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TMEM100 acts as a TAK1 receptor that prevents pathological cardiac hypertrophy progression

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Abstract

Pathological cardiac hypertrophy is the primary cause of heart failure, yet its underlying mechanisms remain incompletely understood. Transmembrane protein 100 (TMEM100) plays a role in various disorders, such as nervous system disease, pain and tumorigenesis, but its function in pathological cardiac hypertrophy is still unknown. In this study, we observed that TMEM100 is upregulated in cardiac hypertrophy. Functional investigations have shown that adeno-associated virus 9 (AAV9) mediated-TMEM100 overexpression mice attenuates transverse aortic constriction (TAC)-induced cardiac hypertrophy, including cardiomyocyte enlargement, cardiac fibrosis, and impaired heart structure and function. We subsequently demonstrated that adenoviral TMEM100 (AdTMEM100) mitigates phenylephrine (PE)-induced cardiomyocyte hypertrophy and downregulates the expression of cardiac hypertrophic markers in vitro, whereas TMEM100 knockdown exacerbates cardiomyocyte hypertrophy. The RNA sequences of the AdTMEM100 group and control group revealed that TMEM100 was involved in oxidative stress and the MAPK signaling pathway after PE stimulation. Mechanistically, we revealed that the transmembrane domain of TMEM100 (amino acids 53–75 and 85–107) directly interacts with the C-terminal region of TAK1 (amino acids 1–300) and inhibits the phosphorylation of TAK1 and its downstream molecules JNK and p38. TAK1-binding-defective TMEM100 failed to inhibit the activation of the TAK1-JNK/p38 pathway. Finally, the application of a TAK1 inhibitor (iTAK1) revealed that TAK1 is necessary for TMEM100-mediated cardiac hypertrophy. In summary, TMEM100 protects against pathological cardiac hypertrophy through the TAK1-JNK/p38 pathway and may serve as a promising target for the treatment of cardiac hypertrophy.

Keywords TMEM100, TAK1, Cardiac hypertrophy, Cardiomyocyte hypertrophy, Protein–protein interaction

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Introduction

Cardiovascular diseases are the leading cause of death, and heart failure (HF) is the end-stage of nearly all cardiovascular diseases [1]. Various drugs have been shown to alleviate the symptoms of HF patients, but the mortality rate of HF patients remains high, suggesting the existence of other regulatory factors that may play crucial roles in HF [2]. There are two types of cardiac hypertrophy, physiologic and pathological, with pathological hypertrophy generally progressing to heart failure [3]. Pathologically, cardiac tissue is characterized mainly by increased heart mass, impaired heart function and aggravated interstitial fibrosis [4]. Despite numerous studies on pathological cardiac hypertrophy, its underlying mechanism has not been fully explored. Therefore, further investigations into the molecular mechanism of pathological cardiac hypertrophy and the identification of new therapeutic targets for HF are urgently needed.

TMEM100 is a highly conserved transmembrane protein that is composed of 134 amino acids and contains two transmembrane domains [5]. TMEM100 is widely located in the cell membrane, endoplasmic reticulum and cytoplasm [6, 7]. In terms of molecular function, TMEM100 is involved in various biological activities, including angiogenesis, protein degradation, the inflammatory response, cell metabolism and signal transduction [7–9]. Previous studies have suggested that TMEM100 is associated with nervous system disease, embryonic growth, tumorigenesis and liver fibrosis [5, 7, 10, 11]. Ken et al. demonstrated that TMEM100 is essential for cardiac morphogenesis, suggesting its potential close relationship with cardiovascular disease [12]. However, it remains unknown whether TMEM100 is involved in the progression of cardiac hypertrophy.

In this study, analysis of the GEO database revealed that TMEM100 was upregulated in patients with cardiac hypertrophy and heart failure. We subsequently verified these findings in different cardiac hypertrophic models. Gain- and loss-of-function experiments revealed that TMEM100 acted as a protective factor in cardiac hypertrophy. In terms of the mechanisms, TMEM100 directly interacts with TAK1 and inhibits the phosphorylation of the TAK1-JNK/p38 axis. TAK1 inhibitor (iTAK1) reversed the effect of TMEM100-mediated cardiac hypertrophy. Overall, these findings provide a theoretical basis for treating cardiac hypertrophy.

Methods and materials

Animal surgery

The animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University (2022-KY-0822).

Eight- to 11-week-old male mice (weight: 25–27 g) were chosen for transverse aortic constriction (TAC) surgery. Male mice were randomly assigned to each group. An intraperitoneal injection of pentobarbital sodium (90 mg/kg, P3761, Sigma–Aldrich) was given to induce anaesthesia. Next, the left chest was opened carefully, and a 7–0 silk suture was tied against a 26-G blunt needle placed parallel to the aortic arch. After ligation, the needle was removed, and the chest was closed. For the sham group, the mice underwent a similar procedure without ligation. Finally, all the mice were placed in a 37° C incubator and allowed to awaken.

Adeno-associated virus 9 (AAV9) mediated TMEM100 overexpression in mice

AAV9-TMEM100 and AAV9-GFP mice were obtained according to previously described methods [13]. In brief, the TMEM100 gene was cloned and inserted into the pAAV vector with the cardiomyocyte-specific promoter cTnT. Next, the pAAV-cTnT-TMEM100 plasmid, pAd Delta plasmid and pAAV2/9 plasmid were cotransfected into 293 T cells. After 72 h, AAV9-TMEM100 was obtained, and the virus was centrifuged and purified. Real time–time PCR was used to determine the TMEM100 titres. The mice were injected with the indicated virus (7.5×10^{11} vg) via the tail vein 2 weeks before TAC surgery. The AAV9-TMEM100 primers used are listed in Supplementary Table 1.

Echocardiography assessment

After 4 weeks of TAC, the mice were fixed on a thermostatic plate, heart structure and function were measured via a small animal ultrasonic imaging system (VEVO2100, FUJIFILM VISUALSONICS, Canada), and a 30-MHz (MS400) transducer was chosen. In M-mode echocardiography, the left ventricle (LV) was evaluated on the short- and long-axis sections. The heart rate, left ventricular end-systolic diameter (LVESd), left ventricular end-diastolic diameter (LVEDd), interventricular septal thickness at diastole (IVSD), left ventricular posterior wall (LVPW), left mass (LV mass correction), ejection fraction (EF) and fraction **s**hortening (FS) were calculated and averaged over three consecutive cardiac cycles.

Animal heart tissues

After the mice were weighed, the hearts were quickly removed and placed in a 10% KCl solution. The dry weights of the hearts were measured prior to fixation in 10% formalin. The dry weights of the lungs and the tibia lengths of the mice were subsequently documented.

Histological examination

The heart tissues were fixed in 10% formalin for 48 h. Haematoxylin (G1004, Servicebio) and eosin (BA-4024, Baso) (H&E) staining and wheat germ agglutinin (WGA, L4895, Sigma) staining were used to determine the cross-sectional areas of the cardiomyocytes. For HE staining, the heart section slices were stained with haematoxylin and eosin for 3 min and sealed. For WGA staining, a diluted WGA dye solution was added, and the heart sectionslices were incubated at 37 °C in a light-free incubator for 1 h. Then, self-fluorescence quenching agent B was added (G1221, Servicebio), and the mixture was incubated for 5 min and washed with PBS. Finally, the heart section slices were sealed. The heart sections were soaked in picrosirius red (PSR, 26357–02, Hede Biotechnology) for 90 min and sealed to observe the degree of cardiac fibrosis. Image-Pro Plus 6.0 software was used to analyze the degrees of cardiomyocyte hypertrophy and cardiac fibrosis shown in the images.

Construction of the plasmids and adenoviruses

The target gene was cloned and inserted into the pcDNA5 vector with a Flag/HA label. The pcDNA5 recombination plasmid was subsequently transformed, sequenced and extracted. Finally, the correct recombinant plasmid was obtained for further experiments.

The TMEM100 gene was cloned from cDNA and ligated to an adenovirus vector with the CMV promoter, pENTR-Flag-GFP. The recombinant adenoviral plasmid was obtained according to the above procedure. After linearization with the PacI enzyme (R0547L, NEB), the plasmid was transfected into 293A cells. After 3 rounds of expansion, TMEM100-overexpressing adenovirus was obtained. AdVector was used as the control. To obtain the TMEM100 knockdown adenovirus, three short hairpin RNAs targeting TMEM100 were cloned and inserted into an adenoviral vector with the U6 promoter (pENTR-U6-CMV-GFP). The rest of the procedure was the same as that for the AdTMEM100 construction. The most efficient knockdown adenovirus was selected through a western blot analysis for further experiments. AdshRNA served as the control group. All primers used in this study are listed in Supplementary Table 1.

