Tripartite motif-containing 14 may aggravate cardiac hypertrophy via the AKT signalling pathway in neonatal rat cardiomyocytes and transgenic mice

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Abstract. Tripartite motif-containing 14 (TRIM14) is an E3 ubiquitin ligase that primarily participates in the natural immune response and in tumour development via ubiquitination. However, the role of TRIM14 in cardiac hypertrophy is not currently clear. The present study examined the role of TRIM14 in cardiac hypertrophy and its potential molecular mechanism. TRIM14 was overexpressed in neonatal rat cardiomyocytes using adenovirus and cardiomyocyte hypertrophy was induced using phenylephrine (PE). Cardiomyocyte hypertrophy was assessed by measuring cardiomyocyte surface area and markers of hypertrophy. In addition, TRIM14-transgenic (TRIM14-TG) mice were created and cardiac hypertrophy was induced using transverse aortic constriction (TAC). Cardiac function, heart weight-to-body weight ratio (HW/BW), cardiomyocyte cross-sectional area, cardiac fibrosis and hypertrophic markers were further examined. The expression of AKT signalling pathway-related proteins was detected. TRIM14 overexpression in cardiomyocytes promoted PE-induced increases in cardiomyocyte surface area and hypertrophic markers. TRIM14-TG mice developed worse cardiac function, greater HW/BW, cross-sectional area and cardiac fibrosis, and higher levels of hypertrophic markers in response to TAC. TRIM14 overexpression also increased the phosphorylation levels of AKT, GSK-3β, mTOR and p70S6K *in vivo* and *in vitro*. To the best our knowledge, the present study was the first to reveal that overexpression of TRIM14 aggravated cardiac hypertrophy *in vivo* and *in vitro*, which may be related to activation of the AKT signalling pathway.

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Introduction

With the development of the social economy and improvements in living standards, the incidence of cardiovascular disease is increasing annually and is an important factor endangering human health (1). The heart develops compensatory cardiac hypertrophy in response to stress, which is primarily characterised by an increase in cardiomyocyte size, protein synthesis, foetal gene re-expression and extracellular matrix (2). Cardiac hypertrophy, as a compensatory mechanism, is beneficial to maintain normal cardiac function in the early stages of stress. However, sustained stress causes cardiac decompensation, which eventually results in cardiac dilation, heart failure and sudden death (3). Cardiac hypertrophy is also an independent risk factor for increased cardiovascular morbidity and mortality (4).

Numerous diseases cause cardiac hypertrophy, including primary or secondary hypertension, valvular heart disease and thyroid disease. The incidence of cardiovascular events, such as myocardial ischaemia, ventricular arrhythmias, heart failure and sudden death, increases with the development of left ventricular (LV) hypertrophy (5-7). At the cellular and molecular level, the cardiomyocyte hypertrophy process primarily includes three aspects: Extracellular hypertrophic signal stimulation, intracellular signal pathway transduction and nuclear gene transcription activation (8,9). The AKT signalling pathway plays a key role in the development of cardiac hypertrophy. Under the stimulation of pressure overload and neurohumoral factors, such as catecholamine hormone, angiotensin II and endothelin, the AKT signalling pathway participates in the pathological process of cardiac hypertrophy by activating specific genes in the nucleus of cardiomyocytes (10,11). However, the mechanism underlying cardiac hypertrophy is not fully understood, and there are no effective prevention or treatment methods. Therefore, it is of theoretical and clinical value to further clarify the molecular mechanism underlying cardiac hypertrophy, and to identify drug targets for the prevention and treatment of cardiac hypertrophy.

Tripartite motif-containing protein (TRIM), also known as N-terminal RING finger/B-box/coiled coil protein, is a ring

component protein of E3 ubiquitin ligase in general. TRIM14 is a member of the TRIM family primarily distributed on the outer membrane of the mitochondria. TRIM14 activates interferon regulatory factor 3 and the NF-κB pathway via recruiting NEMO for promoting ubiquitination reactions that recruited NEMO to the MAVS signaling complex to participate in anti-RNA virus effects (12). TRIM14 is also involved in the DNA signalling pathway mediated by TRIM14 recruiting ubiquitin-specific protease 14 (USP14) to cleave the ubiquitin chains of cGAS at lysine (K) 414 and then inhibiting the degradation of CGAs via autophagy (13). TRIM14 plays an important role in tumours. For example, TRIM14 has been shown to be expressed at high levels in gastric cancer and osteosarcoma, and it may be used as a prognostic indicator for patients. TRIM14 is also related to malignant pathological factors in gastric cancer, and it may be involved in its occurrence and development (14,15). However, TRIM14 inhibits the proliferation of cells in non-small cell lung cancer by promoting the apoptosis of cancer cells and inducing the production of interferon γ (16). TRIM14 also plays an important role in innate immunity to pathogenic microorganisms; it inhibits hepatitis C, Sindbis and influenza viruses (17,18). However, whether TRIM14 is involved in cardiac hypertrophy is not clear.

In the present study, the expression level of TRIM14 protein was first detected in mouse cardiac hypertrophy, and then TRIM14 was overexpressed *in vivo* and *in vitro* to determine its role in cardiomyocyte hypertrophy and cardiac hypertrophy, and its mechanism was further explored.

Materials and methods

Generation of TRIM14-transgenic (TRIM14-TG) mice. Cardiac-specific TG mice with TRIM14 overexpression were established as previously described. Animals were provided by Wuhan University Model Animal Center (19). First, the full-length TRIM14 mouse cDNA was inserted into a vector (α-MHC-DN.JNK2; Addgene) with the mouse α-MHC promoter. TG C57BL/6 mice expressing TRIM14 were generated by microinjecting the α-MHC-TRIM14 vector into fertilized mouse embryos, which were then implanted into pseudopregnant females to obtain the desired TG mice. Mice were housed in a specific pathogen-free environment with free access to food and water. The Animal Care and Use Committee of Zhejiang Chinese Medical University approved the present study (approval no. IACUC-20200120-04; Hangzhou, China). All animal-related procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (19). Roughly 100 embryos and 8 female pseudopregnant mice were used. The mice were raised in a specific pathogen-free (SPF) environment with the conditions of temperature 20-25˚C, humidity of 40-70% and a 12-h light/dark cycle.

