

Reduced expression of transmembrane protein 43 during cardiac hypertrophy leading to deteriorating heart failure in mice

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Research Article

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Abstract

Transmembrane protein 43 (TMEM43), a member of the transmembrane protein subfamily, was found to be associated with arrhythmogenic right ventricular cardiomyopathy. But its role in cardiac hypertrophy have not been elucidated. Here we used a pressure over load induced cardiac hypertrophy model to explore the role of TMEM43 on heart failure. Mice were subjected to aortic banding (AB) to induce cardiac hypertrophy. Mice were also randomly received injection of adeno-associated virus 9 (AAV9)-shTMEM43 or control AAV9 (ScRNA) to knockdown TMEM43 in cardiomyocytes. Four weeks after AB, mice were subjected to echocardiography to evaluate cardiac function. Neonatal rat cardiomyocytes (NRCMs) were stimulated with phenylephrine (PE) and transfected with adenovirus to overexpress TMEM43. As a result, we found that TMEM43 was down-regulated in mice heart and cardiomyocytes post stimulus. Mice with TMEM43 knockdown showed deteriorated heart failure 4 weeks post AB with deteriorating cardiac function and aggregated cardiac hypertrophy and fibrosis. While, NRCMs with TMEM43 overexpression revealed ameliorated hypertrophy response. Moreover, we found that TMEM43 deficiency increased NF- κ B activation in mice heart post AB, while TMEM43 overexpression reduced NF- κ B activation in cardiomyocytes upon PE stimuli. Thus, we conclude that reduced expression of TMEM43 during cardiac hypertrophy leading to deteriorating heart failure in mice.

Introduction

Heart failure is a world pandemic that affects about 1–2% of the global adult population^[1]. It is a systemic disease and the final stage of the disease after heart injury^[2]. Cardiac hypertrophy is the early pathological process of heart failure. In this process, the disorder of neurohumoral, the regulation between cardiac cells, and the activation of molecules in cardiac cells will lead to cardiac volume overload, increased sympathetic activity, circulatory redistribution, and the clinical signs and symptoms of cardiac exhaustion^[3]. At present, it has been confirmed that a variety of inflammatory signaling pathways play an important role in the process of myocardial hypertrophy^[4]. Among them, NF- κ B pathway is activated during the development of various cardiovascular diseases into heart failure, leading to myocarditis reaction, promoting myocardial injury and accelerating the progress of heart failure^[5]. Therefore, targeting NF- κ B pathway is expected to find a new treatment for heart failure.

Transmembrane protein 43 (TMEM43), a member of the transmembrane protein subfamily, was initially found to promote cancer progression in many cancer cells^[6, 7]. In human pancreatic cancer, the expression of TMEM43 is increased and TMEM43 was associated with bad overall survival^[7]. TMEM43 was found to be a crucial part of epidermal growth factor receptor signaling to induce NF- κ B activation thus promotes cancer cells proliferation, survival, migration and epithelial-to-mesenchymal transition^[8]. Recently TMEM43 was found to be associated with arrhythmogenic right ventricular cardiomyopathy. Haywood AF et al found that TMEM43 missense mutation cause arrhythmogenic cardiomyopathy in UK and Canada^[9]. Zheng G also found that TMEM43-S358L mutation leading to increased activation of NF- κ B signaling in heart tissues^[10]. Thus we take hypothesis that TMEM43 may play a role on cardiac

hypertrophy and heart failure. Here in this study, we used an aortic banding model to induce cardiac hypertrophy and heart failure model in mice to explore the role and mechanism of TMEM43 on heart failure.

Methods

Animals and animal mode

C57BL6J were came from the and raised at the SPF Laboratory Animal Center of Nanjing Medical University. All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (Nanjing, China).

