Journal of Molecular Histology (2025) 56:103
https://doi.org/10.1007/s10735-025-10378-7

ORIGINAL PAPER

Protective role of Tongxinluo in mitigating myocardial fibrosis in mice with acute myocardial infarction via neuregulin-1 upregulation and Inhibition of endothelium-interstitial transition

Zhen Li[1] · Yu-jie Yin[2,3,4] · Ya-ru Wei[4] · Yi Liu[1,2,3] · Ning-xin Han[1,2,3] · Xiao-qi Wang[2,3,5] · Yuan-jie Hao[1,2,3] · Ya-fen Wang[5] · Yun-long Hou[1,2,3,5] · Zhen-hua Jia[1,2,3,4,5]

Received: 6 November 2024 / Accepted: 17 February 2025

Abstract

Acute myocardial infarction (AMI) is a leading cause of heart failure, often accompanied by myocardial fibrosis (MF), characterized by excessive extracellular matrix accumulation. Endothelial-to-mesenchymal transition (EndMT) plays a key role in MF progression post-AMI. Neuregulin-1 (NRG-1), a growth factor with cardioprotective properties, has emerged as a potential therapeutic target. Tongxinluo (TXL), a traditional Chinese medicine, mitigates MF by upregulating NRG-1. This study elucidates the mechanisms underlying the protective effects of NRG-1 and TXL against MF following AMI. Left anterior descending artery ligation established a model for mice with AMI. Adeno-associated virus was used to modulate NRG-1 expression in the myocardium. Echocardiography assessed cardiac function, and histological staining was used to evaluate MF. Expression levels of markers for myofibroblasts (α-SMA, FSP-1) and endothelial cells (CD31, VE-cadherin) were analysed to investigate EndMT. The involvement of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signalling pathway in NRG-1's protective mechanism was validated using biochemical methods. Tongxinluo was administered to mice with AMI via gavage for 4 weeks, and its effects on cardiac function, MF and EndMT were assessed. Overexpression of NRG-1 in mice with AMI ameliorated cardiac dysfunction and reduced interstitial and perivascular fibrosis, whereas NRG-1 deficiency exacerbated these effects. NRG-1 protected against EndMT, as evidenced by changes in myofibroblast and endothelial cell markers. The PI3K/AKT signalling pathway was involved in NRG-1's protective mechanism against MF. The administration of TXL to mice with AMI improved cardiac function and reduced MF by activating NRG-1. Furthermore, TXL inhibited EndMT post-AMI through the NRG-1/PI3K/AKT pathway. NRG-1 and TXL protect against MF post-AMI by mitigating EndMT through the PI3K/AKT pathway. These findings suggest that targeting NRG-1 or using TXL may be promising therapeutic strategies for MF following AMI.

Keywords Neuregulin-1 · Acute myocardial infarction · Myocardial fibrosis · Endothelial-to-mesenchymal transition · Tongxinluo

✉ Yun-long Hou
houyl62yl@21cn.com

✉ Zhen-hua Jia
jiazh2024zh@163.com

1 Graduate School, Hebei Medical University, Shijiazhuang, Hebei 050017, China

2 Hebei Academy of Integrated Traditional Chinese and Western Medicine, Shijiazhuang 050035, China

3 National Key Laboratory for Innovation and Transformation of Luobing Theory, Shijiazhuang 050035, China

4 Hebei Yiling Hospital, Shijiazhuang 050091, China

5 Graduate School, Hebei University of Chinese Medicine, Shijiazhuang, Hebei 050090, China

Introduction

Acute myocardial infarction (AMI) is one of the most fatal cardiovascular diseases (Carter et al. 2019). Myocardial fibrosis (MF) is a reparative process after AMI characterised by myocardial fibroblast accumulation and deposition of the extracellular matrix, resulting in cardiac remodelling after AMI (Chaitman et al. 2021; Virani et al. 2020). However excessive MF serves as a pivotal factor in poor prognosis, correlating with increased 30-day readmission rates, reduced 5-year survival rates and elevated risk of heart failure (Gardarsdottir et al. 2022; Ndrepepa et al. 2010;

Published online: 10 March 2025

Berezin and Berezin 2020; Gulati et al. 2018). The main mediators of MF are myofibroblasts, which can be derived from resident fibroblasts, bone marrow-derived fibroblasts, epithelial-mesenchymal transition and endothelial-mesenchymal transition (EndMT). EndMT is a phenotypic switching process where endothelial cells lose their characteristics and acquire mesenchymal features. Studies have shown that approximately 27–35% of fibroblasts in fibrotic tissue originate from endothelial cells through EndMT (Zeisberg et al. 2007). During cardiac fibrosis, endothelial cells undergo EndMT and contribute to cardiac fibroblasts (Jordan et al. 2021; Fan et al. 2012).

