Type III Transforming Growth Factor-β Receptor Drives Cardiac Hypertrophy Through β-Arrestin2–Dependent Activation of Calmodulin-Dependent Protein Kinase II

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Abstract—The role of type III transforming growth factor-β receptor (TβRIII) in the pathogenesis of heart diseases remains largely unclear. Here, we investigated the functional role and molecular mechanisms of TβRIII in the development of myocardial hypertrophy. Western blot and quantitative real time-polymerase chain reaction analyses revealed that the expression of TβRIII was significantly elevated in human cardiac hypertrophic samples. Consistently, TβRIII expression was substantially increased in transverse aortic constriction (TAC)– and isoproterenol-induced mouse cardiac hypertrophy in vivo and in isoproterenol-induced cardiomyocyte hypertrophy in vitro. Overexpression of TβRIII resulted in cardiomyocyte hypertrophy, whereas isoproterenol-induced cardiomyocyte hypertrophy was greatly attenuated by knockdown of TβRIII in vitro. Cardiac-specific transgenic expression of TβRIII independently led to cardiac hypertrophy in mice, which was further aggravated by isoproterenol and TAC treatment. Cardiac contractile function of the mice was not altered in TβRIII transgenic mice; however, TAC led to significantly decreased cardiac contractile function in TβRIII transgenic mice compared with control mice. Conversely, isoproterenol- and TAC-induced cardiac hypertrophy and TAC-induced cardiac contractile function impairment were partially reversed by suppression of TβRIII in vivo. Our data suggest that TβRIII mediates stress-induced cardiac hypertrophy through activation of Ca2+/calmodulin-dependent protein kinase II, which requires a physical interaction of β-arrestin2 with both TβRIII and calmodulin-dependent protein kinase II. Our findings indicate that stress-induced increase in TβRIII expression results in cardiac hypertrophy through β-arrestin2–dependent activation of calmodulin-dependent protein kinase II and that transforming growth factor-β and β-adrenergic receptor signaling are not involved in spontaneous cardiac hypertrophy in cardiac-specific transgenic expression of TβRIII mice. Our findings may provide a novel target for control of myocardial hypertrophy. (Hypertension. 2016;68:654-666. DOI: 10.1161/HYPERTENSIONAHA.116.07420.)

Key Words: cardiac myocyte ■ hypertrophy ■ transforming growth factor ■ transgenic mice

Pathological cardiac hypertrophy is usually induced by pressure overload and sustained β-adrenergic receptor (β-AR) activation. Cardiac hypertrophy is a strong predictor of subsequent cardiovascular events, such as diastolic dysfunction and arrhythmias, which ultimately results in heart failure.1,2 Understanding the key molecular events that mediate pathological hypertrophy is essential for developing new therapeutic strategies to prevent morbidity and mortality associated with cardiac hypertrophy.

Type III transforming growth factor-β (TGF-β) receptor (TβRIII, also known as β-glycan) is the most abundant TGF-β coreceptor in many cell types.3,4 TβRIII is an 851 amino acid (aa) proteoglycan, comprising a large 766 aa extracellular domain, a single-pass hydrophobic transmembrane region, and a short 42 aa cytoplasmic domain.5 Loss or reduced expression of TβRIII has been shown to be an important regulator of cell migration, invasion, cell growth, and angiogenesis in cancer3–5; however, the role of TβRIII in the development of human heart diseases has not been investigated.

TβRIII is classically thought to function as a coreceptor, presenting TGF-β superfamily ligands to their respective
signaling receptors. However, TβRIII has the potential to increase or decrease TGF-β signaling depending on the cell type. Recently, new insights have been gained into the structure and function of cytoplasmic TβRIII domain, which have suggested its essential role in ligand-dependent and ligand-independent functions through interactions with β-arrestin2 and Gα-interacting protein–interacting protein, C terminus. To date, there is no information on the expression levels of TβRIII in normal or pathological human cardiac tissues. Moreover, the role of TβRIII, as well as its downstream effectors, has not been yet studied in mature cardiomyocytes. Accordingly, in this study, we demonstrate that TβRIII expression is increased in human cardiac hypertrophy tissues. Concomitantly, we show that TβRIII is an important regulator in transverse aortic constriction (TAC)– and isoproterenol-induced cardiac hypertrophy in mouse models.

Methods

Detailed and expanded methodology is provided in the online-only Data Supplement.

Human Heart Samples From Patients With Cardiac Hypertrophy

The human study conformed to the principles outlined in the Declaration of Helsinki. The study protocol was approved by the ethics committee of First Affiliated Hospital of Nanjing Medical University (No. 2014-SRFA-128). Written informed consent was obtained from all patients. The tissues were obtained from 14 individuals undergoing heart surgery (8 cardiac hypertrophic samples and 6 noncardiac hypertrophic samples to serve as control). The samples were stored at −80°C until further analyses. Information about the human cardiac samples is listed in Table S1.

Generation of TβRIII Transgenic Mice

TβRIII transgenic (Tg) mice were generated by Cyagen Bioscience Inc (Suzhou, China). Briefly, the mouse TβRIII (GenBank: NM_011578.3) cDNA provided by GeneChem Co (Shanghai, China) was amplified by polymerase chain reaction and cloned into the region between the 5.4-kb mouse α-myosin heavy chain (α-MHC) promoter and the Simian virus 40 (SV40) polyadenylation sequence via Sall sites. The orientation of the inserted TβRIII cDNA was confirmed by sequencing. This construct was microinjected into fertilized eggs of FBV mouse strain. The founders (F0) of TβRIII-Tg lines were backcrossed with C57BL/6 mice for 1 to 5 generations. The positive offspring were identified using polymerase chain reaction with the following oligos (5′–3′): transgene polymerase chain reaction forward, AGCTAAGGGCCACAAGTTC and reverse, GATCTTGAAGTTCACCTTGATGc and internal control polymerase chain reaction forward, TCTTAGCTCTGTCTCCGGT and reverse, CACTGGCTGAGGAAGGAGAC.

In Vivo Adeno-Associated Viral 9 Carrying shRNA for TβRIII Infection

Adeno-associated viral (AAV) 9–mediated cardiac-specific gene knockdown has been previously demonstrated. Green fluorescent protein–tagged AAV9-mediated shRNA for TβRIII (5′-GGGGAGGTTCACCATCTCTG-3′; AAV-shRNA) and green fluorescent protein-tagged AAV9-scramble control (5′-GTTC TCCG AACG TGTC ACGT-3′) were synthesized from Biowit Technologies (Shenzhen, P.R. China). The method to infect mouse heart with virus-mediated shRNA delivery system was previously described by our group. Briefly, after the mice were anesthetized and a thoracotomy was performed, the chest was opened via the fourth intercostal space. The ascending aortic artery and main pulmonary artery were clamped and then AAV9-shRNA or AAV9-scramble control (1×1010 pfu in 100-μL saline) was injected into the left ventricular (LV) cavity through the tip of the heart using a 30-gauge syringe. The occlusion of arteries remained for 10 s after AAV9 injection. Mice in sham group underwent the same procedures and received 100-μL saline. After 7 days of AAV9 infection, mice received either isoproterenol infusion for 2 weeks or TAC for 3 weeks.

Data Analysis

Statistical analysis was performed with SPSS for Windows software version 11.5 (SPSS Inc., Chicago, IL). Comparisons between the 2 groups were determined by a Student t test. Multiple group comparison was performed using 1-way ANOVA with the Bonferroni post hoc test. Comparison between the 2 distinct independent variables, a 2-way ANOVA test was performed. A value of P<0.05 was considered statistically significant.

Results

Increased Expression of TβRIII in Human and Mouse Cardiac Hypertrophic Samples

We initially examined TβRIII expression profiles in human cardiac hypertrophic specimens. The expression levels of TβRIII mRNA and protein were, respectively, increased by 1.88-fold (Figure 1A) and 1.56-fold (Figure 1B and 1C) in human cardiac hypertrophic samples compared with noncardiac hypertrophic samples. Accordingly, the levels of hypertrophic biomarkers including atrial natriuretic peptide, β-MHC (Figure 1A through 1C), and brain natriuretic peptide were significantly elevated (Figure 1A) in human cardiac hypertrophic samples.