For mice aortic banding (AB) model, mice (aged 8-10 week, 23.5-27.5mg) were subjected to aortic banding as previous study described^[11]. Briefly, mice were anesthetized with sodium pentobarbital, and the mice were placed in the left lateral decubitus position after skin preparation. The mouse skin was cut, and the mouse aorta was exposed at the 3rd to 4th costal margins. The aorta was ligated with a 27G needle. After the ligation was successful, the needle was removed. The mice in the sham operation group were only hung up without ligation. The mouse skin was sutured layer by layer, and analgesics were administered three days after the operation. To knockdown TMEM43, mice were injected with adeno-associated virus 9 (AAV9)-shTMEM43 (from Vigene Bio-tek, Shanghai, China) or the control AAV9 (ScRNA) one week before AB surgery.

Adeno-Associated Virus Vector

Recombinant AAV9-shTMEM43 and the control AAV9-scRNA were constructed by Vigene Bioscience Company (Shanghai, China) with a short, small Troponin T (TnT) promoter was used to induce cardiomyocytes specific gene delivery[12]. A total of 60–80 ml of AAV9-shTMEM43 or AAV9-scRNA ($5.0\text{--}6.5 \times 10^{13}$ VG/ml) was injected into the mice via tail vein 1 week before AB surgery.

Echocardiographic evaluation

Transthoracic echocardiography was performed as previously described^[13, 14]. Isoflurane (1.5%) was used to anaesthetize the mice, and echocardiography was performed with a 10-MHz linear-array ultrasound transducer to obtain M-mode echocardiography data. The left ventricle (LV) end-diastolic dimension (LVIDd) and LV end-systolic dimension (LVISd) were obtained, and the LV ejection fraction (LVEF) and LV fractional shortening (LVFS) values were calculated. A total of 10 mice from each group were subjected to transthoracic echocardiography.

Hematoxylin & eosin (HE), PSR staining

Hematoxylin & eosin (HE) staining was used to valuate cross section area as previous described^[15]. Image-Pro Plus 6.0 was used to analyze 10 sections from each heart and 6 hearts from each group. PSR

staining was used to show collagen volume. For the fibrosis area calculation, Image-Pro Plus 6.0 was used to analyze 6 sections from each heart and 6 hearts from each group.

Cardiomyocyte isolation and culture

Neonatal rat cardiomyocyte (NRCM) culture was performed as previously described^[13, 14]. Briefly, the hearts of Sprague-Dawley rats (1-3 days old) were quickly removed, and ventricles were preserved and digested with 0.125% trypsin-EDTA (Gibco) 4 times for 15 min each time. Digestion was halted with DMEM-F12 supplemented with 15% fetal bovine serum (FBS, Gibco, USA). After 5 digestion reactions, the cells were collected and incubated in a 100-mm dish with DMEM-F12 supplemented with 15% FBS. After 90 minutes, the cell culture medium was collected, and NRCMs in the upper layer of the cell medium were removed and seeded onto a 6-well plate to exclude the non-cardiac myocytes adhered to the bottom of the 100-mm dish. NRCMs were identified by α -actin staining.

Cells were transfected with adenovirus (Ad-) to overexpress TMEM43 (Ad-TMEM43, MOI = 50, Vigene Bioscience, Jinan China). Then, the cells were stimulated with 50 μ M phenylephrine (PE) for 48h to induce cardiomyocytes hypertrophy response.

Western blot and qPCR

Total protein was isolated from heart tissues then subjected to SDS-PAGE (50 μ g per sample). After transfer onto Immobilon membranes (Millipore, Billerica, MA, USA), proteins were incubated overnight at 4°C with primary antibodies against total (T) NF- κ B and phosphorylated (P-) NF- κ B purchased from Abcam (1:1000 dilution), and GAPDH (1:1000 dilution) purchased from (Cell Signaling Technology (1:1000 dilution). Blots were developed with enhanced chemiluminescence (ECL) reagents (Bio-Rad, Hercules, CA, USA) and captured by a ChemiDoc MP Imaging System (Bio-Rad). GAPDH served as an internal reference protein.