Adult mouse cardiomyocyte (CM) isolation and culture

C57BL/6 J mice aged 8 to 12 weeks were chosen for CM isolation. The mice were anaesthetized, and the chest was opened to expose the heart. The ascending aorta was clamped, and the heart was fastened onto a heart perfusion apparatus. The heart was digested and cut into several 1-mm^3 pieces. The cell suspension was passed through 100-μm cell strainers (352,360, Corning, USA) and underwent four sequential rounds of gravity settling. After culturing for 24 h, the medium was replaced with fresh media. Angiotensin II (Ang II) (100 nM, Calbiochem Biosciences) was added to the medium to stimulate hypertrophy.

Neonatal rat cardiomyocyte (NRCM) isolation and culture

Sprague–Dawley rats (1–2 days) were chosen for NRCM isolation. The hearts were quickly removed from the SD rats, and the heart sections were then cut into several 1-mm^3 pieces. These pieces were treated with 0.125% trypsin (25300054, 25200056, Gibco) and red blood cell lysis buffer (C3702, Beyotime). After differential adhesion, the fibroblasts were removed, and the NRCMs were cultured in DMEM/F12 medium (C11330500BT-1, Gibco) supplemented with 10% foetal calf serum (10091148, Gibco), BrdU (0.1 mM, ST1056, Beyotime) and PS (15140–122, Gibco).

After the NRCMs were cultured overnight, the corresponding adenovirus was added for 6 h to infect the NRCMs, and the medium was replaced with serum-free medium. After 24 h of infection, PE (50 μM, P6126, Sigma) was added to the medium to stimulate cardiomyocyte hypertrophy. 5Z-7-oxozeaenol (100 Nm, 09890-1MG, Sigma) was used as the TAK1 inhibitor.

Immunofluorescence staining

All operations on NRCMs were performed on glass slides. The glass slides were treated with 4% formaldehyde for 15 min, washed with PBS 3 times, treated with 0.5% Triton X-100 (T8787, Sigma–Aldrich) for 10 min and then washed with PBS 3 times. Next, the NRCMs were incubated in 8% goat serum (BMS0050, Abbkine) for 1 h at 37 °C. An α-actinin (1:100 dilution, A7811, Sigma) antibody was added for 2 h at 37 °C, after which the NRCMs were incubated with the corresponding secondary antibody (1:200 dilution, A11061, Invitrogen) for 1 h at 37 °C. Finally, DAPI (010020, Southern Biotech) was added to the glass slides. Images of the surface areas of the cardiomyocytes were analysed via ImageJ software.

Superoxide dismutase (SOD) enzyme activity assay

The NRCMs were washed with PBS 3 times and lysed with SOD sample preparation solution (Beyotime, S0101S). After centrifugation, the supernatant was used for subsequent detection. After the addition of SOD working buffer, the absorbances were measured at 450 nm.

Lipid peroxidation malondialdehyde (MDA) assay

A lipid peroxidation MDA assay kit (S0131S, Beyotime) was used to determine the MDA contents. The samples were diluted to 1, 2, 5, 10, 20, and 50 μM. The standard

and test samples were subsequently incubated with MDA working solution, and the absorbances were subsequently measured at 532 nm.

RNA sequence and data processing

The RNA-sequencing results for GSE89714, GSE36961, GSE21610 and GSE19210 were downloaded from the GEO database (www.ncbi.nlm.nih.gov), and the differentially expressed genes (DEGs) were analysed online via GEO2R.

Total RNA from the AdTMEM100 and AdVector groups was obtained, and cDNA was generated after PE stimulation. The single-end library was sequenced via MGISEQ 2000. RNA sequence processing was performed by Sangon Biotech Co., Ltd. (Shanghai, China). Principal component analysis (PCA) was performed to analyse the sample distribution on the basis of the RNA sequence data via the VEGAN package in R. For samples with biological duplications, DESeq was used to analyse the differentially expressed genes. Differentially expressed genes (DEGs) must match the following criteria: q value (corrected p value) < 0.05 and |fold change| > 2. Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Kyoto Orthologue Groups (KOGs) functional enrichment analyses of the DEGs were performed via clusterProfiler. Gene set enrichment analysis (GSEA) was performed on gene sets from the Gene Ontology (GO) database. A p/Q value < 0.05 was considered significant. RNA-seq was performed by Sangon Biotech (www.sangon.com).

Quantitative real-time (RT) PCR

The mRNA was extracted with TRIzol reagent (15596–026, Invitrogen). The RNA concentration was measured via a Nanodrop 2000 (Thermo, Madison), and 2 μg of RNA was reverse transcribed into cDNA via the Transcriptor HiScript III RT SuperMix (R323-01, Vazyme). Finally, the cDNA was analysed via a qRT–PCR system (LightCycler 480 Instrument II, Roche Diagnostics, Inc.). GAPDH was used as an internal reference. The primers used in this study are shown in Supplementary Table 2.

Western blotting

Cells and tissues were lysed with RIPA buffer. Next, the protein concentrations were quantified with a BCA kit (23225; Thermo). The protein mixture was boiled at 95℃ for 15 min. The proteins were then separated via 10% SDS–PAGE and transferred to a PVDF membrane (IPVH00010, Millipore, USA). The PVDF membrane was blocked with skim milk for 1 h and washed with TBST buffer. The membrane was incubated with the indicated primary antibodies overnight at 4 °C and washed with TBST buffer 3 times. Finally, the membrane was incubated with the corresponding secondary antibodies for 2 h at 25 °C. A Bio-Rad Chemi Doc XRS + system (1705062, Bio-Rad) was used to detect the protein levels. GAPDH served as an internal reference. All the antibodies used are listed in Supplementary Table 3.

Coimmunoprecipitation (co-IP)

The indicated plasmid was co-transfected into 293 T cells. After 24 h of culture, the 293 T cells were lysed in cold 150 mM IP buffer (20 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; and 1% NP-40 1 × cocktail). After centrifugation at 12,000 g for 10 min, 40 μl of the supernatant that was boiled with 2 × SDS loading buffer served as an INPUT sample, and the remaining supernatants were treated overnight with 20 μl of IP beads (AA104307; Bestchrom) and 0.5–1 μl of the indicated primary antibody at 4 °C. The beads were subsequently washed with 150 mM and 300 mM cold IP buffer several times. Finally, the beads were boiled with loading buffer for western blotting.

Glutathione-S-Transferase (GST) pulldown assay

GST-HA-TAK1, GST-HA-TMEM100, Flag-TAK1 or Flag-TMEM100 was transfected into 293 T cells, and the cells were lysed with T1-PD lysis buffer (50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl, and 1% Triton X-100 1 × cocktail). After centrifugation, 50 μl of the supernatant with 4 × loading buffer was used as an INPUT sample. The GST-HA-protein mixture was treated with GST beads (AA009305; Bestchrom) and mixed with the remaining supernatant overnight at 4 °C. The complexes were centrifugated, and the beads were then washed with cold T1-PD buffer. Finally, the beads were treated with loading buffer for western blotting.

Statistical analysis

All the data are presented as the means ± SDs and were statistically analyzed with SPSS 26.0 software (IBM Corp., Armonk). For data with a normal distribution, the 2-tailed Student's t-test was used for comparisons between two groups, and one-way ANOVA was applied for multiple comparisons with Bonferroni's post hoc analysis (data meeting homogeneity of variance) or with Tamhane's T2 analysis (data of heteroscedasticity). For datasets with skewed distributions, a Kruskal–Wallis nonparametric statistical test was used for multiple comparisons. $p < 0.05$ was considered statistically significant.

Results

TMEM100 was upregulated in cardiac hypertrophy

To explore new targets for treating cardiac hypertrophy, we analyzed the mRNA expressions of genes in patients with hypertrophic cardiomyopathy, patients

with heart failure and rats with heart failure via the GEO database (GSE89714, GSE36961, GSE21610 and GSE19210). Differentially expressed genes (DEGs) were visualized through a Venn diagram, and TMEM100 was highly expressed in all the databases (Fig. 1A). Next, we determined the expressions of TMEM family members (including TMEM100) among these DEGs. The RT–PCR results revealed that the expressions of TMEM2, TMEM119, TMEM229b and TMEM252 did not change in mice after TAC surgery (Figure S1A). Notably, the mRNA and protein expression of TMEM100 was upregulated after TAC for 4 and 8 weeks (Fig. 1B-C). However, the types of cardiac cells in which TMEM100 is upregulated remain unclear. Adult cardiac myocytes were isolated from WT mice and stimulated with angiotensin II (Ang II) for 72 h, and we demonstrated that TMEM100 was upregulated in Ang II-induced cardiomyocyte hypertrophy (Fig. 1D-E). We also obtained the same results for phenylephrine (PE)-induced cardiomyocyte hypertrophy (Fig. 1F-G). On the basis of these findings, we inferred that TMEM100 may play a role in cardiac hypertrophy.

