

Cardiomyocyte overexpression of miR-27b induces cardiac hypertrophy and dysfunction in mice

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Recent studies have begun to reveal critical roles of microRNAs (miRNAs) in the pathogenesis of cardiac hypertrophy and dysfunction. In this study, we tested whether a transforming growth factor- β (TGF- β)-regulated miRNA played a pivotal role in the development of cardiac hypertrophy and heart failure (HF). We observed that miR-27b was upregulated in hearts of cardiomyocyte-specific Smad4 knockout mice, which developed cardiac hypertrophy. *In vitro* experiments showed that the miR-27b expression could be inhibited by TGF- β 1 and that its overexpression promoted hypertrophic cell growth, while the miR-27b suppression led to inhibition of the hypertrophic cell growth caused by phenylephrine (PE) treatment. Furthermore, the analysis of transgenic mice with cardiomyocyte-specific overexpression of miR-27b revealed that miR-27b overexpression was sufficient to induce cardiac hypertrophy and dysfunction. We validated the peroxisome proliferator-activated receptor- γ (PPAR- γ) as a direct target of miR-27b in cardiomyocyte. Consistently, the miR-27b transgenic mice displayed significantly lower levels of PPAR- γ than the control mice. Furthermore, *in vivo* silencing of miR-27b using a specific antagomir in a pressure-overload-induced mouse model of HF increased cardiac PPAR- γ expression, attenuated cardiac hypertrophy and dysfunction. The results of our study demonstrate that TGF- β 1-regulated miR-27b is involved in the regulation of cardiac hypertrophy, and validate miR-27b as an efficient therapeutic target for cardiac diseases.

Keywords: microRNA; cardiac hypertrophy; PPAR- γ ; transgenic mouse; therapeutic target

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Introduction

Heart failure (HF) is one of the most frequent causes of death worldwide. The underlying causes of HF are diverse, but HF is often associated with cardiac hypertrophy. Numerous regulatory pathways are implicated in the transduction of hypertrophic signaling [1]. The members of the transforming growth factor- β (TGF- β) superfamily signal through receptor serine/threonine kinases and intracellular Smad proteins [2], and play a

complex and often contradictory role in the development of cardiac hypertrophy [3]. Although many *in vitro* and *in vivo* studies have shown that TGF- β 1 overexpression leads to cardiac hypertrophy and HF [4, 5], recent studies have shown that TGF- β signaling may play a role in the protective mechanism in cardiac hypertrophy [6-9]. Our previous study reveals that Smad4 deletion in cardiomyocytes results in cardiac hypertrophy characterized by enlargement of cardiomyocytes, age-associated fibrosis and re-expression of certain fetal genes [6]. Growth differentiation factor 15 (GDF15), a member of the TGF- β superfamily, antagonizes the hypertrophic response possibly through a mechanism involving the Smad proteins [7, 8]. In addition, cardiac-specific overexpression of bone morphogenetic protein-10 (BMP-10) impairs the postnatal cardiac hypertrophic growth by activation of

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Smad1/5/8-mediated signaling [9]. However, the molecular mechanisms that regulate the cellular responses of cardiomyocytes to TGF- β signaling, which antagonizes cardiac hypertrophy, still have not been elucidated.

MicroRNAs (miRNAs) are small, endogenous non-coding RNAs, which are approximately 22 nucleotides in length. The miRNA inhibit the expression of specific genes by either degrading the target mRNA or direct translational inhibition [10]. Increasing evidences have revealed that miRNAs play important roles in cardiac hypertrophy and dysfunction [11-14]. Several studies have shown that miRNA expressions are altered in mouse and human HFs. The cardiac-specific knockout of *Dgcr8* or *Dicer*, which are essential for the biogenesis and processing of miRNA, leads to a rapidly progressive dilated cardiomyopathy, HF and postnatal lethality [11-13]. The cardiac overexpression of miR-195, which is upregulated during cardiac hypertrophy, induces pathological cardiac growth and HF in transgenic mice [14]. Similarly, cardiac overexpression of miR-208a, which is located in the non-coding region of the α -myosin heavy chain (α -MHC) gene, is sufficient to induce hypertrophy, while analyses of miR-208a-deficient mice exhibited defects in stress-dependent cardiac growth and proper cardiac conduction [15, 16]. Previous studies have also shown that forced expression of miR-1 or miR-133 prevented hypertrophic cell growth in cultured cardiomyocytes and cardiac hypertrophy in transgenic mice [17, 18]. However, the function of TGF- β -regulated miRNAs in cardiac hypertrophy has not yet been reported.

In this study, we performed miRNA northern blot to examine whether the expression patterns of miRNAs whose expression are changed in mouse models of cardiac hypertrophy [14, 17, 19] were altered in hypertrophic *Smad4*-deficient hearts. We observed that miR-27b was significantly upregulated in *Smad4* mutant hearts, and miR-27b could be inhibited by TGF- β 1. We also observed that overexpression of miR-27b in primary cultured cardiomyocytes promoted hypertrophic cell growth by targeting the peroxisome proliferator-activated receptor- γ (PPAR- γ). Consequently, miR-27b transgenic mice developed dilated cardiomyopathy and cardiac dysfunction. In addition, *in vivo* administration of antagomir-27b in a transverse aortic constriction (TAC) mouse model attenuated cardiac hypertrophy and dysfunction, suggesting miR-27b as a therapeutic target for HF.

Results

miR-27b is upregulated in Smad4-deficient hearts and downregulated by TGF- β 1

Targeted deletion of *Smad4* in cardiomyocytes induces

cardiac hypertrophy and HF [6]. Our previous study has shown that the cluster of miR-23a/miR-27a/miR-24-2 can be inhibited by TGF- β 1 [20]. We hypothesized that the cluster of miR-23b/miR-27b/miR-24-1, which was frequently dysregulated in mouse models of cardiac hypertrophy [14, 19], could also be inhibited by TGF- β signaling. We first performed miRNA northern blot to examine the expression of these miRNAs, and found that miR-27b expression was significantly increased in *Smad4* mutant hearts (Figure 1A); the result was also confirmed by real-time PCR (Supplementary information, Figure S1A). We further examined the expression of miR-23b and miR-24-1 using real-time PCR, and found that they were all upregulated in *Smad4* mutants (Supplementary information, Figure S1B). The expression of miR-27b in a wide range of tissues was also shown by northern blot (Figure 1B). To confirm that miR-27b was regulated by TGF- β signaling, we treated neonatal cardiomyocytes with TGF- β 1. Real-time PCR and northern blot results showed that pre-miR-27b and mature miR-27b were downregulated when treated with TGF- β 1 (Figure 1C and 1D), implying that cardiac miR-27b was inhibited by TGF- β 1. The efficient response of cardiomyocyte to TGF- β 1 was confirmed by plasminogen activator inhibitor-1 (PAI-1) upregulation (Figure 1C and 1D). The expressions of miR-23b and miR-24-1 were also inhibited by TGF- β 1 (Supplementary information, Figure S1C). To examine whether miR-27b could be regulated by TGF- β 1 at the transcriptional level, we used the 2 900-bp promoter of miR-27b, which contained a potential Smad-binding site (CAGACAT), to drive expression of the luciferase reporter gene in NIH3T3 cells. Treatment with TGF- β 1 significantly inhibited the miR-27b promoter activity, whereas no effect was observed when the construct contained the promoter with a mutated Smad-binding site (Figure 1E). All these data corroborate the hypothesis that TGF- β 1 regulated miR-27b transcription.

miR-27b overexpression promotes hypertrophic growth of cardiomyocytes

To investigate the potential function of miR-27b in cardiomyocytes, we performed gain-of-function and loss-of-function experiments by using cultured rat neonatal cardiomyocytes. We designed two adenoviral vectors encoding sense (Ad-miR-27b) and antisense miR-27b (Ad-anti-miR-27b), respectively. Real-time PCR and northern blot analysis showed efficient overexpression of the pre- and mature miR-27b in Ad-miR-27b-infected cardiomyocytes and efficient knockdown of endogenous mature miR-27b in cardiomyocytes with Ad-anti-miR-27b (Figure 2A and 2B).