Total RNA (2 μ g per sample) from frozen mouse heart tissue and cardiomyocytes was reverse transcribed into cDNA using the oligonucleotide (DT) primer and the transcript first strand cDNA synthesis kit (Roche). Then, a light Cyclor 480 instrument (software version 1.5, Roche) and the SYBR green PCR master mix (Roche) was used to perform RT-PCR. All genes were normalized using GAPDH.

Immunofluorescence staining

The cells were seeded on the cell slides, and the treated cells were fixed with 4% formalin, permeabilized with 0.2% Triton X-100, blocked with 8% goat serum, and then given the corresponding primary antibody, such as α -actin or P-NF- κ B, purchased from Abcam (1:100 dilution). Cells were subsequently enriched with fluorescent secondary antibodies, and nuclei were stained with DAPI. Then photographed with a fluorescence microscope (Olympus DX51, Tokyo, Japan).

Statistical analysis

All data are expressed as the mean \pm SD. Differences among groups were analyzed by two-way analysis of variance followed by Tukey's post hoc test. Comparisons between two groups were analyzed by an unpaired Student's t-test. P values less than 0.05 indicated statistical significance.

Results

The expression level of TMEM43 in heart and cardiomyocytes

We explore the expression level of TMEM43 during the process of cardiac hypertrophy. Four weeks post AB, we found that protein level of TMEM43 was down-regulated in heart tissue when compared with sham mice hearts (Figure 1A). We also detected the protein level of TMEM43 in cardiomyocytes stimulated with PE. A decreased expression level of NEK6 was observed in cardiomyocytes exposed to PE (Figure 1B).

TMEM43 knockout deteriorates cardiac hypertrophy

We knockdown TMEM43 level by using AAV9-shTMEM43 with a TnT promoter. As shown in figure 2A, the expression level of TMEM43 was reduced in mice with AAV9-shTMEM43 injection in both sham and AB group when compared with the corresponding ScRNA group. Four weeks after AB, hearts were removed, as shown in figure 2B and 2C, heart weight to body weight ratio (HW/BW), HW to tibia length (TL) were increased, lung weight to body weight ratio (LW/BW) and LW to TL ratio were also increased in AB group; TMEM43 knockout increased HW/BW, HW/ TL, LW/BW and LW/TL ratios when compared with mice in ScRNA-AB group. H&E staining was used to detect cross section area (CSA) about cardiomyocytes. As shown in figure 2D, CSA was increase in AB mice, while TMEM43 deficiency increased this change in AB mice. Cardiac collagen volume was evaluated by PSR staining, AB induced enhanced left ventricular (LV) collagen volume, while TMEM43 deficiency deteriorated collagen deposition (Figure 2E). We also detected transcription level of those heart failure markers: atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), which was proved to further increased in TMEM43 knockout mice heart post AB (Figure 2F).

TMEM43 deficiency aggressive AB induced heart failure

Cardiac function was assessed by echocardiography, 4 weeks after AB, heart rate showed no significant difference among four groups (Figure 3A). However, LV end diastolic diameter (LVIDd), and systolic diameter (LVISd) were increased in two AB groups; systolic internal septal thickness (IVSd) and systolic left ventricular posterior wall thickness (LVPWd) were also increased in AB group. LV ejection fraction (LVEF) and fractional shortening (LVFS) were decreased when compared with the control group. These suggested our heart failure model was successfully established with impaired cardiac systolic and diastolic function. TMEM43 knockout induced increased LVIDd, LVISd, thicker IVSd, LVPWd and dropped LVEF, LVFS, indicating deteriorating cardiac dysfunction (Figure 3B-D).

TMEM43 affects HSP72 phosphorylation

Previous study has found that TMEM43 mutation leading to increased activation of NF- κ B signaling in heart tissues^[10]. We assessed the level of NF- κ B in failure heart tissue as well as cardiomyocytes. As shown in figure 4A, the total level of NF- κ B was unchanged in the two AB mice heart. However, the phosphorylated NF- κ B was up-regulated in AB mice hearts, while TMEM43 knockout further increase the phosphorylation of NF- κ B (Figure 4A and B). We also detected the down-stream inflammation response in heart tissue, as NF- κ B is a crucial inflammatory signaling. As expected, the mRNA level of tumor necrosis factor α (TNF α), interleukin-1 (IL-1), IL-6 was up-regulated in AB hearts while further increased in TMEM43 knockout mice hearts (Figure 4C). These data indicate that TMEM43 deficiency promotes the activation of NF- κ B in failure mice heart.

