Mnk1 (Mitogen-Activated Protein Kinase–Interacting Kinase 1) Deficiency Aggravates Cardiac Remodeling in Mice

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Abstract—Identifying the key factor involved in cardiac remodeling is critically important for developing novel strategies to protect against heart failure. Here, the role of Mnk1 (mitogen-activated protein kinase–interacting kinase 1) in cardiac remodeling was clarified. Cardiac remodeling was induced by transverse aortic constriction in Mnk1-knockout mice and their wild-type control mice. After 4 weeks of transverse aortic constriction, Mnk1-knockout mice developed exaggerated cardiac hypertrophy, fibrosis, dysfunction, and cardiomyocyte apoptosis and showed increased ERK1/2 (extracellular signal–regulated kinase 1/2) activation along with reduced sprouty2 expression. In line with the in vivo studies, Mnk1 knockdown by Mnk1 siRNA transfection induced exaggerated angiotensin II–induced cardiomyocyte hypertrophy in neonatal rat ventricular myocytes (NRVMs). Moreover, adenovirus-mediated overexpression of Mnk1 in NRVMs protected cardiomyocytes from angiotensin II–induced hypertrophy. In addition, overexpression of sprouty2 rescued NRVMs with Mnk1 knockdown from angiotensin II–induced hypertrophy. In accordance with the in vivo studies, as compared with the control group, Mnk1 knockdown led to hyperphosphorylation of ERK1/2 and suppression of the sprouty2 expression in angiotensin II–treated NRVMs; furthermore, Mnk1 overexpression led to hypophosphorylation of ERK1/2 in angiotensin II–treated NRVMs. In addition, sprouty2 overexpression suppressed the activation of ERK1/2 in angiotensin II–treated NRVMs with Mnk1 knockdown. Impressively, Mnk1-knockout mice with overexpression of sprouty2 exhibited signs of a blunted cardiac hypertrophic response. Mnk1 likely carries out a suppressive function in cardiac hypertrophy via regulating the sprouty2/ERK1/2 pathway. It implicates Mnk1 in the development of cardiac remodeling. (Hypertension. 2016;68:1393-1399. DOI: 10.1161/HYPER.116.07906.) ● Online Data Supplement

Key Words: apoptosis ■ cardiac hypertrophy ■ cardiomyocytes ■ fibrosis ■ Mnk1

Cardiac remodeling is regarded as maladaptive changes in response to pathological stimuli, such as neurohumoral factors, pressure or volume overload, biomechanical stress, myocarditis, or inherited mutations, leading to heart failure.1 Cells undergo morphology changes, protein synthesis increase, and cardiac fetal gene expression program reactivation in cardiac remodeling.2,3 Various factors influence cardiac remodeling through their actions on membrane receptors, which transduce signals involving enzymes such as mitogen-activated protein kinases, calcineurin-NFAT (nuclear factor of activated T cells), and Akt (protein kinase B).4,5 Thus, understanding the cellular signaling events regulating cardiac remodeling and finding new molecular targets for the inhibition or reversal of cardiac remodeling may facilitate potential new therapeutic discovery to prevent the development of cardiac remodeling and heart failure.

The Mnk1 (mitogen-activated protein kinase–interacting kinase 1) was discovered as a consequence of bacterial expression libraries screening for proteins regulated by the classical mitogen-activated protein kinases, the ERKs (extracellular signal–regulated kinases).6 Subsequent studies demonstrated that it was activated downstream of the p38 and ERK signaling pathways.7 Mnk1 has low activity in serum-starved cells, and its activity is enhanced abundantly by stimuli that activate either ERK or p38.6,8 Studies have shown that Mnk1 regulates several effector elements, such as eIF4E9–11 and sprouty2.8 It was indicated that sprouty2 could inhibit the Ras/ERK pathway through binding to GRB2 (growth factor receptor–bound protein 2).12,13 Other investigations showed the antiapoptotic activity of eIF4E via promoting the expression of Bcl-2.14 Both sprouty2 and eIF4E can be phosphorylated by Mnk1. The phosphorylation of sprouty2 by Mnk1 may protect sprouty2 from degradation.15 The phosphorylation of eIF4E by Mnk1 facilitates the formation of the eIF4E initiation complex and ribosomal recruitment of specific mRNAs.16

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To date, no studies have investigated the role of Mnk1 in pressure overload–induced cardiac remodeling. We hypothesized that Mnk1 may mediate beneficial effects through its ability to keep the stability of sprouty2 and to promote the translation of Bcl-2. In this study, we investigated one knock-out mouse line globally lacking Mnk1 (Mnk1-KO) and neonatal rat ventricular myocytes (NRVMs) lacking or over-expressing Mnk1 and further aimed to discriminate its role in cardiac remodeling.

**Methods**

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University. An expanded Methods section is available in the online-only Data Supplement, including Animals and Study Design, Echocardiography and Hemodynamics, Histology, Immunofluorescence, Assessment of Apoptosis, Western Blot Analysis, RT-PCR, NRVMs Culture and RNA Interference, Immunoprecipitation, Adenoviral Infection, and Statistical Analysis.

**Statistical Analysis**

Data are presented as mean±SEM. Statistical analysis was performed using SPSS 13.0 (SPSS, Inc) software. Data were analyzed by 1-way ANOVA followed by Tukey post hoc test. P values <0.05 were considered as statistically significant.

