

Transforming Growth Factor- β Receptor III is a Potential Regulator of Ischemia-Induced Cardiomyocyte Apoptosis

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Background—Myocardial infarction (MI) is often accompanied by cardiomyocyte apoptosis, which decreases heart function and leads to an increased risk of heart failure. The aim of this study was to examine the effects of transforming growth factor- β receptor III (TGF β R3) on cardiomyocyte apoptosis during MI.

Methods and Results—An MI mouse model was established by left anterior descending coronary artery ligation. Cell viability, apoptosis, TGF β R3, and mitogen-activated protein kinase signaling were assessed by methylthiazolyldiphenyl-tetrazolium bromide assay, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay, immunofluorescence, electron microscopy, and Western blotting. Our results demonstrated that TGF β R3 expression in the border region of the heart was dynamically changed during MI. After stimulation with H₂O₂, TGF β R3 overexpression in cardiomyocytes led to increased cell apoptosis and activation of p38 signaling, whereas TGF β R3 knockdown had the opposite effect. ERK1/2 and JNK1/2 signaling was not altered by TGF β R3 modulation, and p38 inhibitor (SB203580) reduced the effect of TGF β R3 on apoptosis, suggesting that p38 has a nonredundant function in activating apoptosis. Consistent with the *in vitro* observations, cardiac TGF β R3 transgenic mice showed augmented cardiomyocyte apoptosis, enlarged infarct size, increased injury, and enhanced p38 signaling upon MI. Conversely, cardiac loss of function of TGF β R3 by adeno-associated viral vector serotype 9–TGF β R3 short hairpin RNA attenuated the effects of MI in mice.

Conclusions—TGF β R3 promotes apoptosis of cardiomyocytes via a p38 pathway-associated mechanism, and loss of TGF β R3 reduces MI injury, which suggests that TGF β R3 may serve as a novel therapeutic target for MI. (*J Am Heart Assoc.* 2017;6:e005357. DOI: 10.1161/JAHA.116.005357.)

Key Words: apoptosis • cardiomyocyte • myocardial infarction • transforming growth factor- β receptor III • transgenic mice

Myocardial infarction (MI) is caused by coronary artery stenosis, which leads to the termination of blood flow to the corresponding myocardium. During MI, pathological

changes occur in the heart, including inflammatory reactions, production of reactive oxygen species, and wall stress, resulting in cardiomyocyte apoptosis.¹ Cardiomyocyte apoptosis, which is characterized by the compaction and segregation of chromatin, the fragmentation of nuclei, and the formation of apoptotic bodies, may lead to arrhythmias, cardiac remodeling, and eventually heart failure. Thus, novel targets of cardiomyocyte apoptosis are important for developing therapies for preventing and treating MI.

The transforming growth factor- β (TGF- β) signaling pathway plays an important role in regulating proliferation, migration, invasion, and apoptosis through Smad-dependent and -independent pathways. TGF- β receptor III (TGF β R3) is a transmembrane proteoglycan that functions as a coreceptor of the TGF- β superfamily.² As the most abundant TGF- β receptor, in many cases, TGF β R3 binds and presents TGF- β to TGF β R1 or TGF β R2 in a cell type-specific manner, which is referred to as the ligand-dependent pathway. Recent research suggests that TGF β R3 also plays a critical role in ligand-independent pathways. In L6 myoblasts, for example, TGF β R3 is reported to induce p38 phosphorylation through a ligand-

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Clinical Perspective

What is New?

- The expression of transforming growth factor- β receptor III (TGF β R3) changes during MI and we defined TGF β R3 as a novel and potential regulator of ischemia-induced cardiomyocyte apoptosis.
- p38 mitogen-activated protein kinase pathway is critical for TGF β R3-mediated cardiomyocyte apoptosis induced by MI.
- Cardiac-specific transgenic overexpression of TGF β R3 accelerates myocardial injury during ischemia.

What are the Clinical Implications?

- TGF β R3-p38 mitogen-activated protein kinase pathway plays an important role in cardiomyocyte apoptosis induced by MI; our findings identified TGF β R3 as a potential therapeutic target for the treatment of MI.

independent manner.³ However, the precise role of TGF β R3 in ligand-independent pathways remains to be investigated. Notably, recent studies show that TGF β R3 is critical in the cardiac pathological process. In cardiac fibroblasts, a synthetic peptide from TGF β R3 (P144) and full-length TGF β R3 both reduced collagen production.^{4,5} Notably, we recently demonstrated that TGF β R3 is an important regulator in cardiac hypertrophy.⁶ Moreover, we determined that TGF β R3 prevents apoptosis of cardiac fibroblasts exposed to hypoxia and established a functional link between ERK1/2 and JNK1/2 signaling pathways and TGF β R3.^{5,7} However, the changes in the expression level, functional role, and molecular mechanism of TGF β R3 in cardiomyocyte apoptosis during MI are still largely unclear.

Accordingly, in this study we investigated the potential role of TGF β R3 as a novel target for regulation of cardiomyocyte apoptosis. Our results show that TGF β R3 induces apoptosis of cardiomyocytes during MI via the p38 mitogen-activated protein kinase (MAPK) pathway. Furthermore, knockdown of TGF β R3 reduces the heart injury resulting from MI, which suggests a therapeutic approach for the treatment of heart failure.

Methods

Animal care and experimental protocols in this study conformed to the Institutional Animal Care approval by the ethics committee of the Harbin Medical University and the Guide for the Care and Use of Laboratory Animals by the US National Institutes of Health. In total, 117 adult male wild-type (WT) mice (C57BL/6), 1003 neonatal WT mice (C57BL/6), and 46 cardiac-specific transgenic (Tg) TGF β R3 overexpression mice were used for this study.

Mouse Model of MI

Adult male C57BL/6 mice (25–30 g) were provided by the Experimental Animal Center of Harbin Medical University (grade II) and were fed with food and water freely throughout the experiments.

The mice were anaesthetized with sodium pentobarbitone (30 mg/kg IP) and xylazine (10 mg/kg IP), and the chest skins were shaved and disinfected. Intubation was applied with an artificial respiration machine (UGO Bsile S.R.L. Biological Research Apparatus). An incision was made through the 4th intercostal space, and the heart was exposed. In the MI group, the left anterior descending artery (LAD) was ligated with 7/0 silk thread; infarction was indicated by a significant ST-segment elevation in the ECG. In the sham group, 7/0 silk thread was twined around the LAD and was not tightened.

Isolation of Cardiomyocytes

One- to 3-day-old C57BL/6 mice were anaesthetized by 4% to 5% isoflurane inhalation. The hearts were removed and cut into small pieces. The heart tissues with equirotal shape were digested in 0.25% trypsin, and the isolated cells were collected in 6-well plates with DMEM (HyClone) containing 10% fetal bovine serum (HyClone). Cardiomyocytes were separated from the fibroblasts after 90 minutes of incubation. Then the nonadherent cardiomyocytes were moved into 6-well or 96-well plates with DMEM containing 10% fetal bovine serum and cultured at 37°C in 95% air with 5% CO₂ for 48 hours.

Drug Administration and Transfection of Cardiomyocytes

To assess the effect of TGF β R3 on cardiomyocytes, pc-DNA3.1-mTGF β R3 plasmid (GeneChem) or small interfering RNAs targeting TGF β R3 (GenePharma) were transfected into cells with X-tremeGENE siRNA Transfection Reagent (Roche Molecular Biochemicals). The target sequence of TGF β R3 was 5'GGGA GGUU CACA UCCU AAATT3'. Twenty-four hours after transfection, H₂O₂ (150 μ mol/L) was added to the medium to induce cell apoptosis. A p38 MAPK inhibitor (SB203580, Sigma-Aldrich; 10 μ mol/L) was added to block p38 pathway activation.

Generation of TGF β R3 Tg Mice

TGF β R3-overexpressing Tg mice were generated as previously described.^{6,8} A transgene construct was generated by subcloning the TGF β R3 coding sequence (GenBank: NM_011578.3) into the pRP.Des3d vector backbone, which contains a murine cardiac α -myosin heavy chain gene

promoter. The final targeting vector was electroporated into C57 oosperms, which were then injected into C57 blastocysts, giving rise to a germ-line transmission resulting in TGF β 3^{+/+} Tg mice. Genomic DNA prepared from tails was subjected to polymerase chain reaction for genotyping.