TMEM43 overexpressing protects cardiomyocytes against PE induced hypertrophy

To confirm the protective effect of TMEM43 on hearts. Cardiomyocytes were transfected with Ad-TMEM43 to overexpress TMEM43 (Figure 5A). Cells were exposed to PE to establish cardiomyocytes failing model. PE induced a remarkable hypertrophy response as evidenced by increased cell surface area and increased transcription of ANP and BNP (Figure 5B-C). TMEM43 overexpress caused ameliorated hypertrophy response (Figure 5B-C). Moreover, cells with Ad-TMEM43 revealed reduced expression level of NF- κ B activation and nuclear translocation (Figure 5D) These data indicate that by targeting NF- κ B, TMEM43 exert protection effects in cardiomyocytes.

Discussion

Transmembrane protein 43 (TMEM43) is a member of the TMEM subfamily. This gene is highly conserved and expressed in most species, including bacteria, animals and humans^[16]. TMEM43 protein is an endoplasmic reticulum membrane protein. It was found that TMEM43 gene mutation can cause right ventricular arrhythmia cardiomyopathy^[17]. However, its role in heart failure has not been reported. This study found for the first time that TMEM43 was down-regulated in the development of heart failure - cardiac hypertrophy stage. Deletion of TMEM43 in cardiomyocytes aggravated cardiac hypertrophy and fibrosis induced by pressure overload and deteriorated cardiac dysfunction. However, overexpression of TMEM43 protein in cardiomyocytes delayed PE induced cardiomyocyte hypertrophy response. This suggests that in the process of heart failure, the reduced expression of TMEM43 may accelerate the pathological progress of heart failure.

Inflammation is a biological response of the immune system that can be triggered by a variety of factors, including pathogens, damaged cells and toxic compounds^[18]. Aseptic inflammation plays an important role in the pathological process of a variety of cardiovascular diseases developing into heart failure^[19, 20]. After myocardial injury, the exposed organelles and nuclei of cardiomyocytes reach the danger-associated molecular patterns (DAMPs) on the surface of inflammatory cells or cardiomyocyte membrane, such as toll like receptors (TLRs)^[18, 21]. These receptors activate the downstream classical inflammatory pathway, NF- κ B signaling. NF- κ B transcription factors play an important role in inflammation, immune response, survival and apoptosis^[18]. Under physiological conditions, NF- κ B stays

in the cytoplasm with I κ B protein, which inhibits NF- κ B activation^[18]. TLR activates NF- κ B by phosphorylating NF- κ B. Subsequently, NF- κ B transfers into the nucleus to promote the transcription of inflammation related genes. This signaling causes an inflammatory cascade^[3, 18]. Studies have confirmed that activation of NF- κ B pathway plays a leading role in heart failure induced by various etiologies^[19, 20]. Previous study found that haploinsufficiency of TMEM43 in cardiomyocytes leading to increased DNA damage, caused activation of smad pathway and cardiac fibrosis^[22]. Moreover, arrhythmogenic right ventricular cardiomyopathy patient with TMEM43 mutation was reported to be associated with a high risk of sudden cardiac death^[17]. Takemoto Y also reported that TMEM43 was associated with TGF β signaling, which leading to fibrosis^[23]. Here, we found that TMEM43 deficiency induced more activated NF- κ B signaling and TMEM43 overexpress caused reduced activated NF- κ B in cardiomyocytes. Our founding was consistent with Zheng G's study, which found that during arrhythmogenic right ventricular cardiomyopathy, TMEM43-S358L mutation caused enhanced NF- κ B signaling, leading to TGF β activation^[10]. Thus, by targeting NF- κ B signaling, TMEM43 promotes the progress of heart failure.