**Results**

**Activation of Mnk1 in Myocardium in Response to Pressure Overload**

We first detected the protein expression of total Mnk1 and phosphorylated Mnk1 (phospho-Mnk1) in pressure overload–induced murine hearts. It turned out that the cardiac expression of Mnk1 did not change, but phospho-Mnk1, which indicates the activation state of Mnk1, was upregulated at 2 weeks and 4 weeks after transverse aortic constriction (TAC); however, a subsequent decompensatory decrease in Mnk1 activation occurred from 6 weeks after TAC (Figure 1A).

**Lacking Mnk1 Leads to Deteriorated Cardiac Hypertrophy and Dysfunction**

To test whether Mnk1 contributes to cardiac hypertrophy, we challenged Mnk1-KO mice and their wild-type (WT) control mice (Figure 1B) with pathological pressure overload induced by TAC. The degree of TAC was similar in Mnk1-KO and WT mice as indicated by end-systolic pressure in the 2 groups (Table S1). Four weeks after TAC, Mnk1-KO mice showed a strikingly larger heart and increased cardiomyocyte cross-sectional area than WT animals (Figure 1C and 1D). Mnk1-KO mice also represented increased ratios of heart weight to body weight or tibia length and ratios of lung weight to body weight (Figure 1E). In addition, as shown by increased interventricular septum thickness at diastole, left ventricular end-diastolic diameter, left ventricular end-systolic diameter, and left ventricular end-diastolic posterior wall thickness (Figure S1A in the online-only Data Supplement), the accelerated effect of Mnk1 deficiency on TAC-induced hypertrophic response was confirmed. Moreover, 4 weeks after TAC, Mnk1-KO mice showed a decline in cardiac contractility as indicated by reduced ejection fraction, fractional shortening, maximal rate of pressure development, cardiac output, and stroke volume (Figure S1A; Table S1). Mnk1-KO mice also showed deteriorated cardiac diastolic function as indicated by reduced minimum rate of pressure development and increased time constant of isovolumic pressure decay (Table S1). Consistently, the expression of cardiac fetal genes including atrial natriuretic factor, brain natriuretic peptide, β-myosin heavy chain were significantly augmented in Mnk1-KO mice after TAC, whereas the expression of α-myosin heavy chain was significantly decreased (Figure S1B). Thus, Mnk1 deficiency remarkably renders the heart more susceptible to pressure overload–induced cardiac hypertrophy and dysfunction.

**Mnk1 Suppresses Angiotensin II–Induced Cardiomyocyte Hypertrophy In Vitro**

Western blot analysis showed that angiotensin II (Ang II; 1 μmol/L) treatment for 24 or 48 hours significantly increased phospho-Mnk1 expression while had no effects on total Mnk1 in NRVMs (Figure 2A), suggesting the activation of Mnk1 in response to Ang II stimulation. Next, we performed both gain-of-function and loss-of-function studies using NRVMs. Mnk1 knockdown, which resulted in ≈70% decrease of Mnk1 protein (Figure 2B and 2C), accelerated Ang II–induced cardiomyocyte hypertrophy and activation of fetal gene in NRVMs (Figure 2D through 2F). To further determine whether overexpression of Mnk1 could attenuate cardiomyocyte hypertrophy, we transfected isolated NRVMs with an adenovirus harboring Mnk1 cDNA (AdMnk1) to upregulate Mnk1 expression (Figure S2A). Compared with the AdGFP transfection group, Mnk1 overexpression dramatically blunted the prohypertrophic effect of Ang II, as indicated by cell surface area, expression of atrial natriuretic factor (Figure S2B through S2D). These results indicate an important antihypertrophic role of Mnk1 in cardiomyocytes.

**Mnk1 Inhibits ERK1/2 Signaling Associated With Sprouty2**

We further gained insight into the molecular events mediating the antihypertrophic effect of Mnk1. It was reported that ERK1/2 phosphorylation can be suppressed by sprouty2, which is a substrate of Mnk1. Thus, we detected the protein expression of phospho-ERK1/2 and sprouty2 in sham- and TAC-operated Mnk1-KO/WT mice. We found the pressure overload–induced ERK1/2 activation to be strongly upregulated in Mnk1-KO mice after 4 weeks of TAC (Figure 3A). Meanwhile, we found that sprouty2 expression was markedly downregulated in heart of Mnk1-KO mice subjected to TAC (Figure 3A). In line with this in vivo data, Mnk1 knockdown in NRVMs resulted in hyperphosphorylation of ERK1/2 and decreased sprouty2 expression after Ang II stimulation (Figure 3B). It implies that ERK1/2 and sprouty2 possibly involves in the regulating process of cardiac remodeling by Mnk1. The interaction between Mnk1 and ERK1/2 signaling was further confirmed by immunoprecipitation experiments. Because GRB2 is the link protein between sprouty2 and ERK1/2, we investigated the interaction between sprouty2 and GRB2 in NRVMs. A significant decrease of interaction...
between sprouty2 and GRB2 in NRVMs with Mnk1 knockdown was observed not only in the basal environment but also under the treatment of Ang II (Figure 3C). Consistently, Mnk1 overexpression significantly suppressed ERK1/2 activation in Ang II–stimulated NRVMs (Figure S3A). These data indicate that Mnk1 may specifically inhibit ERK1/2 signaling pathway through the interaction of its substrate sprouty2 with GRB2.

To verify the role of sprouty2, we overexpressed sprouty2 in NRVMs with Mnk1 knockdown (Figure S3B and S3C). After 48 hours of Ang II stimulation, sprouty2 overexpression remarkably suppressed atrial natriuretic factor mRNA levels and ERK1/2 phosphorylation levels (Figure S3D and S3E).