Consistently, TβRIII protein levels of the LV tissue were increased gradually during the time-course experiment in either the Alzet pump application of isoproterenol or the TAC-induced mouse cardiac hypertrophy (Figure 1D and 1E; Figure S1 shows the specificity of TβRIII antibody). Moreover, immunofluorescence staining clearly showed that TβRIII was expressed in mouse LV tissue (Figure 1F, a). Line-scanning analysis of the fluorescence intensity suggested that TβRIII was mainly present in the vicinity of the plasma membrane of mature cardiomyocytes (Figure 1F, b). These results suggested that an increased expression of TβRIII may play a role in the development of cardiac hypertrophy.

We found that the expression levels of TβRIII in primary cultured neonatal mouse cardiomyocytes (NMCs) were upregulated by isoproterenol in a dose- and time-dependent manner (Figure S2). We then examined whether manipulating the expression levels of TβRIII in NMCs affects isoproterenol- or angiotensin II–induced hypertrophic phenotype. Our data clearly show that gain-of-function of TβRIII led to cardiac hypertrophy phenotype without isoproterenol stimulation, whereas loss-of-function of TβRIII attenuated isoproterenol- or angiotensin II–induced cardiomyocyte hypertrophy (Figure S2).

Cardiac-Specific Overexpression of TβRIII Recapitulates the Myocardial Hypertrophy Phenotype

We then examined whether a TβRIII transgenic mouse model carrying the cardiac-specific α-MHC promoter (CS-TβRIII-Tg) could recapitulate the cardiac hypertrophic
phenotype (Figure 2A). Three months after CS-TßRIII-Tg, expression levels of TßRIII were specifically increased in mouse hearts but not in kidneys, suggesting the specificity and successful establishment of the CS-TßRIII-Tg mouse model (Figure 2B and 2C). Relative to wild-type (WT) mouse, the levels of TßRIII and ß-MHC proteins were, respectively, increased ≈2.7-fold and ≈2.4-fold in heterozygous mouse hearts (Figure 2C). The expression levels of TßRIII, atrial natriuretic peptide, brain natriuretic peptide, and ß-MHC mRNA were remarkably increased in 3-month-old CS-TßRIII-Tg mice compared with age-matched WT mice (Figure 2D). Cardiac dimensions were monitored in conscious mice using serial echocardiography. CS-TßRIII-Tg mice showed increased LV wall thickness and LV mass (Figure 2E; Table S4). Comparative analysis of histology (Figure 2F), ratios of heart weight/body weight, and heart weight/tibia length (Figure 2G and 2H) between 3-month-old CS-TßRIII-Tg mice and age-matched WT mice revealed that cardiac-specific overexpression of TßRIII resulted in spontaneous cardiac hypertrophy. Moreover, isoproterenol infusion at a rate of 10 mg/kg per day or TAC produced more severe cardiac hypertrophy in CS-TßRIII-Tg mice than in either WT mice or CS-TßRIII-Tg mice (isoproterenol: Figure 2I through 2K; TAC: Figure 2L through 2N). Notably, after 3 months, there was no alteration of cardiac function in CS-TßRIII-Tg mice; however, cardiac contractility declined dramatically in
these mice 3 weeks post TAC operation or 2 weeks of isoproterenol stimulation, as indicated by the reduced ejection fraction and fractional shortening (Figure 2O and 2P). In addition, Masson staining showed that isoproterenol and TAC stimulation resulted in more severe cardiac fibrosis in CS-TβRIII-Tg mice than their age-matched WT mice (Figure 2Q and 2R).

Taken together, our data suggest that cardiac overexpression of TβRIII was sufficient to induce cardiac hypertrophy and that elevated expression of TβRIII accelerated stress-induced injury of heart function.

Cardiac AAV9-Mediated TβRIII Gene Silencing Attenuates Stress-Induced Myocardial Hypertrophy

We reasoned that if overexpression of TβRIII promoted myocardial hypertrophy, the knockdown of endogenous TβRIII should prevent stress-induced cardiac hypertrophy. We, therefore, used cardiac-targeted AAV9 vector-mediated specific shRNA (AAV9-shTβRIII) to knockdown TβRIII gene in mouse heart. Immunofluorescence staining data suggested that the heart tissue was effectively infected by either AAV9-TβRIII-green fluorescent protein or AAV9-scramble-green fluorescent protein (Figure 3A). Western blot revealed that the expression levels of TβRIII in the LV significantly decreased on infection of hearts with AAV9-shTβRIII in a time-dependent manner (Figure 3B) but not with AAV9-mediated scramble shRNA (data not shown). According to these results, 1 week after infection, mice received isoproterenol infusion (10 mg/kg per day) for 2 weeks or TAC operation for 3 weeks. Echocardiographic examination showed that isoproterenol-induced increase in hypertrophic indices in control mice were greatly decreased in AAV9-shTβRIII-infected mice (Figure 3C, a; Table S5). Moreover, TAC-induced cardiac hypertrophy and impairment of cardiac function were partially rescued by AAV9-shTβRIII infection (Figure 3C, b; Table S6). The ratios of LV mass/body weight and LV mass/tibia length were significantly greater in isoproterenol- or TAC-induced cardiac hypertrophy mice than in sham-operated mice, whereas these indices of isoproterenol- or TAC-induced cardiac hypertrophy were greatly decreased in AAV9-shTβRIII–mediated cardiac-specific knockdown mice compared with the mice infected with AAV9-mediated scramble shRNA (Figure 3D through 3G). Furthermore, 12 weeks after TAC operation, the ejection fraction and fractional shortening declined remarkably in both the WT and the AAV9-mediated scramble shRNA-infected mice; importantly, this TAC-induced impairment of cardiac contractility was partially rescued by AAV9-shTβRIII–mediated cardiac-specific knockdown of TβRIII in mice (Figure 3H and 3I). Consistently, isoproterenol- or TAC-induced increase in the expression levels of β-MHC protein and atrial natriuretic peptide, brain natriuretic peptide, and β-MHC mRNA were significantly decreased by AAV9-shTβRIII–mediated cardiac-specific knockdown of TβRIII (Figure 3J through 3L). These results further confirm that TβRIII plays an important role in stress-induced cardiac hypertrophy.

TβRIII-Mediated Activation of CaMKII Is Linked by β-Arrestin2

Cytoplasmic domain of TβRIII interacts with the scaffolding protein, β-arrestin2, and the consequence of which affects proliferation, mobility, and migration in various cancer cell types.16,17 Mangmool et al17 revealed that β-arrestins are essential for calmodulin-dependent protein kinase II (CaMKII) activation by isoproterenol. We, therefore, examined whether β-arrestin2 served as a scaffold for either TβRIII or CaMKII in NCMCs. The data showed that TβRIII complexed with β-arrestin2 (Figure 4A), and β-arrestin2 also formed a complex with CaMKII (Figure 4B). However, our Co-immunoprecipitation data revealed that there was no direct interaction between TβRIII and CaMKII (Figure S3). Interestingly, the magnitude of these complexes was, respectively, enhanced by isoproterenol stimulation and by transient overexpression of TβRIII in NCMCs (Figure 4C). Conversely, isoproterenol-induced increase in the formation of TβRIII/β-arrestin2 (Figure 4C, a) and β-arrestin2/CaMKII (Figure 4C, b) complexes was remarkably decreased by knockdown of TβRIII in NCMCs. Western blot analysis showed that phosphorylated CaMKII (p-CaMKII) levels were significantly increased by isoproterenol stimulation, an event that was greatly blunted by knockdown of TβRIII in NCMCs (Figure 4D). Interestingly, transient overexpression of TβRIII independently increased expression levels of p-CaMKII and β-MHC in NCMCs (Figure 4D). We next examined whether the scaffold role of β-arrestin2 was required for TβRIII–mediated CaMKII activation and cardiac hypertrophy by knocking down β-arrestin2 (efficiency shown in Figure S4) in TβRIII overexpressed NCMCs. Consistently, knockdown of β-arrestin2 in NCMCs greatly reduced p-CaMKII and β-MHC levels (Figure 4E). KN-93, an inhibitor of CaMKII,
Figure 3. Knockdown of type III transforming growth factor-β receptor (TβRIII) in the heart attenuates isoproterenol (ISO)- or transverse aortic constriction (TAC)-induced cardiac hypertrophy. A, Representative immunostaining demonstrating the effectiveness of cardiac infection with adeno-associated viral 9 (AAV9)-shRNA-green fluorescent protein (GFP) of TβRIII. B, Representative blot (a) and summarized data (b) demonstrating that 3 wk after AAV9-shRNA infection TβRIII protein expression reduced ≈70% in the left ventricle (LV) tissue. C, M-mode echocardiographs representing ISO- and TAC-induced cardiac hypertrophy. D to G, Summarized data representing the calculated ratios of LV mass to body weight (LV mass/BW) and LV mass to tibia length (LV mass/TL) from different experiment groups (n=8–12/group; *P<0.05 vs Wild-type [WT], #P<0.05 vs ISO or TAC). H and I, Summarized data representing ejection fraction (EF) and fractional shortening (FS), respectively, measured 12 wk after differentially operated mice (n=8–9/group; *P<0.05 vs TAC or +AAV9-scr). J, Western blot (a) and summarized data (b) demonstrating β-myosin heavy chain (β-MHC) protein levels obtained from designated experiment conditions (n=7/group). K and L, quantitative real-time polymerase chain reaction demonstrating mRNA expression of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-MHC mRNA from designated experiment conditions (n=7/group; *P<0.05 vs saline or (Continued)
could abrogate the cell surface enlargement (Figure 4F), elevated ratio of protein/DNA ratio (Figure 4G) and up-regulation of β-MHC protein (Figure 4H) caused by TβRIII overexpression in NMCs. These results suggested that the scaffold role of β-arrestin2 between TβRIII and CaMKII was likely necessary for TβRIII-mediated cardiomyocytes hypertrophy.

