-18，和 TNF-9251，TRAF3IP2 过度表达也导致增加的 TWEAK 和其受体，TWEAKR，两者都被认为是促纤维化，促心肌肥大，及功能障碍的贡献。事实上，转基因小鼠过表达可溶性 TWEAK，具体在心肌细胞中，显示出增加的死亡（44, 45），表明其持续诱导促进心力衰竭的发展。TWEAK 也诱导成纤维细胞，纤维细胞增殖，胶原

**FIGURE 5.** Transgenic overexpression of TRAF3IP2 results in spontaneous development of cardiac fibrosis. A, mRNA expression of Colα1, ColIIIα1, and CTGF in LV tissues of 2-month-old Tg and NTg mice as analyzed by RT-qPCR (18S served as an internal control). B, protein levels of Colα1, ColIIIα1, LOX, and CTGF in LV tissues from Tg and NTg mice as analyzed by immunoblotting. Densitometric analysis of immunoreactive bands is summarized on the right. C, deposition and co-localization of Colα1 and ColIIIα1 were analyzed in LV tissues from Tg and NTg mice by immunofluorescence. Relative fluorescent signals were quantified and summarized on the right. D, temporal changes in cardiac fibrosis as analyzed by Masson’s trichrome staining. Mean collagen positive area is summarized on the right (n = 4/group). Error bars represent S.E. *p < at least 0.05 versus NTg littermates.
Our results showed that the male Tg hearts expressed higher contractility (23). Of note, LOX activation is increased during deposition of insoluble collagens that adversely affect cardiac components, including collagens, secreted predominantly by myofibroblasts. Myofibroblasts also express LOX, the collagen cross-linking enzyme. Increased expression of LOX enhances fibroblasts. Myofibroblasts also express LOX, the collagen cross-linking enzyme. Increased expression of LOX enhances cardiac fibrosis (33). It is a progressive process and results from excessive accumulation of extracellular matrix components, including collagens, secreted predominantly by myofibroblasts. Myofibroblasts also express LOX, the collagen cross-linking enzyme. Increased expression of LOX enhances deposition of insoluble collagens that adversely affect cardiac contractility (23). Of note, LOX activation is increased during adverse cardiac remodeling and heart failure development (23). Our results showed that the male Tg hearts expressed higher levels of activated LOX. Of note, both collagens and LOX are NF-κB- and AP-1-responsive genes (50, 51). Thus, TRAF3IP2 overexpression results in the spontaneous induction of various pro-fibrotic mediators and the development of cardiac fibrosis in the male Tg mice.

Interestingly, the expression levels of these remodeling-associated inflammatory mediators remained either unaltered or down-regulated in the hearts of female Tg mice, suggesting that female sex hormones may negatively regulate TRAF3IP2 down-regulated in the hearts of female Tg mice, suggesting that female sex hormones may negatively regulate TRAF3IP2 overexpression in female Tg mice. Proteins that showed a 1.5-fold change or more are depicted. † and ‡ indicate up- or down-regulation, respectively. MCSF, macrophage-colony stimulating factor; TWEAK, TNF-related weak inducer of apoptosis; TWEAKR, TWEAK receptor; BLC, B-lymphocyte chemoattractant; MAdCAM-1, mucosal vascular addressin cell adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; β-FGF, basic fibroblast growth factor; ACE, angiotensin converting enzyme; Ang, angiotensin; ECM, extracellular matrix.
cardiac remodeling. Cardiac-restricted constitutive overexpression of TRAF3IP2 spontaneously resulted in inflammation, myocardial hypertrophy, cardiac fibrosis, and contractile dysfunction.

Future Perspectives—(i) Because the female Tg mice failed to develop myocardial hypertrophy, we will determine whether ovariectomy elicits a hypertrophic response. (ii) The female Tg mice showed increased TRAF3IP2 mRNA, but not protein expression, suggesting its post-transcriptional or post-translational regulation by female sex hormones. Therefore, we will analyze changes in miRNA and LncRNA profiles in hearts from male and female Tg mice, identify the ones that are differentially expressed, investigate their effects on TRAF3IP2 expression, and determine their therapeutic potential. (iii) Despite myocardial hypertrophy and cardiac fibrosis, the male Tg mice failed to develop heart failure. Therefore, we will determine whether additional stressors such as myocardial infarction will result in earlier decompensation and heart failure development or death. (iv) Because pharmacological inhibitors of TRAF3IP2 are not available yet, we will determine whether silencing TRAF3IP2 in the Tg mice with an Adeno-associated virus, serotype 9-based gene therapeutic approach using a truncated cardiac troponin T promoter that provides cardiomyocyte specificity will blunt development of hypertrophy, fibrosis, and contractile dysfunction. (v) Like cardiomyocytes, cardiac fibroblasts are critical in adverse cardiac remodeling. Therefore, we will determine whether transgenic overexpression of TRAF3IP2 in a cardiac fibroblast-specific manner develops cardiac fibrosis and diastolic dysfunction earlier and progresses to heart failure faster. Our long term goal is to develop small molecule or pharmacological TRAF3IP2 inhibitors and investigate their translational potential in cardiac diseases.