To confirm the role of sprouty2 in the hypertrophic response in Mnk1-KO mice, AdSpry2 or AdGFP was injected in the cardiac wall of mice right after TAC surgery (Figure S4A). Left ventricular hypertrophy was evaluated after 4 weeks by echocardiography and hemodynamics. AdSpry2 treatment efficiently reduced cross-sectional area, heart weight to body weight ratio, lung weight to body weight (LW/BW) ratios of WT and Mnk1-KO mice after Sham or TAC operation (n=11–17 mice per experimental group). Data are presented as mean±SEM. *P<0.05 vs Sham group in the same genotype. *P<0.05 vs WT/TAC.
Lacking Mnk1 Leads to Deteriorated Cardiac Fibrosis

The changes in Mnk1-KO hearts after pressure overload also included an increased interstitial fibrosis and perivascular fibrosis as compared with the WT mice (Figure S5A). The mRNA expression levels of fibrotic markers, including connective tissue growth factor, collagen Iα, collagen IIIα, and fibronectin, were significantly augmented in Mnk1-KO mice after TAC (Figure S5B). These results indicate that Mnk1 deficiency leads to deteriorated cardiac fibrosis. Consistently, the transforming growth factor β (TGFβ) and Smad1/5 signaling pathway were significantly enriched (Figure S5C). We further did loss-of-function and gain-of-function studies of Mnk1’s effect on Ang II–induced profibrotic marker expressions in cardiac fibroblasts and found that neither Mnk1 knockdown nor overexpression changed the profibrotic marker expressions (connective tissue growth factor, collagen Iα, collagen IIIα, and fibronectin) in Ang II–treated cardiac fibroblasts (data not shown). However, it was further found that adenovirus transfection–based Mnk1 overexpression led to decreased TGFβ mRNA levels in Ang II–treated NRVMs as compared with the AdGFP group (Figure S5D). However, Mnk1 overexpression did not make a difference to TGFβ mRNA expression in Ang II–treated cardiac fibroblasts as compared with AdGFP group (Figure S5E).

Apoptosis Is Exaggerated in Mnk1-KO Mice After TAC and Mnk1-Silenced NRVMs Stimulated by Ang II

TAC increased the number of TUNEL-positive cells in both WT and Mnk1-KO mice, but the magnitudes of increase were slightly larger in Mnk1-KO mice (Figure S6A). To examine changes in the molecules involved in apoptosis signaling, we quantified cleaved-caspase3, Bax, and Bcl-2 proteins in WT and Mnk1-KO mice and found that the expression levels of cleaved-caspase3 and Bax were significantly increased in Mnk1-KO mice as compared with WT mice. Moreover, the expression of Bcl-2 was significantly decreased in Mnk1-KO mice as compared with WT mice (Figure S6B). Because the eIF4E phosphorylation may link Mnk1 with Bcl-2, we detected total and phosphorylated eIF4E in murine hearts in the indicated groups and found that Mnk1-KO mice displayed decreased expression of phosphorylated eIF4E as compared with the WT mice after TAC (Figure S6C). In line with the in vivo result, Ang II increased the number of TUNEL-positive cells in NRVMs transfected with nontargeting or Mnk1 siRNA, but the magnitudes of increase were much larger in Mnk1 siRNA–transfected NRVMs (Figure S6D). We also found that Mnk1 knockdown led to decreased Bcl-2 expression in Ang II–treated NRVMs as compared with the control cells (Figure S6E). It was further confirmed that Mnk1 overexpression increased Bcl-2 expression in Ang II–treated NRVMs as compared with AdGFP group (Figure S6F).

Discussion

The absence of endogenous Mnk1 results in severe cardiac hypertrophy, fibrosis, dysfunction, and cardiomyocyte apoptosis. Consistent with the in vivo data, siRNA-mediated loss-of-function approaches indicate that decreased expression of endogenous Mnk1 promoted cardiomyocyte hypertrophy in cardiomyocytes after Ang II stimulation, and adenovirus-mediated
Mnk1 overexpression suppresses Ang II–induced cardiomyocyte hypertrophy and cardiomyocyte TGFβ mRNA expression. Impressively, the in vivo and the in vitro studies suggest that the antihypertrophic effect of Mnk1 is likely associated with suppression of ERK1/2 activation via regulation of sprouty2. Our results suggest that Mnk1 may be an indispensable endogenous-negative regulator of cardiac remodeling.

Recent studies have pointed out the significant role of sprouty2, a substrate of Mnk1, during multiple diseases or physiological process such as human mesenchymal stem cell differentiation,17 papillary thyroid cancer,18 and prostate cancer progression19 by regulating ERK signaling pathway. ERK1/2 is one of the most important signaling that could provide new treatment strategies for cardiac hypertrophy and remodeling. In our study, phospho-ERK1/2 expression increased while sprouty2 expression decreased in pressure overload–induced Mnk1-KO mice and Ang II–treated Mnk1-silencing NRVMs as compared with the controls. Consistently, Mnk1 overexpression in NRVMs significantly suppressed the activation of ERK1/2 after Ang II stimulation. Because the phosphorylation of sprouty2 by Mnk1 may protect sprouty2 from degradation,4 and it has been reported that sprouty2 could inhibit the Ras/ERK pathway through binding to GRB2, in this study, it is reasonable that Mnk1 deficiency induces decreased sprouty2 expression and increased ERK1/2 activation. Coimmunoprecipitation assay showed that Mnk1 knockdown decreased the binding of sprouty2 to GRB2 not only in the basal environment but also under the treatment of Ang II. We speculated that a decline in sprouty2 stability because of Mnk1 knockdown leads to decreased binding of sprouty2 to GRB2, resulting in hyper-activation of ERK1/2 signaling pathway. However, in basal environment, phospho-ERK1/2 expression in Mnk1-KO mice and WT ones did not show any differences, indicating