Neuregulin-1 (NRG-1), a member of the epidermal growth factor family, is primarily expressed in the endocardium and cardiac microvascular endothelial cells and is involved in cardiac development, structural maintenance and functional integrity (Geissler et al. 2020). NRG-1 is activated at specific stages of cardiac development to synthesise and secrete the NRG-1 protein. In addition to its expression by cardiomyocytes, NRG-1 can also play a role in the heart through intercellular signalling (Kang et al. 2019). It acts as a signalling molecule that transmits information between cardiomyocytes and other cell types (e.g. endothelial cells and fibroblasts), thus affecting the overall structure and function of the heart (Noll et al. 2024). NRG-1 exhibits diverse biological effects following ischemic heart injury, including anti-inflammatory properties, anti-oxidative stress effects, restoration of electromechanical coupling and regulation of ion channel function (Noll et al. 2024; Shahriary et al. 2019; Kuramochi et al. 2006; Wang et al. 2019). Recent studies have demonstrated the cardioprotective effects of NRG-1 against cardiac ischemic injury and remodelling by improving endothelial injury and dysfunction and inhibiting vascular remodelling (Cui et al. 2016; Wang et al. 2022; Wu et al. 2021). These findings underscore the protective and anti-fibrotic roles of NRG-1 in cardiovascular endothelial injury. However, the precise mechanism by which NRG-1 modulates MF after AMI remains unclear.

Tongxinluo (TXL), a traditional Chinese medicine, has garnered considerable attention in the treatment of cardiovascular and cerebrovascular diseases due to its protective effects on vascular endothelium, plaque stabilisation and enhancement of microcirculation. Numerous clinical studies show that TXL improves cardiac function, reduces infarct size and modulates the expression of collagen and matrix metalloproteinase (Jakob et al. 2017; Parameswarappa et al. 2019). Recent experimental research results also demonstrate that TXL has a significant effect on ischemic injury, possibly through improving microcirculation (Wei and Jiang 2023) and inhibiting inflammation (Liu et al. 2023). Furthermore, TXL has been shown to attenuate MF following AMI by inhibiting EndMT and activating the NRG-1 signalling pathway (Yin et al. 2019). However, further exploration is required to elucidate the precise role of TXL in modulating NRG-1 and its downstream pathway.

This study provides evidence regarding the relationship between NRG-1 and cardiac fibrosis in mice with AMI. It aims to observe the effect of NRG-1 on EndMT-induced interstitial and perivascular fibrosis by interfering with or overexpressing NRG-1 in the myocardium. Additionally, this study explores whether TXL could ameliorate EndMT-induced cardiac fibrosis through the activation of the NRG-1 signalling pathway.

We present the following article following the ARRIVE guidelines 2.0: author checklist.