TMEM100 suppressed cardiomyocyte hypertrophyin vitro

We constructed AdTMEM100 and AdshTMEM100 to investigate the role of TMEM100 in cardiomyocyte hypertrophy. The TMEM100 expressions were determined via western blotting (Fig. 2A). Immunofluorescence staining revealed that TMEM100 suppressed cardiomyocyte hypertrophy after PE stimulation (Fig. 2B). Additionally, the RT–PCR results revealed that TMEM100 inhibited the expressions of hypertrophic biomarkers (*Anp*, *Bnp* and *Myh7)*, as shown in Fig. 2C. In contrast, the knockdown efficiency of TMEM100 was determined via western blotting (Fig. 2D). The results of immunofluorescence staining and the expressions of cardiac hypertrophic markers suggested that TMEM100 knockdown aggravated cardiomyocyte hypertrophy (Fig. 2E-F). These results indicate that TMEM100 serves as a protective factor in CM hypertrophy.

Cardiac-specific overexpression of TMEM100 mitigated cardiac hypertrophy induced by pressure overload in vivo

To further investigate the role of TMEM100 in TAC-induced cardiac hypertrophy in mice, we overexpressed TMEM100 in the heart via the AAV9-cTnT vector [13]. The efficiency of TMEM100 overexpression was detected via western blotting (Fig. 3A). TAC or a sham operation was performed in the indicated groups, and the results were analyzed after 4 weeks. The results revealed that there was no difference in mouse weight (Figure S2A). Echocardiography was used to evaluate heart structure and function. The heart rates of the mice in each group were unchanged after TAC surgery (Figure S2B). Heart structural indicators, including the left ventricular mass (LV mass cor), left ventricular end-diastolic diameter (LVEDd), left ventricular end-systolic diameter (LVESd), interventricular septal thickness at diastole (IVSD) and left ventricular posterior wall (LVPW), were evaluated. The results revealed that the above indicators were lower in AAV9-TMEM100 mice than in AAV9-GFP mice (Fig. 3B-D, Figure S2C-D). In terms of heart function, both EF% and FS% were improved in TMEM100-overexpressing mice compared with those in AAV9-GFP mice (Fig. 3E-F). Next, the heart weight (HW), lung weight (LW) and tibia length (TL) were measured and documented. The heart weight, HW/BW, LW/BW and HW/TL of the AAV9-TMEM100 mice were lower than those of the AAV9-GFP mice (Fig. 3G-I). Moreover, there were no changes in the above indicators under physiological conditions. In summary, TMEM100 overexpression ameliorated cardiac architecture and improved heart function after TAC surgery.

Furthermore, we explored the function of TMEM100 in cardiac hypertrophy by examining the degree of cardiomyocyte hypertrophy and cardiac fibrosis. HE and WGA staining revealed that TMEM100 alleviated the cardiomyocytes enlargement after TAC surgery (Fig. 4A). The qPCR results suggested that the expression of the hypertrophic marker *Myh6* was increased and that the expressions of *Anp*, *Bnp* and *Myh7* decreased in AAV9-TMEM100 (Fig. 4B). Cardiac fibrosis is a crucial pathological basis of cardiac hypertrophy and cardiac remodelling [14]. Images of the PSR indicated that, compared with the control, TMEM100 relieved cardiac fibrosis (Fig. 4C). Similarly, we obtained consistent results by detecting cardiac fibrosis markers (e.g., *Col1a1*, *Col3a1*, *Col8a1* and *Ctgf*), as shown in Fig. 4D. These results demonstrated that TMEM100 mitigated cardiomyocyte hypertrophy, cardiac fibrosis and improved cardiac function.

TMEM100 may participate in oxidative stress and the MAPK signaling pathway after PE stimulation

To determine the underlying mechanism of TMEM100 in cardiac hypertrophy, RNA sequencing of NRCMs infected with AdTMEM100 or AdVector was performed after PE stimulation. The results of hierarchical clustering analysis and PCA indicated that the samples were divided into two clusters (Fig. 5A, Figure S3A). The volcano map and scatter map revealed 383 upregulated genes and 446 downregulated genes (Fig. 5B, Figure S3B). GO functional analysis revealed that TMEM100 was crucial for regulating cardiac hypertrophy, protein synthesis and oxidative stress (Fig. 5C). GSEA further verified that TMEM100 overexpression activated oxidoreductase activity (Fig. 5D). KOG enrichment analysis

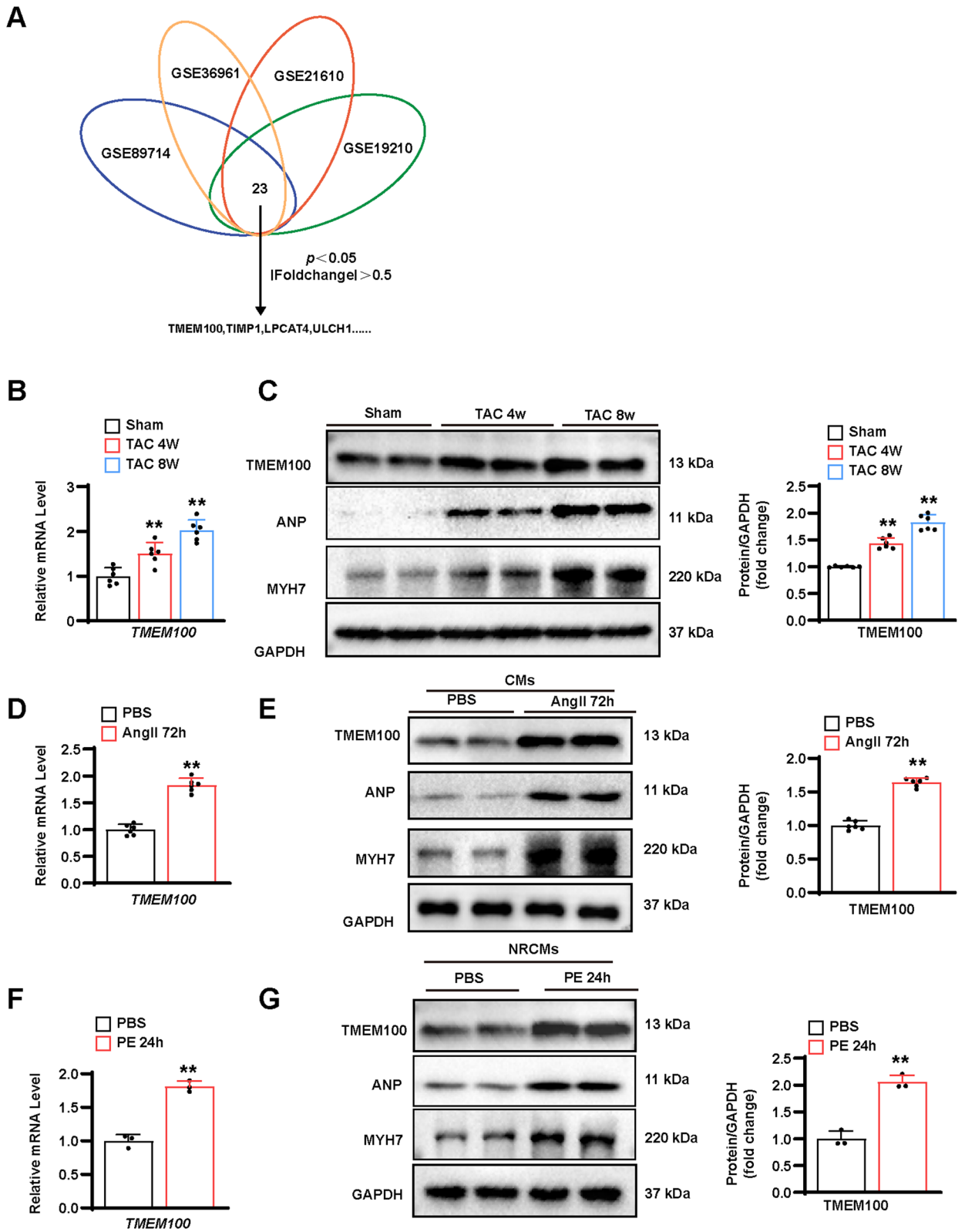

Fig. 1 TMEM100 was up-regulated in cardiac hypertrophy. **A** Venn diagram showing results of TMEM100 expression in cardiac hypertrophy. **B**, **C** The mRNA and protein expression of TMEM100 in mice hearts after TAC 4w, TAC 8w. **D**, **E** The mRNA expression of TMEM100 in cardiac myocytes hearts after Ang II stimulation. **F**, **G** The mRNA expression and protein level of TMEM100 in phenylephrine (PE)-induced cardiomyocytes hypertrophy (For vivo experiments, $n=6$. For vitro experiments, $n=3$ independent experiments). $^{**}p<0.01$. vs Sham/PBS