The cardiomyocytes infected with adenoviruses for

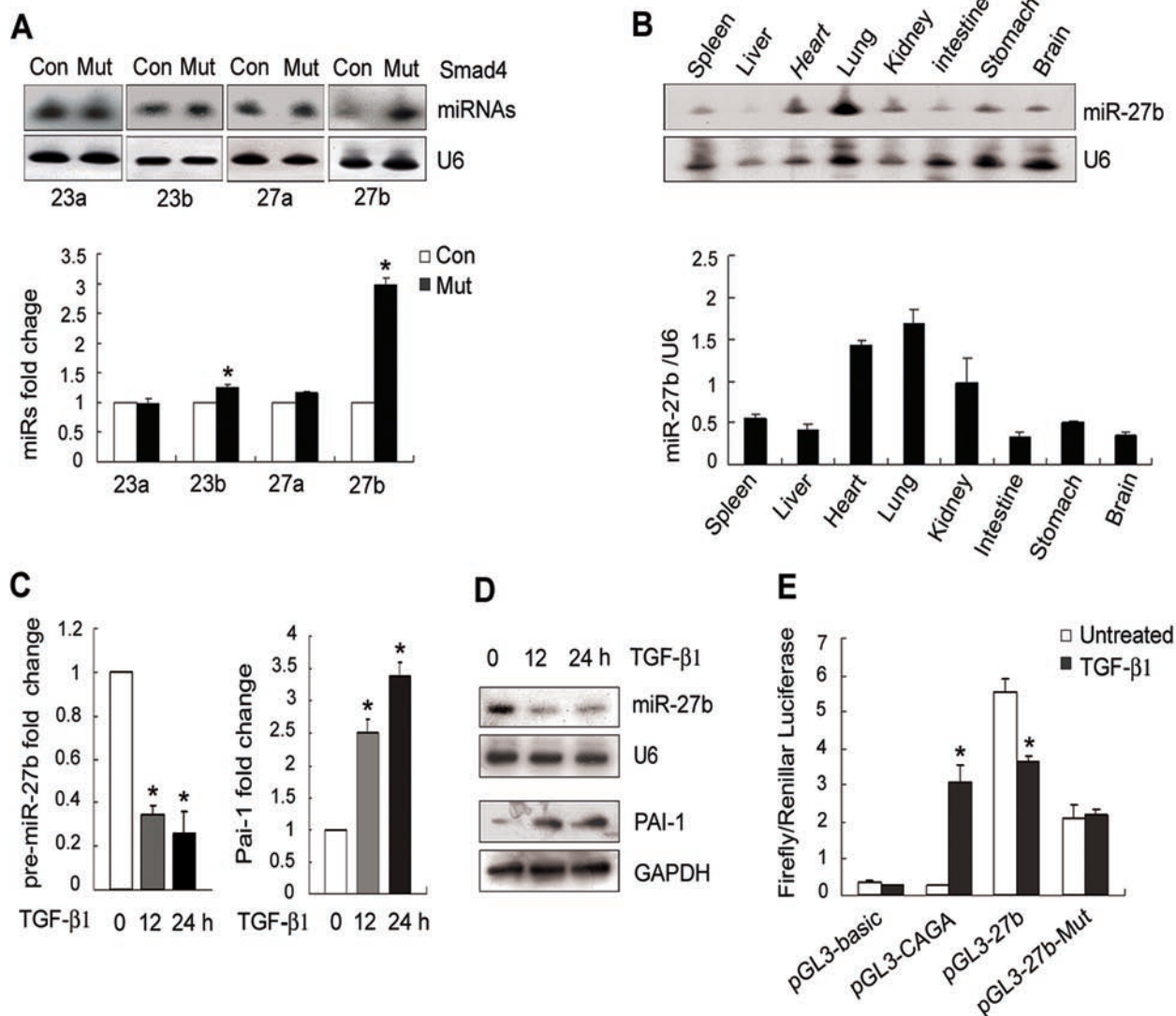


Figure 1 miR-27b upregulated in the hearts of cardiomyocyte-specific Smad4-deficient mice was inhibited by TGF- β 1. **(A)** Northern blot showed that miR-27b was greatly increased in Smad4^{Flox/Flox}; α -MHC-Cre mutant hearts (Mut) compared with Smad4^{Flox/+}; α -MHC-Cre controls (Con). A RNA probe against the small housekeeping RNA U6 (106 nt) was used as a loading control. Lower: quantitative analysis of fold change in expression of miRNAs. * $P < 0.05$ relative to control mice. **(B)** Northern blot showed that miR-27b was expressed in multiple tissues of a 1-month-old mouse. Lower: quantitative analysis of tissue profile for miR-27b. **(C)** Real-time PCR showed that pre-miR-27b was decreased in cardiomyocytes treated with TGF- β 1 for 12 and 24 h, and the TGF- β 1 target molecule, Pai-1, was significantly increased in cardiomyocytes treated with TGF- β 1. **(D)** Northern blot revealed that miR-27b was significantly downregulated upon the treatment of TGF- β 1 for 12 and 24 h, along with the upregulation of PAI-1 shown by western blot. **(E)** NIH-3T3 cells transfected with reporter vectors containing native or mutated miR-27b promoter were treated with or without TGF- β 1 (5 ng/ml) for 24 h, and then harvested for luciferase assay. pGL-3 basic and pGL3-CAGA were used as negative and positive controls, respectively. * $P < 0.05$ relative to respective controls.

48 h were immunostained for α -actinin to study the morphological changes. Immunostaining revealed that cardiomyocytes treated with phenylephrine (PE) or infected with Ad-miR-27b were significantly larger than cardio-

myocytes infected with adenoviruses encoding green fluorescent protein (Ad-GFP) (Figure 2C-2E). Hypertrophy induced by miR-27b overexpression was comparable to that induced by adrenergic agonist PE (Figure 2D and

2E). Conversely, knockdown of miR-27b partially rescued the hypertrophic effects of PE treatment and miR-27b overexpression (Figure 2F-I). Cardiac hypertrophy is always accompanied by upregulation of fetal genes, such as atrial natriuretic factor (ANF) and skeletal muscle and cardiac actin (SKA). Real-time PCR showed that

miR-27b overexpression could significantly upregulate ANF and SKA transcripts, while miR-27b knockdown could partially rescue the upregulation of these genes in the cardiomyocytes treated with PE or cardiomyocytes overexpressing miR-27b (Figure 2J and 2K). Quantitative assessment of cardiomyocyte proliferation by using