Consistent with the in vitro study results, immunofluorescence staining revealed that the colocalizations of TβRIII-mediated cardiomyocytes hypertrophy from the hearts of 3-month-old CS-TβRIII-Tg mice using Western blot analysis. The data show that expression levels of TGF-β1 (Figure 4G) and up-regulation of β-arrestin2/CaMKII complexes significantly increased in LVs of CS-TβRIII-Tg mice compared with WT mice (Figure 5C). Accordingly, expression levels of p-CaMKII were significantly elevated in LVs of 3-month-old CS-TβRIII-Tg mice compared with age-matched WT mice (Figure 5D).

We next examined whether isoproterenol-stimulation or TAC operation affected the abundance and localization of TβRIII/β-arrestin2 and β-arrestin2/CaMKII complexes in WT hearts. The fluorescent intensity, an indicator of the abundance of TβRIII expression in the heart tissue, was greatly enhanced by either isoproterenol or TAC induction. Most importantly, the merged images demonstrated that there was an increased fluorescent intensity near the plasma membrane compartment of cardiomyocytes in response to isoproterenol or TAC, reflecting that isoproterenol or TAC promoted the formation of TβRIII/β-arrestin2 (Figure 5E) and β-arrestin2/CaMKII complexes (Figure 5F). Co-immunoprecipitation data further confirmed the important role of TβRIII in stress-induced cardiac hypertrophy, given that isoproterenol- or TAC-induced increase in the magnitude of TβRIII/β-arrestin2 and β-arrestin2/CaMKII complexes was greatly decreased by AAV9-mediated specific knockdown of TβRIII in the heart tissue (Figure 5G and 5H). Furthermore, isoproterenol- or TAC-induced elevation of p-CaMKII levels was substantially attenuated by cardiac-specific inhibition of TβRIII (Figure 5I and 5J). These data together suggested that TβRIII interfered with the process of cardiac hypertrophy through the interaction with β-arrestin2 and activation of CaMKII.

TβRIII-Induced Spontaneous Cardiac Hypertrophy Is Independent to TGF-β1 and Smad2/3

We next examined whether an increased TGF-β1 and Smad2/3 are involved in spontaneous cardiac hypertrophy in 3-month-old CS-TβRIII-Tg mice using Western blot analysis. The data show that expression levels of TGF-β1 (Figure 6A) and ratio of phosphorylated Smad2/3 (p-Smad2/3)/total Smad2/3 (T-Smad2/3; Figure 6B) were not altered in LV tissues dissected from the hearts of 3-month-old CS-TβRIII-Tg mice compared with age-matched mice. Furthermore, the expression levels of TGF-β1 in TAC- or isoproterenol-induced hypertrophic heart tissues were significantly elevated, although isoproterenol- or TAC-induced increase in TGF-β1 levels was not attenuated by cardiac-specific knockdown of TβRIII (Figure 6C and 6D). These results suggest that spontaneous cardiac hypertrophy observed in CS-TβRIII-Tg mice was most likely because of ligand-independent signaling.

β-ARs Are Not Involved in Cardiac TβRIII Overexpression-Induced Hypertrophy

We first detected whether there is a direct interaction between TβRIII and β1-AR or between TβRIII and β2-AR. Co-immunoprecipitation data show that TβRIII did not assemble to form a complex with either β1-AR (Figure 7A) or β2-AR (Figure 7B) in mouse LV tissues. Moreover, the downstream signaling of β1-AR, cAMP, and protein kinase A levels in LV tissues, dissected from 3-month-old CS-TβRIII-Tg mouse hearts (Figure 7C and 7D), were not altered compared with age-matched WT mice. Interestingly, isoproterenol-induced increase in cAMP and protein kinase A levels in LV tissues of age-matched WT mice were not attenuated by AAV9-shTβRIII–mediated cardiac-specific knockdown (Figure 7E and 7F). These results strongly suggest that manipulating the expression levels of TβRIII did not affect the downstream signaling of β-AR in overexpression-induced spontaneous cardiac hypertrophy.

Discussion

We demonstrated, for the first time, that (1) increased expression of TβRIII in the cardiomyocytes lead to cardiac hypertrophy independent to the downstream signaling of TGF-β1 and β1-AR; (2) cardiac-specific overexpression of TβRIII accelerates stress-induced cardiac hypertrophy and impairment of cardiac function; (3) β-arrestin2, respectively, links TβRIII and CaMKII and is required for TβRIII-mediated activation of CaMKII and cardiac hypertrophy. These findings may provide a novel therapeutic strategy for preventing cardiac hypertrophy.

To date, investigations on the role of TβRIII in the heart have been focused on noncardiomyocytes. Townsend et al. indicated that endocardial cell epithelial–mesenchymal transformation required TβRIII. We have previously shown that TβRIII was a direct target for microRNA-21, and the expression of TβRIII in cardiac fibroblasts, through an extracellular TβRIII receptor, can be upregulated by TGF-β signaling. We have recently reported that simvastatin alleviates cardiac fibrosis, in the case of myocardial infarction mouse model, via upregulation of TβRIII expression in cardiac fibroblasts, through an enhanced interaction between TβRIII and Gαt-interacting protein–interacting protein, C terminus to inhibit mitogen-activated protein kinase signaling. However, no experimental investigation was conducted to clarify whether TβRIII is expressed in cardiomyocytes and whether altered expression of TβRIII in cardiomyocytes may affect cardiac function. In
the present experimental model, TβRIII was overexpressed specifically in cardiomyocytes both in vivo and in vitro, whereas the elevated expression of TβRIII in cardiomyocytes leading to cardiac hypertrophy there was no cardiac fibrosis found in CS-TβRIII-Tg mice, albeit stresses accelerated cardiac fibrosis in these mice. This paradox phenomenon with our previous results may attribute to following factors: (1) overexpression of TβRIII in different cell types may trigger