Figure 3. Mnk1 deficiency induces ERK1/2 (extracellular signal–regulated kinase 1/2) activation associated with sprouty2 (Spry2). A, Representative blots and quantitative results for phospho-ERK1/2 (p-ERK1/2), total ERK1/2, and Spry2 protein expression in myocardium in each group (n=6 per group). *P<0.05 vs Sham group in the same genotype. #P<0.05 vs wild-type (WT)/transverse aortic constriction (TAC). B, Representative blots and quantitative results for p-ERK1/2, total ERK1/2, and Spry2 protein expression in NRVMs transfected with Mnk1 (mitogen-activated protein kinase–interacting kinase 1) siRNA or CON siRNA treated with Ang II or not for the indicated time points. The data are representative of 3 independent experiments. *P<0.05 vs CON siRNA/Ang II 24 h or CON siRNA/Ang II 48 h. C, Representative results of immunoprecipitation experiments (IP) depicting the decreased interaction of Spry2 with GRB2 (growth factor receptor–bound protein 2) in NRVMs transfected with Mnk1 siRNA treated with Ang II or not. The data are representative of 3 independent experiments. Data are presented as mean±SEM.
that the misbalance of ERK1/2 in heart only appeared under stress. To verify the role of sprouty2, we simultaneously overexpressed sprouty2 in NRVMs with Mnk1 knockdown. After 48 hours of Ang II treatment, sprouty2 overexpression remarkably suppressed atrial natriuretic factor mRNA levels and ERK1/2 phosphorylation levels. Sprouty2 overexpression in Mnk1-KO mice consistently caused a blunted hypertrophic response. Collectively, these findings suggest that the antihypertrophic effect of Mnk1 is associated with suppression of ERK1/2 activation, which is likely mediated by sprouty2.

Chronic pressure overload is associated with excessive forms of collagen deposition or fibrosis, which affects myocardial compliance characteristics with a resulting increase in myocardial stiffness.\(^{23,24}\) Results of this study impressively show that Mnk1 deficiency dramatically increase interstitial fibrosis and perivascular fibrosis and compromise cardiac diastolic dysfunction. This is plausible when considering that ERK1/2 serves as an important pathway in the fibrotic response by promoting activation of fibrotic pathways like smad1/5 pathways\(^{25,26}\) and production of profibrotic factors such as TGF-\(\beta\).\(^{27}\) Here, we observed increased TGF-\(\beta\) expression and activation of smad1/5 pathways in Mnk1-KO murine hearts subjected to TAC. We also found that Mnk1 overexpression led to decreased TGF-\(\beta\) mRNA levels in Ang II–treated NRVMs but did not affect TGF-\(\beta\) mRNA levels in Ang II–treated cardiac fibroblasts, as compared with the AdGFP group. Therefore, the accelerated fibrosis in the hearts of Mnk1-KO mice after TAC is likely because of the increased TGF-\(\beta\) expression derived from cardiomyocytes.

Studies also have provided evidence for an important role of Mnk1 in several pathophysiological processes of several diseases via phosphorylation of eIF4E, such as pancreatic acinar cell maturation,\(^{28}\) multiple myeloma expansion,\(^{29}\) and nasopharyngeal carcinoma.\(^{30}\) eIF4E is an oncogenic rate-limiting factor of cap-dependent translation of several mRNA including Bcl-2.\(^{31}\) Mnk1-eIF4E axis and Bcl-2 expression are intimately associated with apoptosis in several types of cells.\(^{32-34}\) Our studies suggest that Mnk1-eIF4E axis and Bcl-2 may affect cardiomyocytes apoptosis. However, effect of Mnk1-eIF4E axis on mRNA translation and protein synthesis is complex. In vascular smooth muscle cells, the activation of Mnk1/eIF4E pathway contributes to Ang II–induced protein synthesis and hypertrophy.\(^{35}\) One recent research demonstrated that Mnk inhibitor CGP57380 affected translation of only those mRNAs that contain both a cap and a hairpin in the 5′-untranslated regions.\(^{36}\) Mnk1 may affect translation of specific mRNAs in different cells. In our research, we cannot exclude the possibility that Mnk1 affect the expression of Bcl-2 via another molecule or signaling pathway.

**Perspectives**

Our study provides evidence that Mnk1 may be a critical negative regulator of cardiac remodeling via inhibition of sprouty2-dependent ERK1/2 signaling and promoting Bcl-2 expression. These findings expand our understanding of the roles of Mnk1 in the heart. We propose that targeting Mnk1 may develop novel promising strategies for reversing cardiac remodeling.

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**Disclosures**

None.

**References**

Novelty and Significance

What Is New?