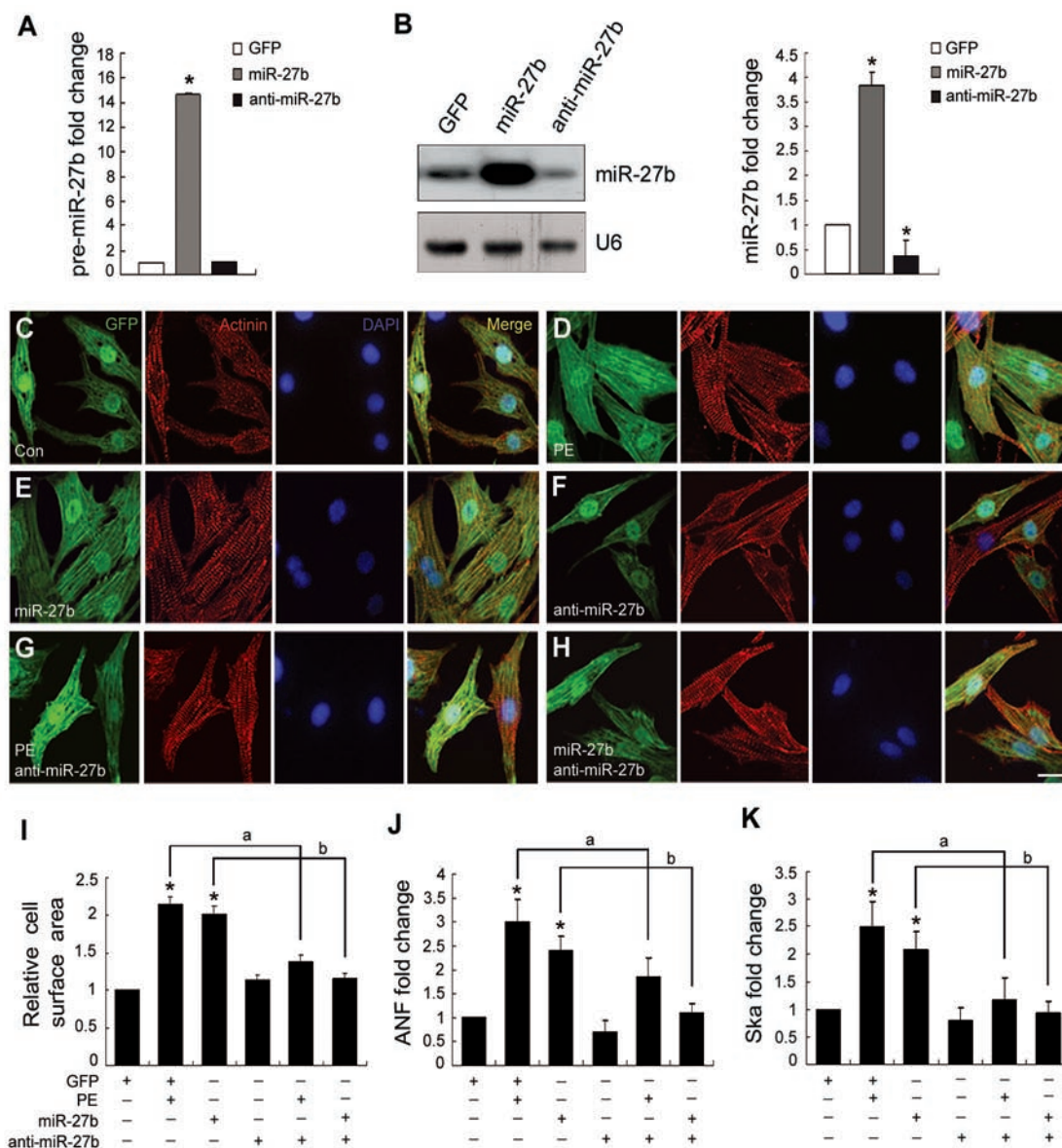


Figure 2 Overexpression of miR-27b promoted hypertrophic growth of cardiomyocytes. **(A)** Real-time PCR showed that miR-27b adenovirus effectively increased the expression level of pre-miR-27b. **(B)** Northern blot and quantification results showed that miR-27b adenovirus increased the miR-27b level in cardiomyocytes, while anti-miR-27b adenovirus decreased the endogenous expression level of miR-27b. **(C-H)** Isolated rat neonatal cardiomyocytes were infected with Ad-miR-27b, Ad-anti-miR-27b or control adenoviruses, and treated with or without PE. Cardiomyocytes were stained for GFP (green) and α -actinin (red). DAPI staining was done to visualize the nuclei (blue). Scale bar, 15 μ m. **(I)** Fold change in mean cell surface area of α -actinin-immunostained cardiomyocytes ($n = 120$ cells/condition). **(J, K)** ANF and SKA transcripts were examined by real-time PCR. * $P < 0.05$ relative to respective controls. ^a $P < 0.05$ vs Ad-GFP treated with PE. ^b $P < 0.05$ vs miR-27b overexpression.

Ki67 immunofluorescence showed that cardiomyocyte proliferation was not affected by miR-27b overexpression (Supplementary information, Figure S2A). These findings indicate that miR-27b could induce hypertrophy of cardiomyocytes.

Cardiomyocyte-specific overexpression of miR-27b induces cardiac hypertrophy

To investigate the role of miR-27b in cardiac remodeling *in vivo*, we generated two transgenic mouse lines carrying the mouse miR-27b DNA under the control of α -MHC promoter. Northern blot results revealed that miR-27b was successfully overexpressed in the transgenic hearts from lines #1 and #2 (3-4-fold increase, Figure 3A). We selected line #1 for further studies. All these miR-27b transgenic mice were born normally and had normal cardiac structure (data not shown). They survived to adulthood and were fertile. However, 30% of transgenic mice died suddenly between 6 and 12 months of age. The miR-27b transgenic mice developed significant cardiac hypertrophy at 3 months of age (Figure 3B). They showed higher heart weight/body weight (HW/BW) and heart weight/tibia length (HW/TL) ratios than the littermate controls (Figure 3C). The fetal genes such

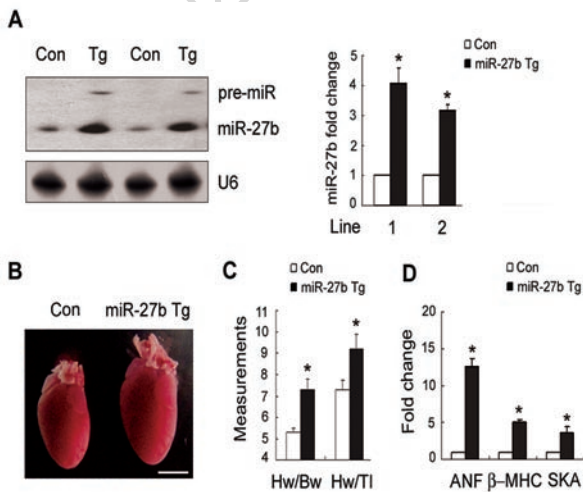


Figure 3 Overexpression of miR-27b induced cardiac hypertrophy *in vivo*. (A) Northern blots and quantification data ($n = 3$) showed an approximately 3-4-fold increase of miR-27b expression in hearts of miR-27b transgenic (Tg) mice compared with control (Con) littermates. A RNA probe against U6 was used as a loading control. (B) Gross morphology of miR-27b Tg hearts was enlarged compared with control hearts at 3 months. Scale bar, 5 mm. (C) Heart weight to body weight or tibia length of 3-month-old miR-27b Tg mice ($n = 7$) was significantly higher than controls ($n = 8$). (D) Analysis of ANF, β -MHC and SKA by real-time PCR in hearts from miR-27b Tg and control mice ($n = 6$ /genotype). * $P < 0.05$ vs control mice.