Experimental Procedures

Animal Studies—All animal studies were approved by the Institutional Animal Care and Use Committees at Tulane University, New Orleans, LA and the Harry S. Truman Memorial Veteran’s Hospital/University of Missouri, Columbia, MO, and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Wild type FVB mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Generation of TRAF3IP2-Tg Mice and Copy Number Determination—Mus musculus TRAF3IP2 cDNA was amplified by RT-PCR using heart RNA isolated from C57BL/6 mice as the starting template. The PCR product was cloned into α-MyHC promoter vector (Fig. 1A; a kind gift from Jeffrey Robbins, University of Cincinnati, Cincinnati, OH; α-MyHC promoter GenBank™ accession #U714441), and the nucleotide sequence was confirmed. The resultant construct of ~7.8 kb (Fig. 1B) was used to generate Tg mice. PCR screening was used to identify the founder animals, and three Tg lines (lines 1, 2, and 3) were generated by breeding the founder mice with wild type FVB mice (The Jackson Laboratory). Line 1 was used in the present study. Transgene copy number was determined by Southern blot analysis using genomic DNA isolated from tail snips and digested with EcoRV and NotI (Fig. 1C). Genotyping was performed by PCR using genomic DNA isolated from tail snips and the following primers: forward, 5′-TGT CTG ATT CAC GGC GCA-3′; reverse, 5′-CGG CAC TCT TAG CAA ACC TCA GCC AC-3′ (Fig. 1D).

Temporal Studies—TRAF3IP2-Tg mice (hereafter referred to as Tg) were bred with wild type FVB mice. The offspring were screened for the transgene by PCR. Naïve Tg mice along with the littermates were investigated for up to 8 months of age. Age- and gender-matched NTg littermates served as controls. Blood pressure was recorded between 9 and 11 a.m. using tail-cuff plethysmography (BP 2000 Blood Pressure Analysis System, Visitech).

Echocardiography—Both male Tg and age-matched littermates (n = 6 in each group) were subjected to transthoracic echocardiography under isoflurane anesthesia using a Vevo 770 high resolution ultrasound system (FUJIFILM VisualSonics, Toronto, ON) with a 30-MHz frequency real-time microvisuализation scan head (RMV707). Motion (M)-mode and bidimensional (B)-mode tracings were taken, and the parameters for anatomical alterations in left ventricle (LV anterior and posterior wall thickness, internal diameters at systole and diastole) and function (EF and FS) were evaluated. FS was calculated using the equation %FS = LVEDD − LVESD/LVEDD × 100, where LVEDD is LV end diastolic diameter, and LVESD is LV end systolic diameter.

In Vivo High Resolution Cine-MRI—Non-invasive MRI was performed on anesthetized mice (1.8−2.7% isoflurane) using a small bore 7-tesla Bruker BioSpec MRI scanner equipped with a phased array mouse heart coil combined with an 86-mm volume coil (Bruker Biospin Inc., Billerica, MA) (54). Body temperature was maintained with warm air, and respiration rate was monitored using a small animal monitoring system (SA Instruments, Inc., Stony Brook, NY). A retrospective FLASH
cine pulse sequence was applied with the following parameters: 1-mm slice thickness and 30 × 30-mm² field of views for LV in short-axis images. LV functional parameters were determined using a series of cine images of the LV in short-axis view acquired at 20 equally spaced time points throughout the entire cardiac cycle with a frame rate of 7–9 ms/frame. Five to six short-axis slices were acquired over the whole LV. At each time point, the endocardial borders were traced to measure the LV chamber area for each short-axis slice using Segment version 1.8 R0736 (see Segment at Medviso) software (14). LV volumes at each phase were calculated as the sum of chamber areas of all slices multiplied by 1 mm of the slice thickness. The systolic and diastolic functions were calculated using a previously established protocol (15).

LV Chamber Volume and Contractile Function—Mice anesthetized with isoflurane were subjected to in vivo LV chamber volume and function analysis using a Scisense ADVantage™ pressure volume conductance catheter (FTS-1912B; Scisense Inc.) inserted into the LV via the right carotid artery (55). Data were analyzed using iWorx® Labscribe Instrument software (Dover, NH, USA) (16).

Cardiac Hypertrophy—In addition to echocardiography and cine-MRI, myocardial hypertrophy was analyzed by (i) heart to body weight ratios, (ii) cardiomyocyte cross-sectional area by WGA staining, and (iii) fetal gene (ANP, BNP, β-MHC) expression. Cardiomyocyte size was determined as the mean area of 10–15 randomly selected cells/field from a total of 8–10 fields per section.

Cardiac Fibrosis—Fibrosis was characterized by (i) immunofluorescence for Collα1 and Collα1 and (ii) Masson’s trichrome staining.