Figure 4. Type III transforming growth factor-β receptor (TβRIII) activates calmodulin-dependent protein kinase II (CaMKII) signaling via enhanced physical interaction with β-arrestin2 in vitro. A and B. Co-immunoprecipitation (Co-IP) data demonstrate that TβRII and β-arrestin2, β-arrestin2 with CaMKII form physical complexes in neonatal mouse cardiomyocytes (NMCMs); cell lysates and n-IgG served as positive and negative control, respectively (n=3 repeats). C. Representative Co-IP experiments demonstrating the magnitude of TβRIII/β-arrestin2 (a) and β-arrestin2/CaMKII complex (b) in NMCMs under the indicated conditions (n=3 repeats). D. Representative Western blot (a) and summarized (b) data showing relative expression levels of p-CaMKII in NMCMs under the indicated conditions. Isoproterenol (ISO); NMCMs stimulated with ISO; +siTβRIII: NMCMs transfected with TβRIII siRNA + ISO stimulation; +NC (siTβRIII): NMCMs transfected with scrambled TβRIII siRNA to serve as negative control+ISO stimulation; TβRIII: NMCMs transfected with plasmid containing TβRIII cDNA; EV: NMCMs transfected with empty vector pcDNA3.1 to serve as negative control for TβRIII overexpression (n=4; *P<0.05 vs Ctrl, #P<0.05 vs ISO). E. Representative Western blots (a) and summarized data (b) demonstrating knocking down of β-arrestin2, in the TβRIII-overexpressed NMCMs, resulted in a decreased expression of β-MHC and p-CaMKII (n=3/group; *P<0.05 vs Ctrl, #P<0.05 vs TβRIII overexpression). F. Surface area and protein/DNA ratio measured, respectively, in wild-type NMCMs (Ctrl) and in TβRIII-overexpressed NMCMs either in the absence of KN93 (TβRIII) or in the presence of 1 µmol/L KN93 (+KN93) and vehicle (+dimethyl sulphoxide [DMSO]; n=4/group; *P<0.05 vs Ctrl, #P<0.05 vs TβRIII overexpression). G. Representative Western blots (a) and summarized data (b) demonstrating inhibition of CaMKII resulted in decreased expression of β-MHC and p-CaMKII in the TβRIII-overexpressed NMCMs (n=3/group; *P<0.05 vs Ctrl, #P<0.05 vs TβRIII overexpression). All data presented as mean±SD.
diverse intracellular signaling, as evident in that TβRIII coupled with Go-interacting protein–interacting protein, C terminus in fibroblast cells to inhibit mitogen-activated protein kinases and that TβRIII linked with β-arrestin2 in cardiomyocytes to activate CaMKII; (2) the effects of TβRIII may also depend on experimental models; with regard to pathological processes, myocardial infarction model is distinct to TAC or isoproterenol stimulation; (3) the data generated, from 3-month-old to 1-year-old CS-TβRIII-Tg mouse (data not shown), revealed that while developing cardiac hypertrophy, there was no concomitant cardiac fibrosis. Collectively, the experimental evidences provided in this study do not support the notion that TβRIII has profibrotic effects in hearts when TβRIII overexpressed in cardiomyocytes. However, the mechanisms by which TAC and isoproterenol accelerated cardiac fibrosis in CS-TβRIII-Tg mice may involve in complicated intracellular signaling and need further investigation.

Importantly, we found that mRNA and protein expression levels of TβRIII were significantly increased in the patients’ hypertrophic heart tissue. Consistently, the expression levels of TβRIII in mouse hearts were greatly increased by either isoproterenol stimulation or TAC operation. These results together suggest that TβRIII is a critical regulator for stress-induced cardiac hypertrophy. The use of these animal models in our study allowed the minimization of confounding factors for dissecting the potential molecular mechanisms by which TβRIII regulates cardiac hypertrophy.

Figure 5. Type III transforming growth factor-β receptor (TβR android mediated activation of calmodulin-dependent protein kinase II (CaMKII) is through its interaction with β-arrestin2 in vivo. A and B, Immunofluorescence staining showing enhanced colocalization of TβRIII and β-arrestin2 (A) and β-arrestin2 with CaMKII (B) in transgenic (Tg) mice as indicated by the white arrow head (n=4/genotype). C, Representative Co-immunoprecipitation (Co-IP) demonstrating formation of TβRIII/β-arrestin2 and β-arrestin2/CaMKII complex in Tg mice and wild-type (WT) mice (n=4/genotype). D, Representative Western blot (a) and summarized data (b) showing an increased p-CaMKII activity in the left ventricle tissue of Tg mice than that of WT mice (n=7/genotype; *P<0.05 vs WT). E and F, Immunofluorescence images showing isoproterenol (ISO) stimulation or transverse aortic constriction (TAC) operation resulted in the increased formation of TβRIII/β-arrestin2 and β-arrestin2/CaMKII complexes near the plasma membrane compartment of the left ventricle tissue from WT mice as indicated by the white arrow head (n=4/group). G and H, Representative Co-IP data demonstrating the altered magnitude of TβRIII/β-arrestin2 (a) and β-arrestin2/CaMKII (b) complexes in left ventricle tissue from adeno-associated viral (Continued)
It seems that deletion of TβRIII by the disruption of its exon 2 or exon 3 resulted in embryonic lethality for the majority of the null mice or defects in heart development.22,23 These mouse models support essential and nonredundant roles for TβRIII in heart development. In this study, we used cardiac-specific overexpression of TβRIII mouse to determine whether gain-of-function may lead to cardiac hypertrophy and may affect cardiac function. Although CS-TβRIII-Tg mice clearly showed cardiac hypertrophic phenotype, echocardiography did not show cardiac dysfunction in 3-month-old to 1-year-old CS-TβRIII-Tg mouse compared with age matched WT littermate controls (data not shown). However, 3 weeks after TAC operation, CS-TβRIII-Tg mice developed exaggerated cardiac hypertrophy and displayed a strong decrease in ejection fraction and fractional shortening compared with TAC-treated WT mice. Finally, our results show that cardiac-specific AAV9-mediated knockdown of TβRIII partially rescued isoproterenol- or TAC-induced cardiac hypertrophy and preserved ejection fraction and fractional shortening compared with TAC-treated WT mice. These results demonstrate that TβRIII is a potential regulator of the development of cardiac hypertrophy and heart failure.

Notably, the highly conserved cytoplasmic domain of TβRIII between species has been shown to greatly affect TβRIII function.4 Specifically, identified sequences within the cytoplasmic tail of TβRIII associate with β-arrestin2, a scaffolding protein.3,12 β-arrestins were originally identified as regulators of G-protein–coupled receptors, which bind to activated receptors, targeting them for internalization and desensitization.24 Surprisingly, a series of studies using a variety of cell types showed that via interaction with β-arrestin2, TβRIII regulates several signaling pathways, including both Smad-dependent and Smad-independent signaling.7,11,25 Collectively, there seems to be considerable cell type specificity in the mechanisms of the TβRIII/β-arrestin2 interaction resulting in signaling output. Our data show that TβRIII influences CaMKII activation via interaction with β-arrestin2 in mouse cardiomyocytes.

CaMKII seems to play an important pathogenic role in the development of cardiac hypertrophy and heart failure.26 β-arrestin2 scaffolds CaMKII and is required for isoproterenol-stimulated CaMKII activation in mouse heart.17 Moreover, knockdown of β-arrestin2 prevented cardiac myocyte hypertrophy on β-AR activation.27 We show here that β-arrestin2 serves as a scaffolding protein between TβRIII and CaMKII, suggesting that these proteins are assembled within a TβRIII/β-arrestin2/CaMKII complex. More importantly, isoproterenol- or TAC-induced increase in expression level of TβRIII is paralleled with cardiac hypertrophy and with the elevated magnitudes of TβRIII/β-arrestin2 and β-arrestin2/CaMKII complexes, as well as with the enhanced activation of CaMKII. Moreover, overexpression of TβRIII both in vitro and in vivo resulted in a spontaneous phenotype of cardiac hypertrophy. Conversely, knockdown of TβRIII, both in vitro and in vivo, greatly attenuated stress-induced activation of CaMKII and cardiac hypertrophy. The novel finding of this study implies that β-arrestin2-dependent CaMKII activation is most likely required for stress-induced TβRIII-mediated development of cardiac hypertrophy. Specifically,
we identified that TβRIII, a multifunctional sensor, serves as upstream signaling for β-arrestin2-dependent activation of CaMKII.

It is well documented in several studies that TGF-β or β-AR signaling play crucial role in cardiac hypertrophy. Therefore, we tested whether TβRIII overexpression-induced spontaneous hypertrophy is also linked to downstream signaling of TGF-β1 because TβRIII functions as a coreceptor, presenting ligands to their respective signaling receptors. Our data demonstrate that downstream signaling of TGF-β1 are most likely not involved cardiac hypertrophy because of CS-TβRIII-Tg, because neither TGF-β1 nor p-Smad2/3 levels, in LV tissues, were enhanced in these mice compared with WT mice. Furthermore, the stimulatory effects of TAC or isoproterenol on expression levels of TGF-β1 were not blunted by cardiac-specific AAV9-mediated knockdown of TβRIII.