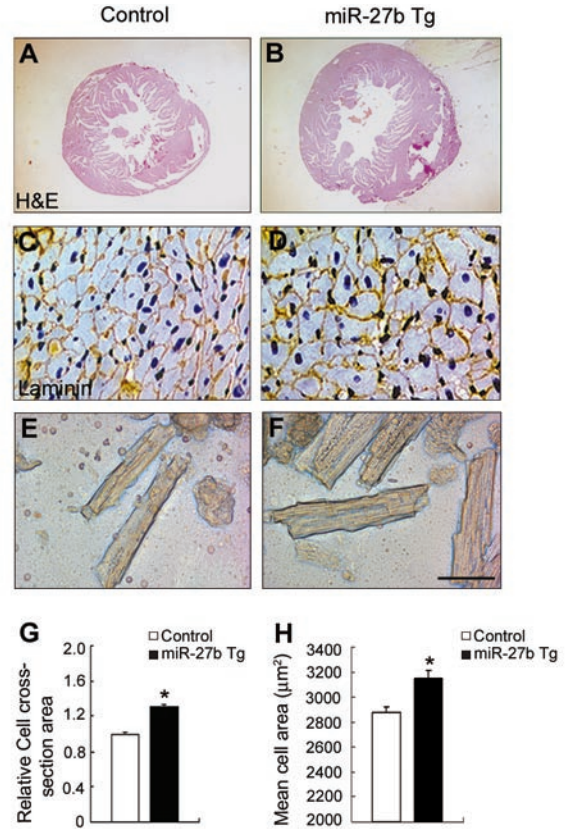


Figure 4 Overexpression of miR-27b *in vivo* induced cardiac hypertrophy. (A, B) Macroscopic view of H&E-stained transverse sections from control (A) and miR-27b Tg hearts (B). (C, D) Immunostaining with a rabbit polyclonal anti-laminin antibody was performed on transverse sections of myocardium from littermate control (C) and miR-27b Tg mice (D). (E, F) Representative images of isolated cardiomyocytes from 3-month-old littermate control (E) and miR-27b Tg mice (F). (G) Histological sections were detected by laminin staining to determine cell size. Mean cardiomyocyte cross-sectional area of miR-27b Tg hearts ($n = 500$) was significantly larger than control cardiomyocytes ($n = 500$). * $P < 0.001$. (H) The mean cell area of the cardiomyocytes ($n = 150$) isolated from 3-month-old miR-27b transgenic mice was greater than control cardiomyocytes ($n = 150$; * $P < 0.001$). Scale bars, A, B: 2 mm and C-F: 50 μm .

as ANF, β -MHC and SKA were also significantly up-regulated in the heart tissues of miR-27b transgenic mice (Figure 3D).

We performed further studies to ascertain whether the increase of the heart size was due to cell growth (increase in cell size) or cell proliferation (increase in cell number). Cardiomyocyte proliferation was assessed by BrdU staining. The cardiomyocyte proliferation in the miR-27b transgenic mice and the littermate controls were not significantly different (Supplementary information, Figure S2B). Cross-sections of hearts confirmed left ventricle

(LV) enlargement (Figure 4A and 4B), and showed that the cardiomyocytes of the transgenic mice were larger than those of the control mice using laminin immunohistochemistry (Figure 4C and 4D). Quantitative measurement of miR-27b transgenic cardiomyocytes revealed a 32% increase in cell cross-sectional area relative to controls (Figure 4G). We also measured the cell size of the individual cardiomyocytes in isolated adult myocyte preparations (Figure 4E and 4F). The mean cell area of the cardiomyocytes ($n = 150$) obtained from three-month-old miR-27b transgenic mice ($n = 4$) was greater than that

of those obtained from the control mice (Figure 4H).

These observations indicated that miR-27b overexpression *in vivo* is sufficient to induce cardiac hypertrophy, which may be largely due to the increased size of cardiomyocyte.

miR-27b transgenic mice developed impaired cardiac function

To evaluate the effect of miR-27b overexpression on cardiac function, we first assessed the LV dimensions and the systolic functions of 3-month-old and 6-month-

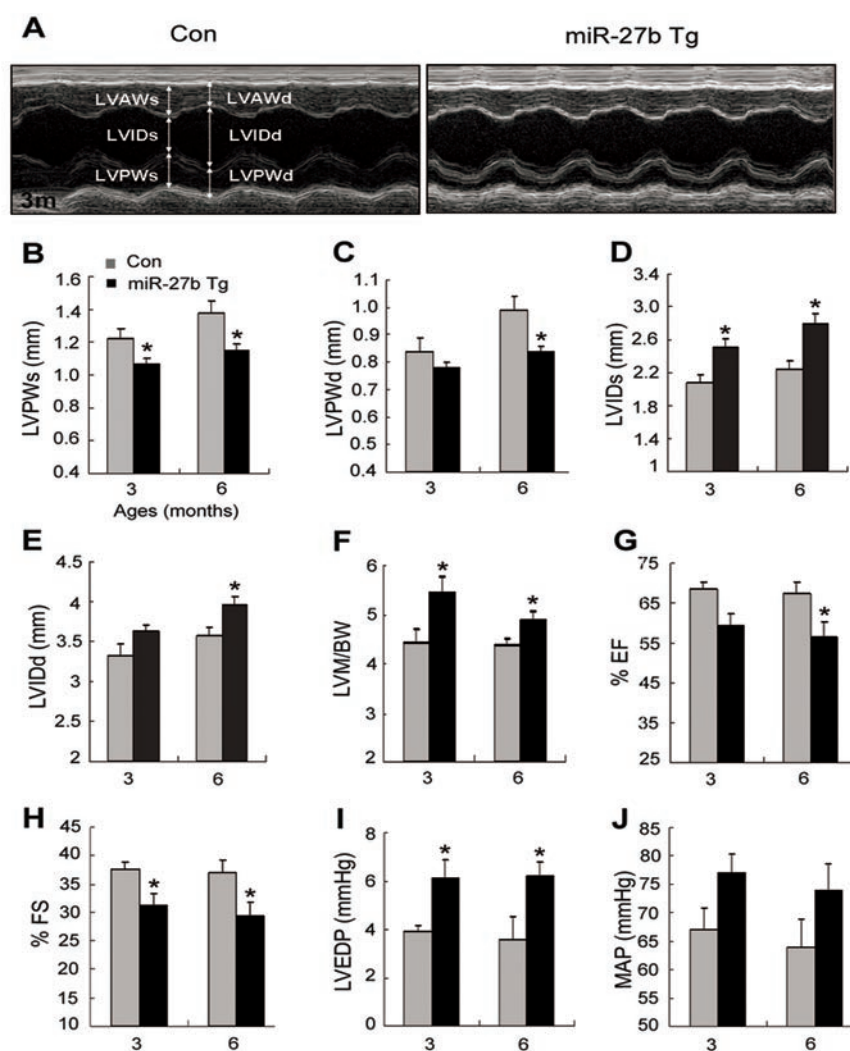


Figure 5 Cardiac dysfunction in miR-27b transgenic mice. **(A)** Representative M-mode images of miR-27b Tg mice (right) and littermate controls (left). **(B, C)** Measurements of the LV wall thickness in systole (LVPWs) and diastole (LVPWd). **(D, E)** Measurements of the LV internal diameters in systole (LVIDs) and diastole (LVIDd). **(F-H)** Quantification of ratio of LV mass to body weight (LVM/BW), ejection fraction (EF), and fractional shortening (FS). Mean values \pm SEM were determined by echocardiography. **(I, J)** Quantification of the LV diastolic end pressure (LVEDP), the mean arterial blood pressure (MAP) in hearts from miR-27b Tg mice and control mice at the age of 3 and 6 months. Mean values \pm SEM were determined by hemodynamic analysis. * $P < 0.05$, significantly different from age-matched controls.

old miR-27b transgenic mice by using M-mode echocardiography (Figure 5A and Supplementary information, Table S1). We observed thinning of the LV walls (Figure 5B and 5C), an increase in the LV internal diameter in diastole and systole (Figure 5D and 5E) and an increase in the ratio of the LV mass (LVM) to the body weight (BW) (Figure 5F). In addition, the ejection fraction (EF) showed a declining trend (Figure 5G). Fractional shortening (FS), an echocardiographic index of LV contractile function, was also decreased in the miR-27b transgenic mice (Figure 5H).

Functional invasive hemodynamic measurements were performed to confirm the occurrence of echocardiographic alterations in miR-27b transgenic mice. The LV diastolic end pressure (LVEDP), measured after inducing anesthesia and LV catheterization, was found to be increased (Figure 5I). The mean arterial blood pressure (MAP) was also slightly increased (Figure 5J). However, the heart rates and other hemodynamic variables of the control and transgenic mice were not different (Supplementary information, Table S2). All these results reflected the impairment of cardiac function in miR-27b transgenic mice.