Transverse aortic constriction (TAC) surgery. 8-10-week-old male C57BL/6J mice weighing 25 g were subjected to sham or TAC surgery as previously described (19). Mice were anaesthetised via a 50 mg/kg intraperitoneal injection of sodium pentobarbital. With the mouse in the supine position and in the presence of the toe-pinch reflex, the skin over the middle chest was opened, the aortic arch was exposed through the right side of the clavicle, and a 26-gauge needle was used to ligate the aortic arch with 7-0 silk sutures. The needle was removed quickly and the chest was closed. A self-regulating heating pad maintained the mouse body temperature at ~37˚C during this process. Mice were placed at 37˚C until recovery. Sham-operated mice underwent the same surgery with the exception of aortic constriction. The mice were divided into the following 4 groups: 10 mice in the NTG Sham group, 10 mice in the TRIM14-TG Sham group, 11 mice in the NTG TAC group and 11 mice in the TRIM14-TG TAC group.

Echocardiography measurements. After 4 weeks of surgery, echocardiography (Vevo2100; FUJIFILM VisualSonics) measurements were taken from mice under anaesthesia with isoflurane inhalation; the induction and maintenance concentrations of isoflurane were 3 and 1.5-2%, respectively, as previously described (19). M-mode tracings from more than three consecutive cardiac cycles were recorded at the papillary muscle level of the LV short axis. LV end-diastolic/end-systolic dimensions (LVEDd/LVESd) were measured, and LV fractional shortening (FS) was calculated. FS was calculated as FS (%)=(LVEDd-LVESd)/LVEDd x100.

Histological analysis. Histological analysis was performed as previously described (19). After the echocardiography measurements were completed, mice were euthanized by cervical dislocation after being anesthetized with 3% isoflurane inhalation. The death of mice was confirmed by respiratory and cardiac arrest, and pupil dilation. The body weight (BW) was measured. Hearts were excised, washed with saline. The heart weight (HW), lung weight (LW) and HW/tibia length (TL) were measured. The ratios of HW/BW, LW/BW and HW/TL were calculated to evaluate the degree of cardiac hypertrophy. Subsequently, hearts were fixed with 4% paraformaldehyde at room temperature for 24 h and embedded in paraffin following standard histological procedures. Sections (5 μm) were taken at the papillary muscle level of the heart and stained with H&E according to standard protocols to assess tissue morphology, and picrosirius red (PSR) to assess collagen deposition. Images of H&E staining and PSR staining from more than five samples per group were captured using light microscopy (Olympus Corp.), and cardiomyocyte cross-sectional area and cardiac fibrosis were measured using image analysis software (Image-Pro Plus 6.0; Media Cybernetics). The partially dissected heart tissue of each mouse was fixed for pathological analysis, while the remaining portion was snap-frozen in liquid nitrogen and stored at -80˚C for reverse transcription-quantitative PCR (RT-qPCR) and western blotting.

Neonatal rat cardiomyocyte (NRCM) culture, TRIM14 overexpression and phenylephrine (PE) treatment. A total of 45 neonatal Sprague Dawley rats were used for the cell experiment. Neonatal Sprague Dawley rats (age, 1-3-days), were euthanized by decapitation following anaesthesia with 1% isoflurane inhalation and the heart was rapidly excised. Subsequently, primary NRCMs were isolated from ventricles and cultured at 37˚C and 5% CO_2 in high-glucose DMEM (HyClone) containing 15% foetal bovine serum (Gibco; Thermo Fisher Scientific,

Table I. Antibodies used in the present study.

Antibody	Manufacturer	Catalogue number	Species raised in	Dilution
TRIM14	Santa Cruz Biotechnology, Inc.	sc-79761	Goat	1:1,000
p-AKT	CST	4060	Rabbit	1:1,000
AKT	CST	4691	Rabbit	1:1,000
p-GSK-3β	CST	9322s	Rabbit	1:1,000
GSK-3β	CST	12456s	Rabbit	1:1,000
p-mTOR	CST	5536s	Rabbit	1:1,000
mTOR	CST	2983s	Rabbit	1:1,000
p-p70 S6K	CST	9208	Rabbit	1:1,000
p70 S6K	CST	2708	Rabbit	1:1,000
GAPDH	Proteintech	HRP-60004	Mouse	1:10,000

CST, Cell Signaling Technology; p, phosphorylated; TRIM14, tripartite motif-containing 14.

Inc.), 1% penicillin/streptomycin and 0.1 mM 5-bromodeoxyuridine, as previously described (19). After 24 h, NRCMs were infected with adenovirus at a multiplicity of infection of 50 and for 12 h at 37˚C. NRCMs were placed in serum-free medium for starvation, 50 μmol/l PE (Abcam) was added, and the cells were cultured for another 24 h and harvested (20). The control group was treated with the same volume of PBS. To overexpress TRIM14, the entire coding region of the rat TRIM14 gene (NM_001399174.1) was inserted into a replication-defective adenovirus vector (AdTRIM14) under the control of the cytomegalovirus promoter. An adenovirus vector containing the gene encoding green fluorescent protein was used as a control (AdGFP). The pcDNA 3.1-TRIM14 plasmid was synthesized and subsequently packaged into adenovirus by Shanghai Shenggong Corporation.