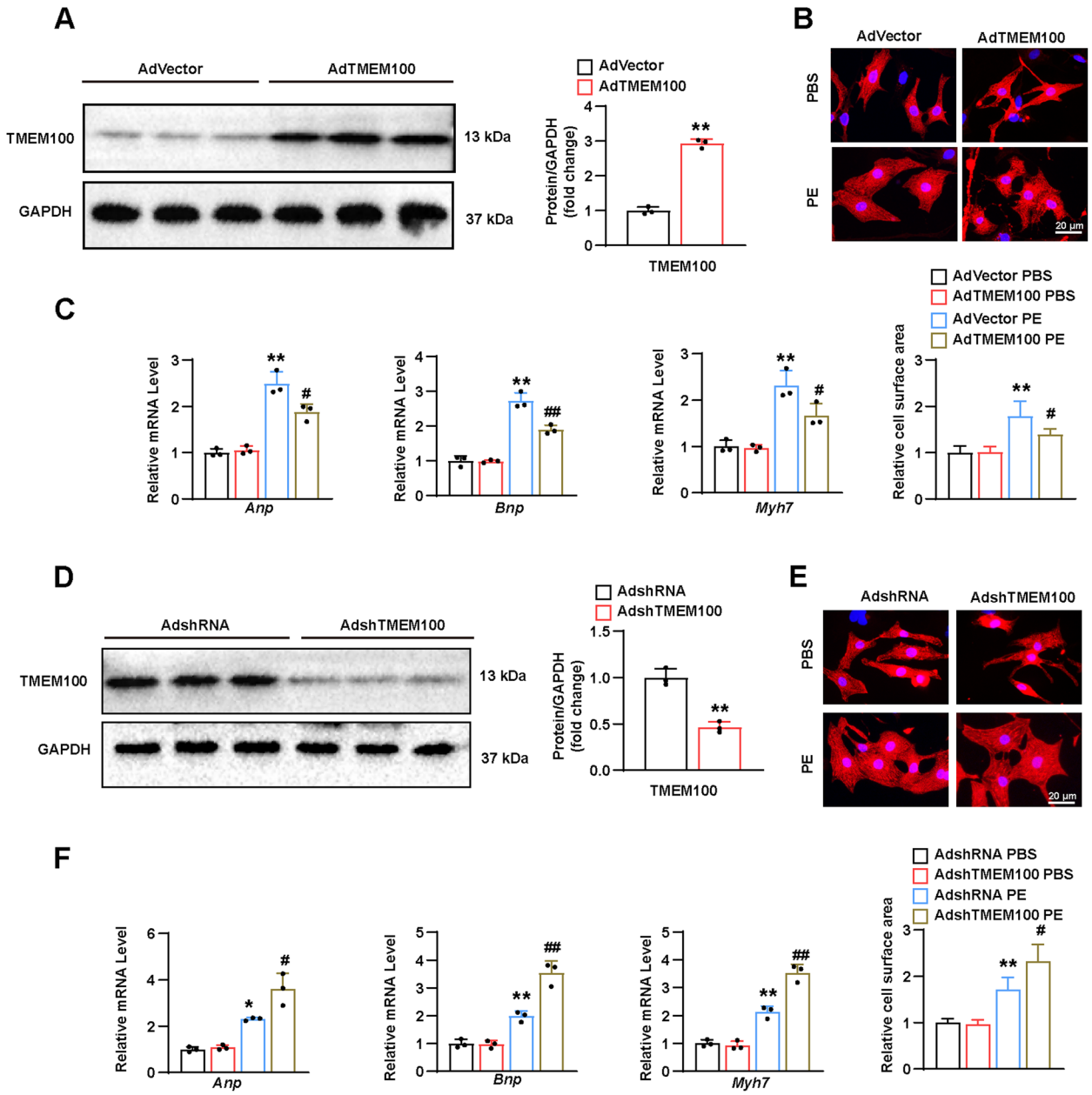

Fig. 2 TMEM100 served as a protective factor in PE-induced cardiomyocytes hypertrophy. **A** The protein expression of AdTMEM100 and AdVector was verified by western blot ($n = 3$ independent experiments). **B** Representative images of immunofluorescence staining of AdTMEM100 and AdVertor group (left) and NRCMs cross-sectional-area ($n =$ at least 30 cells, right) after PE (50 μM)/PBS stimulation. **C** The mRNA level of cardiac hypertrophic markers (*Anp*, *Bnp* and *Myh7*) was analyzed by RT-PCR assay ($n = 3$ independent experiments). **D** The protein expression of AdshTMEM100 and AdshRNA was verified by western blot ($n = 3$ independent experiments). **E** Representative images of immunofluorescence staining and cross-sectional-area in every group ($n =$ at least 30 cells). **F** The mRNA level of cardiac hypertrophic markers was analyzed by RT-PCR ($n = 3$ independent experiments). $^{*}p < 0.05$ vs. AdshRNA PBS, $^{**}p < 0.01$ vs. AdVector/AdshRNA PBS, $^{\#}p < 0.05$ vs. AdVector/AdshRNA PE, $^{\#\#}p < 0.01$ vs. AdVector/AdshRNA PE

revealed that TMEM100 overexpression was associated mainly with signal transduction mechanisms (Figure S3C). To search for the details of the signaling pathways, KEGG enrichment analysis was performed on the up- and downregulated genes, and the results revealed that mitogen-activated protein kinase (MAPK) was the most enriched pathway contributing to TMEM100-mediated cardiac hypertrophy (Fig. 5E). The heatmap presented MAPK axis-related genes (Fig. 5F). On the basis of the RNA sequence, we hypothesized that the MAPK pathway

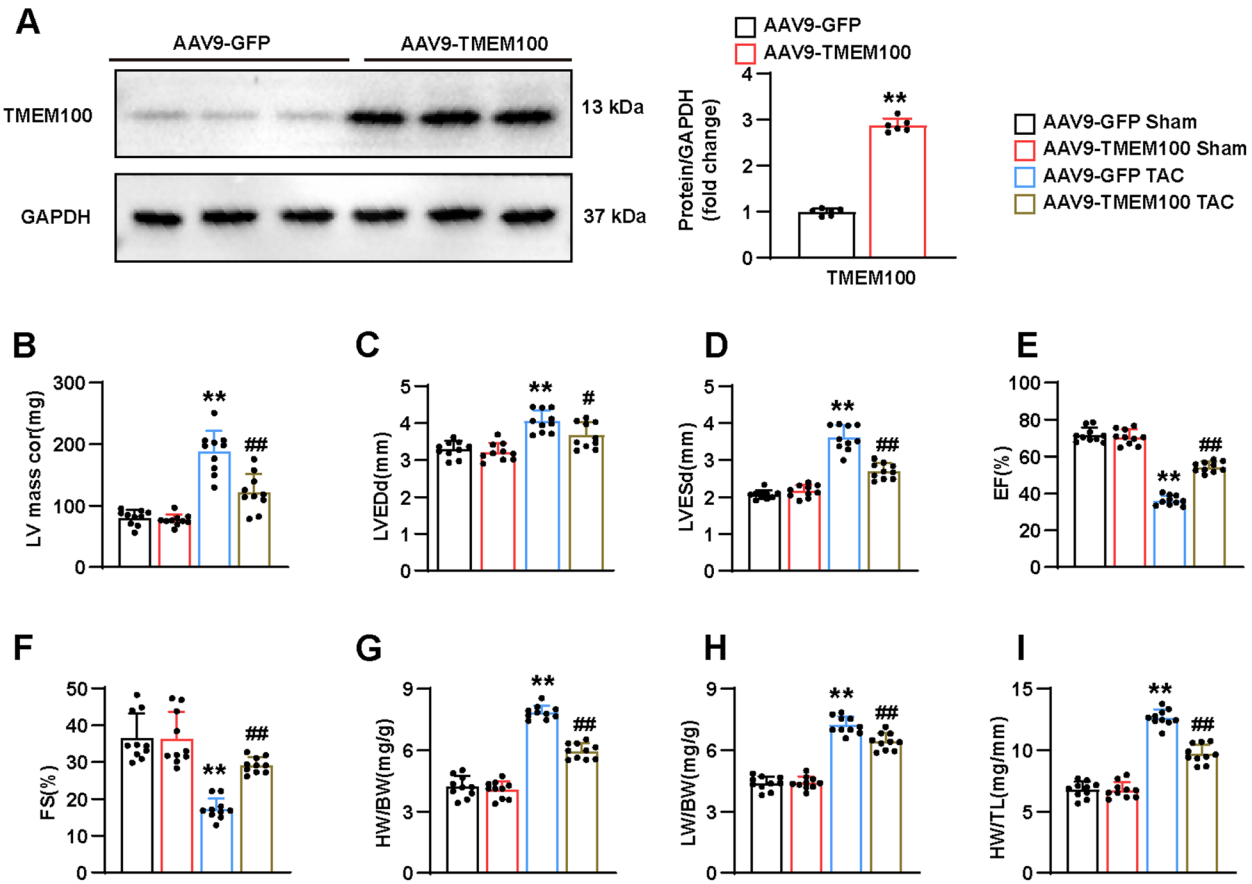

Fig. 3 TMEM100 overexpression improved the cardiac structure and function in vivo. **A** The TMEM100 expression in AAV9-GFP and AAV9-TMEM100 group ($n=3$). **B-F** The ultrasonic evaluation results of left ventricular mass correction (LV mass cor), LV end diastolic diameter (LVEDd), LV end systolic diameter (LVESd), shortening fraction (FS%) and ejection fraction (EF%) in each group ($n=10$). **G-I** The measurement results of heart weight (HW) / body weight (BW), lung weight (LW)/BW and HW/tibia length (TL) ratios in each group ($n=10$). $^{**}p<0.01$ vs. AAV9-GFP sham, $^{\#}p<0.05$ vs. AAV9-GFP TAC, $^{\#\#}p<0.01$ vs. AAV9-GFP TAC

and oxidative stress were associated with the regulatory role of TMEM100 in cardiac hypertrophy.