PPAR- γ is a direct target of miR-27b in cardiomyocytes

To reveal the potential mechanism of miR-27b in cardiac hypertrophy, we screened the candidate miR-27b target mRNAs that have been reported to be involved in cardiac hypertrophy using a bioinformatic approach, as detailed at TargetScan (<http://genes.mit.edu/targetscan/>). This analysis led to the identification of PPAR- γ , in which mRNA 3'-UTR regions comprise the seed sequences and flanking nucleotides matching miR-27b, which is highly conserved among the human, mouse, rat and dog species (Supplementary information, Figure S3A-S3C). To confirm whether PPAR- γ is a direct target of miR-27b, we performed luciferase reporter assays in HEK293 cells. Cotransfection of miR-27b with the luciferase reporter gene linked to the wild-type 3'-UTR of PPAR- γ strongly inhibited the luciferase activity, while cotransfection with anti-miR-27b rescued this effect (Figure 6A). However, no effect was observed with the construct harboring a mutant segment of PPAR- γ 3'-UTR (seed sequence 5'-ACUGUGA-3' was mutated to 5'-AGAAUUC-3'). Moreover, cotransfection of miR-24 (control miRNA; not complementary to the 3'-UTR of PPAR- γ) with the wild-type PPAR- γ 3'-UTR construct did not alter the luciferase activity, indicating that miR-27b specifically targeted PPAR- γ (Figure 6A). The RT-PCR and real-time PCR results showed that the miR-27b overexpression did not affect the transcriptional level of PPAR- γ in cardiomyocytes (Figure 6B). However,

the infection of HEK293 cells and rat neonatal cardiomyocytes with Ad-miR-27b reduced the PPAR- γ protein levels, whereas knockdown of endogenous miR-27b in these cells increased the PPAR- γ protein levels (Figure 6C). We further measured the PPAR- γ expression level in Smad4 mutant heart tissues, which showed increased miR-27b levels, and observed that PPAR- γ was significantly decreased in Smad4 mutants (Figure 6D). Consistently, PPAR- γ levels were significantly reduced in miR-27b transgenic hearts (Figure 6E).

To test the effect of PPAR- γ inhibition on cardiomyocytes, we infected cardiomyocytes with adenovirus that interfere with PPAR- γ (Ad-si-PPAR- γ ; Supplementary information, Figure S4A-S4C). Knockdown of PPAR- γ in cardiomyocytes increased the cell size (Supplementary information, Figure S4D) and upregulated the expression of fetal genes (Supplementary information, Figure S4E), indicating that PPAR- γ interference induced hypertrophy of cardiomyocytes.

Furthermore, we developed Ad-PPAR- γ to overexpress PPAR- γ in cardiomyocytes. Western blot results showed that PPAR- γ was successfully overexpressed in cardiomyocytes infected by Ad-PPAR- γ (Figure 6F). In addition, PPAR- γ overexpression could reduce the increased cell surface area caused by miR-27b overexpression (Figure 6F). All these observations suggest that miR-27b acts as a negative regulator of PPAR- γ at post-transcriptional levels, and PPAR- γ mediates the cardiac hypertrophic effect of miR-27b.

Inhibition of miR-27b attenuated cardiac hypertrophy and dysfunction in a TAC mouse model

To test whether miR-27b inhibition was truly beneficial for cardiac diseases, we injected antagomir-27b into mice subjected to pressure overload of the LV by TAC and followed the disease for additional 3 weeks (Supplementary information, Figure S5A). First, we tested the effect of antagomir-27b *in vivo* and found that mature miR-27b was significantly downregulated in hearts by antagomir-27b administration (Supplementary information, Figure S5B). In addition, measurements of HW/BW, HW/TL, fetal genes and histological examination indicated that antagomir-27b administration did not affect the morphology of hearts in unstressed mice (Supplementary information, Figure S5C-S5F). Expression levels of miR-27b were increased in mice after the TAC surgery (Figure 7A). The TAC-induced miR-27b overexpression was effectively downregulated by antagomir-27b treatments as shown by real-time PCR (Figure 7B). In the myocardium, antagomir-27b treatment significantly reversed changes in PPAR- γ expression caused by pressure overload (Figure 7C). TAC-operated mice treated with