Immunofluorescence. Immunofluorescence staining was performed as previously described (19). NRCMs used for immunofluorescence were grown in dishes containing coverslips. At the end of NRCM treatment, immunofluorescence was performed as follows: NRCMs were fixed with 4% formaldehyde at room temperature for 15 min, permeabilised with 0.1% Triton X-100 and blocked in a 10% BSA solution at room temperature for 30 min. NRCMs were incubated with primary antibodies against α-actinin (1:100 dilution; Abcam) at 4˚C overnight, followed by incubation with fluorescent secondary antibodies (1:200 dilution; Thermo Fisher Scientific, Inc.) at room temperature for 1 h and staining with DAPI. Details of all antibodies are provided in Table I. In total, ≥100 cells were selected from five different fields of view in a single experiment to calculate the average cell size. A fluorescence microscope (Olympus Corp.) was used to collect the fluorescence staining images of NRCMs. The cell surface areas of >100 NRCMs were calculated using Image-Pro Plus 6.0. The experiment was repeated twice and data from three biological replicates were used for statistical analysis.

RT-qPCR. RT-qPCR was performed as previously described (19). Total RNA was extracted from cultured cells or the left ventricles of mice using TRIzol® reagent (Thermo Fisher Scientific, Inc.). cDNA was synthesised using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) according to manufacturer's instructions. SYBR™ Green PCR Master Mix (Roche Diagnostics) was used for qPCR, and the products were quantified using the LightCycler 480 System (Roche Diagnostics). The thermal cycling conditions for qPCR are as follows: denaturation at 95˚C for 10 sec, annealing at 60˚C for 10 sec, extension at 72˚C for 10 sec, for a total of 40 cycles. GAPDH was used as a reference gene. The primers used in RT-qPCR are provided in Table II.

Western blot analysis. Western blotting was performed as previously described (19). Total protein was extracted from cultured cardiomyocytes or left ventricle tissues using RIPA lysis (Beyotime Institute of Biotechnology) buffer containing PMSF, protease inhibitor and phosphatase inhibitor. The BCA method was used to determine protein concentration. Equal amounts of protein (20 μg) were separated by SDS-PAGE (10%) and transferred to PVDF membranes (Millipore-Sigma). Membranes were blocked with 5% skimmed milk at room temperature for 1 h and incubated with primary antibodies (Table I) at 4˚C overnight. After incubation with secondary antibodies conjugated with horseradish peroxidase (HRP) (1:10,000 dilution; Proteintech) at room temperature for 1 h, protein bands were visualised using an ECL imaging system (Bio-Rad Laboratories, Inc.). Protein expression levels were quantified using ImageJ v1.48 (National Institutes of Health). GAPDH was used as an internal reference.

Statistical analysis. Data are presented as the mean ± SD (n=3). Student's t-test was used for comparisons between two groups. Comparisons among more than two groups were carried out using one-way ANOVA followed by Tukey's post-hoc test. All statistical analyses were performed using SPSS (version 21.0; IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

TRIM14 increases cardiac hypertrophy. A model of cardiac hypertrophy was created in mice that underwent TAC. The

Table II. Sequences of primers used for PCR (5'-3').

A, Mouse primers

Gene name	Forward	Reverse primer
ANP	TCGGAGCCTACGAAGATCCA	TTCGGTACCGGAAGCTGTTG
BNP	GAAGGACCAAGGCCTCACAA	TTCAGTGCGTTACAGCCCAA
MYH7	CAACCTGTCCAAGTTCCGCA	TACTCCTCATTCAGGCCCTTG
Collagen Iα	TGCTAACGTGGTTCGTGACCGT	ACATCTTGAGGTCGCGGCATGT
Collagen III	ACGTAAGCACTGGTGGACAG	CCGGCTGGAAAGAAGTCTGA
CTGF	TGACCCCTGCGACCCACA	TACACCGACCCACCGAAGACACAG
GAPDH	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA
TRIM14	GAGAATGGCGAGCGAGACTA	TTGGCGTACGGGCGTATTT

B, Rat primers

Gene name	Forward	Reverse
ANP	AAAGCAAACTGAGGGCTCTGCTCG	TTCGGTACCGGAAGCTGTTGCA
MYH7	GTTTGCTGAAGGACACTCAAATCC	TTCTTCTTCTGGTTGATGAGGCTGG
GAPDH	TGTGAACGGATTTGGCCCTA	GATGGTGATGGGTTTCCCGT
TRIM14	ATGAAAAATGGCGAGCGGGA	TTGAGTCTGCAGAAACCTGCG

cardiac tissue of mice was collected and the protein expression levels of TRIM14 were detected. The results showed that the expression levels of TRIM14 were significantly upregulated in the cardiac tissue of mice that underwent TAC surgery compared with those in mice that received sham surgery (Fig. 1A and B). These findings suggested that TRIM14 was involved in the pathogenesis of cardiac hypertrophy.

TRIM14 aggravates PE-induced cardiomyocyte hypertrophy. To further assess the role of TRIM14 in cardiac hypertrophy, primary cardiomyocytes were isolated from neonatal rats. Cardiomyocyte hypertrophy was induced by PE stimulation (Fig. 2A) and overexpression of TRIM14 was mediated by adenovirus infection, which was confirmed by a significant increase in mRNA levels (Fig. 2C). Following PE stimulation, the surface area of cardiomyocytes in the AdTRIM14 group was significantly higher than that in the AdGFP control group (Fig. 2B). The mRNA expression levels of ANP and MYH7 in the after PE treatment AdTRIM14 group were also significantly upregulated compared with those in the AdGFP group (Fig. 2D). These results indicated that TRIM14 promoted cardiomyocyte hypertrophy *in vitro*.