Generation of Adeno-Associated Viral Vectors and In Vivo Vector Delivery

As previously described by our group, adeno-associated viral vector (AAV) serotype 9 (AAV9)-mediated shRNA for type III TGF- β coreceptor was purchased from Biowit Technologies.⁶ Sh-TGF β 3 (5'-GGGAGGTTACATCCTAAA-3')-GFP was cloned into a self-complementary AAV vector backbone. Iodixanol gradient ultracentrifugation was used to purify the recombinant AAV9 vectors.^{6,9} Briefly, a thoracotomy was performed on mice to open the chest through the 4th intercostal space under a general anesthetic. The ascending aortic artery and the main pulmonary artery were occluded. The AAV9 was injected into the left ventricular cavity via the tip of the heart as a 100- μ L bolus (1×10^9 pfu). Then the arteries were clamped for 10 seconds after AAV9 injection. LAD ligation was performed on the 2nd week after AAV9 injection.

Measurement of Cell Viability

According to the manufacturer's instructions, cells were seeded at a concentration of 10 000 per well. After experimental treatment, the cells were incubated with 10 μ L of methylthiazolyldiphenyl-tetrazolium bromide solution (Sigma-Aldrich) (0.5 mg/mL) for 4 hours at 37°C. One-hundred microliters of dimethyl sulfoxide were added into each well, and the plates were shaken for 10 minutes to completely dissolve the formazan. A microplate spectrophotometer (Tecan) was used to read the absorbance value for each well at 490 nm.

Measurement of Cell Apoptosis

To measure apoptotic activity of cardiomyocytes indicated by DNA strand breaks, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; In Situ Cell Death Detection FITC Kit or TMR red, Roche) assays were performed on cardiomyocytes or heart tissues, which were immunostained with anti- α -actinin antibody (Sigma-Aldrich). Cells planted on cover slips or cryosections of mouse heart were fixed in 4% paraformaldehyde for 1 hour, blocked with methanol with 3% H₂O₂ for 10 minutes, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 minutes. Cryosections were incubated with anti- α -actinin antibodies (1:200 dilution; Sigma-Aldrich) in a humidified chamber for 1 hour, followed by TUNEL staining for 1 hour and 4',6-diamidino-2-phenylindole staining for nuclei for 5 minutes.

Laser scanning confocal microscopy (FV300, Olympus) was used to detect TUNEL-positive cells in randomly selected fields.

Electron Microscopy

The collected cardiomyocytes or heart tissues were fixed in 2% glutaraldehyde and immersed in 2% osmium tetroxide. Samples were then dehydrated by a graded series of ethanol (30%, 50%, 70%, 80%, and 90%) and pure acetone, embedded in Araldite (Serva) and cut into ultrathin sections using an FCR Reichert Ultracut ultramicrotome (Leica Microsystems). Sections were contrasted with uranyl acetate and lead acetate. A JEOL 1200 electron microscope (JEOL Ltd) was used to observe the micromorphological changes in cardiomyocytes.

Western Blotting

In brief, the quantities of protein in samples extracted from cardiomyocytes or the peri-infarct region of mice were determined using a bicinchoninic acid kit. Equivalent amounts of protein were loaded in equal volumes and fractionated by SDS-PAGE (10–15% polyacrylamide gels). GAPDH was used as the internal control. The antibodies were as follows: anti-TGF β 3, anti-phospho-p38, anti-phospho-ERK1/2, anti-phospho-JNK1/2, anti-p38, anti-ERK1/2, anti-JNK1/2 (1:1000 dilution; Cell Signaling Technology), and anti-GAPDH (1:500 dilutions; Research Diagnostics).

Immunofluorescence

Cryosections of the border region (4 μ m) were fixed in 4% paraformaldehyde for 2 hours, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 10 minutes, and blocked with 5% bovine serum albumin in PBS with Tween 20 for 1 hour. Cryosections were incubated with anti- α -actinin antibody (1:500; Sigma-Aldrich) and anti-TGF β 3 antibody (1:100; Sigma-Aldrich) in a humidified chamber overnight at 4°C, followed by 4',6-diamidino-2-phenylindole staining for nuclei for 5 minutes. Laser scanning confocal microscopy (FV300, Olympus) was used to image slides in randomly selected fields.

Measurement of Infarction Size

Infarct size was measured by the triphenyl tetrazolium chloride (Sigma-Aldrich) assay as previously described.⁶ In brief, hearts were excised and dissected into 1 mm-thick sections. The sections were immersed in 1% triphenyl tetrazolium chloride for 25 minutes at 37°C and fixed with 3.5% methanol overnight. Viable tissues stained red and infarct tissues remained uncolored. The infarct size was calculated with image analysis software (Image-Pro Plus v4.0; Media Cybernetics) as previously described.⁶

Echocardiography

Transthoracic echocardiography using an echocardiogram (Vivid 7, GE Medical) with a 10-MHz linear transducer was performed to determine left ventricular function as previously described.⁶

Data Analysis

The data were processed with GraphPad Prism 5.0 analysis software and are presented as means \pm SEM. Multiple comparisons were determined by 1-way ANOVA with the Bonferroni's post hoc test. We considered a 2-tailed value of $P<0.05$ as statistically significant.

Results

Dynamic Expression of TGF β 3 in the Border Region During MI and in Cardiomyocytes Stimulated With H₂O₂

Several reports have substantiated the loss of TGF β 3 expression in human prostate cancer and in epithelial-derived ovarian cancer and have identified a suppressive role for TGF β 3 in the migration and invasion of these tumors. To characterize the possible role of TGF β 3 in MI, we removed the hearts from mice that had received LAD ligation for 3, 6, 9, and 12 hours, and we examined the expression of TGF β 3 in the border region by Western analysis (Figure 1A). The protein levels of TGF β 3 were elevated initially and peaked at 6 hours postinfarction, gradually decreasing to baseline levels by 12 hours postinfarction. Findings from immunostaining images further confirmed that TGF β 3 was mainly expressed in membrane of cardiomyocytes and showed increased TGF β 3 protein in the border region of the heart after 6 hours infarction (Figure 1B).

Consistent with the *in vivo* data, TGF β 3 levels were increased in cardiomyocytes exposed to short-term treatment with H₂O₂ (3 and 6 hours) and decreased to baseline from 6 to 12 hours (Figure 1C). The dynamic TGF β 3 expression, which undergoes transient elevation both *in vivo* and *in vitro* suggests the possibility that TGF β 3 might regulate the response to MI.

TGF β 3 is Involved in Regulating Apoptosis of Cardiomyocytes Exposed to H₂O₂

TGF β 3 is known to regulate cell proliferation, migration and invasion, and apoptosis in tumor cells.¹⁰ To determine whether it also plays a role in apoptosis in cardiomyocytes, we overexpressed TGF β 3 or a negative control (NC) vector in cardiomyocytes for 24 hours (Figure S1A) and also treated them with or without H₂O₂ (150 μ mol/L) for 6 hours.

Western blotting showed that H₂O₂ increased the expression of TGF β 3 by 1.9-fold and TGF β 3 plasmid increased the expression of TGF β 3 by 2.1-fold; a combination of TGF β 3 plasmid and H₂O₂ treatment increased expression of TGF β 3 by 4-fold (Figure 2A). Methylthiazolyldiphenyl-tetrazolium bromide and TUNEL assays revealed that overexpression of TGF β 3 directly impaired cell viability and led to cell apoptosis. Furthermore, compared with the damage in the H₂O₂+NC group, the damage was accumulatively enhanced in cardiomyocytes that both overexpressed TGF β 3 and were treated with H₂O₂ for 24 hours (Figure 2B through 2D). To verify these findings, we performed electron microscopy. Distinct subcellular morphological changes of apoptosis (chromosome condensation and apoptotic bodies) were evident in H₂O₂-treated and TGF β 3-overexpressing cells, while cells with both TGF β 3 overexpression and H₂O₂ treatment had the most dramatic effects (Figure 2E). These data suggest that H₂O₂ and TGF β 3 overexpression mediate increases in both the TGF β 3 protein level and apoptosis, and that the increased level of TGF β 3 tended to parallel the degree of apoptosis.