In conclusion, our study for the first time found that TMEM43 was down-regulated in cardiac hypertrophy in mice heart and cardiomyocytes. reduced expression of TMEM43 accelerates the progress of heart failure in mice. TMEM43 may increase the activation of NF- κ B signaling in heart failure which leading to inflammatory cascade.

Declarations

Funding sources

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Figures

Figure 1

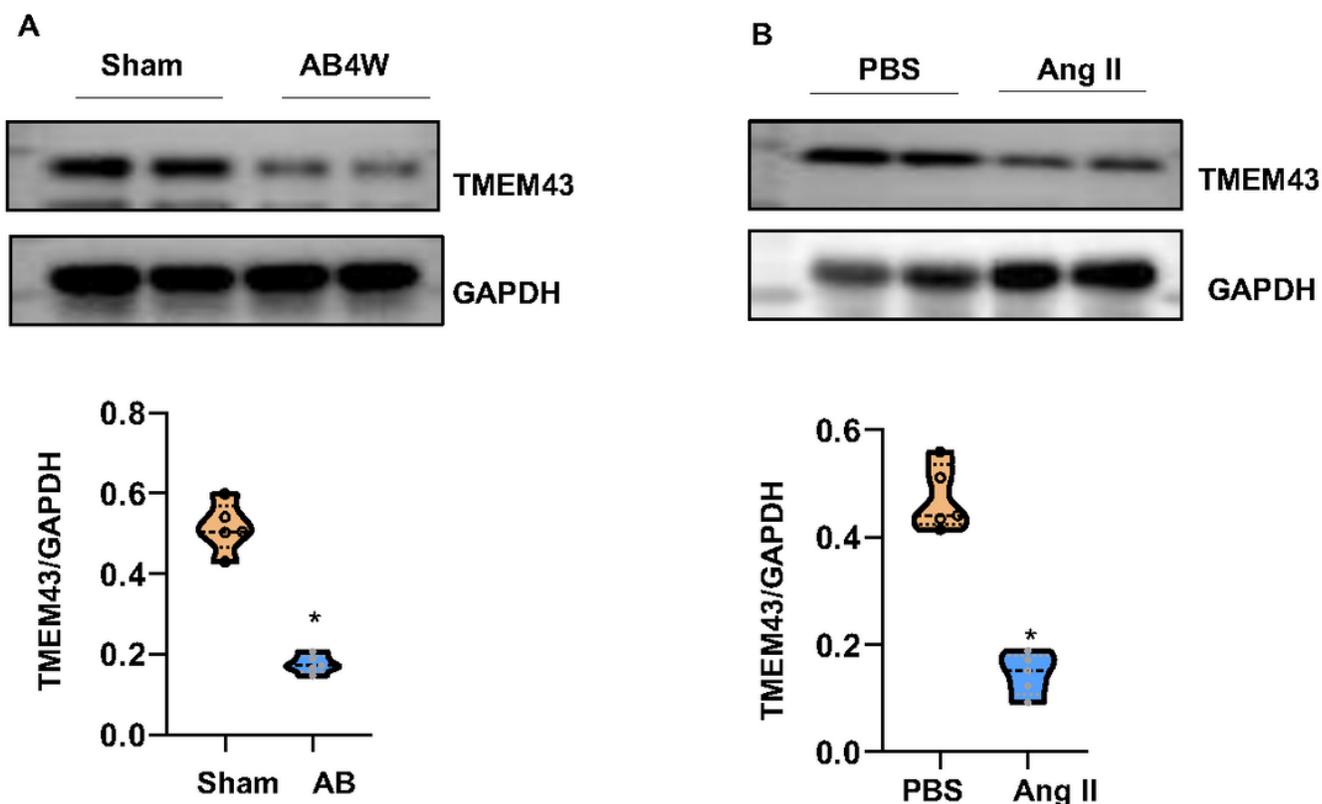


Figure 1

The expression level of TMEM43 in heart and cardiomyocytes