Materials and methods

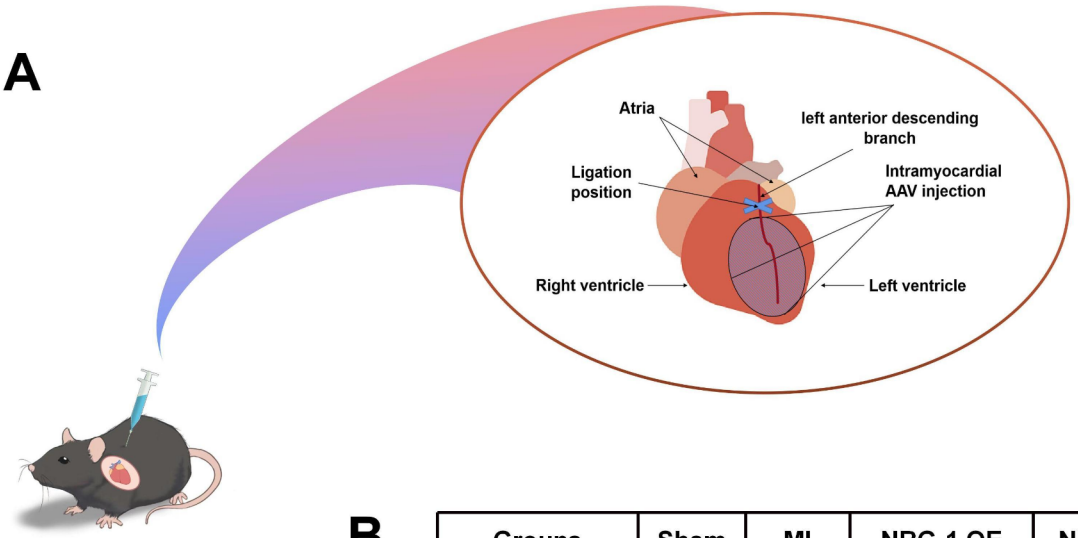
Animals and drugs

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (NO. N2021065) granted by the ethics committee of Hebei Academy of Integrated Traditional Chinese and Western Medicine, in compliance with the guidelines for the care and use of animals. Specific-pathogen-free male C57BL/6 N mice (18–22 g) were purchased from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. (Beijing, China). The mice were kept in the animal room of the Hebei Academy of Integrated Traditional Chinese and Western Medicine, maintained at a constant temperature (24 °C ± 2 °C) with a 12-hour light–dark cycle and had ad libitum access to water and food.

Myocardial infarction model and adeno-associated virus injection

The mice were anaesthetised with 2.5 ml/kg pentobarbital. Throughout the procedure, the mice were placed on a heat preservation board, and a ventilator was connected to the trachea to maintain stable temperature and breathing. Chest hair was gently removed using depilatory cream, and the surgical area was disinfected with iodophor. To expose the heart at the fourth intercostal space, intercostal muscles were bluntly dissected following a skin incision. Under a microscope, the pericardium was carefully torn with blunt forceps, and the left anterior descending (LAD) artery was permanently ligated using 8 − 0 silk ligatures. The ligation site was approximately 1–2 mm below the left auricle, as illustrated in Fig. 1A. Electrocardiogram monitoring confirmed ST-segment elevation coinciding with pallor of the

A



B

Groups	Sham	MI	NRG-1 OE	NRG-1 KD	NC
AMI	-	+	+	+	+
AAV Injection	-	-	+	+	+

Groups	Sham	MI	TXL	TXL+ NRG-1 KD	NC
AMI	-	+	+	+	+
AAV Injection	-	-	-	+	+
TXL	-	-	+	+	-
CMC-Na	+	+	-	-	+

C

Fig. 1 Groups of mice were treated with different intervention. (**A**) Ligation site: left anterior descending branch of the left auricle 1–2 mm. AAV injection location was around the shaded area. (**B**) Sham group was only threaded without ligation, other groups were ligation of the LAD. NRG-1 OE group, NRG-1KD group and NC group were injected with the empty AAV vector. (**C**) After one week of adaptive feeding, the mice were modeled, then mice were sacrificed 4 weeks after modeling

anterior left ventricular (LV) wall post-ligation. Immediately after inducing AMI in C57BL/6 mice, adeno-associated virus (AAV) for NRG-1 overexpression or knockdown was injected into the heart. Adeno-associated virus is a small, non-pathogenic virus commonly used for gene delivery and gene therapy. This injection involves the direct delivery of an AAV vector (viral particles carrying a target gene) into a specific tissue or organ for efficient gene delivery and expression. The AAV used in this study was provided by Hanbio Tech (Shanghai, China), with titres of 1.4×10^{12} vg/mL stored at −80 °C. Prior to surgery, the AAV and microinjector were kept on ice to maintain virus activity. Following LAD ligation, the AAV was injected using the microinjector at four sites around the LV below the left atrial appendage,

with 10 µl per site, as shown in the shaded area in Fig. 1A. The surgical site was sterilised with iodophor, followed by suturing of muscle and skin layers with 4−0 silk sutures. Upon recovery from anaesthesia, the mice were returned to their cages.