To further confirm the protective role of TGF β 3, we prepared knockdown cardiomyocytes (Figure S1B). Compared with cardiomyocytes exposed to H₂O₂, TGF β 3 knockdown cells with H₂O₂ treatment showed decreased injury as evidenced by the effects on cell viability (Figure 3A) and apoptosis (Figure 3B and 3C). Furthermore, knockdown of TGF β 3 in H₂O₂-treated cells alleviated changes in the microstructure (Figure 3D). Notably, TGF β 3 knockdown alone did not alter cell apoptosis. Thus, loss of TGF β 3 partially reduced the effect of H₂O₂ on cardiomyocytes apoptosis, suggesting that TGF β 3 is involved in regulation of cell apoptosis.

TGF β 3 Mediates Cardiomyocyte Apoptosis via p38 Signaling Upon H₂O₂ Treatment

The MAPK signaling pathway has a well-established role in the pathogenesis of cardiovascular diseases, including MI.^{11,12} The ERK1/2, JNK1/2, and p38 signaling pathways are activated in the infarcted myocardium zone and border regions upon MI.¹³ Furthermore, the p38 signaling pathway is activated in the basal state in the heart, and the p38 inhibitor RWJ-67657 can restrict the infarction size in the rat following MI.¹⁴ To explore the effects of TGF β 3 on the MAPK pathway, we performed Western analysis of cardiomyocytes with or without TGF β 3 overexpression and before and after H₂O₂ exposure. Our results suggest that under both the normal and H₂O₂ conditions, TGF β 3 enhances p38 signaling but does not influence ERK1/2 or JNK1/2 signaling (Figure 4A through 4C). In addition, while H₂O₂ and TGF β 3 overexpression activated p38 signaling separately, p38

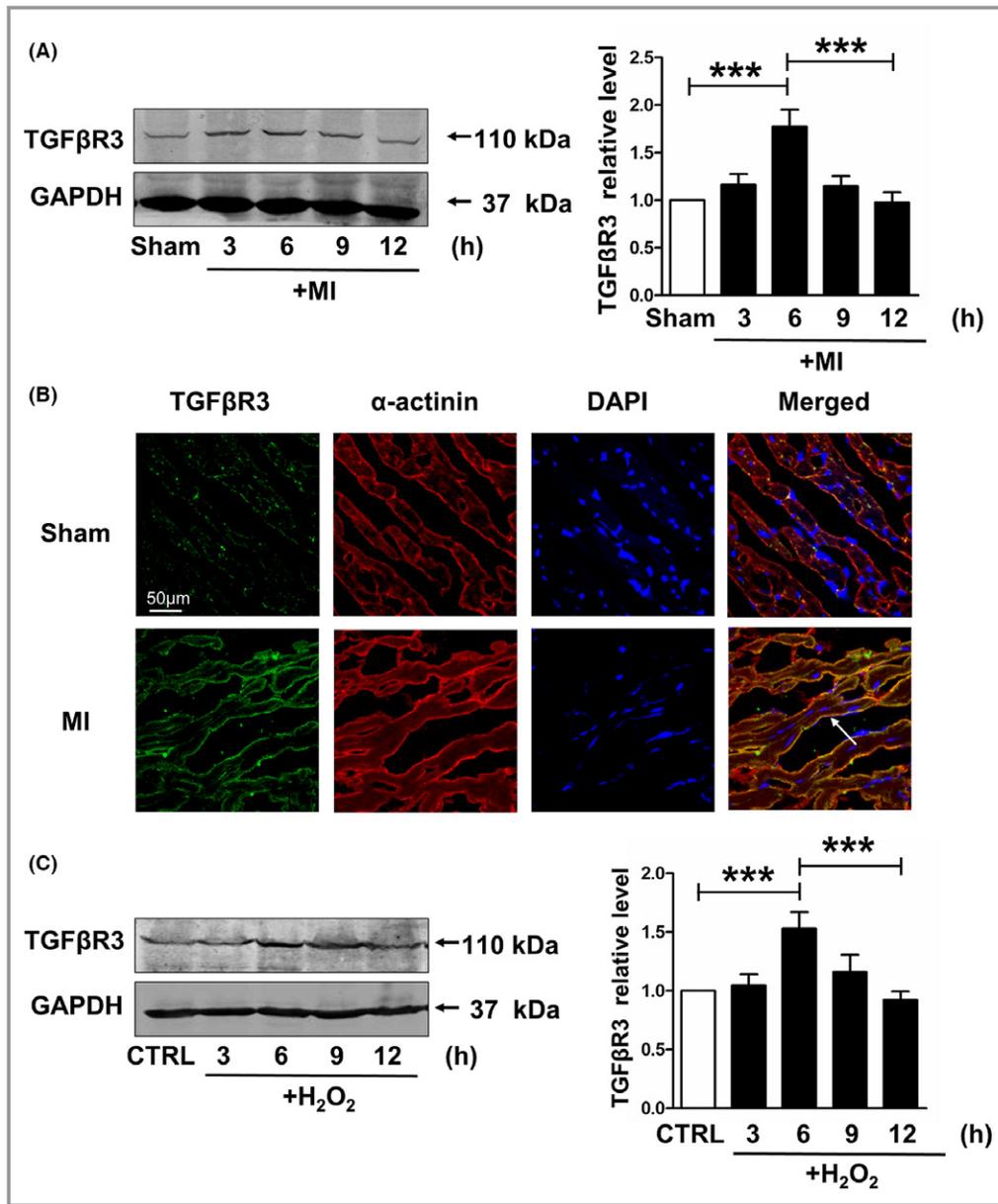


Figure 1. Expression of transforming growth factor-β receptor III (TGFβR3) is dynamically changed in the border region after myocardial infarction (MI) and in cardiomyocytes stimulated with H₂O₂. A, Western blot of TGFβR3 in the border region at 0, 3, 6, 9, and 12 hours after MI (***P*<0.001; n=6). B, Representative immunofluorescence staining images of heart tissue sections of border regions from mice at 6 hours after MI induction. TGFβR3 is indicated by the white arrow. The scale bar represents 50 μm and is applicable to all panels (n=6). C, Western blot of TGFβR3 in cardiomyocytes stimulated with H₂O₂ (150 μmol/L) for 0, 3, 6, 9, and 12 hours (***P*<0.001; n=6). Data are shown as means±SEM. CTRL indicates control; DAPI, 4',6'-diamidino-2-phenylindole.

phosphorylation was further activated by the combination of TGFβR3 and H₂O₂. The converse effect was observed when TGFβR3 was knocked down (Figure 4D). These findings suggest that TGFβR3 is critical for the activation of p38 signaling in cardiomyocytes treated with H₂O₂. To determine whether the p38 pathway is involved in regulation by TGFβR3 of apoptosis, we used a p38 inhibitor (SB203580, 10 μmol/L)

to block the p38 pathway. Methylthiazolyldiphenyl-tetrazolium bromide and TUNEL assays demonstrated that blocking p38 signaling inhibits the effect of TGFβR3 on apoptosis, confirming the key role of the p38 pathway in TGFβR3-dependent induction of apoptosis (Figure 5A through 5C). Thus, our results provide further verification of the role of the TGFβR3-p38 pathway in regulating cardiomyocyte apoptosis.