Cardiac-specific overexpression of TRIM14 promotes dysfunction induced by pressure overload. To evaluate whether the overexpression of TRIM14 in the heart aggravated cardiac hypertrophy, cardiac-specific TRIM14-TG mice were created and bred as experimental animals, and wild-type littermates [non-TG mice (NTG)] were used as controls. Cardiac hypertrophy was induced by TAC surgery and echocardiography was performed after 4 weeks. In total, ~10% of the mice that underwent TAC surgery were excluded due to procedure failure and other surgical

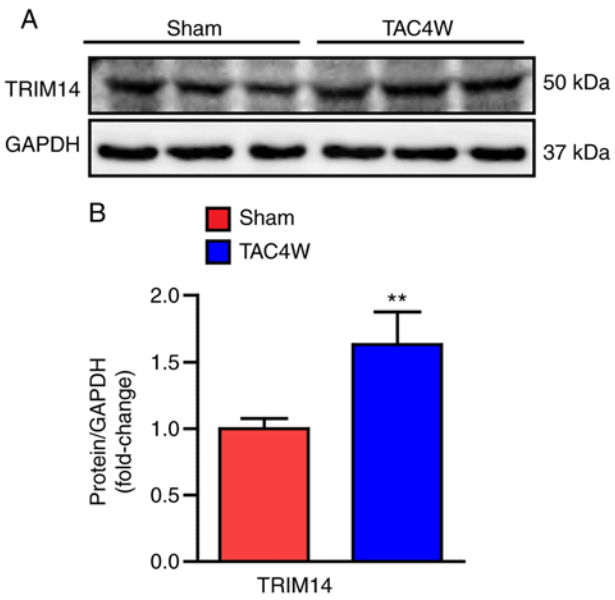

Figure 1. TRIM14 is increased in TAC-induced cardiac hypertrophy. (A) Western blot analysis of TRIM14 protein in the heart tissues of the sham and TAC groups. (B) Semi-quantification of TRIM14 protein expression in the heart tissues of the sham and TAC groups. $^{**}P<0.01$ vs. sham; n=6. TRIM14, tripartite motif-containing 14; TAC, transverse aortic constriction; 4W, 4 weeks.

complications, including acute heart failure and wound infection caused by surgery. LVEDd, LVESd and FS were measured. The results showed that LVEDd and LVESd were significantly higher in the TRIM14-TG group than those in the NTG group, and the FS percentage was lower than that in the NTG group (Fig. 3A-C). After echocardiography, the mice were sacrificed. The body weight (BW), heart weight (HW) and lung weight (LW) of the

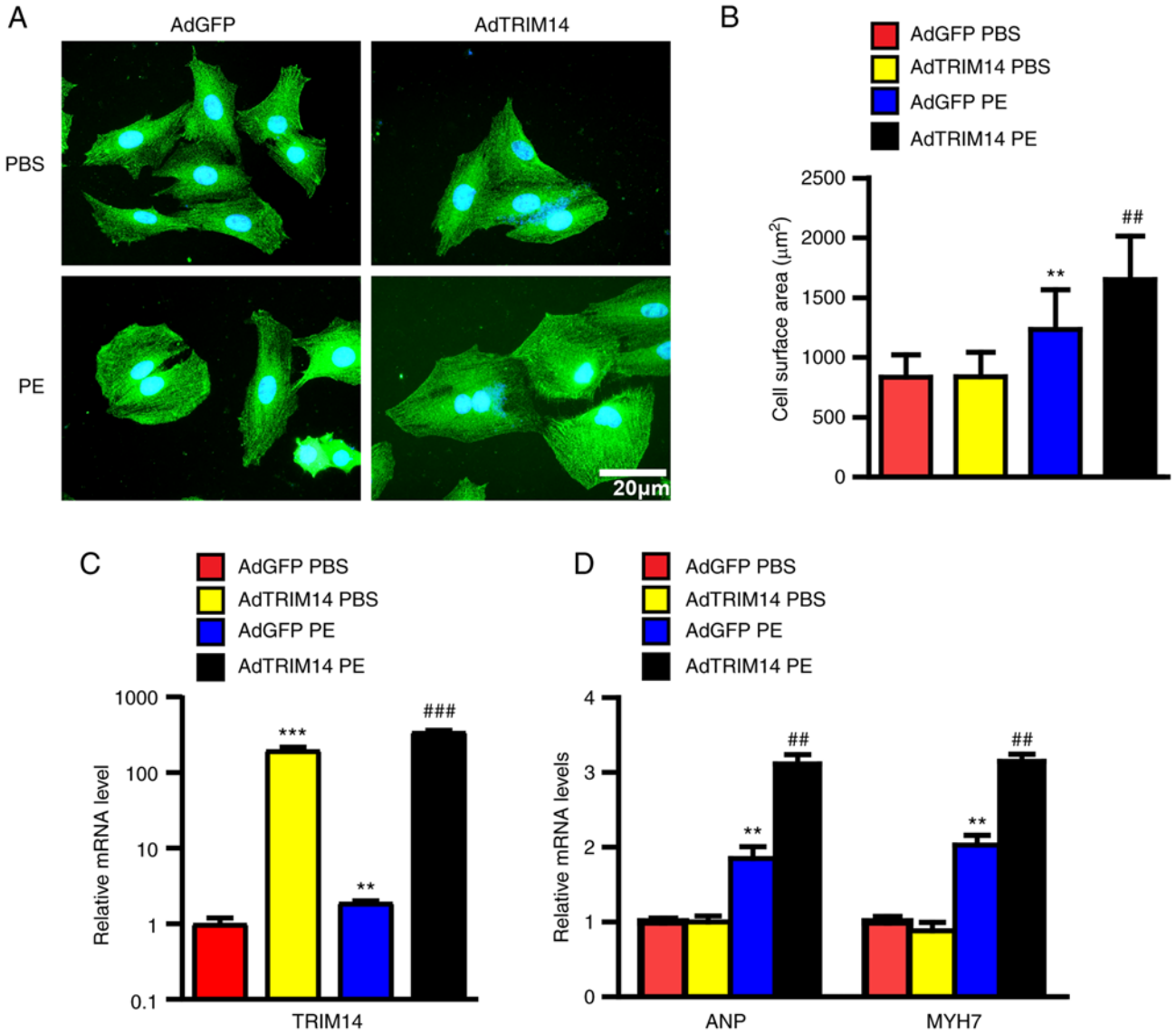

Figure 2. TRIM14 aggravates PE-induced cardiomyocyte hypertrophy. (A) Representative images of cardiomyocytes infected with AdGFP or AdTRIM14 and treated with PBS or PE (50 μmol/l). (B) Quantitative results of cardiomyocyte surface area. **P<0.01 vs. AdGFP PBS; $^{\#\#}$P<0.01 vs. AdGFP PE; ≥100 cells in each experimental group; scale bar, 20 μm; n=3. The relative mRNA expression levels of (C) TRIM14, (D) ANP and MYH7 in neonatal rat cardiomyocytes after infection with AdGFP or AdTRIM14 and treatment with PBS or PE. **P<0.01, ***P<0.001 vs. AdGFP PBS; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. AdGFP PE; n=3. TRIM14, tripartite motif-containing 14; PE, phenylephrine; AdGFP, adenovirus GFP; AdTRIM14, adenovirus TRIM14.