The TAK1-JNK/p38 *axis*, oxidative stress and inflammation are involved in TMEM100-regulated cardiac hypertrophy in vivo

We detected the phosphorylation of the core downstream molecules of the MAPK pathway, JUN N-terminal kinases (JNK), extracellular regulated protein kinases (ERK) and p38 in pressure overload induced cardiac hypertrophy. As shown in Fig. 6A, compared with AAV9-GFP mice, AAV9-TMEM100 inhibited the phosphorylation of JNK and p38 after TAC surgery, while the phosphorylation of ERK was unchanged. Furthermore, we investigated the possible upstream molecules contributing to the TMEM100-mediated inhibition of the MAPK pathway. Some crucial MAP3Ks that interact with TMEM100 were detected via a Co-IP assay. The results revealed that TMEM100 could interact with apoptosis signal-regulating kinase 1 (ASK1) and transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) but not with TPL2 (Fig. 6B-D). Next, the phosphorylation of ASK1 and TAK1 was detected in AAV9-TMEM100-treated and control mice. Notably, TMEM100 inhibited the phosphorylation of TAK1 but not that of ASK1 after TAC surgery (Fig. 6E). Since TMEM100 participates in oxidative stress in cardiac hypertrophy, we detected the activity of the antioxidant, SOD (superoxide dismutase), and found that TMEM100 overexpression increased its enzyme activity (Fig. 6F). We also obtained similar results through a lipid peroxidation malondialdehyde (MDA) assay (Fig. 6G). Moreover, numerous studies have reported that TAK1 is associated with the inflammatory response [15]. The inflammatory signaling pathway NF-κB was detected, and the results showed that TMEM100 inhibited the activation of p65 (Fig. 6H). Inflammatory factors (e.g., *Il6*, *Il1β* and *Tgfα*) were detected, and the results revealed that TMEM100

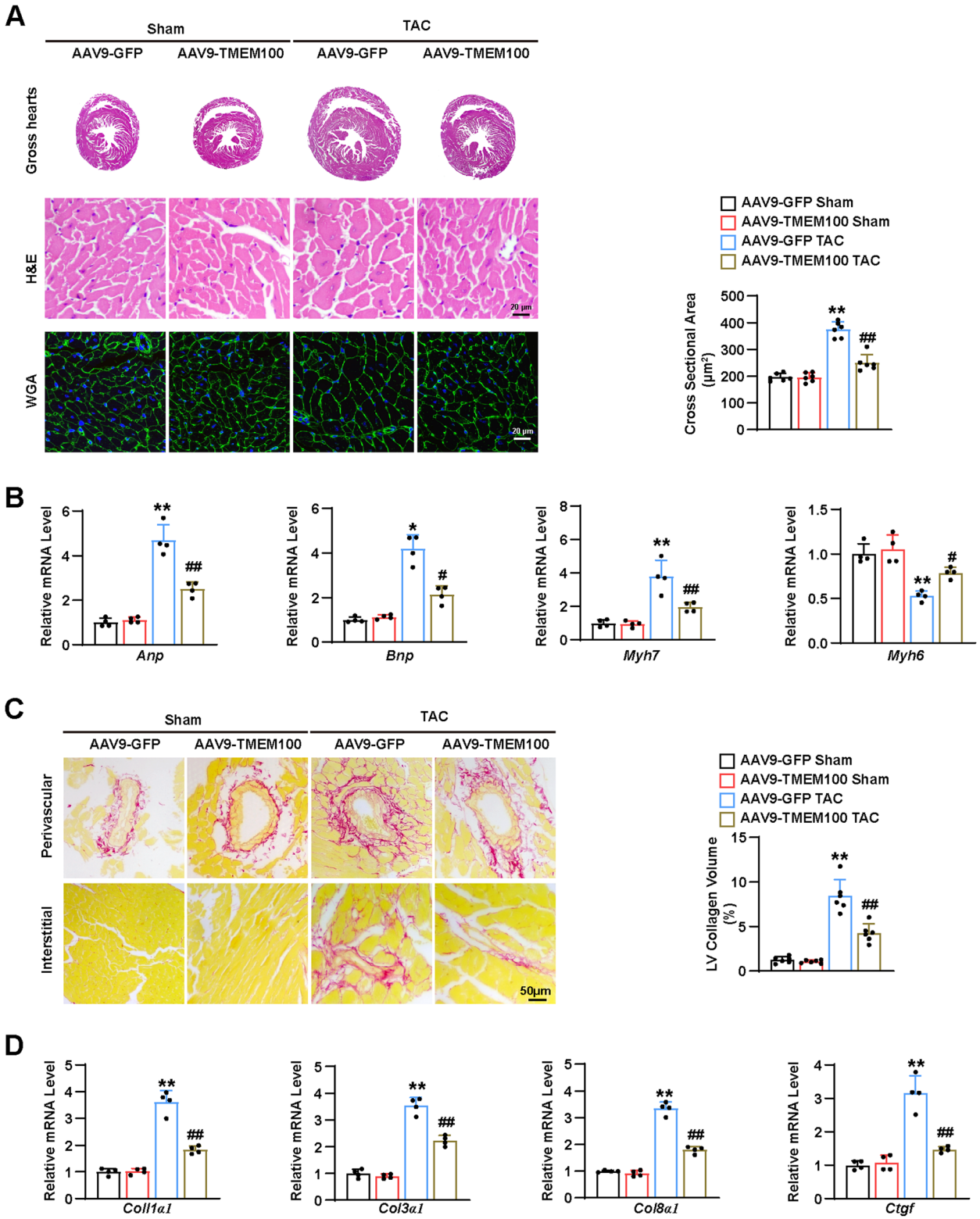

Fig. 4 TMEM100 overexpression attenuated cardiomyocytes enlargement and cardiac fibrosis in vivo. **A** Representative images of hematoxylin & eosin (HE) staining and wheat germ agglutinin (WGA) staining (left, $n=6$) and LV cardiomyocytes' cross-sectional-area (right, $n=120$ cells) in every group. **B** The mRNA level of cardiac hypertrophic markers was analyzed by RT-PCR ($n=4$). **C** Representative images of picrosirius red (PSR) staining ($n=6$) and LV collagen volume (right) in every group. **D** The mRNA level of cardiac fibrosis markers was analyzed by RT-PCR ($n=4$). $^{*}p<0.05$ vs. AAV9-GFP sham, $^{**}p<0.01$ vs. AAV9-GFP sham, $^{\#}p<0.05$ vs. AAV9-GFP TAC, $^{\#\#}p<0.01$ vs. AAV9-GFP TAC