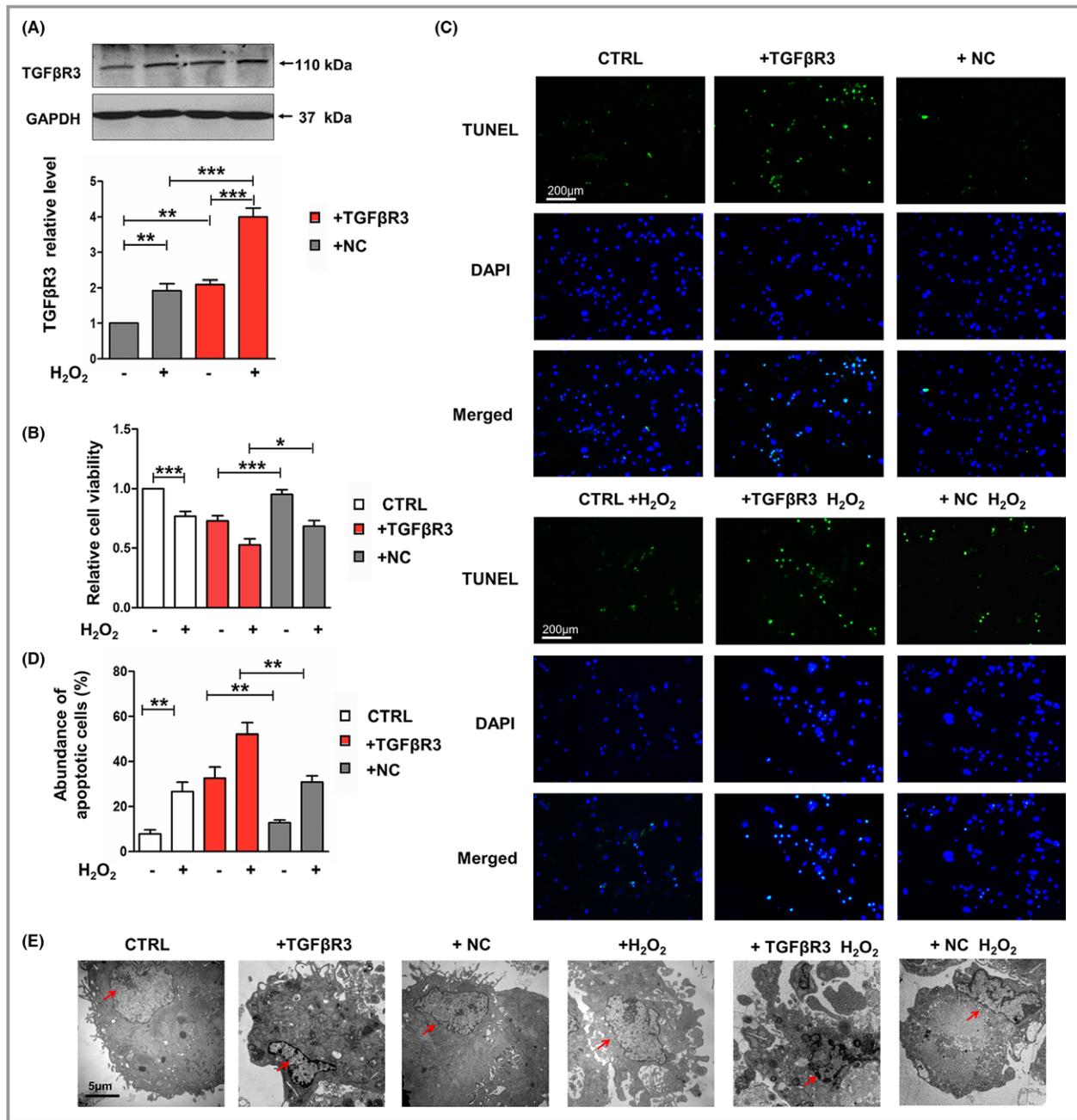


Figure 2. Transforming growth factor- β receptor III (TGF β R3) induces cardiomyocyte apoptosis and enhances the effect of H₂O₂ on cardiomyocytes. A, Western blotting of TGF β R3 in cardiomyocytes overexpressing TGF β R3 and/or treated with H₂O₂. (** P <0.01; *** P <0.001; n =6). B, The cell viability of cardiomyocytes overexpressing TGF β R3 and/or treated with H₂O₂ was measured by methylthiazolyldiphenyl-tetrazolium bromide assay (* P <0.05; *** P <0.001; n =7). C and D, Quantification of cardiomyocyte apoptosis assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and 4',6-diamidino-2-phenylindole (DAPI) staining. The scale bar represents 200 μ m and is applicable to each panel (** P <0.01; n =7). E, Representative electron microscopy images of cardiomyocytes with different treatments. Nuclei are indicated by the red arrows. The scale bar represents 5 μ m and is applicable to each panel (n =6). Data are shown as means \pm SEM. CTRL indicates control; NC, negative control.

Overexpression of TGF β R3 Augments MI Injury

To study the in vivo function of TGF β R3, we next generated TGF β R3^{+/+} Tg mice driven by cardiac specific α -myosin heavy-chain promoter (Figure 6A and 6B). After LAD ligation for 24 hours, the infarct size was greater for TGF β R3^{+/+} Tg mice

than for WT mice (Figure 6C). In addition, the Tg mice exhibited a deterioration in heart function after 24 hours of MI: the fractional shortening was decreased to 9.5 \pm 1.4% and the ejection fraction was decreased to 21.5 \pm 2.5% (P <0.05) in TGF β R3^{+/+} Tg mice subjected to LAD ligation (Figure 6D and 6E).