Figure 6. Transforming growth factor-β1 (TGF-β1) signaling is not involved in cardiomyocyte-specific overexpression of type III transforming growth factor-β receptor (TβRIII)–induced hypertrophy. A, Representative Western blots (a) and summarized data (b) of TGF-β1 levels in left ventricular tissues of cardiac-specific transgenic expression of TβRIII mice and wild-type (WT) mice (n=6/genotype). B, Representative Western blot (a) and summarized data (b) of p-Smad2/3:t-Smad2/3 ratio in levels in left ventricular tissues of and WT mice (n=6/genotype). C and D, Representative Western blot (a) and summarized data (b) for TGF-β1 in isoproterenol (ISO)- or transverse aortic constriction (TAC)-treated mice with or without adeno-associated viral 9 (AAV9)-mediated TβRIII shRNA infection (n=4/group; *P<0.05 vs Ctrl; saline or sham). Data presented as mean±SD.

Figure 7. Type III transforming growth factor-β receptor (TβRIII) does interact with β1-adrenergic receptor (β1-AR) and β2-AR, and manipulating expression levels of TβRIII does not affect downstream signaling of β1-AR1. Co-immunoprecipitation data demonstrate that TβRIII does not form a complex with either β1-AR (A) or β2-AR (B; n=3 repeats). cAMP (C) and protein kinase A (PKA) (D) levels were not increased in the left ventricular tissues of cardiac-specific transgenic expression of TβRIII mice compared with wild-type (WT) mice (n=5–6). Enhanced level of cAMP (E) and PKA (F) levels in the left ventricle tissues mouse heart by isoproterenol (ISO) infusion were not affected by adeno-associated viral 9 (AAV9)-shRNA-mediated knocking down of TβRIII (n=5). All data presented as mean±SD.
which further confirmed that manipulating TβRIII levels in the hearts does not affect TGF-β1 expression.

β-arrestin2 is known to mediate translocation of CaMKII and Epac to agonist occupied β1-AR complex and then brings these molecules in close proximity to the location of cAMP generation by adenylyl cyclase. cAMP directly binds to and stimulates Epac, leading to CaMKII activation via a Rap-PLC-PKC mechanism. β-arrestins also scaffold β-ARs and regulate their internalization and desensitization, however, our data show that there is no direct interaction between TβRIII and β-ARs in mouse hearts. More importantly, the levels of cAMP and protein kinase A were virtually the same in LV tissues between 3-month-old CS-TβRIII-Tg mice and age-matched WT mice. TAC- or isoproterenol-induced increase in cAMP and protein kinase A levels was not decreased by car

The novel mechanisms by which TβRIII drives cardiac hypertrophy and dysfunction may be a potential approach for the prevention or therapy of myocardial hypertrophy.

Perspectives

The novel mechanisms by which TβRIII drives cardiac hypertrophy provide new mechanistic insights into the cardiac hypertrophy discovery. Of a small molecule to inhibit TβRIII-initiated signaling pathway may be a new strategy for preventing and curing prohypertrophic stresses-induced cardiac hypertrophy and dysfunction.

Sources of Funding

This work was supported, in part, by the National Basic Research Program of China (973 program, 2012CB517803/2014CB542401), and the National Nature Science Foundation of China (31100826/81370340/81330004), and Heilongjiang Chang Jiang Scholar Candidates Program for Provincial Universities (2013CHB004).

Disclosures

None.

References


### Novelty and Significance

**What Is New?**

- **We report here, for the first time, that there is an enhanced expression of TβRIII in both the human and the mouse cardiac hypertrophy and that elevated expression of TβRIII in the heart independently lead to cardiac hypertrophy.**

- **Our findings disclose a novel regulatory complex that is composed of TβRIII, β-arrestin2, and CaMKII, where the scaffolding role of β-arrestin2 is required for TβRIII-mediated activation of CaMKII and cardiac hypertrophy.**

**What Is Relevant?**

- **Manipulation of TβRIII expression levels in heart affects distinct prohypertrophic stresses–induced cardiac hypertrophic phenotype, where an increased TβRIII expression promotes cardiac remodeling and the impairment of cardiac function; conversely, stresses-induced cardiac hypertrophy and declined cardiac function are significantly attenuated by downregulation of TβRIII.**

**Summary**

Increased expression of TβRIII leads to cardiac hypertrophy via β-arrestin2-dependent activation of CaMKII pathway; modulation of their levels may provide an attractive therapeutic target for controlling myocardial hypertrophy.
Type III Transforming Growth Factor-β Receptor Drives Cardiac Hypertrophy Through β-Arrestin2–Dependent Activation of Calmodulin-Dependent Protein Kinase II

Jie Lou, Dan Zhao, Ling-Ling Zhang, Shu-Ying Song, Yan-Chao Li, Fei Sun, Xiao-Qing Ding, Chang-Jiang Yu, Yuan-Yuan Li, Mei-Tong Liu, Chang-Jiang Dong, Yong Ji, Hongliang Li, Wenfeng Chu and Zhi-Ren Zhang

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Type III TGF-β Receptor Drives Cardiac Hypertrophy Through β-arrestin2-dependent Activation of CaMKII

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Running title- TβRIII governs cardiac hypertrophy

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Supplement Methods

Reagents. Unless otherwise noted, all chemicals were purchased from Sigma (Sigma Aldrich, USA). KN93 was dissolved in DMSO and the final dilution rate of DMSO was 1:1000.

Animals. Adult C57BL/6 mice were provided by the Experimental Animal Center of Harbin Medical University (Grade II). Food and water were provided ad libitum throughout the experiments. The animals were kept under standard animal room conditions (temperature 21 ± 1°C; humidity 55–60%; 12 h light: dark cycles). The mice were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) and xylazine (12.5 mg/kg, i.p.). The adequacy of anesthesia was monitored by the absence of withdrawal reflex to tail pinch. All animal experiments were approved by the Animal Experiments Committee of the Harbin Medical University and conformed to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health).

Isoproterenol (ISO)-induced Cardiac Hypertrophy in vivo. Adult male mice (22–26 g body weight) were anesthetized and placed in the prone position. Then, a 1-cm incision was prepared to subcutaneously implant the osmotic mini-pump (Alzet model 1004; DURECT Corporation) over the dorsal thorax. Pumps contained ISO (Sigma-Aldrich, USA) which was dissolved in saline solution and the infusion rate was 10 mg/kg per day. At the pre-determined time points (day 3, 7, 14, 21, and 28), the mice were sacrificed and the heart was quickly excised, flushed, and weighed. Heart samples were stored in cold (4°C) buffer for other experiments. Alzet 1002 osmotic mini-pumps were used for the mice infused with ISO (10 mg/kg per day) for two weeks, i.e. AAV9 injected mice, Tg mice and their WT control mice.

Pressure Overload-induced Cardiac Hypertrophy in vivo. Adult male mice (22–26 g body weight) were anesthetized and placed in the supine position. A midline cervical incision was made to expose the trachea. After successful endotracheal intubation, the cannula was connected to a volume cycled rodent ventilator (UGO BASILE S.R.L. Italy). The chest was opened and the thoracic aorta was identified. A 7-0 silk suture was placed around the transverse aorta and tied around a 26-gauge blunt needle which was subsequently removed. This maneuver increased systolic pressure about 1.5-fold and induced cardiac hypertrophy. At the pre-determined time points (1, 2, 3, and 4 weeks), the surviving mice were sacrificed and the hearts were quickly excised, weighed, and stored in cold (4°C) buffer for other experiments.

Echocardiography. Evaluation of cardiac function was assessed by echocardiography (VEVO 770 micro-ultrasound, Visual Sonics Inc, Toronto, Canada) with a 30-MHz linear array ultrasound transducer. The left ventricle (LV) was assessed in both parasternal long-axis and short-axis views at a frame rate of 120 Hz. The calculated LV mass, left ventricular anterior wall-diastole (LVAWd), left ventricular anterior wall-systole (LVAWs), left ventricular posterior wall-diastole (LVPWd), left ventricular posterior wall-systole (LVPWs), left ventricular internal
dimension in diastole (LVIDd), and left ventricular internal diameter in systole (LVIDs) were calculated with VEVO Analysis software (version 2.2.3).