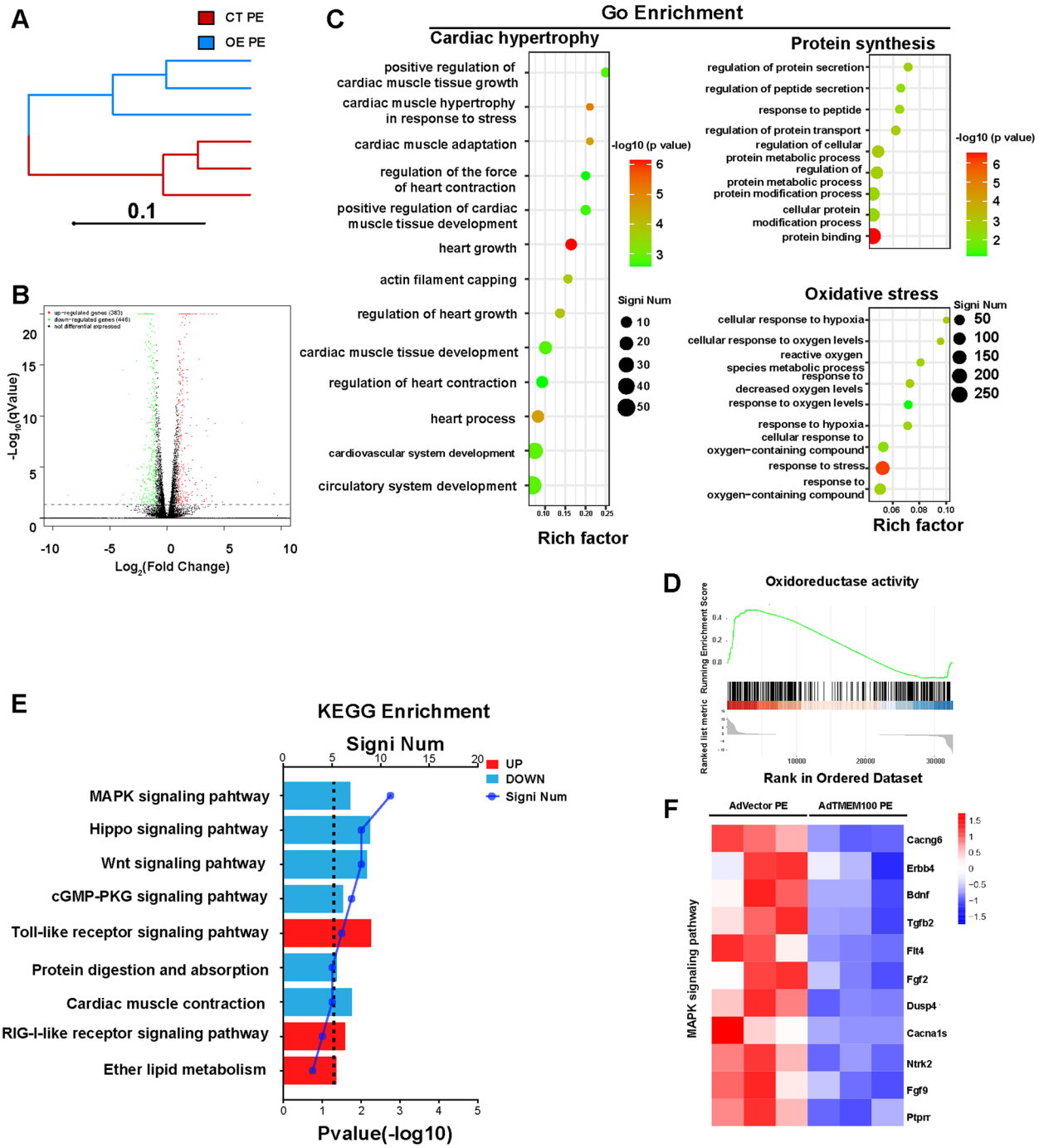

Fig. 5 RNA-sequence results of AdTMEM100 and AdVector group after PE stimulation. **A** The results of hierarchical clustering analysis of the AdTMEM100 and AdVector sample distribution. **B** The volcano map revealed the differential genes in AdTMEM100 and AdVector group after PE stimulation. **C** GO analysis of molecular events involved in cardiac hypertrophy, protein synthesis and oxidative stress. **D** GSEA analysis showing role of TMEM100 in cardiac hypertrophy. **E** The results of KEGG pathway enrichment through analyzing up- and down-regulated genes. **F** The heatmap showing the significant genes associated with MAPK signaling pathway

inhibited inflammatory activation in hypertrophic cardiac hearts (Fig. 6I). Together, TMEM100 inhibited the phosphorylation of the TAK1-JNK/p38 axis, oxidative

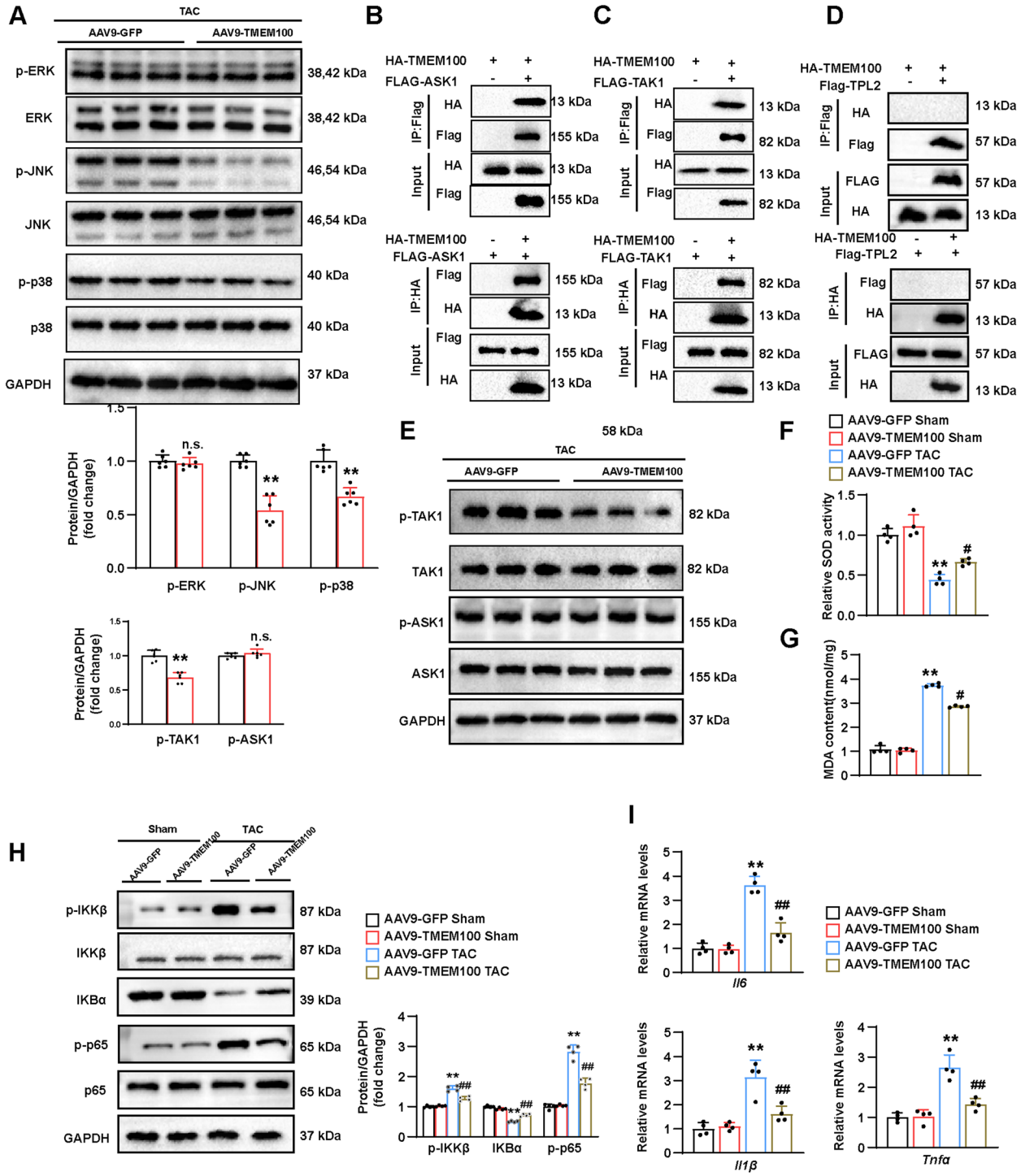

Fig. 6 TMEM100 inhibited the phosphorylation of TAK1-JNK/p38 in vivo. **A** The total and phosphorylated expression of JUN N-terminal kinases (JNK), extracellular regulated protein kinases (ERK) and p38 in heart tissues after TAC/sham surgery ($n=6$). **B** The interaction between TMEM100 and apoptosis signal-regulating kinase 1 (ASK1) in 293 T cells. **C** The interaction between TMEM100 and transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1) in 293 T cells. **D** The interaction between TMEM100 and TPL2 in 293 T cells. **E** The phosphorylation of ASK1 and TAK1 in heart tissues after TAC/sham surgery ($n=6$). **F**, **G** Relative SOD activity and MDA in each group ($n=4$). **H** The expression of NF-κB axis in each group ($n=4$). **I** The mRNA level of inflammatory factor in every group ($n=4$). $^{**}p<0.01$ vs. AAV9-GFP sham, $^{\#}p<0.05$ vs. AAV9-GFP TAC, $^{\#\#}p<0.01$ vs. AAV9-GFP TAC

stress and inflammation in pressure overload-induced cardiac hypertrophy.

TMEM100 directly combines with TAK1 and inhibits its phosphorylation in vitro

The phosphorylation of MAPKs was further detected in PE-induced cardiomyocyte hypertrophy. TMEM100 overexpression inhibited the phosphorylation of the TAK1-JNK/p38 axis, whereas TMEM100 knockdown activated the TAK1-JNK/p38 signaling pathway (Fig. 7A-B). Consistent with the in vivo results, TMEM100 overexpression increased SOD enzyme activity, whereas TMEM100 knockdown inhibited SOD activity (Fig. 7C-D). Next, we demonstrated that TMEM100 could directly bind to TAK1 via a GST pulldown assay (Fig. 7E-F). We constructed truncated forms of TMEM100 and TAK1 and then performed IP mapping to search for the exact domains of TMEM100 and TAK1 that interact. The results revealed that amino acids from at least 53–75 and 85–107 of TMEM100 and from 1 to 300 of TAK1 were required for its interaction (Fig. 7G-H). Is a direct interaction between TMEM100 and TAK1 necessary for TMEM100 to inhibit the phosphorylation of TAK1? A TMEM100-truncated adenovirus (AdTMEM100 mutant) lacking the TAK1-binding domain was constructed. The results revealed that the AdTMEM100 mutant (AdTMEM100M) failed to inhibit the phosphorylation of the TAK1-JNK/p38 axis (Fig. 7I). These results showed that TMEM100 could directly interact with TAK1 and inhibit the phosphorylation of the TAK1-JNK/p38 pathway.