Immunofluorescence—A thin midsection of heart was fixed overnight in 4% buffered paraformaldehyde, embedded in paraffin, cut into 4-μm-thick sections, and used for immunofluorescence. The following targets were analyzed: (i) TRAF3IP2 using anti-TRAF3IP2 antibody (1:25; #sc-100647, Santa Cruz Biotechnology, Inc.) and Alexa Fluor® 488-tagged donkey anti-mouse secondary antibody (1:400; #A-21202; Thermo Fisher Scientific). Hoechst (1:400; #H-3570, Thermo Fisher Scientific) was used to visualize nuclei. To identify and quantify cardiomyocyte cell size, tissue sections were permeabilized with 0.4% TBS/Tween following antigen retrieval (citrate buffer; 45 min in a steamer) and incubated with Alexa Fluor® 594-conjugate (1:400; #S32356; Thermo Fisher Scientific). Pho- tomicrographs were obtained using a Nikon Eclipse 80i microscope and a Spot RT digital camera and analyzed by SPOT Advanced Software (Sterling Heights, MI).

mRNA Expression—Total RNA was isolated from frozen LV tissue using TRIzol reagent (Sigma), and 0.5 μg of RNA was reverse-transcribed into cDNA using a reverse transcription kit (Agilent Technologies). mRNA expression was quantified by RT-qPCR using the following Applied Biosystems™ Taq-Man™ probes: ANP (assay ID Mm01255748), BNP (assay ID Mm01255770_g1), β-MHC (Myh7; assay ID Mm01319006_g1), Collα1 (assay ID Mm00801666), CollIIIα1 (assay ID Mm1254476), CTGF (assay ID Mm01192932_g1), IL-6 (assay ID Mm00446191), IL-18 (assay ID Mm00434226), LOX (assay ID Mm00495386), and TRAF3IP2 (assay ID Mm00506094_m1). 18S rRNA (assay ID Hs99999901) served a loading control. All data were normalized to corresponding 18S levels and analyzed using 2⁻ΔΔCT method.

Protein Quantibody Array—A Quantibody® Mouse Cytokine Antibody Array 4000 (#QAM-CAA-4000; RayBiotech) was used to analyze changes in protein expression in LV homogenates. The assay is a multiplexed sandwich ELISA-based quantitative array platform that simultaneously detects and quantifies 200 cytokines. Results are expressed as -fold change compared with the corresponding NTg littersmates of similar age and gender. A -fold change of 1.5 was used as a reference standard.

Immunoblotting—Preparation of LV homogenates, electrophoresis, and immunoblotting were described previously (18, 40–42). The following antibodies were used: TRAF3IP2 (1:500; #NB100-56740, Novus), ANP (1:400; #NB2P-14873, Novus), IL-6 (1:200; #AF-406-NA, R&D Systems), α-tubulin (1:1000; #2144, Cell Signaling Technology or CST), phospho-p65 (Ser536; 1:1000; #3031, CST), p65 (1:1000; #8242, CST), phospho-c-Jun (Ser63, 1:1000; #9165, CST), JNK (1:1000; #9252, CST), phospho-JNK (Thr183/Tyr185; 1:1000; #9261, CST), c-Jun (1:1000; #9245, CST), and phospho-JNK (Thr183/Tyr185; 1:1000; #2678, CST), and phospho-IKKβ (Ser176/Ser180; 1:1000; #2694, CST), LOX (1:500; #sc-373995, Santa Cruz Biotechnology or SCB), IL-18 (1:100; #sc-7954, SCB), BNP (1:400, #sc-67455, SCB), Collα1 (1:2000; #ab34170, Abcam), CollIIIα1 (1:2000; #ab77778, Abcam), and Collα1 (1:2000; #ab34170, Abcam), CollIIIα1 (1:2000; #ab77778, Abcam), CTGF (1:500; #210303, United States Biological), and β-MHC (1:400, #M9850-11C, United States Biological).

Statistical Analysis—Data were analyzed using Microsoft Excel, Clampfit (Molecular Device, Sunnyvale, CA), and Origin 7 (OriginLab Corp., Northampton, MA) programs. Normality criteria were evaluated to select the correct parametric or non-parametric test using the Shapiro-Wilk estimator. Because the sample size was small (n = 4–8/group), the test determined that a non-parametric approach should be used to avoid type II errors. Because two groups were used (Tg and NTg), pairwise comparisons were made using the Tukey adjustment. The statistical tests were performed using the software SPSS 23.0.0.1 (Chicago, IL). The overall significance level was set at 0.05. The results are presented as the mean ± S.E.
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Author Contributions—B. C. designed the experimental strategy and interpreted the data. Y. M., A. J. V., T. Y., H. K. K., S. S., and Z. S. performed the experiments. T. Y., S. S. V. P. S., J. M. S., and U. S. contributed to vector generation, sequencing, restriction digestion, purification, Southern blots, characterization, and breeding of the transgenic mouse model. Y. M., T. Y., and A. J. V. analyzed the data. Y. M., T. Y., A. J. V., and B. C. wrote the manuscript. J. D. G. performed echocardiography and pressure-volume conductance catheterization. I. M. performed cardiac MRI.

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