Groups

In the first part of this study, the mice were randomly divided into 5 groups: (Carter et al. 2019) Sham group: underwent a sham procedure without LAD ligation; (Chaitman et al. 2021) Model group: underwent LAD ligation; (Virani et al. 2020) NRG-1 overexpression group: underwent LAD ligation+AAV (AAV2/Vec-TIE-m-NRG-1); (Gardarsdottir et al. 2022) NRG-1 knockdown group: underwent LAD ligation+AAV (AAV2/Vec-TIE-mir30-sh6-m-NRG-1), where the knockdown of NRG-1 was achieved through the use of an AAV vector expressing miR30-based short hairpin RNA (shRNA) targeting NRG-1. The miR30 sequence was selected because of its ability to effectively silence target genes by inducing RNA interference, specifically targeting NRG-1 mRNA for degradation, leading to reduced NRG-1 expression in the myocardium; and (Ndrepepa et al. 2010) Negative Control (NC) group: underwent LAD ligation+AAV (AAV2/Vec-TIE NC). The Sham group served as the control group to isolate the effects of thoracotomy and ligation on cardiac function and structure in mice. Mice in the Sham group only underwent threading without ligation to simulate the surgical process without inducing myocardial damage (Fig. 1B).

In the second part of this study, the mice were randomly divided into 5 groups: (Carter et al. 2019) Sham group: underwent a sham procedure without LAD ligation; (Chaitman et al. 2021) Model group: underwent LAD ligation; (Virani et al. 2020) TXL group: underwent LAD ligation+TXL; (Gardarsdottir et al. 2022) TXL+NRG-1 knockdown group: underwent LAD ligation+TXL+AAV (AAV2/Vec-TIE-mir30-sh6-m- NRG-1); and (Ndrepepa et al. 2010) NC group: underwent LAD ligation+AAV (AAV2/Vec-TIE NC). Tongxinluo was provided by Shijiazhuang Yiling Pharmaceutical Co., Ltd (Hebei, China). Mice in the TXL group and TXL+NRG-1 knockdown group received 0.75 g/kg/d TXL diluted with 0.5% carboxymethyl cellulose sodium via gavage for 4 weeks. The Sham group, Model group and NC group were given an equal volume of carboxymethyl cellulose sodium via gavage for 4 weeks (Fig. 1C).

Echocardiography

Four weeks after modelling, cardiac function was evaluated using a Vevo 3100 small animal ultrasound system (VisualSonics Inc., Canada) with a 30 MHz centre frequency scan head. Isoflurane (2% vol/vol) was administered using a small animal anaesthesia machine until the heart rate stabilised between 350 and 450 beats per minute in mice. M-mode images were obtained from a parasternal long-axis view at the level of the papillary muscles, and several parameters, including LV ejection fraction (EF), LV fractional shortening and diastolic and systolic LV diameters were measured. Subsequently, the hearts were collected for further analysis following echocardiography.

Cardiac hypertrophy index

The mice were weighed using a balance, and then their chests were opened to remove the hearts and weigh them. The heart weight index (HWI) was calculated by dividing the heart weight by the body weight.

Haematoxylins-eosin staining and Masson's trichrome staining

The tissues were fixed with 10% formalin and embedded in paraffin. Sections were cut into 4-micron thicknesses. Haematoxylin-eosin staining (Sigma-Aldrich) was performed on all sections using an automated staining machine. Masson's trichrome staining was conducted according to the manufacturer's instructions using the kit (G1340, Solarbio, China). Images were captured using a microscope (magnification = ×400). Fibrotic areas in each section were quantified using Image J software. The percentage of fibrosis was calculated as the fibrosis area divided by the total LV area, multiplied by 100%.

Immunohistochemistry and Immunofluorescence

The tissue sections were dewaxed using xylene and treated with gradient ethanol, followed by antigen retrieval with citric acid. After blocking with 5% bovine serum albumin in phosphate buffered saline (PBS), the sections were incubated overnight at 4 °C with the primary antibody. Subsequently, secondary antibodies were applied for 1 h at room temperature, followed by three 5-minute washes with PBS. Nuclei were counterstained with DAPI or haematoxylin. Images were captured using a confocal microscope and analysed using Image-Pro Plus 6.0 software. Antibodies were sourced as follows: CD31 (1:2000, ab182981, Abcam, UK), α-SMA (1:2000 ab186981 Abcam, UK), Anti-Collagen I (1:100, ab270993-100 Abcam, UK) and Collagen Type III (N-Terminal) Rabbit Polyclonal Antibody (1:300, 22734-1-AP-150, China).