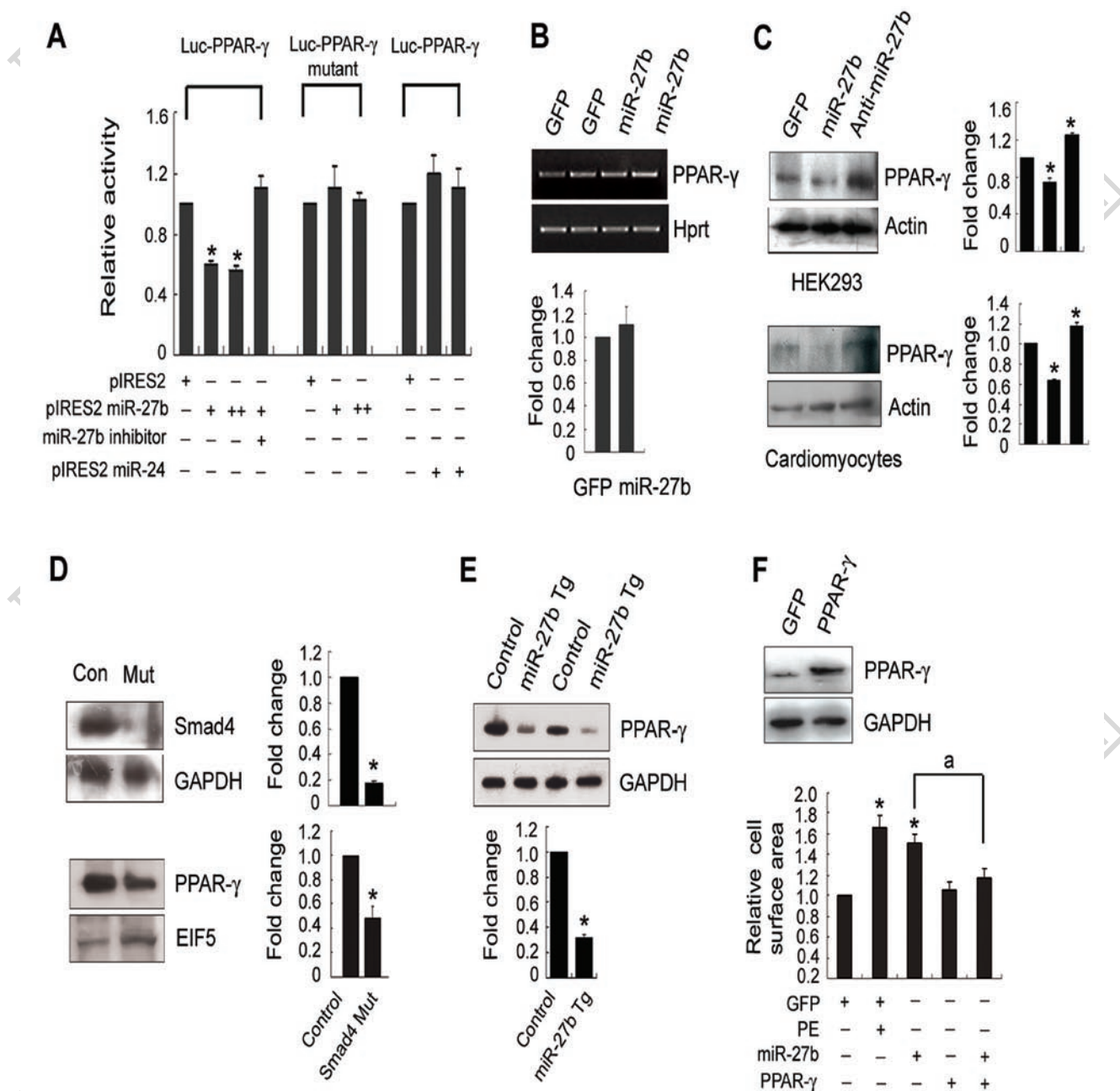


Figure 6 PPAR- γ is a target of miR-27b in cardiomyocytes. **(A)** Dual luciferase activity assay of HEK293 cells cotransfected with a luciferase reporter plasmid containing the naive or mutant PPAR- γ 3'-UTR and a miR-27b or miR-24 expression plasmid or a miR-27b inhibitor. * $P < 0.05$ relative to respective controls. + Represents 200 ng/well and ++ represents 400 ng/well. **(B)** RT-PCR and real-time PCR showed that miR-27b did not affect the mRNA level of PPAR- γ . **(C)** Western blots for PPAR- γ proteins from HEK293 cells and cardiomyocytes infected with Ad-miR-27b or Ad-anti-miR-27b. Right: quantitative analysis of fold change in expression of PPAR- γ . **(D)** Western blot showed that PPAR- γ level was decreased in the heart tissues of Smad4-deficient mice. Right: quantitative analysis of fold change in the expression of Smad4 and PPAR- γ . **(E)** PPAR- γ was decreased in the heart tissues of miR-27b transgenic mice revealed by western blot. GAPDH was used as a loading control. Lower: quantitative analysis of fold change in the expression of PPAR- γ . **(F)** PPAR- γ overexpression rescued hypertrophic growth of cardiomyocytes caused by miR-27b overexpression. Upper: western blot for PPAR- γ proteins from cardiomyocytes infected with Ad-PPAR- γ or Ad-GFP. Lower: Quantitative analysis of fold change in mean cell surface area of α -actinin-immunostained cardiomyocytes infected with adenoviruses ($n = 160$ cells/condition). * $P < 0.05$ relative to Ad-GFP. ^a $P < 0.05$ vs miR-27b overexpression.

saline displayed progressive cardiac hypertrophy and interstitial fibrosis, whereas TAC-operated mice treated with antagomir-27b showed significant attenuation of the effects (Figure 7D-7G). Analysis of cardiac function by echocardiography showed that antagomir-27b treatment significantly reversed LV wall thickness and LV mass and normalized fractional shortening (Figure 7H-7J and Supplementary information, Table S3).

Discussion

In this study, we investigated the physiological functions of miR-27b and the molecular mechanisms by which miR-27b induces cardiac hypertrophy. The important novel findings of our study are as follows: (1) miR-27b is upregulated in Smad4-deficient hypertrophic heart; (2) miR-27b is significantly inhibited by TGF-

$\beta 1$ in cardiomyocytes; (3) cardiac overexpression of miR-27b is sufficient to induce cardiac hypertrophy and dysfunction; (4) PPAR- γ , the target gene of miR-27b, is downregulated in miR-27b transgenic mice, and PPAR- γ overexpression could partially rescue the effects caused by miR-27b overexpression; (5) *in vivo* inhibition of miR-27b attenuates structural and functional deterioration in a TAC-induced HF mouse model. Thus, our results provide the first and critical genetic evidences showing that TGF- β -regulated miR-27b plays an important role in cardiac growth and hypertrophy.

Increasing evidences from experimental studies have shown that Smad-mediated responses to TGF- β signaling have cardioprotective effects in cardiac hypertrophy [21]. Cardiomyocyte-specific deletion of Smad4 induces cardiac hypertrophy and HF [6], while transaortic constriction leads to a significant increase in cardiac hypertrophy

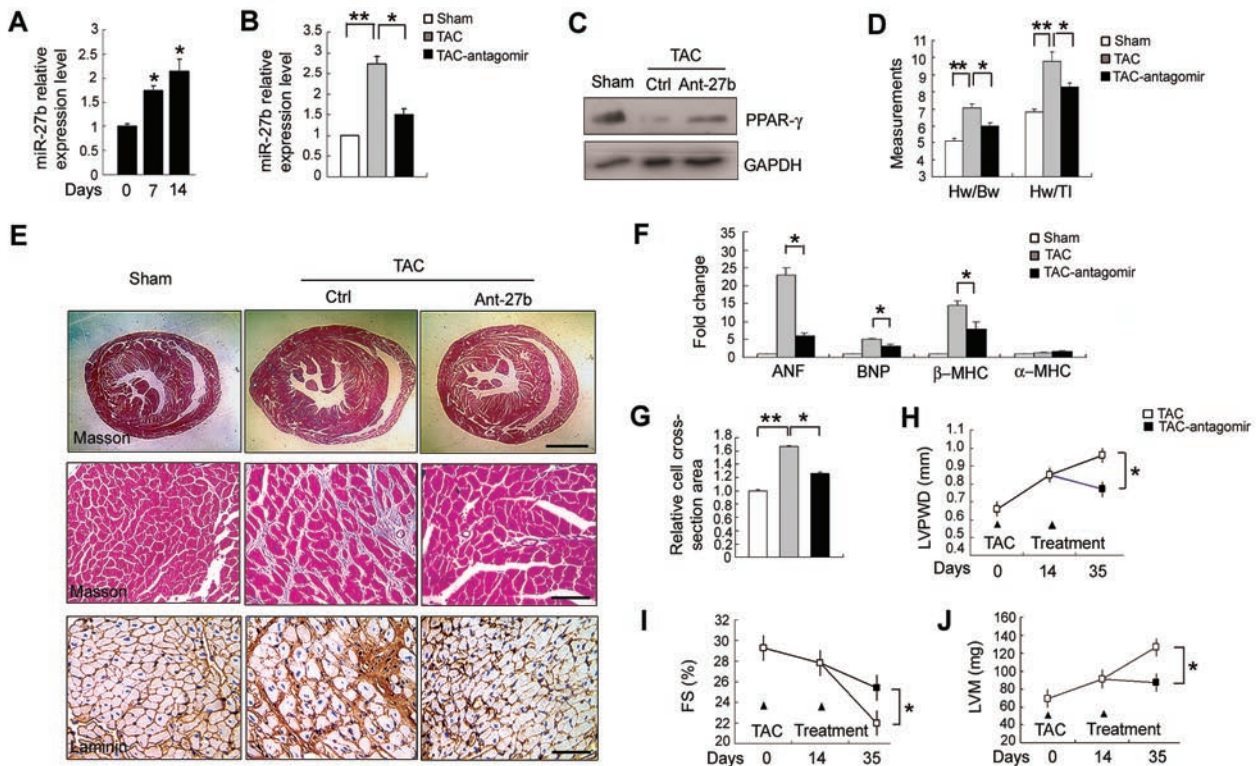


Figure 7 Antagomir-27b attenuated cardiac hypertrophy and dysfunction in a TAC mouse model. **(A)** Detection of miR-27b expression level in TAC models by real-time PCR. * $P < 0.05$ relative to control. **(B)** Detection of miR-27b expression level 3 weeks after treatment with saline or antagomir-27b by real-time PCR. * $P < 0.05$ and ** $P < 0.01$. **(C)** PPAR- γ expression level was determined in hearts by western blot ($n = 3$ /condition). **(D)** Heart weight to body weight or tibia length of mice from sham ($n = 11$), TAC treated with saline ($n = 7$) or with antagomir-27b ($n = 5$). * $P < 0.05$ and ** $P < 0.01$. **(E)** Histological analysis of hearts from different groups using masson staining and Laminin immunostaining. Scale bar, upper panel: 2 mm and middle and lower panel: 50 μ m. **(F)** Analysis of ANF, BNP, β -MHC and α -MHC in hearts from different groups by real-time PCR ($n = 3$ /condition). * $P < 0.05$. **(G)** Cross-sectional areas were analyzed after laminin immunostaining ($n = 400$ cells/condition). * $P < 0.05$ and ** $P < 0.01$. **(H-J)** LVPWD, FS and LVM were analyzed from TAC mice treated with saline ($n = 8$) or antagomir-27b ($n = 5$). * $P < 0.05$.