A. Protein level of TMEM43 in mice heart 4 weeks post aortic banding (AB) (n=5).

B. Protein level of TMEM43 in cardiomyocytes stimulated with PE (n=5).

* $P < 0.05$ vs. sham/PBS group

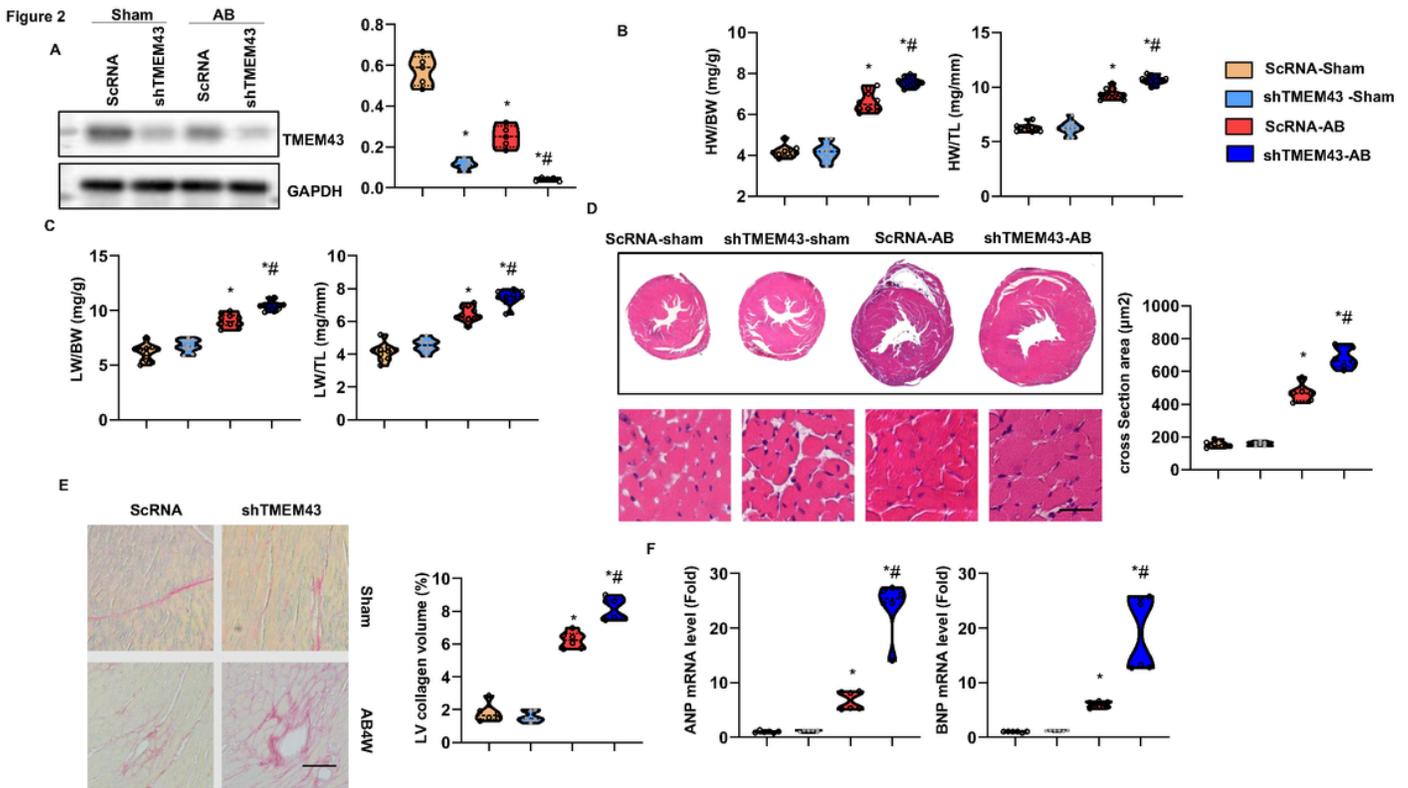


Figure 2

TMEM43 knockout deteriorates cardiac hypertrophy

A. Protein level of TMEM43 in mice heart 4 weeks post aortic banding (AB) (n=5).

B and C. heart weight to body weight ratio (HW/BW), HW to tibia length (TL) ratio (HW/TL), lung weight to body weight ratio (LW/BW) and LW to TL ratio (LW/TL) in mice heart (n=10). D. Image