- Mnk1 (mitogen-activated protein kinase–interacting kinase 1) deficiency accelerated pressure overload–induced cardiac hypertrophy, fibrosis, dysfunction, and cardiomyocyte apoptosis.
- Mnk1 knockdown exaggerated cardiomyocyte hypertrophy and apoptosis in angiotensin II–treated neonatal rat ventricular myocytes. Sprouty2 overexpression rescued the neonatal rat ventricular myocytes with Mnk1 knockdown from angiotensin II–induced hypertrophy.
- Mnk1 overexpression in neonatal rat ventricular myocytes protected the cells from angiotensin II–induced cardiomyocyte hypertrophy. Sprouty2 overexpression in Mnk1-knockout mice caused a blunted hypertrophic response.

What Is Relevant?

- This study indicates that therapeutic approaches to regulate Mnk1/sprouty2 signaling pathways may be useful for prevention and treatment of cardiac remodeling and heart failure.

Summary

Mnk1 may play a crucially protective role in the progression of cardiac remodeling, and this likely occurs through the suppression of sprouty2/ERK1/2 (extracellular signal–regulated kinase 1/2) pathway.
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Mitogen-activated protein kinase interacting kinase 1 (Mnk1) Deficiency Aggravates Cardiac Remodeling in mice

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Supplemental Methods

Animals and study design
All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University. Male Mnk1-KO mice (C57BL/6 background) and their WT littermates aged 8 to 10 weeks were used in this study. Mnk1-KO mice was kindly provided by Professor Rikiro Fukunaga From Graduate School of Frontier Biosciences, Osaka University. Transverse aortic constriction (TAC) was performed as described previously.

Echocardiography and hemodynamics
Cardiac function was determined by transthoracic echocardiography and hemodynamic analysis. Echocardiography was performed in mice anesthetized with 1.5% isoflurane, using a Mylab 30CV (ESAOTE S. P. A) equipped with a 10-MHz linear-array ultrasound transducer. Body temperature was maintained at 37°C using a heating pad. The left ventricle (LV) dimensions were assessed in the parasternal short-axis view. M-mode tracings were used to measure interventricular septal thickness at diastole (IVSd), left ventricular end-diastolic diameter (LVEDd), left ventricular end-systolic diameter (LVESd) and left ventricular end-diastolic posterior wall thickness (LVPWd), ejection fraction (EF), and fractional shortening (FS). End-diastole was defined as the phase in which the largest area of the LV was obtained.

For hemodynamic measurements, a microtip catheter transducer (SPR-839, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the left ventricle of mice anesthetized with 1.5% isoflurane. The signals were continuously recorded using a Millar Pressure-Volume System (MPVS-400, Millar Instruments, Houston, TX, USA), and the data were processed by PVAN data analysis software. All measurements were made by the observer who was blinded with respect to the identity of the tracings.

After the invasive hemodynamic measurements, mice were euthanized by cervical dislocation. Hearts, lungs and tibiae of the sacrificed mice were dissected and weighed to compare the heart weight/body weight (HW/BW, mg/g), lung weight/body weight (LW/BW, mg/g), and heart weight/tibia length (HW/TL, mg/mm) ratios in KO and WT mice.

Histology
Excised hearts were arrested in diastole with 10% KCl, weighed, fixed by perfusion with 10% formalin, and embedded in paraffin. The hearts were cut transversely close to the apex to visualize the left and right ventricles. Several sections of each heart (4-5 μm thick) were prepared, stained with hematoxylin-eosin (HE) and picrosirius red (PSR) to determine the cardiomyocyte cross-sectional area (CSA) and collagen deposition, and were measured using a quantitative digital image analysis system (Image Pro-Plus, version 6.0).
Assessment of apoptosis

Apoptosis was assessed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining according to the protocol of ApopTag® Plus Fluorescein In Situ Apoptosis Detection Kit (S7111, Chemicon, Temecula, CA, USA). Briefly, 4 μm thick paraffin-embedded sections were prepared from mouse hearts of each group and deparaffinized sequentially. Cells on coverslips were fixed in 1% paraformaldehyde in PBS, stained with TUNEL reagents and the nuclei were stained by DAPI.

Western Blot Analysis

Myocardium or cardiomyocytes were lysed in RIPA lysis buffer, and the protein concentration was measured with the BCA protein assay kit (Themo, 23227) by an ELISA reader (Synergy HT, Bio-tek). Protein lysates were electrophoresed on 10 % SDS-PAGE gels, transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, IPFL00010), and blocked with 5 % non-fat milk for 2 h. The PDVF membranes were incubated with the appropriate primary antibodies anti-phospho-Mnk1, anti-Mnk1, anti-phospho-ERK1/2, anti-ERK1/2, anti-sprouty2, anti-phospho-eIF4E, anti-eIF4E, anti-Bax, anti-Bcl-2, anti-cleaved-caspase3, anti-phospho-smad1/5, anti-smad1/5, anti-TGFβ (Cell Signaling Technology), GAPDH (Santa Cruz), and anti-GRB2 (Abcam). The membranes were then incubated with IRDye® 800CW conjugated secondary antibodies (LI-COR). The blots were scanned and analyzed using a two-color infrared imaging system (Odyssey, LI-COR).

RT-PCR

mRNA expression levels of hypertrophic and fibrotic markers were determined by RT-PCR. Briefly, total RNA was isolated from snap-frozen tissues and cardiomyocytes using the RNA isolation kit (Roche). Their yields and purities were spectrophotometrically estimated using the A260/A280 and A230/260 ratios via a SmartSpec Plus Spectrophotometer (Bio-Rad). The RNA (2μg of each sample) was reverse-transcribed into cDNA using oligo (DT) primers and the Transcriptor First Strand cDNA Synthesis Kit (Roche). The PCR amplifications were quantified using a LightCycler 480 SYBR Green 1 Master Mix. The expression levels of the target genes were normalized by GAPDH.