TAK1 was necessary for the regulatory effects of TMEM100 on cardiac hypertrophy

An inhibitor of TAK1 (iTAK1, 5Z-7-ox) was used to identify whether the phosphorylation of TAK1 is required for TMEM100 to suppress cardiac hypertrophy. After PE stimulation, TMEM100 knockdown increased the phosphorylation and activation of the TAK1 pathway, whereas iTAK1 significantly suppressed TAK1 activation (Fig. 8A). As shown in Fig. 8B, iTAK1 and TMEM100 knockdown had no effect on cardiomyocyte hypertrophy in the PBS group. In the PE group, TMEM100 knockdown aggravated cardiomyocyte hypertrophy, whereas the application of iTAK1 blocked the harmful effect of TMEM100 knockdown on cardiomyocyte hypertrophy (Fig. 8B). Consistent with the results of the immunofluorescence staining, treatment with iTAK1 reversed the effect of TMEM100 on the cardiac hypertrophic markers, *Anp*, *Bnp* and *Myh7* (Fig. 8C).

Discussion

Although numerous studies have been conducted on HF and cardiac hypertrophy, the morbidity and mortality of HF remain high [1]. Therefore, new therapeutic targets for treating HF and cardiac hypertrophy are urgently needed. In this study, we reported some new findings demonstrating the function of TMEM100 in pathological cardiac hypertrophy. First, several GEO databases and cardiac hypertrophic models revealed that TMEM100 was upregulated in patients with cardiac hypertrophy. Next, we determined that TMEM100 suppressed cardiac hypertrophy and oxidative stress through gain- and loss-of-function experiments. Regarding the mechanism by which TMEM100 regulates cardiac hypertrophy, the RNA-seq results suggested that the MAPK pathway was associated with TMEM100-mediated cardiac hypertrophy. Furthermore, these findings were verified by western blot in vivo *and* in vitro. Molecular experiments revealed that TMEM100 (amino acids 53–75 and 85–107) directly combine with TAK1 (amino acids 1–300), and TMEM100 lacking the binding domain fails to inhibit phosphorylation of the TAK1-JNK/p38 axis. Finally, we demonstrated that the phosphorylation of TAK1 via iTAK1 was required for the suppression of cardiac hypertrophy by TMEM100.

Some TMEM family members have been reported to play a role in cardiac hypertrophy. Downregulated TMEM43 activates the NF-κB pathway and exacerbates HF [16]. Other studies have shown that TMEM43 is involved in arrhythmogenic right ventricular cardiomyopathy and cardiotoxicity [17, 18]. Yang et al. reported that TMEM117 deficiency suppresses endoplasmic reticulum stress and oxidative stress in cardiac hypertrophy [19]. These studies on TMEM family proteins and cardiac hypertrophy suggest a significant role for TMEM family members in cardiovascular disorders. Previous studies have shown that TMEM100 is involved in many diseases. For example, it has been reported that TMEM100 can

(See figure on next page.)

Fig. 7 TMEM100 directly bound to TAK1 and inhibited the phosphorylation of TAK1 axis in vitro. **A**, **B** The total and phosphorylated expression of TAK1 axis in NRCMs treated with indicated adenovirus. **C**, **D** SOD enzyme activity in TMEM100 overexpression and knockdown group. **E**, **F** The direct interaction between TMEM100 and TAK1. **G**, **H** Schematic diagram and IM-mapping results showing the domains of TMEM100 and TAK1. **I** The total and phosphorylated expression of TAK1 axis in NRCMs treated with AdVector, AdTMEM100 and AdTMEM100 mutant (TMEM100 M) adenovirus. For C-D, $n = 4$ independent experiments. For others, $n = 3$ independent experiments. $^{*}p < 0.05$ vs. AdshRNA PBS, $^{**}p < 0.01$ vs. AdVector PBS, $^{\#}p < 0.05$ vs. AdVector PE, $^{\#\#}p < 0.01$ vs. AdshRNA PE

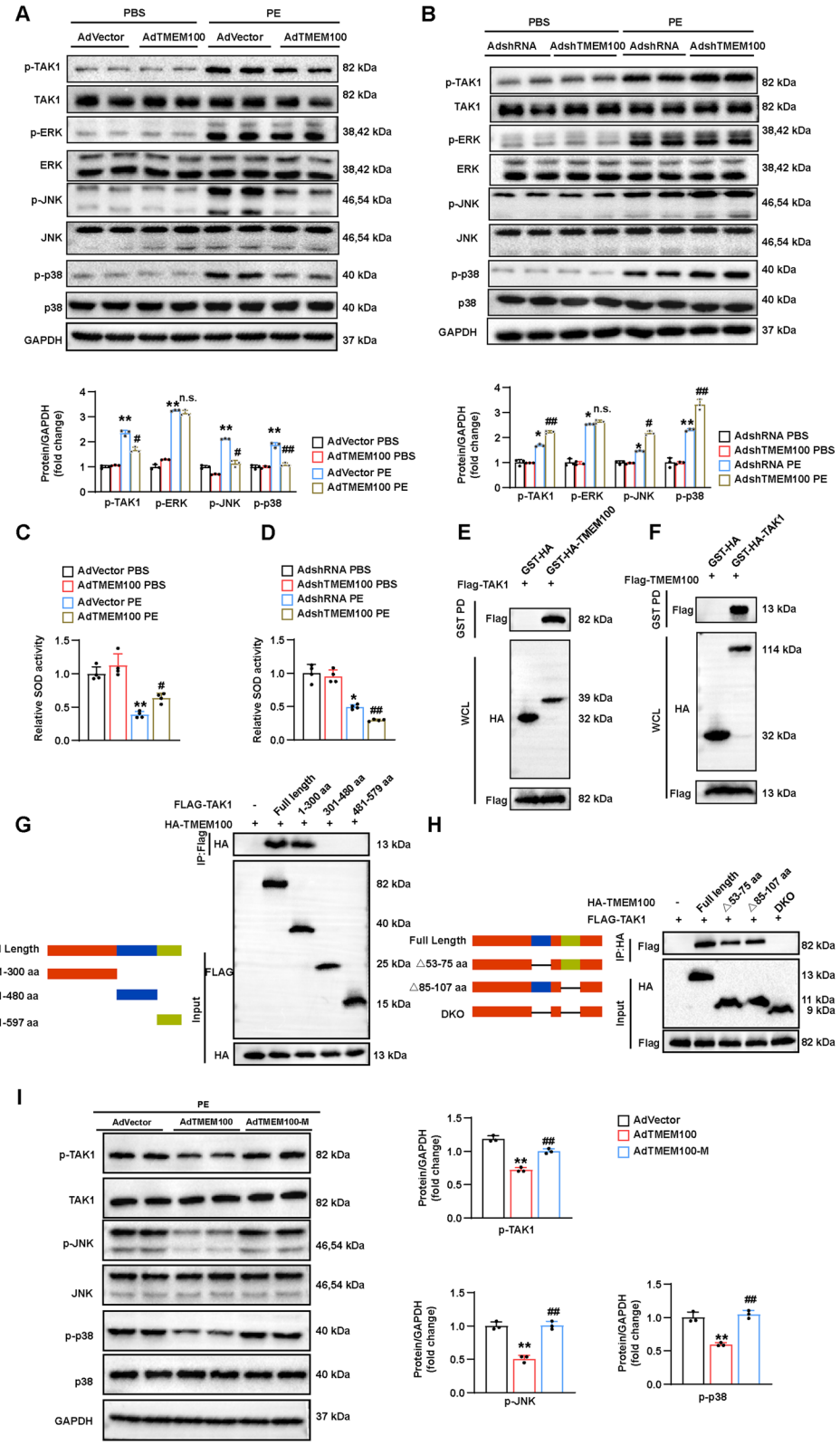

Fig. 7 (See legend on previous page.)