of H&E staining and quantified cross section area in mice heart (n=6). E. Image of PSR staining and quantified LV collagen volume in mice heart (n=6). F. Transcription level of ANP and BNP in mice heart (n=6). * $P < 0.05$ vs. ScRNA-sham group; # $P < 0.05$ vs. ScRNA-AB group.

Figure 3

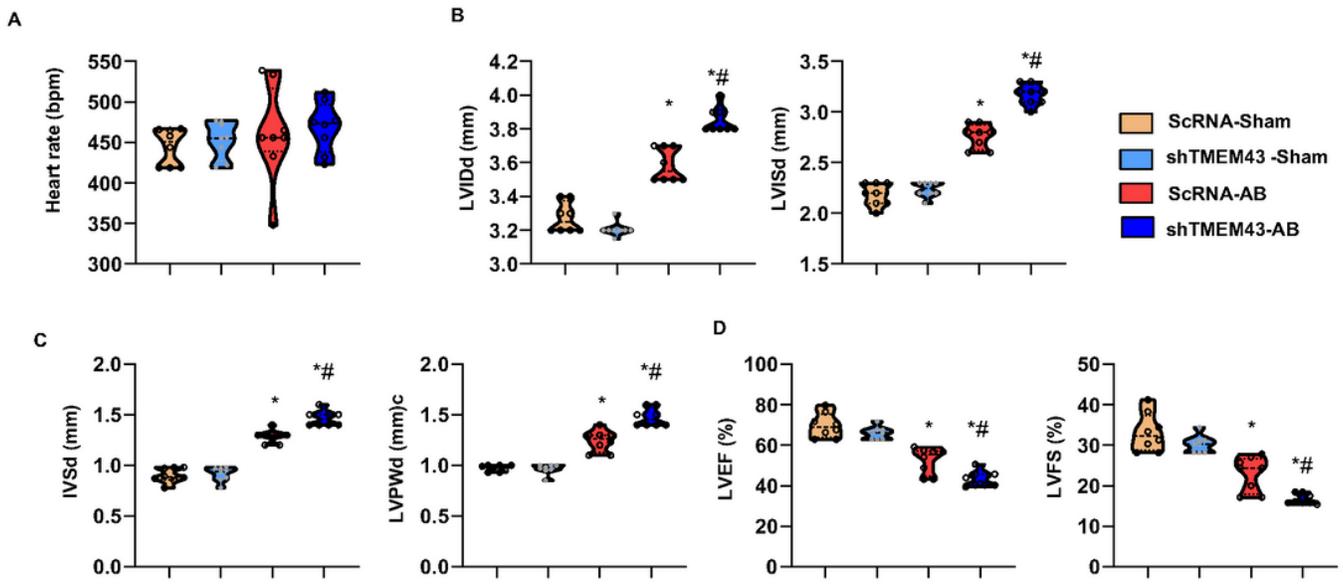


Figure 3

TMEM43 deficiency aggressive AB induced heart failure

A-D. Echocardiography results in mice heart 4 weeks post aortic banding (AB) (n=8).

* $P < 0.05$ vs. ScRNA-sham group; # $P < 0.05$ vs. ScRNA-AB group.