Neonatal rat ventricular myocytes (NRVMs) culture and RNA interference

Briefly, hearts were removed from 1- to 2-day-old Sprague-Dawley rats under aseptic conditions and placed in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (Gibco, C11330). The hearts were washed with DMEM/F12 medium, and the atria and aorta were discarded. The ventricles were minced with scissors into 1 mm³ fragments, and they were enzymatically digested five times for 15 min each with 8 mL of D-Hanks containing 0.125% trypsin (Gibco, 25200). The harvested cells were centrifuged, and the sediment was resuspended in DMEM/F12 medium supplemented with 15% fetal bovine serum (FBS; HyClone, SH30406.02E) and 0.1 mmol/L bromodeoxyuridine (BrdU; Sigma, B5002). The fibroblast content of the cell suspension was removed by a differential attachment technique. Cell-rich medium was planted to 35-mm dishes coated with gelatin. After 48 h, the cardiomyocytes were transfected with gene-specific siRNA (Dharmacon, siGENOME SMART pool) or...
nontargeting siRNA at a concentration of 25 nM using DharmaFECT 1 Transfection Reagent according to the manufacturer’s instructions. After 24h, the culture medium was changed to serum-free DMEM/F12 for 12 h, and then, the cultured cardiomyocytes were stimulated with 1 μM Ang II (Sigma, A9525). To identify the cardiomyocytes and assess cardiomyocyte hypertrophy, we characterized cells by immunocytochemistry for cardiac α-actinin. The cells were washed with PBS, fixed with RCL2 (ALPHELYX, RCL2-CS24L), permeabilized in 0.1% Triton X-100 in PBS, and stained with anti-α-actinin (Millipore, 05-384) at a dilution of 1:100 in 1% goat serum. The secondary antibody was Alexa Fluor® 568 goat anti-mouse IgG (Invitrogen, A11004). The cardiomyocytes on coverslips were mounted onto glass slides with SlowFade Gold antifade reagent with DAPI (Invitrogen, S36939).

**Immunoprecipitation**

Immunoprecipitation (IP) was performed for analysis of the association of sprouty2 with GRB2. Briefly, cells were lysed at 4°C in Tris (50 mM, pH7.4), NaCl (150–900 mM), EDTA (1 mM), and Triton X-100 (1%) supplemented with protease inhibitors (Complete; Roche Diagnostics) and PMSF (1 mM) and then sonicated. The resulting lysates were incubated for 20 minutes at 4°C and then centrifuged at 13,000×g for 15 minutes at 4°C. 40μL supernatant of the indicate groups were separated and boiled for 10 min as input. For each IP, the rest supernatant were separated equally into two pipes and added 0.5μg IgG or sprouty2 respectively with 30μL Protein G-Sepharose on a rocking platform overnight at 4°C. And then the beads were thoroughly washed for 5–6 times with cold PBS. Bound proteins were resolved by SDS/PAGE, transferred to PVDF membranes, and immunoblotted as indicated with anti-GRB2 antibody (Abcam), anti-IgG (Cell Signaling Technology), and anti-sprouty2 (Santa Cruz). The membranes were then incubated with IRDye® 800CW conjugated secondary antibodies (LI-COR). The blots were scanned and analyzed using a two-color infrared imaging system (Odyssey, LI-COR).

**Adenoviral infection**

Adenoviruses bearing human Mnk1 (AdMnk1) or Sprouty2 (AdSpry2) under the control of cytomegalovirus (CMV) promoter were constructed and amplified by ViGene Biosciences Company (Shandong, China). Adenovirus (shMnk1) was engineered to targeting rat Mnk1 transcripts by Hanbio Company (CCGAGGCCAAAAGACCTCATCTCTAA) (Shanghai, China). The non-targeting shRNA was used as a control. 48 h after plating, cardiomyocytes were incubated for 2 h with recombinant adenoviruses. After removal of the virus suspension, cells were replaced in serum-free medium for 12 h and then stimulated with 1 μM Ang II (Sigma, A9525). Viruses were used at a multiplicity of infection (MOI) of 100.

**Intramyocardial injection**

For high transduction efficiency in cardiac tissue, adenovirus-mediated intramyocardial gene transfer was carried out³. Briefly, mice were anesthesized with sodium pentobarbital, and lungs were mechanically ventilated. A horizontal skin incision was made at the level of 2–3 intercostal space, the aortic constriction was performed. After stitching the 2–3 intercostals space, the 3-4 intercostal space was opened, and the heart was smoothly and gently “popped out”. Adenoviruses bearing
AdSpry2 or AdGFP \(1 \times 10^9\) pfu\(^4\) were administered by direct injection in the left ventricular free wall \(1 \times 10^{11}\) pfu/ml, 10 µl) with a syringe equipped with 31-gauge needle. After injection, the heart was immediately placed back, followed by manual evacuation of pneumothoraces and closure of muscle and the skin suture, and the mice were allowed to recover. Four weeks later, the echocardiography and hemodynamics were performed, and the Hearts, lungs and tibiae of the sacrificed mice were dissected and used for further analyses.

**Statistical analysis**

Data are presented as mean ± SEM. Statistical analysis was performed using SPSS 13.0 (SPSS Inc.) software. Data were analyzed by one-way ANOVA followed by Tukey’s post-hoc test. P values<0.05 were considered as statistically significant.