**Neonatal Mice Cardiomyocyte Culture and Transfection.** Neonatal C57BL/6 mice were obtained from the Experimental Animal Center of Harbin Medical University. Neonatal mouse cardiomyocytes were isolated by enzymatic digestion from the ventricles of 1–3-day-old neonatal mice. Briefly, each pup was placed on a 37°C temperature-controlled pad under general anesthesia from spontaneous respirations of 3.0% isoflurane in oxygen. After anesthesia, the hearts were quickly excised, washed, and minced in ice-cold serum-free DMEM. The myocardial cells were dispersed by incubating with 0.25% Trypsin EDTA (~1 mL of Trypsin for every 100 mg of tissue), which was then mixed by intermittent pipetting along with stirring at 37°C in a water bath for 2 min. The cell suspension was allowed to stand for 1 min. The supernatant containing single cells was collected into 15-mL Falcon tubes kept on ice, to which 7 mL media supplemented with 10% fetal calf serum was added, and the digestion step was repeated five to ten times until tissue was digested completely. The cell suspensions from each digestion was pooled and centrifuged at 2,500 rpm for 10 min at 4°C. Cells were suspended in DMEM with 10% FBS, and pre-cultured in a humidified incubator (95% air-5% CO2) for 2 hours to remove fibroblasts. The non-adherent cardiomyocytes were plated in another dish. Forty-eight hours after plating, the neonatal ventricular myocytes were placed into serum-free medium for the designated experiments and the cells were kept in serum-free medium for 4-6 h before transfection. Cardiomyocytes were transfected with pc-DNA3.1-mTβRIII plasmid (GeneChem Co.) or empty vector as control at a dosage of 1 μg/mL; and mouse TβRIII siRNA (GenePharma, Shanghai) or scrambled siRNA as control at a dosage of 100 pmol/mL for 6 hours using Lipofectamine™ 2000 (Invitrogen). Following these procedures, the Lipo-containing medium was replaced by serum-free medium for 24-48 h and the cells were ready for experiments. The sequences of the siRNAs were shown in Table S2. Cells were harvested 24 or 48 hours after ISO treatment for immunohistochemistry and Western blot analyses.

**Western Blot.** For Western blot analyses, cell extractions or tissue lysates were cleared by 12,000 x g centrifugation for 10 min at 4°C. Protein concentration was determined by BCA Protein Assay Kit (APPLYGEN, Beijing, China). Equal protein samples were subsequently separated by SDS/PAGE and transferred to PVDF membranes. After blocking with 5% non-fat dry milk in phosphate-buffered saline (PBS) for 2 hours at room temperature, the membranes were incubated with primary antibodies at 4°C overnight. The primary antibodies used in the present study included anti-TβRIII antibody (1:1000 dilution, Cell signaling #2519; 1:200 dilution, Santa Cruz, sc-28975, sc-6199), anti-β-arrestin2 antibody (1:1000 dilution, Cell Signaling #3857; 1:200 dilution, Santa Cruz, sc-13140), anti-p-CaMKII antibody (1:1000 dilution, Cell Signaling, #3361), anti-CaMKII antibody (1:200 dilution, Santa Cruz, sc-13082), anti-ANP antibody (1: 200 dilution, Santa Cruz, sc-20158), anti-β-MHC (1:5000 dilution, Sigma-Aldrich, M8421), anti-β1-AR antibody (1: 200 dilution, Santa Cruz, sc-568), anti-β2-AR antibody (1:1000 dilution, Abcam,
ab182136), phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) antibody (1:1000 dilution, Cell Signaling, #8828), Smad2/3 antibody (1:1000, Cell Signaling, #5678), anti-TβRII antibody (1:200 dilution, Santa Cruz, sc-399), anti-TβRII antibody (1:200 dilution, Santa Cruz, sc-17792), anti-TGF-β antibody (1:1000 dilution, Cell Signaling, #3711), anti-GAPDH antibody (1:5000 dilution, Abcam, ab8245). After washing with PBS-0.1% Tween 20 (PBST), membranes were incubated with goat anti-rabbit IRDye® 800 CW (1:10000 dilution, LI-COR, P/N 926-32211) or goat anti-mouse IRDye® 800 CW (1:10000 dilution, LI-COR, P/N 926-32210) at room temperature for 1 h. The bands were quantified by using Odyssey infrared imaging system (LI-COR) and Odyssey v3.0 software.

**Quantitative Real-time RT-PCR.** Total RNA was extracted from the heart tissue or mouse cardiac myocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with the RT system protocol in a 20 μL reaction mixture. Total RNA (1 μg) was used in the reaction, and a random primer was used for the initiation of cDNA synthesis. The reaction mixture was incubated at 25°C for 10 min, 37°C for 120 min and 85°C for 5 min, respectively. Quantitative Real-time PCR (qRT-PCR) was performed on the ABI Prism 7500 sequence detection system using SYBR Green PCR core reagents (Applied Biosystems). PCR was performed following the manufacturer’s recommendations for a 25 μL reaction volume. Transcript quantities were compared by using the relative quantitative method, where the amount of detected mRNA was normalized to the amount of endogenous control (GAPDH). The relative value to the control sample is given by $2^{-\Delta\Delta CT}$. The primer sequences are listed in Table S3.

**Immunofluorescence.** Mouse heart was fixed with 4% paraformaldehyde for 2 hrs followed by 18% sucrose for 16 hours, and preserved in optimum cutting temperature (OCT) compound (−80°C). Longitudinal sections of the left ventricular wall were cut with a cryostat microtome (CM 3500 cryostat, Israel) at a thickness of 4 μm and permeabilized with 0.2% Triton X-100 (PBST) for 10 min and blocked with 5% bovine serum albumin (BSA) PBST for 1 hour. Mouse monoclonal antibody against α-actinin (1:500 dilution, Sigma-Aldrich, A7811), rabbit polyclonal anti-TβRIII antibody (1: 100 dilution, Sigma-Aldrich, SAB4502962), mouse monoclonal anti-β-arrestin2 antibody (1:50 dilution; Santa Cruz, sc-13140) and rabbit polyclonal anti-CaMKII antibody (1: 50 dilution, Santa Cruz, sc-13082) were prepared in 1% BSA/PBST for overnight incubation at 4°C. The sections were washed in PBST and incubated with secondary antibodies coupled to Alexa Fluor® 594 donkey anti-mouse IgG (1:1000 dilution, Invitrogen, A-21203) or Alexa Fluor® 488 donkey anti-rabbit IgG (1:1000 dilution, Invitrogen, A-21206). All slides were imaged using a confocal microscope (Olympus, Fluoview1000, Japan). Identical acquisition settings were used for all images.

**Co-immunoprecipitation.** Immunoprecipitation was performed by overnight incubation/rotation 250 μg protein with 2 μg of rabbit polyclonal anti-TβRIII antibody (Santa Cruz, sc28975), goat polyclonal anti-TβRIII antibody (Santa Cruz, sc6199),
goat polyclonal anti-β-arrestin2 antibody (Santa Cruz, sc6386), mouse monoclonal anti-β-arrestin2 antibody (Santa Cruz, sc13140), rabbit polyclonal anti-CaMKII antibody (Santa Cruz, sc13082), rabbit polyclonal anti-β1-AR antibody (Santa Cruz, sc-568), rabbit polyclonal anti-TβRI antibody (Santa Cruz, sc-399), mouse monoclonal anti-TβRII antibody (Santa Cruz, sc-17792); or with 1:10 diluted rabbit monoclonal anti-β2-AR antibody (Abcam, ab182136). The control (n-IgG) was also included for each sample. After incubation, protein A/G plus agarose beads were added to immunoprecipitated samples and incubated for 2 h with rotation. Beads were washed twice with lysis buffer, and once with PBS. Bound immunocomplex proteins were eluted by the addition of SDS loading buffer and analyzed by western blotting assay.

**Cell Surface Area Measurement.** The surface area of α-actinin-stained NMCMs was measured after employing hypertrophic stimuli by the computer-assisted planimetry. Cardiomyocytes in 20 - 30 fields were examined in each experiment.