Figure 4

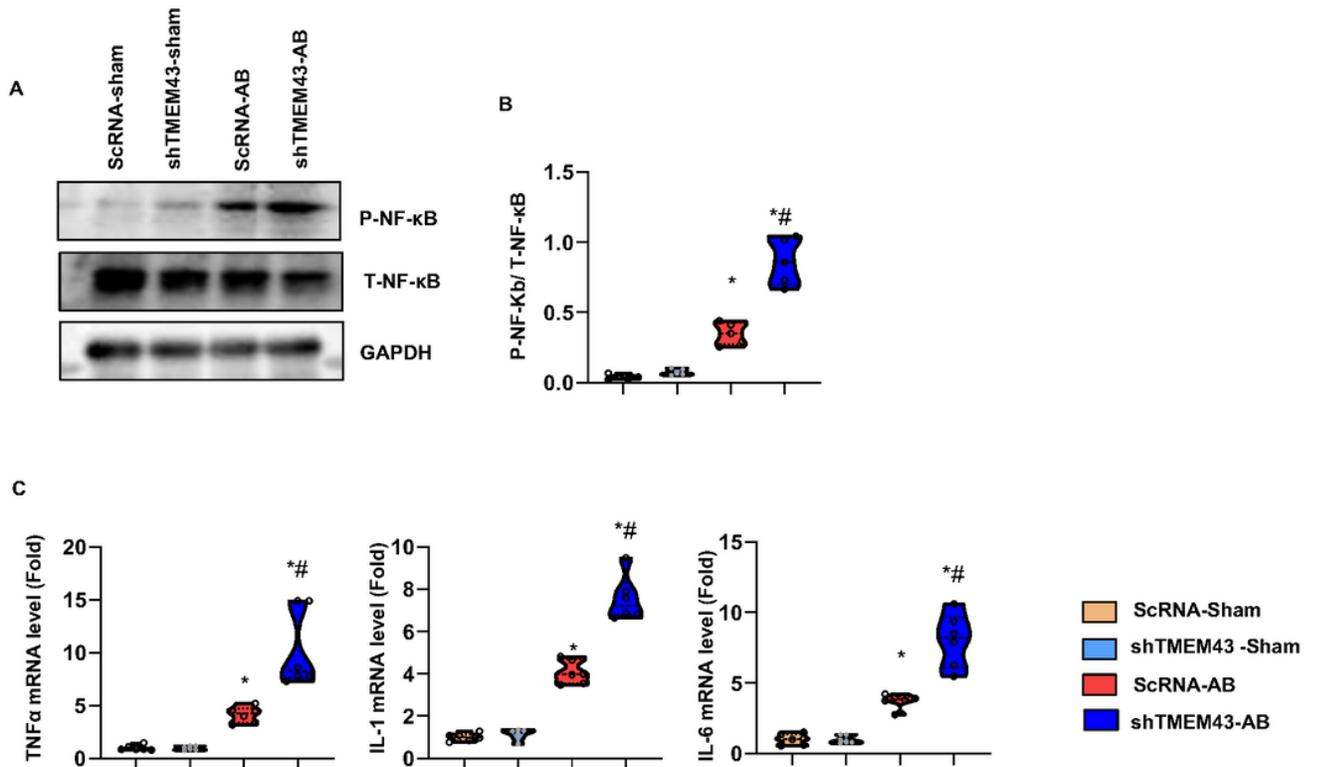


Figure 4

TMEM43 affects HSP72 phosphorylation

A and B. Protein level of total NF- κ B and phosphorylated NF- κ B in mice heart 4 weeks post aortic banding (AB) (n=5). C. Transcription level of TNF α , IL-1 and IL-6 in mice heart (n=6). * $P < 0.05$ vs. ScrNA-sham group; # $P < 0.05$ vs. ScrNA-AB group.

Figure 5

TMEM43 overexpressing protects cardiomyocytes against PE induced hypertrophy

A. Protein level of TMEM43 in cardiomyocytes transfected with Ad-TMEM43 (n=5). B. Image of α -actin staining and quantified cell section area in cardiomyocytes treated with PE (n=6). C. Transcription level of ANP and BNP in cardiomyocytes treated with PE (n=6). D. Image of phosphorylated NF- κ B staining. * $P < 0.05$ vs. Ad-NC-PBS group; # $P < 0.05$ vs. Ad-NC-PE group.