**References:**

Table S1. Hemodynamic parameters in mice after 4 weeks of TAC

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT(n=7)</td>
<td>KO(n=8)</td>
</tr>
<tr>
<td>HR(min⁻¹)</td>
<td>461.9±25.7</td>
<td>428.1±14.0</td>
</tr>
<tr>
<td>ESP(mmHg)</td>
<td>113.8±4.8</td>
<td>112.7±4.2</td>
</tr>
<tr>
<td>EDP(mmHg)</td>
<td>12.3±2.2</td>
<td>9.6±1.2</td>
</tr>
<tr>
<td>ESV(μL)</td>
<td>13.7±1.1</td>
<td>12.9±1.4</td>
</tr>
<tr>
<td>EDV(μL)</td>
<td>26.1±0.9</td>
<td>26.2±1.7</td>
</tr>
<tr>
<td>dP/dTmax(mmHg/s)</td>
<td>9735.9±442.6</td>
<td>9507.8±908.0</td>
</tr>
<tr>
<td>CO(μL/min)</td>
<td>7804.8±405.1</td>
<td>6452.5±428.8</td>
</tr>
<tr>
<td>Stroke volume(μL)</td>
<td>17.0±0.9</td>
<td>15.0±0.6</td>
</tr>
<tr>
<td>dP/dTmin(mmHg/s)</td>
<td>-7935.9±357.9</td>
<td>-7920.4±591.2</td>
</tr>
<tr>
<td>Tau_w(ms)</td>
<td>9.3±1.0</td>
<td>8.5±0.9</td>
</tr>
</tbody>
</table>

HR, heart rate; ESP, end-systolic pressure; EDP, end-diastolic pressure; ESV, endsystolic volume; EDV, end-diastolic volume; dp/dtmax, maximal rate of pressure development; CO, cardiac output; dp/dtmin, maximal rate of pressure decay; Tau_w, time constant of isovolumic pressure decay.

*P<0.05 vs WT/sham. †P<0.05 vs WT/TAC.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT+AdGFP</th>
<th>KO+AdGFP</th>
<th>KO+AdSpry2</th>
</tr>
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<tbody>
<tr>
<td>HR (min⁻¹)</td>
<td>449.8±20.8</td>
<td>451.4±21.3</td>
<td>474.4±14.4</td>
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<tr>
<td>ESP (mmHg)</td>
<td>147.3±6.8</td>
<td>155.7±6.9</td>
<td>151.3±3.8</td>
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<tr>
<td>EDP (mmHg)</td>
<td>14.7±3.0</td>
<td>37.3±4.5*</td>
<td>12.1±0.6†</td>
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<tr>
<td>ESV (μL)</td>
<td>21.9±0.4</td>
<td>28.1±1.6*</td>
<td>24.3±0.3†</td>
</tr>
<tr>
<td>EDV (μL)</td>
<td>30.5±0.3</td>
<td>32.9±0.6*</td>
<td>30.1±0.6†</td>
</tr>
<tr>
<td>dP/dTmax (mmHg/s)</td>
<td>7229.8±208.6</td>
<td>6139.1±146.5*</td>
<td>8113.7±594.3†</td>
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<tr>
<td>CO (μL/min)</td>
<td>5106.4±461.6</td>
<td>3167.4±228.4*</td>
<td>6529.5±968.3†</td>
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<tr>
<td>Stroke volume (μL)</td>
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<td>7.48±0.4*</td>
<td>13.5±1.6†</td>
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<tr>
<td>dP/dTmin (mmHg/s)</td>
<td>-7291.8±371.7</td>
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<td>-7274.9±446.7</td>
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<tr>
<td>Tau_w (ms)</td>
<td>9.7±0.5</td>
<td>22.7±4.7*</td>
<td>8.4±0.6†</td>
</tr>
</tbody>
</table>

HR, heart rate; ESP, end-systolic pressure; EDP, end-diastolic pressure; ESV, end-systolic volume; EDV, end-diastolic volume; dp/dtmax, maximal rate of pressure development; CO, cardiac output; dp/dtmin, maximal rate of pressure decay; Tau_w, time constant of isovolumic pressure decay.