**Protein/DNA Ratio Measurement.** Protein/DNA ratio was measured as previously described. For the quantitative analysis of total cellular protein and DNA content, NMCMs were washed twice with phosphate-buffered saline. After wash, 1 mL of 0.2 N perchloric acid was added to NMCMs. The samples were collected and centrifuged for 10 min at 10,000 g. The precipitates were resuspended in 250 μL of 0.3 N KOH and incubated for 20 min at 60°C. Protein content was analyzed by the Lowry method using bovine serum albumin as a standard. DNA content was detected using Hoechst dye 33258 (Life Technologies) with salmon sperm DNA as a standard.

**Detection of Intracellular PKA and cAMP.** PKA Kinase Activity Kit (Abcam, ab139435) and cAMP Direct Immunoassay Kit (Abcam, ab65355) were respectively used for the detection of intracellular PKA and cAMP levels, according to manufacturer’s instruction. For the detection of PKA, in brief, tissue lysates were washed with ice-cold PBS twice and 1 mL lysis buffer containing 1 mM PMSF was added to 200 mg tissues, and then kept on ice for 10 min. The lysates were centrifuged at 13,000 rpm for 15 min. Protein concentration was determined using BCA method. The activities of PKA were determined and calculated according to the manufacturer’s instruction. To assess the concentration of cAMP, 1 mL 0.1 M HCl was added to the 200 mg heart tissue, and then incubated at room temperature (22-24°C) for 20 min. The lysates were centrifuged at 13,000 rpm for 10 min at 4 °C to collect the supernatant. 100 μL supernatant was used for the detection of cAMP level according to the manufacturer’s instruction. The concentrations of PKA and cAMP measured spectrophotometrically, at a wavelength of 450 nm, at room temperature (22-24°C), and their respective standard curves were generated concurrently.

**Reference**
**Table S1.** Description of human samples used in present study

<table>
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<td>3</td>
<td>Tetralogy of Fallot</td>
<td>Right ventricular outflow tract</td>
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<td></td>
<td></td>
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<td>reconstruction</td>
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<td>59</td>
<td>Hypertrophic obstructive cardiomyopathy</td>
<td>Septal myectomy</td>
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<tr>
<td>CH3</td>
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<td>57</td>
<td>Hypertrophic obstructive cardiomyopathy</td>
<td>Septal myectomy</td>
</tr>
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<td>CH4</td>
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<td>Dilated cardiomyopathy</td>
<td>Heart transplantation</td>
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<td>Heart transplantation</td>
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<td>NCH1</td>
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**Table S2.** Sequences of the specific siRNAs used in present study

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<th>Antisense (5’-3’)</th>
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<td>TβRIII (siRNA1)</td>
<td>CUGGAGUGGUAGUGUUUAATT</td>
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<tr>
<td>TβRIII (siRNA2)</td>
<td>CCUCCAGGUGGAAUAUAAUTT</td>
<td>AUUAUACCACUGGAAGGTT</td>
</tr>
<tr>
<td>TβRIII (siRNA3)</td>
<td>GGUCCUCCAAGUAGUUAUATT</td>
<td>AUAAUCACUUGGAGGACCTT</td>
</tr>
<tr>
<td>TβRIII (siRNA4)</td>
<td>GGGAGGUCCACAUCUAAATT</td>
<td>UUAGGAUGUGAACUCCTT</td>
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<tr>
<td>β-arrestin2 (siRNA1)</td>
<td>CGAGCUUUCUGUGCCAAAUTT</td>
<td>AUUUGCACAGAAAGCACGTT</td>
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<tr>
<td>β-arrestin2 (siRNA2)</td>
<td>GUGCGCUUAUCAUCAGAATT</td>
<td>UUCUGAUGAUAAAGCCGACTT</td>
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<tr>
<td>β-arrestin2 (siRNA3)</td>
<td>GGCACCUACACUGUGAATT</td>
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<tr>
<td>β-arrestin2 (siRNA4)</td>
<td>GGCUCAGCUACAGAAGAATT</td>
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<tr>
<td>Negative control</td>
<td>UUCUCCGAACGUGUCACGUTT</td>
<td>ACGUGACACGUGACCGAATT</td>
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</tbody>
</table>

All the siRNAs correspond to *mus musculus*. 

7
Table S3. The primers used for qRT-PCR to detect the mRNAs of targeting genes

<table>
<thead>
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<th>Gene</th>
<th>Sense (5'-3')</th>
<th>Antisence (5'-3')</th>
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<tr>
<td>mANP</td>
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<tr>
<td>mBNP</td>
<td>TGATTCTGCTCTGCTTTTTT</td>
<td>GTGGATTGTCTGGAGACTG</td>
</tr>
<tr>
<td>mβ-MHC</td>
<td>CCAGAAGCCTCGAAATGTC</td>
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<td>mGAPDH</td>
<td>CTGGAGAAGCCTGCAAGTA</td>
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<tr>
<td>hANP</td>
<td>CAGCAAGCAGTGGGATGCTCCT</td>
<td>TCTGCGTTGAGACACGGCATTGT</td>
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<tr>
<td>hBNP</td>
<td>TGGAAACGTCCGGTTACAGGA</td>
<td>TCCGGTCCATCTCTCCCTCCAAA</td>
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<td>hβ-MHC</td>
<td>GGGCAAAAGGCAAGGGCAAGAAA</td>
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<td>GGTCCGGATGGCGTAGTTTT</td>
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<tr>
<td>hGAPDH</td>
<td>CATCACCATCTTCCAGAGCAGAGA</td>
<td>TGCAGGAGGACATTGCTATGAC</td>
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</table>

Primers for ANP, BNP and β-MHC correspond to *mus musculus* (m) or *homo sapiens* (h).
**Table S4.** Echocardiographic analysis of TβRIII WT and Tg mice

<table>
<thead>
<tr>
<th>Index</th>
<th>WT (n=10)</th>
<th>Tg (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVAWd (mm)</td>
<td>0.70±0.07</td>
<td>0.90±0.14*</td>
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<tr>
<td>LVAWs (mm)</td>
<td>1.05±0.10</td>
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</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.72±0.08</td>
<td>0.83±0.06*</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
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<td>1.14±0.23*</td>
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<tr>
<td>LVIDd (mm)</td>
<td>3.26±0.31</td>
<td>3.51±0.37</td>
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<tr>
<td>LVIDs (mm)</td>
<td>2.31±0.28</td>
<td>2.42±0.46</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>72.73±18.38</td>
<td>106.53±20.39*</td>
</tr>
</tbody>
</table>

LVAWd, left ventricle anterior wall diameter in end-diastole; LVAWs, left ventricle anterior wall diameter in end-systole; LVPWd, left ventricle posterior wall diameter in end-diastole; LVPWs, left ventricle posterior wall diameter in end-systole; LVIDd, left ventricular internal dimension in diastole; LVIDs, the left ventricular internal diameter in systole; Tg, TβRIII transgenic mice and WT, wild-type. Data presented as mean ± SD. * p < 0.05 vs. respective WT control.
Table S5. Echocardiographic parameters of cardiac-specific TβRIII knock-down mice upon ISO stimulation

<table>
<thead>
<tr>
<th>Index</th>
<th>Control (n=12)</th>
<th>ISO (n=9)</th>
<th>+AAV9-shTβRIII (n=12)</th>
<th>+AAV9-scr (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVAWd (mm)</td>
<td>0.77±0.22</td>
<td>1.26±0.13*</td>
<td>1.01±0.17†</td>
<td>1.34±0.31*</td>
</tr>
<tr>
<td>LVAWs (mm)</td>
<td>1.09±0.28</td>
<td>2.02±0.34*</td>
<td>1.51±0.30†</td>
<td>1.88±0.44*</td>
</tr>
<tr>
<td>LVPWd (mm)</td>
<td>0.70±0.13</td>
<td>1.20±0.18*</td>
<td>0.95±0.19†</td>
<td>1.31±0.33*</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>1.01±0.17</td>
<td>1.75±0.33*</td>
<td>1.43±0.31†</td>
<td>1.66±0.21*</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.23±0.33</td>
<td>3.42±0.62</td>
<td>3.26±0.57</td>
<td>3.13±0.90</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.07±0.40</td>
<td>1.75±0.80</td>
<td>1.94±0.57</td>
<td>1.94±0.48</td>
</tr>
</tbody>
</table>