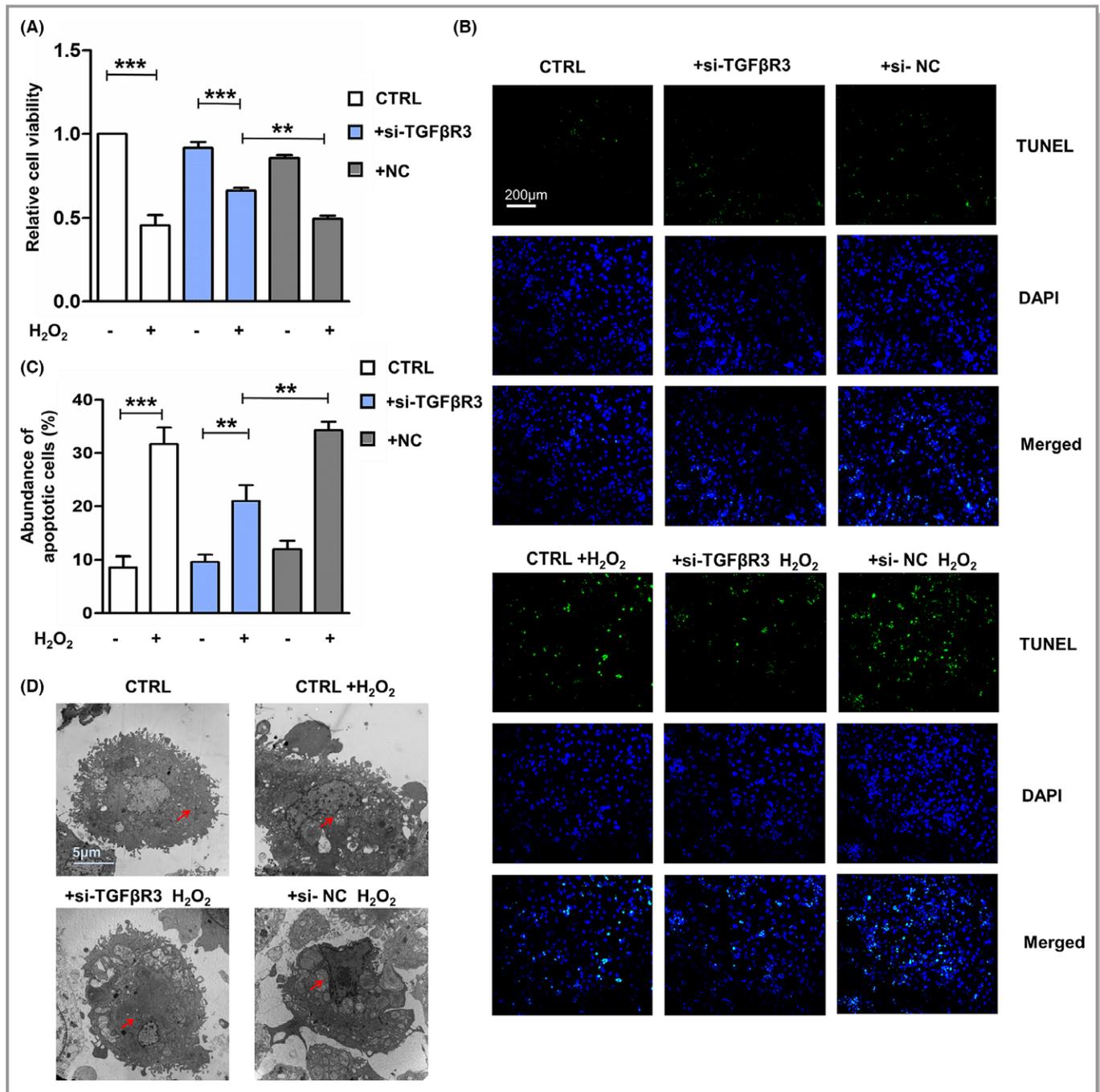


Figure 3. Loss of transforming growth factor-β receptor III (TGFβR3) attenuates apoptosis of cardiomyocytes after H₂O₂ treatment. A, The cell viability of cardiomyocytes transfected with small interfering RNA-TGFβR3/small interfering RNA-negative control (NC) and/or treated with H₂O₂ was measured by methylthiazolyldiphenyl-tetrazolium bromide assay (***P*<0.01; ****P*<0.001; n=8). B and C, Cardiomyocyte apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and 4',6-diamidino-2-phenylindole (DAPI) staining. The scale bar represents 200 μm and is applicable to each panel (***P*<0.01; ****P*<0.001; n=6). D, Representative electron microscopy images of cardiomyocytes with different treatments. Nuclei are indicated by the red arrows. The scale bar represents 5 μm and is applicable to each panel (n=7). Data are shown as means±SEM. CTRL indicates control.

To reveal the changes in apoptosis and the microstructure of the myocardium that are associated with TGFβR3 overexpression, the border regions of mice were examined by TUNEL staining and electron microscopy. Compared with

WT mice, TGFβR3^{+/+} Tg mice had more apoptotic cells (red) and the sarcomere structures were disordered with condensation of chromatin after 24 hours of MI (Figure 6F through 6H). Furthermore, the phosphorylation of p38 was

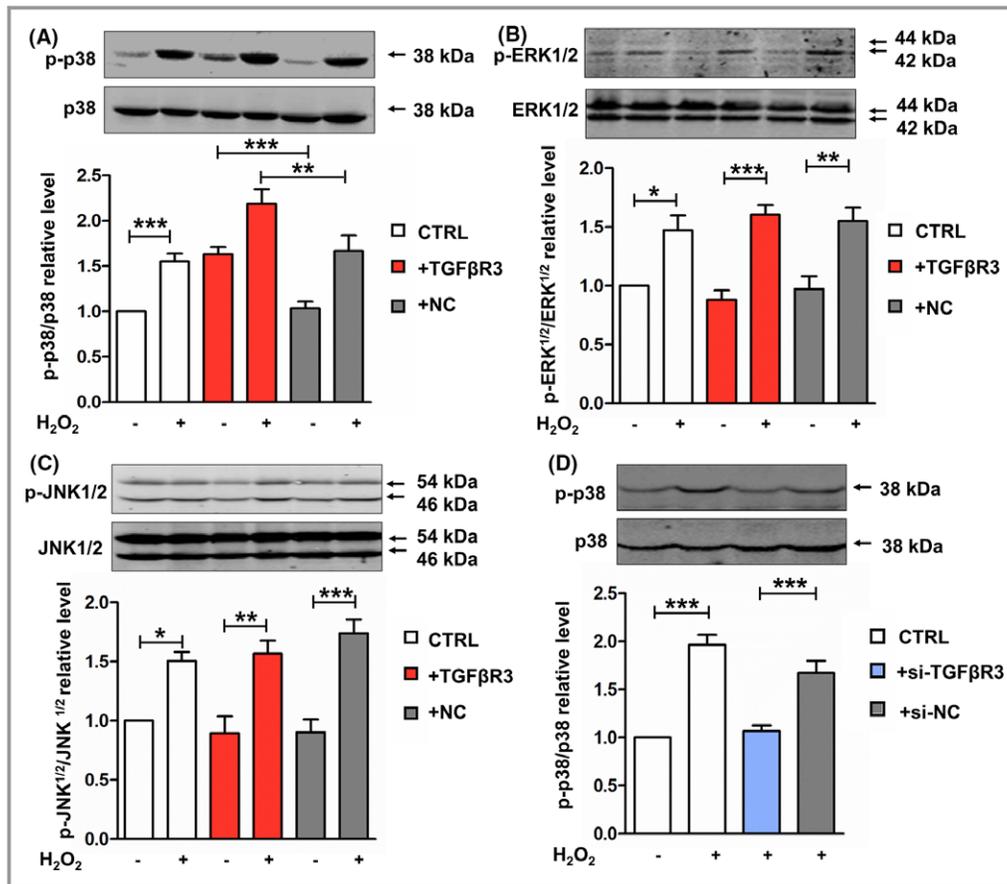


Figure 4. Effects of transforming growth factor- β receptor III (TGF β R3) on mitogen-activated protein kinase signaling pathway in cardiomyocytes stimulated with H₂O₂. A through C, Protein expression of phospho-ERK1/2, total ERK1/2, phospho-JNK1/2, total JNK1/2, phospho-p38, and total p38 was detected by Western blotting for cardiomyocytes overexpressing TGF β R3 and/or treated with H₂O₂ (* P <0.05; ** P <0.01; *** P <0.001; n =7). D, Protein expression of phospho-p38 and total p38 was detected by Western blotting for cardiomyocytes transfected with small interfering RNA (si)-TGF β R3 and/or treated with H₂O₂ (** P <0.001; n =6). Data are shown as means \pm SEM. CTRL indicates control; NC, negative control.

enhanced in TGF β R3+/+ mice compared with WT mice at 6 hours after MI, although the 2 groups of mice had similar levels of p38 activation before MI (Figure 6I). These data suggest that overexpression of TGF β R3 enhances the sensitivity of the myocardium to MI injury, indicating the possibility that loss of TGF β R3 may mitigate the effects of MI on the heart.

Knockdown of TGF β R3 Alleviates MI Injury

To determine whether loss of TGF β R3 mitigates the effects of MI on the heart, we delivered AAV9 vectors carrying a short hairpin RNA of TGF β R3 (AAV-shTGF β R3) by injection. AAV9 is a cardiac-specific AAV with high efficiency of transduction in the heart and coronary artery, and injection of AAV has successfully delivered genes into the myocardium of rats, pigs, and hamsters.^{15,16} The effectiveness of cardiac infection with AAV-shTGF β R3-GFP is shown (Figure 7A). Knockdown of

TGF β R3 in cardiomyocytes was confirmed by Western blotting (Figure 7B). The infarct size after 24 hours of MI was decreased to 32.5 \pm 3.0% (P <0.05) in AAV-shTGF β R3 mice (Figure 7C). We examined the heart function after 4 weeks of MI because several reports have shown that the muridae ischemia heart shows characteristics of heart failure at this time point.^{5,17} Fractional shortening and ejection fraction were significantly improved in AAV-shTGF β R3 mice compared with AAV-scramble mice (Figure 7D and 7E).

To verify the in vivo protective effects of TGF β R3 shRNA, we examined the number of apoptotic cells and the microstructure of heart tissue from AAV-shTGF β R3 mice. Fewer apoptotic cells were observed in AAV-shTGF β R3 mice compared with AAV-NC mice after 24 hours of MI. Furthermore, consistent with the in vitro observations, silencing of TGF β R3 attenuated the damage to the microstructure at 24 hours after MI (Figure 7F and 7G). AAV-shTGF β R3 also partially restored the sarcomere disruption and chromatin