*P<0.05 vs WT+AdGFP group. †P<0.05 vs KO+AdGFP group.
Figure S1. Electrocardiographic data and cardiac hypertrophy-related transcripts detection. (A) Echocardiographic parameters in Mnk1-KO and WT mice after 4 weeks of Sham or TAC (n=7-8 mice per experimental group). (B) Levels of cardiac hypertrophy-related transcripts (ANP, BNP, β-MHC, α-MHC) quantified by RT-PCR (n=6 mice per experimental group). Data are presented as mean ± SEM. * $P<0.05$ vs Sham group in the same genotype. $^\# P<0.05$ vs WT/TAC.
Figure S2. Overexpression of Mnk1 protected NRVMs from Ang II-induced hypertrophy. (A) Expression and activation state of Mnk1 in NRVMs after transfection with 50 MOI and 100 MOI Mnk1 adenovirus (AdMnk1). *P<0.05 vs AdGFP. (B) Representative imaging of immunostaining NRVMs for α-actinin (green) in each group. (C) Quantification of cell surface area (n=50 cells per group). *P<0.05 vs the No drug group transfected with the same type of adenovirus, #P<0.05 vs AdGFP/Ang II 48h. (D) ANP mRNA levels in NRVMs with overexpression of Mnk1 after Ang II treatment. *P<0.05 vs the No drug group transfected with the same type of adenovirus, #P<0.05 vs AdGFP/Ang II 48h. Data are presented as mean ± SEM.
Figure S3. Mnk1 suppresses ERK1/2 activation dependent on sprouty2 (Spry2). (A) Representative blots and quantitative results for p-ERK1/2, total ERK1/2 expression in NRVMs transfected with AdMnk1 or AdGFP treated with Ang II for 48 h or not. The data are representative of 3 independent experiments. $^#P<0.05$ vs. AdGFP/Ang II 48h. (B) Mnk1 mRNA expression in NRVMs after Mnk1 shRNA adenovirus (shMnk1) infection. $^*P<0.05$ vs. non-targeting shRNA (shRNA) group. (C) Spry2 protein expression in NRVMs after Spry2 adenovirus (AdSpry2) infection. $^#P<0.05$ vs. AdGFP group. (D) ANP mRNA expression in Ang II-treated NRVMs transfected with shMnk1 with or without Spry2 overexpression. $^#P<0.05$ vs. NRVMs transfected with shMnk1 without Spry2 overexpression. (E) Representative blots and quantitative results for p-ERK1/2, total ERK1/2 expression in Ang II-treated NRVMs transfected with shMnk1 with or without Spry2 overexpression. $^#P<0.05$ vs. NRVMs transfected with shMnk1 without Spry2 overexpression. Data are presented as mean ± SEM.
Figure S4. Spry2 overexpression blunted the hypertrophic response in Mnk1-KO mice hearts 4 weeks after TAC. (A) Spry2 expression after myocardial injection using AdSpry2 (n=4). *P<0.05 vs AdGFP group. (B) Hematoxylin- and eosin-stained heart sections (n=4-6 mice per experimental group) and quantification of cardiomyocyte cross-sectional area in each group (n=100-200 cells per experimental group). (C) HW/BW, HW/TL and LW/BW ratios of mice after TAC operation (n=8 mice per experimental group). (D) Echocardiographic parameters. (n=6-7 mice per experimental group). (E) Levels of cardiac hypertrophy-related transcripts (ANP, BNP, β-MHC, α-MHC) quantified by RT-PCR (n=6 mice per experimental group). (F) Representative blots and quantitative results for p-ERK1/2 expression in the indicated groups. Data are presented as mean ± SEM. *P<0.05 vs WT+AdGFP group. #P<0.05 vs KO+AdGFP group.
Figure S5. Effect of Mnk1 on cardiac fibrosis. (A) PSR staining for detecting fibrosis in WT and Mnk1-KO mice subjected to Sham or TAC (n=5-7 mice per group). Quantification of fibrotic area in each group. n>25 for each group. *P<0.05 vs sham-operated (Sham) group in the same genotype. #P<0.05 vs WT/TAC. (B) mRNA expression levels of CTGF, Collagen Iα, Collagen IIIα and Fibronectin in each group (n=6 per group). *P<0.05 vs Sham group in the same genotype. #P<0.05 vs WT/TAC. (C) Representative blots and quantitative results for TGFβ, phospho-smad1/5 (p-smad1/5), total smad1/5 protein expression in each group (n=6 per group). *P<0.05 vs Sham group in the same genotype. #P<0.05 vs WT/TAC. Data are presented as mean ± SEM. (D) TGFβ mRNA expression in Ang II-treated NRVMs infected with AdMnk1 or AdGFP. *P<0.05 vs the No drug group transfected with the same type of adenovirus, #P<0.05 vs AdGFP/Ang II 48h. (E) Representative blots and quantitative results for Mnk1 protein expression in cardiac fibroblasts after transfection with 50
MOI or 100 MOI of AdMnk1. *P<0.05 vs AdGFP group. (F) TGFβ mRNA expression in Ang II-treated cardiac fibroblasts infected with AdMnk1 or AdGFP. *P<0.05 vs the No drug group transfected with the same type of adenovirus. Data are presented as mean ± SEM.
Figure S6. Effect of Mnk1 on cardiomyocyte apoptosis. (A) Representative images of terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay in a section of mice hearts (n=3 per group). (B) Cardiac expression of Bax, Bcl-2, cleaved-caspase3 (c-caspase3), phospho-eIF4E (p-eIF4E), and total eIF4E proteins in each group (n=6 per group). (C) Cardiac expression of phospho-eIF4E (p-eIF4E), and total eIF4E proteins in each group (n=6 per group). P<0.05 vs Sham group in the same genotype. #P<0.05 vs WT/TAC. (D) Representative images of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in NRVMs infected with Mnk1 siRNA or CON siRNA treated with Ang II for 48h (n=3 per group). (E) Representative blots and quantitative results for Bcl-2 expression in NRVMs infected with Mnk1 siRNA or CON siRNA treated with Ang II for the indicated time points or not. The data are representative of 3 independent experiments. *P<0.05 vs the No drug group transfected with the same type of siRNA, #P<0.05 vs CON siRNA/Ang II 24h or CON siRNA/Ang II 48h. (F) Representative blots and quantitative results for Bcl-2 expression in NRVMs infected with AdMnk1 or AdGFP treated with Ang II for 48 h. The data are representative of 3 independent experiments. *P<0.05 vs the No drug group transfected with the same type of adenovirus, #P<0.05 vs the No drug group transfected with the same type of adenovirus.
vs AdGFP/Ang II 48h. Data are presented as mean ± SEM.