sacrificed mice was measured. The ratios of HW/BW, LW/BW and HW/tibia length (TL) were calculated to evaluate the degree of cardiac hypertrophy. The results showed that HW/BW, LW/BW and HW/TL in the TRIM14-TG group were significantly higher than those in the NTG group (Fig. 3D-F). These results showed that the degree of cardiac hypertrophy and deterioration of cardiac function in TRIM14-TG mice after TAC was higher than that in NTG mice, which indicated that TRIM14 had a positive regulatory effect on cardiac hypertrophy and cardiac dysfunction caused by pressure load.

Cardiac-specific overexpression of TRIM14 facilitates cardiomyocyte hypertrophy and fibrosis in response to pressure overload. To further examine whether TRIM14 exacerbated TAC-induced cardiac hypertrophy, histopathological and molecular analyses were performed on mouse heart tissue. H&E and PSR staining was used to observe cardiac tissue morphology, cardiomyocyte cross-sectional area and the percentage of collagen area. The results showed that the heart size, cardiomyocyte cross-sectional area and percentage of collagen area (perivascular and interstitial collagen; the statistical results of interstitial fibrosis were not presented) of TRIM14-TG mice were significantly increased after TAC compared with those in the NTG group (Fig. 4A-D). The mRNA levels of TRIM14 in TRIM14-TG mice are significantly higher than those in NTG mice, indicating the successful establishment of the TRIM14-TG mice (Fig. 4E). The mRNA expression levels of the hypertrophic markers ANP, BNP and MYH7, and those of the fibrosis-related genes collagen I α, collagen III and CTGF, were also detected in the left ventricle of mice. The mRNA expression levels of ANP, BNP, MYH7, collagen I α, collagen III and CTGF were significantly upregulated in TRIM14-TG mice after TAC compared with those in NTG mice (Fig. 4F and G). These