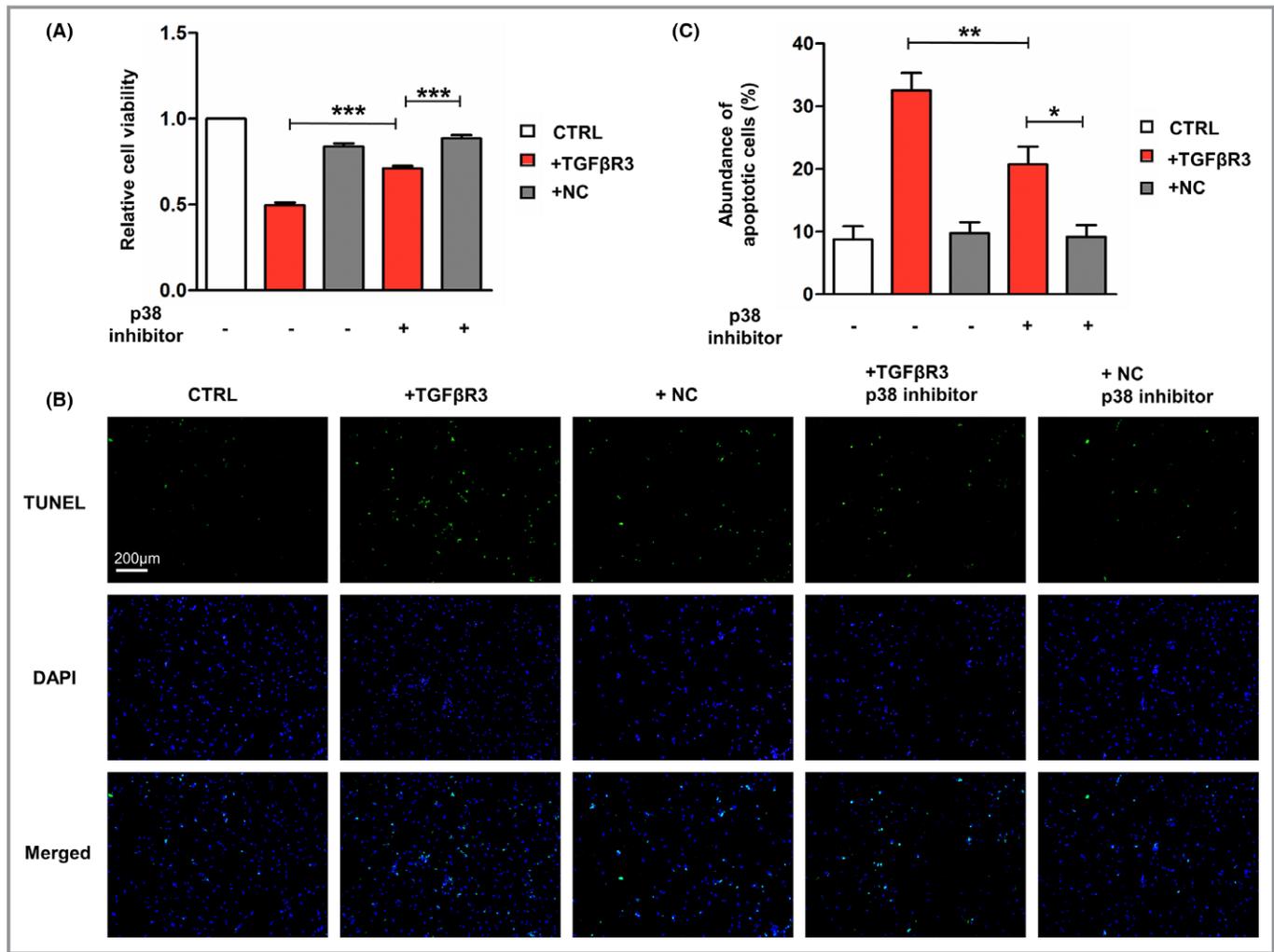


Figure 5. p38 signaling is involved in the upregulation by transforming growth factor-β receptor III (TGFβR3) of apoptosis. A, The cell viability of cardiomyocytes overexpressing TGFβR3 and/or treated with p38 inhibitor was measured by methylthiazolyldiphenyl-tetrazolium bromide assay (***P*<0.001; n=6). B and C, Cardiomyocyte apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and 4',6-diamidino-2-phenylindole (DAPI) staining. The scale bar represents 200 μm and is applicable to each panel (**P*<0.05; ***P*<0.01; n=6). CTRL indicates control; NC, negative control.

condensation observed in AAV-shNC mice after MI (Figure 7H). Finally, p38 activation after 6 hours of MI was significantly suppressed in AAV-shTGFβR3 mice but not in AAV-shNC mice (Figure 7I). Collectively, these data suggest that loss of function of TGFβR3 partially protects the heart from MI injury.

Discussion

We demonstrated that TGFβR3 expression is dynamically regulated in the heart during MI and in cardiomyocytes stimulated with H₂O₂. Furthermore, using both in vitro and in vivo assays with both gain-of-function and loss-of-function experiments, we demonstrated that TGFβR3 potentiates cell apoptosis, increases infarct size, and impairs heart function by regulating the p38 pathway during MI. Our findings provide

novel insight for the development of therapeutic strategies for MI.

Previous findings have shown that changes in TGFβR3 affect cell homeostasis.^{2,18} In the early stage of some tumors, the expression of TGFβR3 is suppressed, and replenishment of TGFβR3 inhibits tumor metastasis, invasion, and growth.¹⁹ TGFβR3 mRNA is also induced by glucocorticoids, especially aldosterone, dexamethasone, and hydrocortisone, in hepatic stellate cells, fibroblasts, and osteoblasts, which suggests a critical role for TGFβR3 in controlling pleiotropic cellular effects.²⁰ During coronary vessel development, TGFβR3 is dynamically regulated and is required; TGFβR3-null mice cannot survive after embryonic day 14.5.²¹ In addition, β-arrestin2 promotes the endocytosis of TGFβR3, and TGFβR3 and β-arrestin2 coordinately function to regulate epithelial and cancer cell migration.²² Interestingly, TGFβR3 expression is