in the Smad3-deficient mice [22]. Studies on cardiomyocyte-specific Smad5 mutant mice have also suggested that Smad5 is required for cardiac homeostasis [23]. In this study, we observed that miR-27b was upregulated in Smad4-deficient hypertrophic heart tissues. We also observed that TGF- β 1 significantly inhibited miR-27b at the transcriptional level. Furthermore, we provided the first direct evidence to prove that miR-27b overexpression is sufficient to induce cardiac hypertrophy both *in vitro* and *in vivo*. All these findings suggest that TGF- β signaling might protect cardiomyocytes from hypertrophic growth by downregulating miRNAs that cause cardiac hypertrophy when overexpressed. This new finding has increased our knowledge on the complex molecular mechanisms of TGF- β in maintaining cardiac homeostasis. Recent studies have shown that miRNA mediates TGF- β signaling for modulating several biological processes [20, 24]. miR-23b cluster miRNAs, including miR-27b, have been shown to regulate liver stem cell differentiation by targeting Smads [25]. Our data suggest that miR-23b cluster miRNAs could be inhibited by TGF- β 1. Whether there is a reciprocal feedback loop between TGF- β signaling and miR-23b/miR-27b/miR-24-1 cluster in cardiomyocytes needs to be further investigated.

It is noteworthy that miR-27b is frequently upregulated in pressure-overloaded hypertrophic hearts [14, 17, 19]. Recent studies also demonstrated that miR-27b displayed an overt myocardial expression during heart development [26]. However, the miR-27b function in cardiac hypertrophy has not been elucidated yet. In the present study, we elucidated the *in vivo* physiological and pathological consequences of miR-27b in cardiac hypertrophy using both gain-of-function and loss-of-function approaches. Most importantly, we performed curative experiment to demonstrate that miR-27b inhibition was indeed beneficial for pressure overload-induced cardiac hypertrophy and HF. Previous studies have revealed that a limited number of miRNAs may constitute new therapeutic targets for ischemic heart diseases [27-30] as well as hypertension-induced HFs [31, 32]. Our study showed that *in vivo* knockdown of miR-27b significantly attenuated hypertrophic effects caused by pressure overload, providing a new potential therapeutic target for pressure overload heart diseases.

In this study, we identified that PPAR- γ was the target of miR-27b and partially mediated cardiac hypertrophy that resulted from miR-27b overexpression. PPAR- γ is a well-known critical regulator during cardiac hypertrophy. PPAR- γ agonists inhibit mechanical stress-induced hypertrophy of cultured neonatal rat ventricular cardiomyocytes [33]. Cardiac hypertrophy induced by the pressure overload in heterozygous PPAR- γ -deficient mice

is slightly more pronounced than that in the wild-type controls [34]. Studies on cardiomyocyte-specific PPAR- γ knockout mice have shown that PPAR- γ suppresses cardiac growth and embryonic gene expression and protects the heart from oxidative damage [35, 36]. Previous studies have shown that miR-27b regulates adipocyte differentiation and modulates inflammatory responses by targeting PPAR- γ [37, 38]. In our study, we observed that miR-27b targeted PPAR- γ in cardiomyocytes. Supportively, PPAR- γ expression was significantly downregulated in hypertrophic hearts of miR-27b transgenic mice. This was further confirmed by the rescue experiment, which showed that overexpression of PPAR- γ decreased the hypertrophic growth caused by miR-27b overexpression. As miR-27b might target hundreds of mRNAs simultaneously, our results demonstrate that PPAR- γ is an important, but possibly not the only, target gene responsible for cardiac hypertrophy in miR-27b transgenic mice.

In conclusion, our study identified that TGF- β -regulated miR-27b is a regulator of cardiac hypertrophy, suggesting that antagonizing miR-27b might be a new therapeutic entry point for cardiac diseases.

Materials and Methods

Mouse strains

To obtain mice with targeted deletion of *Smad4* gene in heart, homozygous mice for the floxed *Smad4* allele (*Smad4^{fllox/fllox}*, referenced as *Smad4^{Co/Co}* before) [39] were bred with α -MHC-Cre transgenic mice, in which the expression of Cre recombinase is driven by the α -MHC promoter [6]. The genotyping was performed as described [6].

Cloning and expression of miRNAs

A 500 bp genomic fragment encompassing the miR-27b coding region was amplified by PCR and ligated into adenoviral vectors to generate Ad-miR-27b using 293A cells. PCR primers for miR-27b were as follows: miR-27b sense: 5'-TTTCTCGAGGAAGATGCT-CACCAGCCCTTA-3' and miR-27b antisense: 5'-TTTTCTA-GAGCATCATCTTGCCAGCGACT-3'. The inhibitor of miR-27b used for target analysis was synthesized by Shanghai GenePharma Co., Ltd.

Isolation, culture and treatment of neonatal cardiomyocytes

Neonatal rat ventricular myocytes were isolated from 2-day-old pups, infected and stained as described [40].

Generation of miR-27b transgenic mice

A mouse genomic fragment flanking the miR-27b was amplified using primers 5'-TCACATTGCCAGGGATTACCAC-3' and 5'-TCAGCACGCTGTTTGCCTCTT-3', then subcloned into a cardiac-specific expression plasmid containing the α -MHC promoter and human growth hormone (GH) poly (A) signal. To genotype the mouse, genomic DNA was isolated from mouse-tail biopsies and analyzed by PCR using specific primers: 5'-

ATGACAGACAGATCCCTCCTATCTCC-3' and 5'-TCAGCACG CTGTTTGCCTCTT-3'.

Western blot analysis

Western blot was carried out on myocardial extracts as described [41]. 30 µg of proteins were electrophoresed on 12% SDS-PAGE and transferred onto PVDF membranes. Immunoblotting was performed according to the manufacturer's instructions using the following antibodies: anti-PPAR-γ, anti-Smad4, anti-PAI-1 and anti-GAPDH (Santa Cruz).

Transthoracic echocardiography and in vivo blood pressure measurement

Cardiac function and heart dimensions were evaluated by echocardiography and *in vivo* blood pressure measurements as described [6].

Antagomir application on TAC models

TAC surgeries were performed on male C57BL/6 mice (8 weeks old) as described [42]. Chemically modified antisense oligonucleotides (antagomir) were synthesized by GenePharma Co., Ltd. Treatments started 2 weeks after TAC, and animals received 0.2 ml saline, antagomir-27b (three injections in three consecutive days, 80 mg/kg body weight) via tail vein injections [31, 32].

Statistical analysis

All statistical analyses were performed using SPSS software. Results are means ± SEM. Statistical differences were determined by Student's *t*-test. *P*-value < 0.05 was considered significant.

Other methods including miRNA northern blot, plasmid and adenovirus constructions, isolation of cardiomyocytes, real-time PCR, luciferase reporter assay, histology and immunohistology are described in Supplementary information, Data S1.