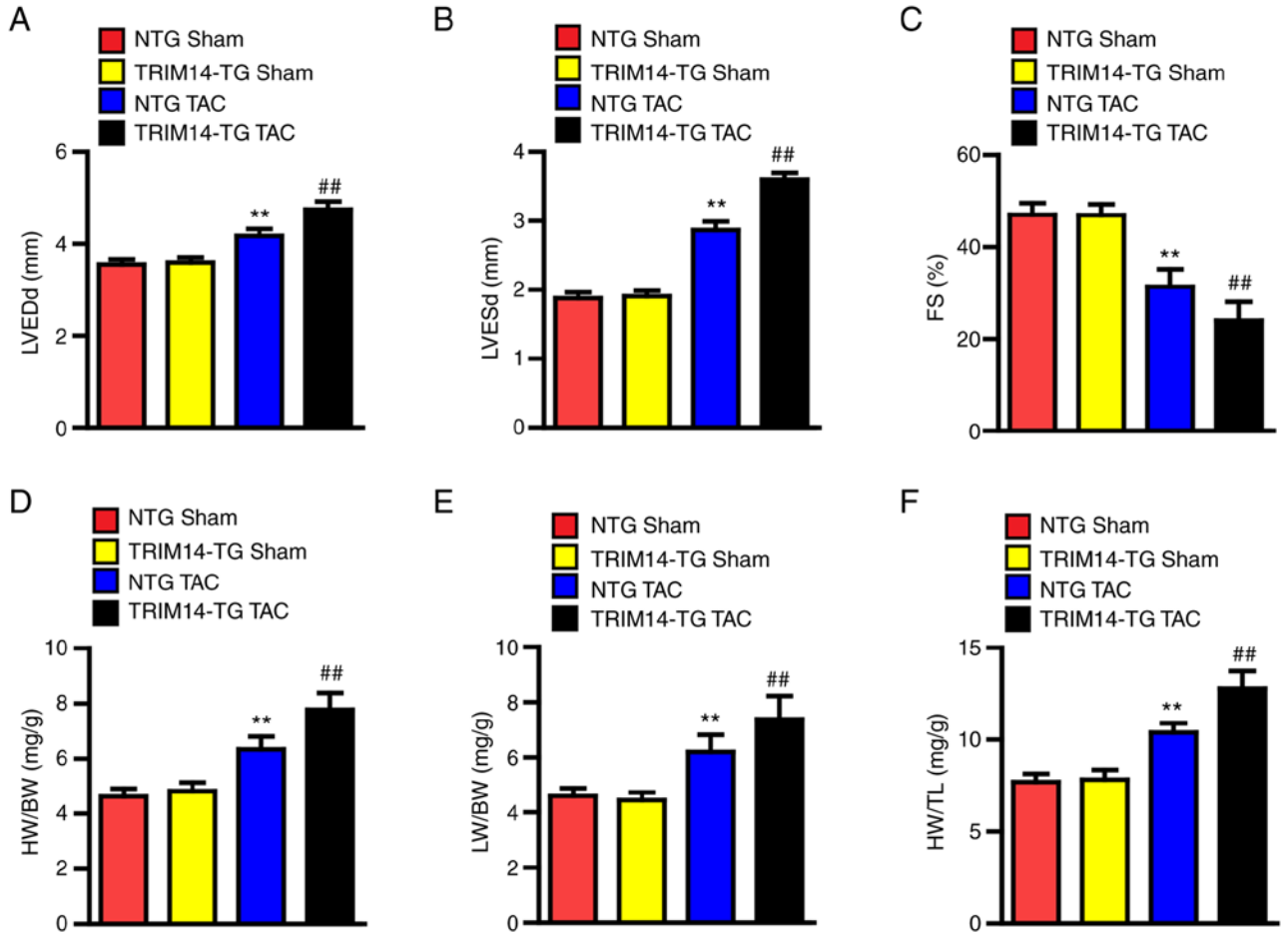

Figure 3. TRIM14 overexpression exacerbates cardiac hypertrophy and dysfunction caused by pressure overload. A total of 4 weeks after sham or TAC surgery, the statistical results of (A) LVEDd, (B) LVESd and (C) FS were obtained in 10 NTG mice and 10 TRIM14-TG mice. A total of 4 weeks after sham or TAC surgery, the (D) HW/BW, (E) LW/BW and (F) HW/TL ratios of 10 NTG mice and 10 TRIM14-TG mice were analysed. **P<0.01 vs. NTG sham; $^{\#\#}$P<0.01 vs. NTG TAC. TRIM14, tripartite motif-containing 14; TAC, transverse aortic constriction; LVEDd, left ventricular end diastolic diameter; LVESd, left ventricular end systolic diameter; FS, fractional shortening; NTG, non-transgenic; TG, transgenic; HW, heart weight; BW, body weight; LW, lung weight; TL, tibia length.

results suggested that TRIM14 contributed to the development of cardiac hypertrophy and fibrosis.

TRIM14 enhances activation of the AKT signalling pathway under pressure overload. The results of the present study indicated that TRIM14 promoted cardiac hypertrophy *in vivo* and *in vitro*; however, the molecular mechanism underlying the effects of TRIM14 in cardiac hypertrophy is not clear. Previous studies have shown that activation of the AKT signalling pathway is closely related to cardiac hypertrophy and that TRIM14 regulates the AKT signalling pathway (15,21). Therefore, it was hypothesised that TRIM14 may regulate cardiac hypertrophy via the AKT signalling pathway. AKT signalling pathway-related proteins and their phosphorylation levels were detected in cardiomyocytes and cardiac tissues. Western blotting showed that the phosphorylation levels of AKT, GSK-3β, mTOR and P70S6K in the AdTRIM14 group were significantly elevated following PE treatment compared with those in the AdGFP group (Fig. 5A and B). The same trend was also observed in TRIM14-TG mice in response to pressure overload (Fig. 5C and D). These results suggested that TRIM14 overexpression promoted cardiac hypertrophy by activating the AKT signalling pathway.

Discussion

The present study showed that TRIM14 played an important role in cardiac hypertrophy and this effect may be mediated by the AKT signalling pathway. To the best of our knowledge, the current study is the first to propose the role of TRIM14 in cardiac hypertrophy via activation of the AKT signalling pathway. Findings revealed that the overexpression of TRIM14 increased cardiomyocyte surface area and hypertrophic markers in NRCMs treated with PE, and it impaired cardiac function and increased HW/BW, cardiomyocyte cross-sectional area, hypertrophic markers, the percentage of collagen area and fibrosis-related genes in mice following TAC surgery. In addition, the results revealed that TRIM14 promoted cardiac hypertrophy by activating the AKT signalling pathway *in vivo* and *in vitro*. The results of the current study demonstrated that TRIM14 has a positive regulatory effect on cardiac hypertrophy.

TRIM14 participates in multiple cell processes, such as proliferation (22), transformation (23), migration (14), apoptosis (24), natural immune response (25) and protein degradation (17). Therefore, TRIM14 is associated with a variety of pathological processes. TRIM14 is upregulated by interferon I, and it is found in infection with human immunodeficiency virus-1, hepatitis B