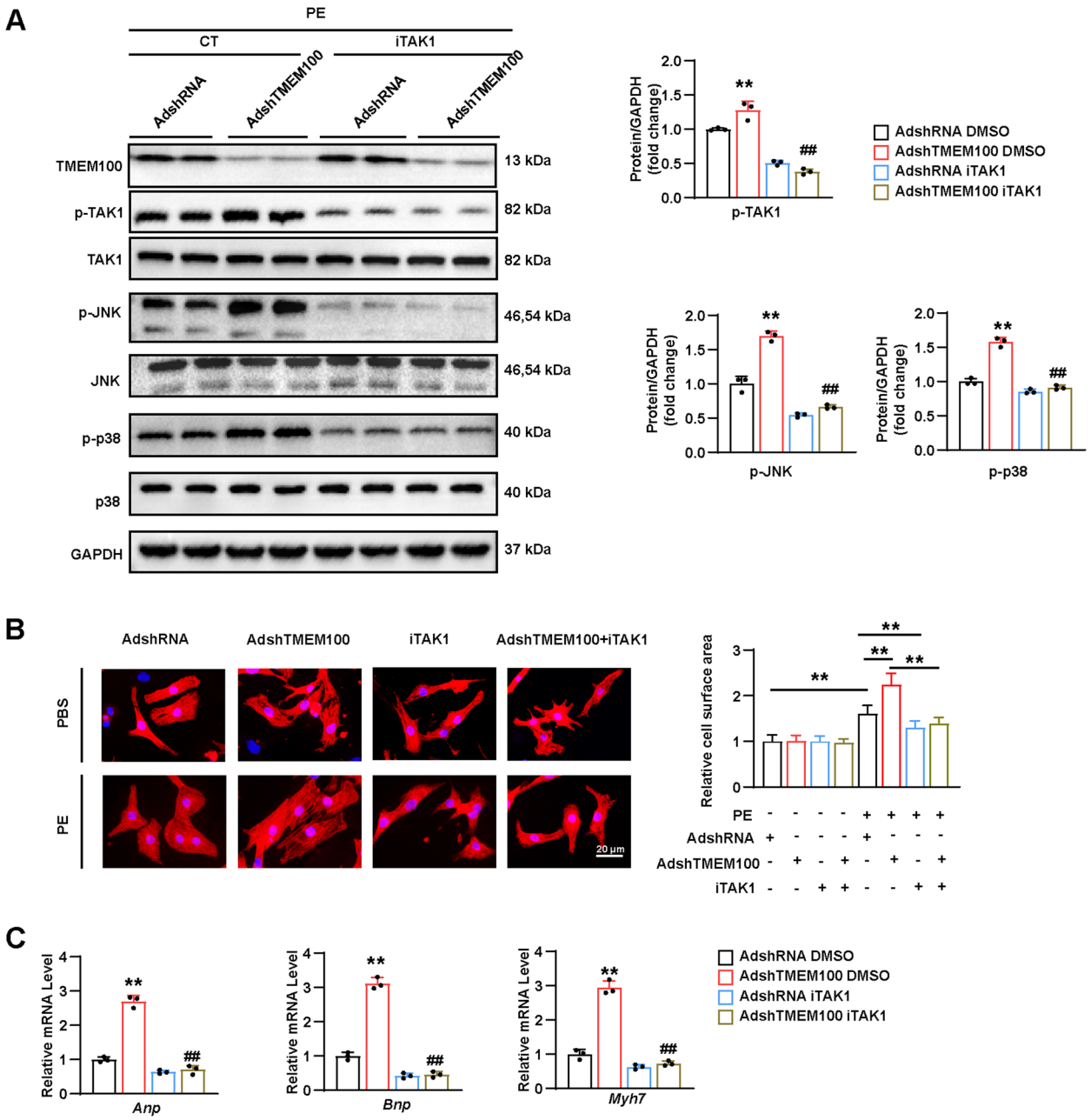

Fig. 8 TAK1 was necessary for TMEM100-mediated cardiac hypertrophy. **A** The protein expression of TAK1-JNK/p38 in NRCMS in indicated group treated with iTAK1 or DMSO after PE stimulation. **B** Representative images of NRCMs cross-sectional-area in every group (n = at least 30 cells). **C** The mRNA level of cardiac hypertrophic markers (*Anp*, *Bnp* and *Myh7*). $n = 3$ independent experiments. $^{**}p < 0.01$ vs. AdshRNA DMSO, $^{\#\#}p < 0.01$ vs. AdshTMEM100 DMSO

alleviate pain and pruritus [10, 20]. TMEM100 also acts as a tumor suppressor gene in lung, liver, stomach, prostate and breast cancer [11, 21–24]. However, its role in pathological cardiac hypertrophy remains unknown. In this study, we screened the differentially expressed genes from the GEO database and identified the upregulation of TMEM100 in cardiac hypertrophy. We further demonstrated that TMEM100 served as a protective factor in TAC-induced cardiac hypertrophy and PE-induced cardiomyocyte hypertrophy. Oxidative stress is known to be involved in the process of cardiac hypertrophy [25, 26]. Our study is the first to reveal that TMEM100 serves as an antioxidative stress molecule in cardiac hypertrophy. However, the mechanism by which TMEM100 functions

in the response to antioxidant stress may require further exploration in future work.

The MAPK cascade is the classic signaling pathway that is associated with cardiac hypertrophy. Luo et al. reported that E26 transformation-specific sequence 2 (ETS2) combines with MAPK phosphatase 3 (MKP3) to form a feedback loop with ERK, modulating cardiac hypertrophy [27]. The E3 ubiquitin ligase RNF207 exacerbates cardiac hypertrophy by promoting the K63-linked ubiquitination of TAK1-binding protein 1 (TAB1), which triggers the activation of the TAK1-JNK/p38 axis [28]. Our RNA sequence study revealed the potential involvement of MAPK in the process of cardiac hypertrophy. We found that TMEM100 could bind to both ASK1 and TAK1, which are significant upstream molecules of MAPK [29]. We subsequently revealed that TMEM100 inhibited the phosphorylation of TAK1 but not of ASK1. TAK1 is a classic molecule of the MAPK and NF-κB pathways; it can be activated in response to various pathological stimuli and is involved in biological processes such as inflammation, cell death and oxidative stress [30]. Interestingly, we observed that the overexpression of TMEM100 mitigated the inflammatory response in mouse hearts.

TAK1 contains a kinase domain, a TAB1 binding domain and a TAB2/3 binding domain, all of which are essential for the phosphorylation and activation of TAK1 [30]. How does TMEM100 modulate the phosphorylation of TAK1? The results of the GST pulldown assay revealed that TMEM100 directly interacted with TAK1. IP mapping revealed that the transmembrane domain of TMEM100 and the N-terminal 1–300 amino acids of TAK1 were essential for its interaction. We constructed AdTMEM100M and demonstrated that the dissociation of TMEM100 from TAK1 failed to inhibit the phosphorylation of TAK1. 5Z-7-ox is a selective inhibitor of TAK1 [31]. We demonstrated that TAK1 was necessary for TMEM100-mediated cardiac hypertrophy via iTAK1. On the basis of the above results, we concluded that TMEM100 directly interacted with TAK1 and inhibited the phosphorylation of the TAK1-JNK/p38 axis in cardiac hypertrophy.

Conclusions

In summary, TMEM100 is upregulated in response to cardiac hypertrophy. Subsequent functional experiments revealed that TMEM100 overexpression alleviated oxidative stress and cardiac hypertrophy, whereas the opposite results were obtained in the TMEM100-knockdown group. Finally, molecular investigations revealed that TMEM100 modulates cardiac hypertrophy by directly binding to TAK1 and inhibiting the phosphorylation of the TAK1-JNK/p38 axis. Therefore, targeting TMEM100 may become a novel strategy for the treatment of cardiac hypertrophy.

Abbreviations

AAV9	Adeno-associated virus 9
ASK1	Apoptosis signal-regulating kinase 1
DEGs	Differentially expressed genes
ERK	Extracellular regulated protein kinases
EF	Ejection fraction
FS	Fraction **s**hortening
GO	Gene ontology
GSEA	Gene set enrichment analysis
HF	Heart failure
iTAK1	TAK1 inhibitor
JNK	JUN N-terminal kinases
KEGG	Kyoto Encyclopedia of Genes and Genomes
KOG	EuKaryotic Orthologous Groups
LV	Left ventricular
MDA	Malondialdehyde
NRCMs	Neonatal rat cardiomyocytes
PE	Phenylephrine
SOD	Superoxide dismutase
TMEM100	Transmembrane protein 100
TAC	Transverse aortic constriction
TAK1	Transforming growth factor-β (TGF-β)-activated kinase 1

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12964-024-01816-2.

Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions

X.T.Z. and Z.Y.H. designed and supervised the study and revised the manuscript; B.B.Z., S.G., Y.L.Z., Y.Y.L., F.Y., Y.G.W and G.L. performed the experiments; X.Y.Y., J.H.S., H.Y.L, H.X.Z and W.C.Z. analyzed the experimental data.

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Availability of data and materials

The datasets during the present study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Animal experiments are carried out according to the Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committee of the First Affiliated Hospital of Zhengzhou University.
Not applicable.

Consent for publication

All of the authors participated in the study and have agreed to the content of the manuscript.

Competing interests

The authors declare no competing interests.

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