Mice were treated with 10 mg/kg ISO for two weeks; one week before ISO administration, the mice were injected with AAV9-mediated shTβRIII (+shTβRIII) or scramble control (+AAV9-scr). Data presented as mean ± SD. * p < 0.05 vs. respective control mice, † p < 0.05 vs. respective ISO-treated mice.
<table>
<thead>
<tr>
<th>Index</th>
<th>Control (n=8)</th>
<th>TAC (n=9)</th>
<th>+AAV9-shTβRIII (n=8)</th>
<th>+AAV9-scr (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 weeks LWAWd (mm)</td>
<td>0.75±0.12</td>
<td>1.57±0.18*</td>
<td>1.19±0.20†</td>
<td>1.55±0.19*</td>
</tr>
<tr>
<td>LWAWs (mm)</td>
<td>1.24±0.21</td>
<td>2.24±0.22*</td>
<td>1.71±0.31†</td>
<td>2.30±0.24*</td>
</tr>
<tr>
<td>LWPWd (mm)</td>
<td>0.73±0.13</td>
<td>1.47±0.20*</td>
<td>1.05±0.19†</td>
<td>1.62±0.32*</td>
</tr>
<tr>
<td>LWPWs (mm)</td>
<td>1.14±0.22</td>
<td>2.07±0.22*</td>
<td>1.55±0.30†</td>
<td>2.25±0.35*</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.71±0.24</td>
<td>3.23±0.34</td>
<td>3.72±0.43</td>
<td>3.39±0.51</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>2.33±0.35</td>
<td>1.74±0.46*</td>
<td>2.39±0.54†</td>
<td>1.70±0.66*</td>
</tr>
</tbody>
</table>

One week before TAC operation, the mice were injected with AAV9-mediated shTβRIII (+shTβRIII) or scramble control (+AAV9-scr). Mouse echocardiography was performed at three weeks after TAC operations. Data presented as mean ± SD. * p < 0.05 vs. respective control mice, † p < 0.05 vs. respective TAC-operated mice.
Figure S1. The specificity and exact size of TβRIII were determined using a variety of tissues and cells known to express TβRIII. The data show that TβRIII, with a molecular weight of 110 kDa, was detected. Heart and lung tissue were from C57BL/6 mouse; CM: C57BL/6 mouse cardiomyocytes; CF: C57BL/6 mouse cardiac fibroblasts; L5178Y: mouse lymphoma cell line; NB4: human leukemia cell line; L5178 and NB4 cells served as positive control of TβRIII expression. TβRIII band (110 kDa) was not shown in the blots where the lysates treated with secondary antibody alone incubating blot.
Figure S2. Results: TβRIII plays a key role in development of hypertrophy in neonatal mouse cardiomyocytes

To examine the potential role of TβRIII in cardiac hypertrophy, we performed gain- and loss-of-function studies in neonatal mouse cardiomyocytes (NMCMs). The efficiencies of overexpression of TβRIII and knocking down of TβRIII expression are shown in Figures S2A and S2B. Consistent with the in vivo studies, TβRIII protein level was increased in NMCMs upon application of ISO, in a dose- and time-dependent manner (Figures S2C & S2D). The surface area, protein and DNA ratio were significantly increased in NMSCs stimulated by ISO; however, the stimulatory effects of ISO on the surface area (Figure S2E) and ratio of protein/ DNA (Figure S2F) were greatly diminished by knocking down of TβRIII in NMCMs. Importantly, overexpression of TβRIII in NMCMs without ISO stimulation was sufficient to significantly increase surface area (Figure S2E), as well as the ratio of protein/DNA (Figure S2F). ISO-induced increase in mRNA expression levels of ANP, BNP, and β-MHC (Figure S2G), expression levels of β-MHC protein (Figure S2H) was also greatly abrogated by knocking down in NMCMs. Consistently, overexpression of TβRIII in NMCMs independently led to significantly elevated expression levels of ANP, BNP, and β-MHC mRNA (Figure S2G) and β-MHC protein (Figure S2H).

In addition, the protein expression levels of TβRIII and β-MHC were significantly increased by treating the NMCMs with 100 nM AngII (Figure S2I). AngII-induced up-regulation of β-MHC was almost abolished by knocking down of TβRIII in NMCMs (Figure S2J). These data were consistent with the hypothesis that TβRIII plays a key role in stress-induced cardiac hypertrophy.
Figure S2

**Figure S2. TβRIII acts as a key regulator in ISO-induced hypertrophy of NMCMs.** (A) Representative Western blotting (a) and summarized data (b) demonstrating the efficiency of TβRIII expression in the NMCMs transfected with plasmid containing TβRIII cDNA or empty vector (EV) to serve as negative control (n = 3; p < 0.05 vs. EV group). (B) Representative Western blot (a) and summarized data (b) showing the TβRIII gene silencing efficiency by specific small interference RNAs (siRNA) in NMCMs; siRNA (S4) was used in all subsequent experiments (n = 3; p < 0.05 vs. NC group). (C & D) Representative blots (a) and summarized data demonstrating the expression profiles of TβRIII in NMCMs stimulated with different doses of ISO for 48 h and a variety of time points with 10 µM ISO (n = 5/group; *p < 0.05 vs. Ctrl group). (E) Relative mRNA level of ANP, BNP, and β-MHC. (F) Protein/DNA ratio (fold change).
0.05 vs. 0 µM ISO group). Cell surface area (E) and protein/DNA ratio (F) of NMCMs under indicated experimental conditions (n = 4/group; *p < 0.05 vs. Ctrl; †p < 0.05 vs. ISO). (G) qRT-PCR demonstrating mRNA expression of ANP, BNP, and β-MHC mRNA in differentially manipulated NMCMs treated with no ISO (Ctrl) or with 10 µM ISO for 24 hrs (n = 4/group; *p < 0.05 vs. Ctrl; †p < 0.05 vs. ISO). (H) Representative blot (a) and summarized data (b) indicating the expression profiles of β-MHC in differentially manipulated NMCMs, in the absence (Ctrl) or in the presence of 10 µM ISO for 24 hrs (n = 4/group; *p < 0.05 vs. Ctrl; †p < 0.05 vs. ISO). Ctrl: control; ISO: stimulated with ISO; +siTβRIII: TβRIII gene silenced NMCMs stimulated with ISO; +NC: NMCMs transfected with scrambled siRNA against TβRIII serves as negative control and stimulated with ISO; TβRIII: NMCMs over-expressing TβRIII but without ISO stimulation; EV: empty vector pcDNA3.1 transfecting NMCMs without ISO stimulation. (I) Representative Western blot (a) and summarized data (b) of TβRIII protein expression in cultured NMCMs in the absence (Ctrl) or in the presence of 100 nM Ang II, at the indicated time points (n = 4/group; *p < 0.05 vs. Ctrl). (J) Western blot (a) and summarized data (b) represent the expression levels of β-MHC respectively in NMCMs without (Ctrl) or with AngII stimulation (AngII) and in NMCMs transfected with either siTβRIII (+siTβRIII) or scramble siTβRIII (+NC). 48 h after transfection, 100 nM AngII was administrated to the NMCMs and cultured for 6 h (n = 4/group; *p < 0.05 vs. Ctrl; † p < 0.05 vs. AngII). All data presented as mean ± SD.
Figure S3. TβRIII does not directly interact with CaMKII in neonatal mouse cardiomyocytes (NMCMs). Representative co-IP experiments demonstrate that the magnitude of TβRII/β-arrestin2 (a) and β-arrestin2/CaMKII complex (b) in NMCMs under the indicated experimental conditions. However, there is no interaction between TβRIII and CaMKII (n = 3 repeats). ISO: NMCMs stimulated with ISO; +siTβRIII: NMCMs transfected with TβRIII siRNA + ISO stimulation; +NC(si TβRIII): NMCMs transfected with scrambled TβRIII siRNA to serve as negative control + ISO stimulation; TβRIII: NMCMs transfected with plasmid containing TβRIII cDNA; EV: NMCMs transfected with empty vector pcDNA3.1 to serve as negative control for TβRIII overexpression.
Figure S4. Representative western blot (a) and summarized data (b) showing the β-arrestin2 gene silencing efficiency in NMCMs; siRNA (S2) was used in subsequent experiments (n = 3; * p < 0.05 vs. NC group). All data presented as mean ± SD.