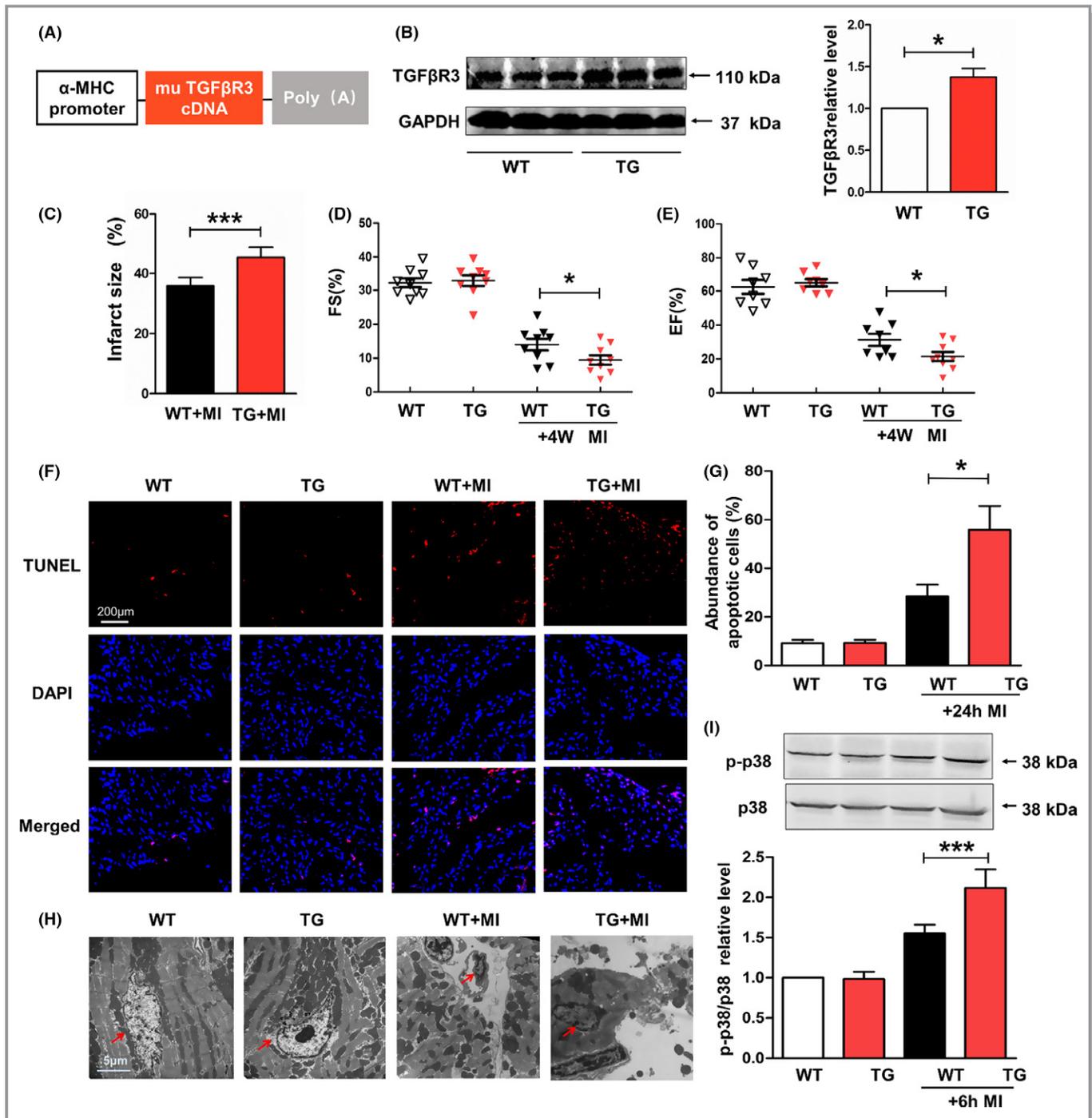


Figure 6. Overexpression of transforming growth factor- β receptor III (TGF β R3) in vivo enhances heart injury after myocardial infarction (MI). A, The establishment of the TGF β R3 transgenic mouse line. TGF β R3 expression was driven by the α -myosin heavy chain (α -MHC) promoter. B, Western blotting of TGF β R3 in wild-type (WT) mice and transgenic mice overexpressing TGF β R3 (Tg) (* $P < 0.05$; n=9). C, Quantification of the infarct size of WT and Tg mice as assessed by 2,3,5-Triphenyltetrazolium chloride assay at 24 hours after MI induction (*** $P < 0.001$; n=6). D and E, Quantification of the fractional shortening (FS) and ejection fraction (EF) of WT and Tg mice with or without MI by echocardiography at 24 hours after treatment (* $P < 0.05$; n=8). F, Representative terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) images of heart tissue sections of border region from WT and Tg mice with or without MI after 24 hours. The scale bar represents 200 μ m and is applicable to each panel (n=6). G, Quantification of cardiomyocyte apoptosis assessed by TUNEL assay and 4',6-diamidino-2-phenylindole (DAPI) staining. The scale bar represents 200 μ m and is applicable to each panel (* $P < 0.05$; n=6). H, Representative electron microscopy images of heart tissue sections of border regions from WT and Tg mice with or without MI 24 hours after treatment. Nuclei are indicated by the red arrows. The scale bar represents 5 μ m and is applicable to each panel (n=6). I, Protein expression of phospho-p38 and total p38 in border regions from WT and Tg mice with or without MI after 6 hours of treatment (*** $P < 0.001$; n=9). Data are shown as means \pm SEM.

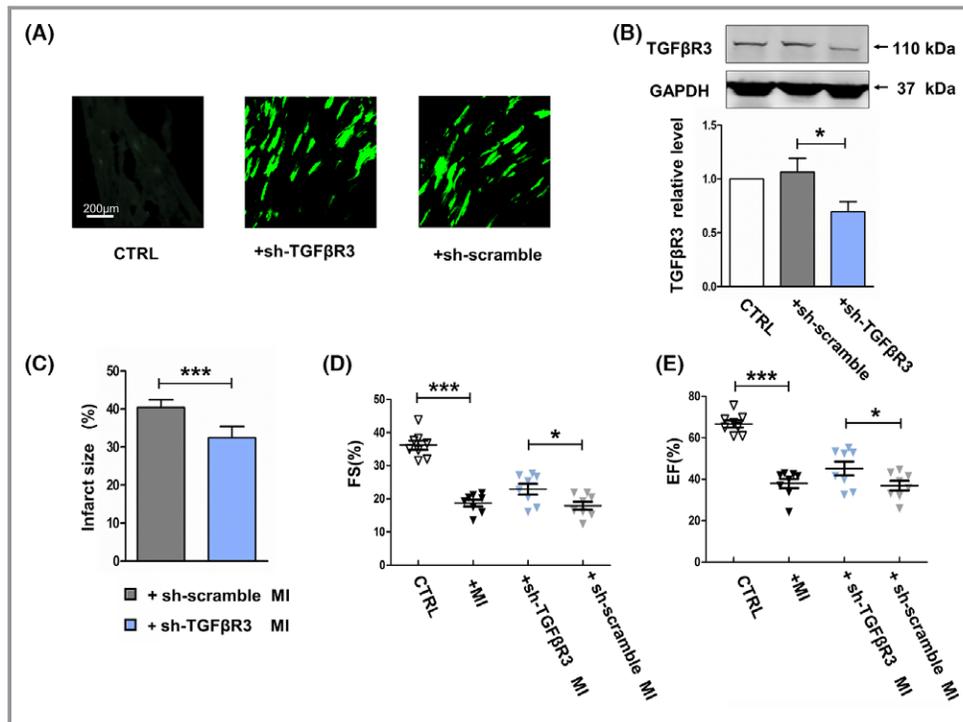


Figure 7. Knockdown of transforming growth factor- β receptor III (TGF β 3) in vivo alleviates heart injury after myocardial infarction (MI). Mice were injected with adeno-associated viral vector (AAV) serotype 9 carrying TGF β 3 short hairpin (sh) RNA or scramble RNA. A, Representative immunostaining images showing the effectiveness of heart infection with AAV-shTGF β 3-GFP. The scale bar represents 200 μ m and is applicable to all panels. B, Protein expression of TGF β 3 in mice treated with AAV-shTGF β 3 or AAV-scramble ($*P < 0.05$; $n = 6$). C, Quantification of the infarct size of mice at 24 hours after MI induction as assessed by 2,3,5-triphenyltetrazolium chloride assay ($***P < 0.001$; $n = 6$). D and E, Quantification of the fractional shortening (FS) and ejection fraction (EF) of mice with different treatments by echocardiography ($*P < 0.05$; $***P < 0.001$; $n = 9$). F, Representative terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) images of heart tissue sections of border regions from mice at 24 hours after MI induction. The scale bar represents 200 μ m and is applicable to each panel ($n = 6$). G, Quantification of cardiomyocyte apoptosis assessed by TUNEL assay and 4',6-diamidino-2-phenylindole (DAPI) staining ($***P < 0.001$; $n = 7$). H, Representative electron microscopy images of heart tissue sections of border regions from mice at 24 hours after MI induction. Nuclei are indicated by the red arrows. The scale bar represents 5 μ m and is applicable to each panel ($n = 7$). I, Protein expression of phospho-p38 and total p38 in border regions from mice at 6 hours after MI induction ($***P < 0.001$; $n = 6$). Data are shown as means \pm SEM. CTRL indicates control.

significantly suppressed in rat lungs during long-term anoxia (day 14).²³ In contrast, our results suggest that TGF β 3 is upregulated in mice during MI. This difference may be explained by the acute course of MI injury as compared with the long-term course of anoxia. In our study, the expression of TGF β 3 peaked at 6 hours after MI and then fell to baseline levels at 12 hours after MI. We also demonstrated that targeting TGF β 3 at an early stage of MI (within 6 hours) may ameliorate the injury, which suggests that TGF β 3 participates in the regulation of cardiomyocyte behavior during the early stage of MI. Consistently, the expression of TGF β 3 was upregulated by H₂O₂ and was further increased by the combination of H₂O₂ and TGF β 3 overexpression plasmid. In cultured cardiomyocytes, TGF β 3 directly leads to cell apoptosis and enhances the cell ultrastructural changes under normal conditions. Under

H₂O₂ conditions, an increase in TGF β 3 levels exerts a cumulative effect on the degree of apoptosis. Consistently, TGF β 3 is also known to enhance apoptosis in some tumor cells; however, TGF β 3 suppresses the apoptosis of cardiofibroblasts,^{7,10} potentially by deactivating the TGF β 1/TGF β 2 complex to downregulate cell apoptosis.²⁴ According to our data, TGF β 3 promotes cardiomyocyte apoptosis induced by myocardial infarction. The induction of apoptosis by TGF β 3 is observed in some tumor cells, while TGF β 3 is also reported to suppress apoptosis of cardiofibroblasts. And the discrepant effect of TGF β 3 on apoptosis in various context may be explained by the difference of disease models or cell type-specific signaling pathways.