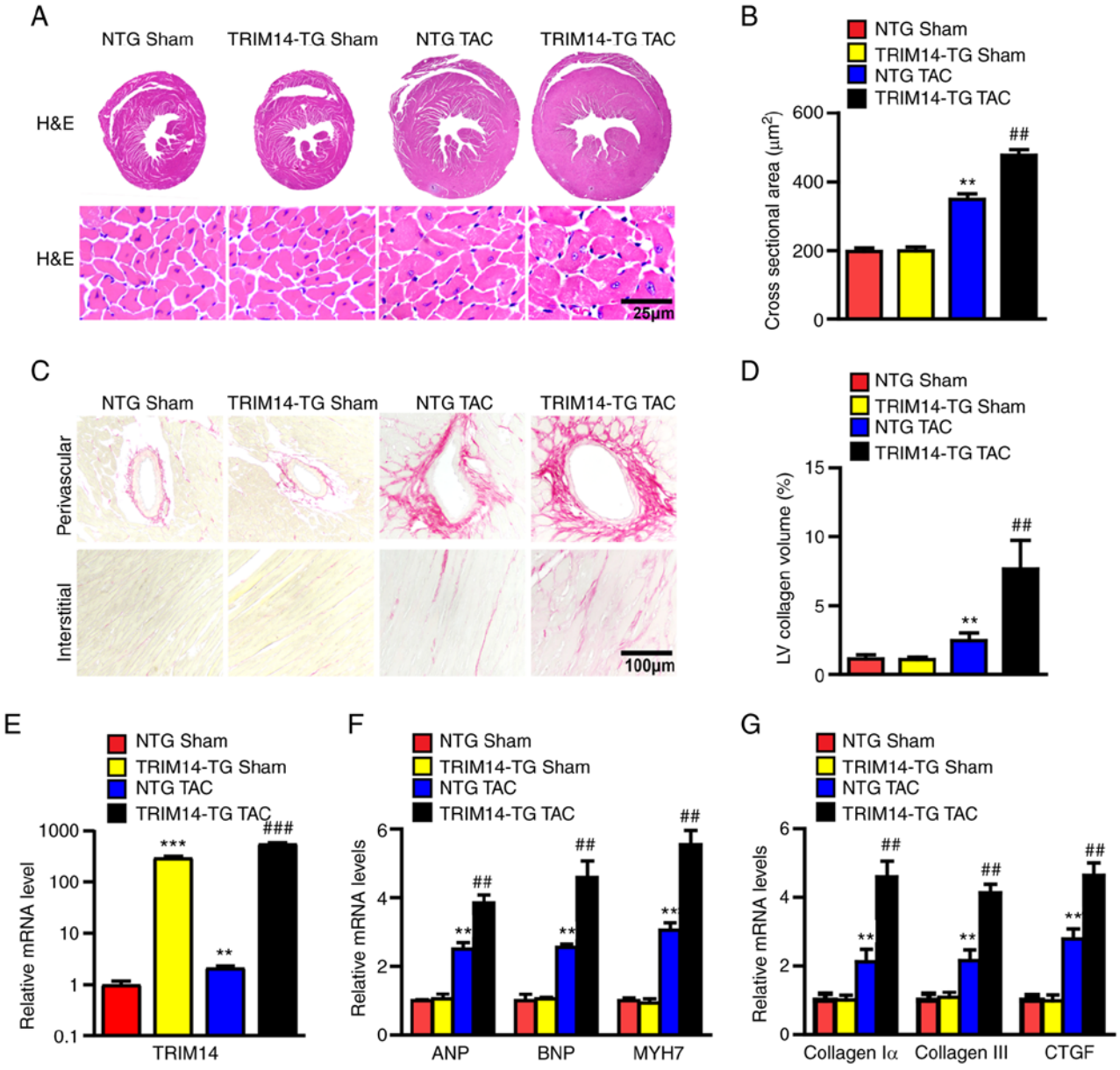

Figure 4. Overexpression of TRIM14 promotes cardiac hypertrophy and fibrosis. (A) Representative H&E staining images of cardiac and cardiomyocyte cross-sectional area; n=5. (B) Statistical results of cardiomyocyte cross-sectional area; ≥100 cells per group; scale bar, 25 μm; n=5. (C) Representative PSR staining images of cardiac perivascular and interstitial collagen fibres; scale bar, 100 μm; n=5. (D) Statistical results of cardiac interstitial fibrosis; ≥40 fields per group. The relative mRNA expression levels of (E) TRIM14, (F) ANP, BNP and MYH7, and (G) collagen Iα, collagen III and CTGF were determined using reverse transcription-quantitative PCR. n=6; **P<0.01, ***P<0.001 vs. NTG sham; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. NTG TAC. TRIM14, tripartite motif-containing 14; PSR, picrosirius red; NTG, non-transgenic; TAC, transverse aortic constriction; TG, transgenic; LV, left ventricular.

virus, hepatitis C virus, influenza A virus and other viruses, which suggests that TRIM14 participates in a wide range of natural immune regulatory responses (17,18,26,27). TRIM14 is also involved in cancer metastasis, invasion and cell resistance (14,28). An increasing number of studies have shown that tumour and immune regulatory factors, such as p53 and IL6, have an important role in cardiac hypertrophy (29,30). TRIM8 aggravates cardiac hypertrophy by enhancing the TAK1-dependent signalling pathway, and TRIM32 suppresses cardiac hypertrophy by inhibiting the AKT signalling pathway (31,32). However, little attention has been given to TRIM14 in cardiac hypertrophy. Molecules with similar structures generally have similar functions, such as MYH6 and MYH7. Therefore, it was hypothesised that TRIM14 may regulate cardiac hypertrophy. It was observed that the protein expression levels of TRIM14 were increased in mouse hypertrophic hearts, and that overexpression of TRIM14 in NRCMs treated with PE increased cardiomyocyte surface area and hypertrophic markers. HW/BW, LW/BW and HW/TL were measured in TRIM14-TG mice following TAC surgery to test the proposed hypothesis. It was demonstrated that the aforementioned ratios were significantly increased, which suggested that TRIM14 promotes pathological cardiac hypertrophy. Further experiments revealed that the cardiomyocyte cross-sectional area, the percentage of collagen area and the mRNA expression levels of the hypertrophic markers ANP, BNP and MYH7, and of the fibrosis-related genes collagen I α, collagen III and CTGF, were also significantly elevated in TRIM14-TG mice. Under the stimulation of inappropriate body