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References

- 1 Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* 2006; **7**:589-600.
- 2 Azhar M, Schultz Jel J, Grupp I, et al. Transforming growth factor beta in cardiovascular development and function. *Cytokine Growth Factor Rev* 2003; **14**:391-407.
- 3 Schmierer B, Hill CS. TGF-β-SMAD signal transduction: molecular specificity and functional flexibility. *Nat Rev Mol Cell Biol* 2007; **8**:970-982.
- 4 Schultz Jel J, Witt SA, Glascock BJ, et al. TGF-beta1 mediates the hypertrophic cardiomyocyte growth induced by angiotensin II. *J Clin Invest* 2002; **109**:787-796.
- 5 Rosenkranz S, Flesch M, Amann K, et al. Alterations of

- β-adrenergic signaling and cardiac hypertrophy in transgenic mice overexpressing TGF-β₁. *Am J Physiol Heart Circ Physiol* 2002; **283**:H1253-H1262.
- 6 Wang J, Xu N, Feng X, et al. Targeted disruption of Smad4 in cardiomyocytes results in cardiac hypertrophy and heart failure. *Circ Res* 2005; **97**:821-828.
- 7 Ago T, Sadoshima J. GDF15, a cardioprotective TGF-β superfamily protein. *Circ Res* 2006; **98**:294-297.
- 8 Xu J, Kimball TR, Lorenz JN, et al. GDF15/MIC-1 functions as a protective and antihypertrophic factor released from the myocardium in association with SMAD protein activation. *Circ Res* 2006; **98**:342-350.
- 9 Chen H, Yong W, Ren S, et al. Overexpression of bone morphogenetic protein 10 in myocardium disrupts cardiac postnatal hypertrophic growth. *J Biol Chem* 2006; **281**:27481-27491.
- 10 Carrington JC, Ambros V. Role of microRNAs in plant and animal development. *Science* 2003; **301**:336.
- 11 da Costa Martins PA, Bourajjaj M, Gladka M, et al. Conditional *Dicer* gene deletion in the postnatal myocardium provokes spontaneous cardiac remodeling. *Circulation* 2008; **118**:1567-1576.
- 12 Chen JF, Murchison EP, Tang R, et al. Targeted deletion of *Dicer* in the heart leads to dilated cardiomyopathy and heart failure. *Proc Natl Acad Sci USA* 2008; **105**:2111-2116.
- 13 Rao PK, Toyama Y, Chiang HR, et al. Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. *Circ Res* 2009; **105**:585-594.
- 14 van Rooij E, Sutherland LB, Liu N, et al. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc Natl Acad Sci USA* 2006; **103**:18255-18260.
- 15 van Rooij E, Sutherland LB, Qi X, Richardson JA, Hill J, Olson EN. Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* 2007; **316**:575-579.
- 16 Callis TE, Pandya K, Seok HY, et al. MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. *J Clin Invest* 2009; **119**:2772-2786.
- 17 Sayed D, Hong C, Chen IY, Lypowy J, Abdellatif M. MicroRNAs play an essential role in the development of cardiac hypertrophy. *Circ Res* 2007; **100**:416-424.
- 18 Care A, Catalucci D, Felicetti F, et al. MicroRNA-133 controls cardiac hypertrophy. *Nat Med* 2007; **13**:613-618.
- 19 Cheng Y, Ji R, Yue J, et al. MicroRNAs are aberrantly expressed in hypertrophic heart: do they play a role in cardiac hypertrophy? *Am J Pathol* 2007; **170**:1831-1840.
- 20 Sun Q, Zhang Y, Yang G, et al. Transforming growth factor-β-regulated *miR-24* promotes skeletal muscle differentiation. *Nucleic Acids Res* 2008; **36**:2690-2699.
- 21 Xiao H, Zhang YY. Understanding the role of transforming growth factor-β signaling in the heart: overviews of studies using genetic mouse models. *Clin Exp Pharmacol Physiol* 2008; **35**:335-341.
- 22 Divakaran V, Adrogue J, Ishiyama M, et al. Adaptive and maladaptive effects of SMAD3 signaling in the adult heart after hemodynamic pressure overloading. *Circ Heart Fail* 2009; **2**:633-642.
- 23 Umans L, Cox L, Tjwa M, et al. Inactivation of *Smad5* in endothelial cells and smooth muscle cells demonstrates that *Smad5* is required for cardiac homeostasis. *Am J Pathol* 2007; **170**:1460-1472.

- 24 van Rooij E, Sutherland LB, Thatcher JE, et al. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci USA* 2008; **105**:13027-13032.
- 25 Rogler CE, LeVoci L, Ader T, et al. MicroRNA-23b cluster microRNAs regulate transforming growth factor-beta/bone morphogenetic protein signaling and liver stem cell differentiation by targeting Smads. *Hepatology* 2009; **50**:575-584.
- 26 Chinchilla A, Lozano E, Daimi H, et al. MicroRNA profiling during mouse ventricular maturation: a role for miR-27b modulating Mef2c expression. *Cardiovasc Res* 2011; **89**:98-108.
- 27 Ren XP, Wu J, Wang X, et al. MicroRNA-320 is involved in the regulation of cardiac ischemia/reperfusion injury by targeting heat-shock protein 20. *Circulation* 2009; **119**:2357-2368.
- 28 Lin Z, Murtaza I, Wang K, Jiao J, Gao J, Li PF. miR-23a functions downstream of NFATc3 to regulate cardiac hypertrophy. *Proc Natl Acad Sci USA* 2009; **106**:12103-12108.
- 29 Wang X, Zhang X, Ren XP, et al. MicroRNA-494 targeting both proapoptotic and antiapoptotic proteins protects against ischemia/reperfusion-induced cardiac injury. *Circulation* 2010; **122**:1308-1318.
- 30 Hu S, Huang M, Li Z, et al. MicroRNA-210 as a novel therapy for treatment of ischemic heart disease. *Circulation* 2010; **122**:S124-S131.
- 31 Thum T, Gross C, Fiedler J, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signaling in fibroblasts. *Nature* 2008; **465**:980-985.
- 32 Da Costa Martins PA, Salic K, Gladka MM, et al. MicroRNA-199b targets the nuclear kinase Dyrk1a in an auto-amplification loop promoting calcineurin/NFAT signaling. *Nat Cell Biol* 2010; **12**:1220-1230.
- 33 Yamamoto K, Ohki R, Lee RT, Ikeda U, Shimada K. Peroxisome proliferator-activated receptor γ activators inhibit cardiac hypertrophy in cardiac myocytes. *Circulation* 2001; **104**:1670-1675.
- 34 Asakawa M, Takano H, Nagai T, et al. Peroxisome proliferator-activated receptor γ plays a critical role in inhibition of cardiac hypertrophy *in vitro* and *in vivo*. *Circulation* 2002; **105**:1240-1246.
- 35 Duan SZ, Ivashchenko CY, Russell MW, Milstone DS, Mortensen RM. Cardiomyocyte-specific knockout and agonist of peroxisome proliferators-activated receptor- γ both induce cardiac hypertrophy in mice. *Circ Res* 2005; **97**:372-379.
- 36 Ding G, Fu M, Qin Q, et al. Cardiac peroxisome proliferator-activated receptor γ is essential in protecting cardiomyocytes from oxidative damage. *Cardiovasc Res* 2007; **76**:269-279.
- 37 Jennewein C, Von Knethen A, Schmid T, Brune B. MicroRNA-27b contributes to lipopolysaccharide-mediated peroxisome proliferator-activated receptor γ mRNA destabilization. *J Biol Chem* 2010; **285**:11846-11853.
- 38 Karbiener M, Fischer C, Nowitsch S, et al. MicroRNA miR-27b impairs human adipocyte differentiation and targets PPARgamma. *Biochem Biophys Res Commun* 2009; **390**:247-251.
- 39 Yang X, Li C, Herrera PL, Deng CX. Generation of Smad4/Dpc4 conditional knockout mice. *Genesis* 2002; **32**:80-81.
- 40 Murakami M, Nakagawa M, Olson EN, Nakagawa O. A WW domain protein TAZ is a critical coactivator for TBX5, a transcription factor implicated in Holt-Oram syndrome. *Proc Natl Acad Sci USA* 2005; **102**:18034-18039.
- 41 Teng Y, Sun AN, Pan XC, et al. Synergistic function of Smad4 and PTEN in suppressing forestomach squamous cell carcinoma in the mouse. *Cancer Res* 2006; **66**:6972-6981.
- 42 Hill JA, Karimi M, Kutschke W, et al. Cardiac hypertrophy is not a required compensatory response to short-term pressure overload. *Circulation* 2000; **101**:2863-2869.

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