To examine the signaling pathways that mediate TGF β 3-dependent apoptosis in cardiomyocytes, we evaluated the

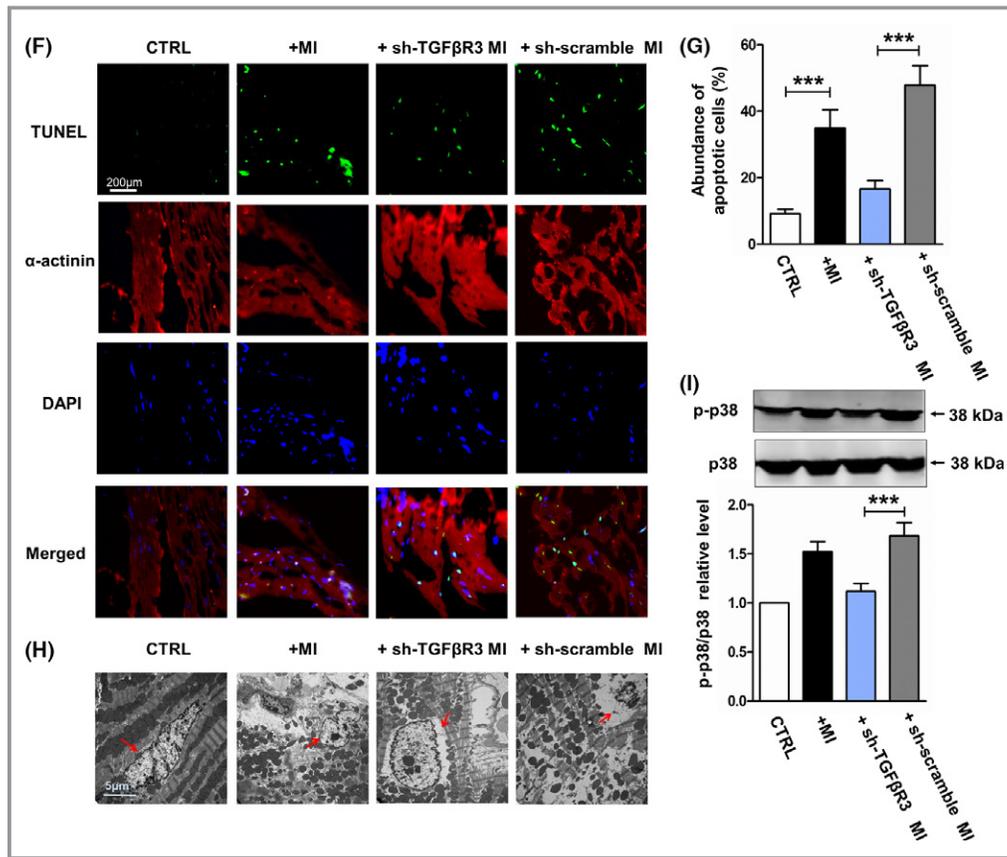


Figure 7. Continued.

activation of MAPKs after MI. In most cases, TGF β 3 serves as a coreceptor in TGF- β signaling and binds and presents ligands such as TGF- β 1 to specific receptors, leading to the activation of downstream Smad-dependent and Smad-independent signaling.²¹ The Smad-independent activation of MAPK signaling by TGF- β has been extensively studied. For example, in mouse mesangial cells, the p38 pathway is activated by TGF- β 1 to induce collagen and fibronectin expression. We demonstrated that overexpression of TGF β 3 during MI activates the p38 signaling pathway but does not alter the JNK1/2 or ERK1/2 pathway. Interestingly, TGF β 3-induced activation of p38 signaling has also been detected in colon cancer cells, fibroblasts, and L6 myoblasts.^{24,25} In the infarct heart, cytokines such as tumor necrosis factor and interleukin 6 and injury of ischemia-reperfusion activate the p38 signaling pathway.²⁶ Furthermore, as a critical part of the response to MI in the heart, several investigations have confirmed that the p38 pathway sensitizes the caspase cascade to induce cell apoptosis, which supports our findings.^{27,28} We observed that the activation of p38 was attenuated by knockdown of TGF β 3 under H₂O₂ or MI conditions. As a target of H₂O₂, TGF β 3 is involved in the regulation of H₂O₂ on cardiomyocyte apoptosis, which is also influenced by some other factors. Thus, loss of TGF β 3 in

knockdown cells partially impaired the effect of H₂O₂. Moreover, p38 inhibitor abolished the effects of TGF β 3 on apoptosis, suggesting that TGF β 3 mediates cardiomyocyte apoptosis at least partially via p38 signaling. This is consistent with previous reports demonstrating that p38 signaling mediates cell apoptosis during ischemia reperfusion injury.²⁹ In our recent study, calmodulin-dependent protein kinase II, a regulator of the p38 pathway, was confirmed to function downstream of TGF β 3.⁶ However, whether calmodulin-dependent protein kinase II also functions in the response to MI warrants further investigation.

To dissect the in vivo role of TGF β 3, we generated cardiac-specific Tg mice overexpressing TGF β 3. Notably, the infarct size of TGF β 3+/+ Tg mice was significantly increased compared with that of WT mice. In addition, forced expression of TGF β 3 impaired heart function after 24 hours of MI, as evidenced by the reduced ejection fraction and fractional shortening, suggesting the increased sensitivity to MI injury by Tg mice. Although p38 signaling was upregulated in mice after MI, in this study, p38 signaling was not altered in TGF β 3+/+ Tg mice compared with WT mice before MI induction, which is different from the in vitro results. This difference may be attributed to the following factors: first, the expression of TGF β 3 was increased less in the in vivo study

(1.5-fold increase) than in the in vitro study (2-fold increase); second, the complex in vivo microenvironment makes it difficult to eliminate interference; finally, the activation of p38 in TGF β R3+/+ Tg mice during MI is less dramatic than the activation in cardiomyocytes after H₂O₂ exposure, which is consistent with the suppressed heart function and augmented infarct size. Nevertheless, p38 activation was statistically enhanced in both TGF β R3-overexpression cardiomyocytes and in TGF β R3+/+ mice, and the link between TGF β R3 expression and p38 was verified in loss-of-function experiments, which supports the role for p38 in mediating the effects of MI injury.

Loss-of-function experiments in mice were performed by left ventricular cavity injection of cardiac-specific AAV expressing shTGF β R3 rather than producing TGF β R3 null mice because TGF β R3 plays a critical role in heart growth. Coronary artery development is blocked in TGF β R3 null mice, which cannot survive after E14.5 during embryonic development.³⁰ The high efficiency for myocardial transduction of AAV9 makes it a strong tool in Tg research of the heart, and it has been successful in previous investigations.³¹ Our results show that AAV-shTGF β R3-mediated knockdown of TGF β R3 in the myocardium alleviates the injury of MI on the heart, as exemplified by improved heart function and decreased apoptosis, which was verified by echocardiography. During the development of MI, the ventricular wall became thicker as a result of myocardial hypertrophy and collagen deposition. The inflexible myocardium impaired the systolic and diastolic function of the heart.^{32,33} Thus, we confirmed that knockdown TGF β R3 in the myocardium protects the heart from injury caused by MI, providing a novel target of MI therapy.

Conclusions

We present evidence of the critical role of TGF β R3 in the pathogenesis of MI. We show that TGF β R3 may mediate heart ischemic injury by inducing apoptosis, which may be promoted in part by hyperactivated p38 signaling. Although the underlying mechanisms of p38 in mediating the effects of MI need to be further investigated, our present work identifies TGF β R3 as a potential therapeutic target for MI.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Figure S1. Efficacy of TGF β R3 overexpression or knockdown in cardiomyocytes and heart tissues. **(A)** Protein expression of TGF β R3 in cardiomyocytes transfected with pc-DNA3.1-m TGF β R3 plasmid or NC plasmid (*denotes $P < 0.05$; $n = 6$). **(B)** Protein expression of TGF β R3 in cardiomyocytes transfected with TGF β R3 siRNA or NC siRNA (**denotes $P < 0.01$; $n = 6$). Data are shown as the means \pm SEM. CTRL indicates control; NC, negative control; si, small interfering RNA; TGF β R3, transforming growth factor β receptor III.