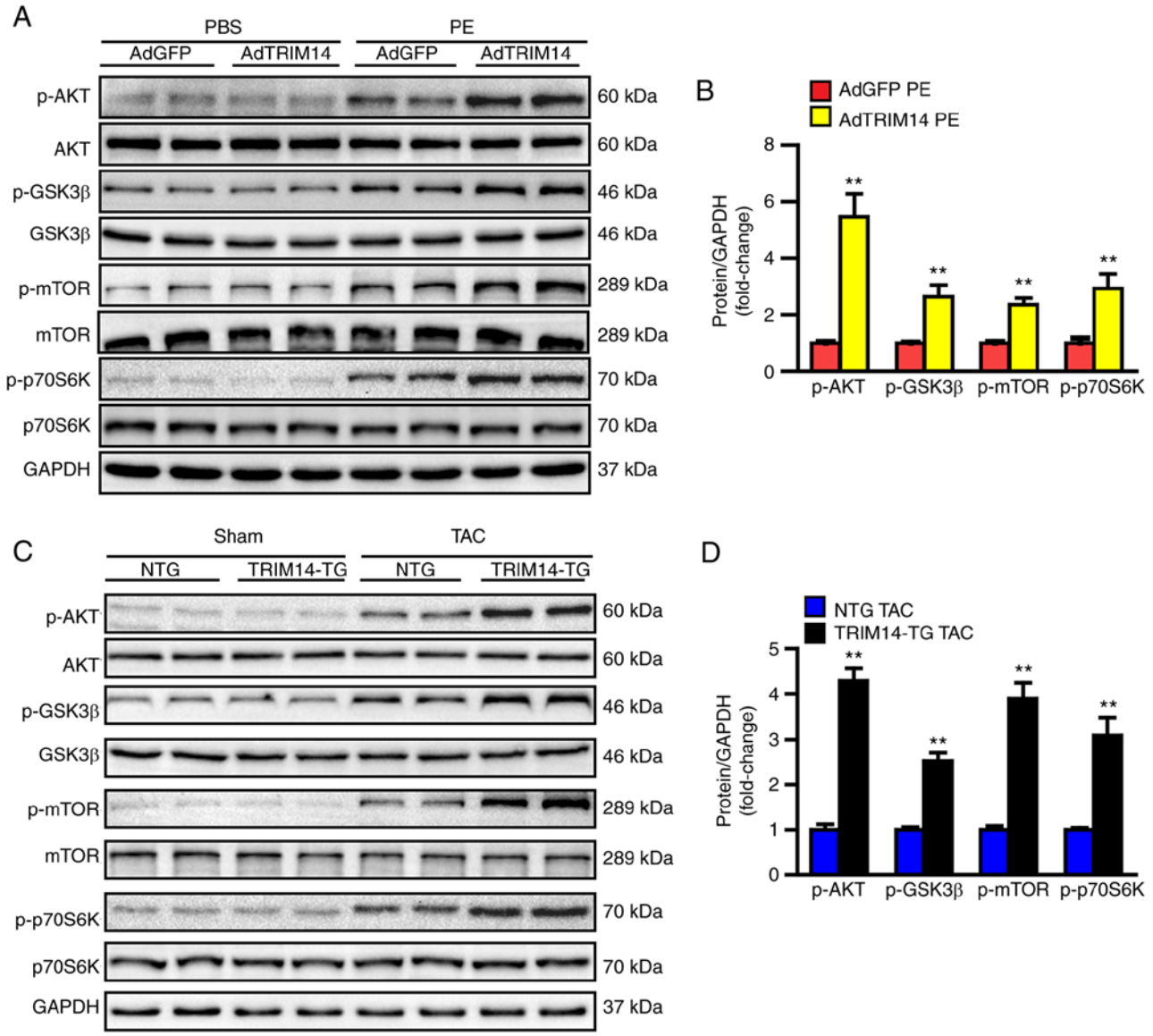

Figure 5. TRIM14 enhances activation of the AKT signalling pathway in response to hypertrophic stimulation. (A) Western blotting of total and p-AKT, p-GSK-3β, p-mTOR and p-P70S6K in NRCMs after infection with AdGFP or AdTRIM14 and treatment with PBS or PE. (B) Semi-quantification of the protein expression levels of p-AKT, p-GSK-3β, p-mTOR and p-P70S6K in NRCMs. **P<0.01 vs. AdGFP PE; n=3. (C) Western blotting of total and p-AKT, p-GSK-3β, p-mTOR and p-P70S6K in NTG and TRIM14-TG mice with TAC surgery. (D) Semi-quantification of the protein expression levels of p-AKT, p-GSK-3β, p-mTOR and p-P70S6K in mice. **P<0.01 vs. NTG TAC; n=6. TRIM14, tripartite motif-containing 14; NRCMs, neonatal rat cardiomyocytes; AdGFP, adenovirus GFP; AdTRIM14, adenovirus TRIM14; PE, phenylephrine; p, phosphorylated; NTG, non-transgenic; TAC, transverse aortic constriction; TG, transgenic.

fluid or pressure factors, such as increased cardiac volume load due to aortic valve regurgitation and increased cardiac afterload due to hypertension, TRIM14 may activate factors of cardiac hypertrophy and fibrosis via its ubiquitination of these factors, which leads to pathological changes in cardiac hypertrophy (11,22). However, the mechanism underlying the effects of TRIM14 on regulation of cardiac hypertrophy is not clear.

Numerous signalling pathways are involved in cardiac hypertrophy, such as IGF-I/PI3K/AKT/PKB signalling pathway, mTOR pathway and the CaN-NFAT (calcineurin-nuclear factor of activated T cell) and MAPK (mitogen-activated protein kinase) pathways (33-35). Yan *et al* (36) revealed that AKT1 was ubiquitinated and simultaneously interacted with k63, which promoted AKT1 accumulation at the plasma membrane. AKT was activated via phosphorylation of T308, which further activated the downstream GSK-3β/mTOR/p70S6K signalling pathway to promote the synthesis of hypertrophic factors and fibrin, which in turn led to cardiac hypertrophy and dysfunction. A previous study reported that TRIM14 contributes to osteosarcoma cell proliferation by upregulating the AKT signalling pathway, and inhibition of AKT activity reverses the effect of TRIM14 on cell proliferation (15). Since the AKT signalling pathway plays a key role in cardiac hypertrophy, it was hypothesized that TRIM14 regulated cardiac hypertrophy via the AKT signalling pathway. It was shown that the levels of phosphorylated (p)-p-AKT, p-GSK-3β, p-mTOR and p-p70S6K were significantly increased in the cardiac tissue of TRIM14-TG mice following TAC surgery and in the cardiomyocytes infected with AdTRIM14 and treated with PE. These findings suggested that TRIM14 may promote cardiac hypertrophy by activating the AKT signalling pathway. However, the limitations of the present study include the lack of verification as to whether TRIM14 knockdown suppressed

cardiac hypertrophy, and whether inhibition of AKT reversed the pro-cardiac hypertrophy effect of TRIM14. Therefore, further research is needed to investigate these issues.

In the current study, the focus was primarily on the effect of TRIM14 on the heart, and not on blood lipid levels; therefore, serum samples were not collected. This is another limitation of the present study and monitoring changes in blood lipids will be taken under consideration in future studies.

In conclusion, the current study suggested that TRIM14 may promote cardiac hypertrophy by activating the AKT signalling pathway. Therefore, TRIM14 may be a novel target for preventing cardiac hypertrophy.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HH performed the experiments, analysed the data and wrote the manuscript. YC and XF analysed the data. GX performed the experiments. MY designed the study and performed most of the experiments. HH and MY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Animal experiments were approved by the Animal Care and Use Committee of The Third Affiliated Hospital of Zhejiang Chinese Medical University (approval no. IACUC-20200120-04; Hangzhou, China). All animal-related procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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