Western blot

The hearts were homogenised in RIPA buffer, and protein concentrations were determined using the BCA Protein Assay Kit (SW101-02, Beyotime Biotechnology, China). Proteins were separated on 4–20% SDS-PAGE gels and transferred to PVDF membranes (ISEQ00010, Millipore, USA). Rapid blocking solution (P30500, New Saimei, China) was used for blocking at 37 °C for 10 min. Primary antibodies were incubated overnight at 4 °C, followed by washing 3 times with TBST for 10 min each time the next day. Secondary antibodies were incubated at 37 °C for 1 h and membranes were washed 3 times with TBST for 10 min each time.

Primary antibodies were sourced as follows: Anti-phosphatidylinositol 3(PI3)-Kinase p85 alpha (1:1000, ab191606, Abcam, UK), Anti-CD31 (1:2000, ab222783, Abcam, UK), Anti-S100A4 (1:1000, ab197896, Abcam, UK), Anti-NRG-1 (1:1000, ab180808, Abcam, UK), Anti-VE-Cadherin (1:1000, ab205336, Abcam, UK), Anti-alpha smooth muscle Actin (1:5000, ab32575, Abcam, UK), Anti-eNOS (1:2000, ab199956, Abcam, UK), phosphorylated-protein kinase B (p-AKT) (1:2000, CST4060, Cell Signalling Technology, USA) and AKT (1:1000, CST4685, Cell Signalling Technology, USA). Each band was normalised using Anti-GAPDH (1:5000, ab181602, Abcam, UK) as the loading control. Secondary antibodies used were Goat Anti-Rabbit IgG H&L Antibody (1:10000, ab216773, Abcam, UK) and Goat Anti-Mouse IgG H&L Antibody (1:10000, ab216772, Abcam, UK). Pre-stained Protein Marker II (10–200 kDa) (G2058-250UL, Servicebio, China) was also used.

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA), and data were presented as mean ± standard deviation. Group comparisons were assessed using the one-way analysis of variance method, followed by the Student–Newman–Keuls test for parametric data. Non-parametric data were analysed using the Kruskal–Wallis test for multiple comparisons. A significance level of $P < 0.05$ was considered statistically significant.

Results

Neuregulin-1 deficiency aggravated cardiac dysfunction following acute myocardial infarction

The AMI model was induced by ligating the LAD coronary artery, confirmed by ST-segment elevation (Fig. 2A). Adeno-associated virus was injected into mouse hearts to overexpress and knock down NRG-1 levels. Four weeks post-surgery, the hearts exhibited myocardial hypertrophy and pallor in the infarcted area (Fig. 2B). Myocardial hypertrophy significantly increased (Fig. 2C). Echocardiography findings (Fig. 2D) revealed ventricular wall thinning, reduced EF and impaired ventricular wall motion in both the Model and NC groups compared with the Sham group. Notably, comparisons between the NC and NRG-1 overexpression groups revealed significant improvements in EF and reduced myocardial fibrosis in the NRG-1 overexpression group, indicating the protective effect of NRG-1 overexpression. Conversely, comparisons between the NC and NRG-1 knockdown groups showed exacerbated ventricular wall thinning and reduced EF following NRG-1 knockdown, emphasizing the detrimental effect of NRG-1 deficiency on cardiac function. Therefore, these results confirmed that intervention with NRG-1 altered both cardiac structure and function in mice following AMI.

Neuregulin-1 regulated secondary myocardial fibrosis following acute myocardial infarction

Haematoxylin staining revealed evident collagen fibre hyperplasia in the Model and NC groups (Fig. 3A). Compared with the NC group, NRG-1 overexpression reduced fibrosis and area, whereas NRG-1 knockdown increased fibrosis and area, accompanied by myocardial cell deformation and reduction. Masson's trichrome staining showed tightly arranged myocardial fibres in the Sham group, with no notable collagen fibre hyperplasia. In the NC group, fibrosis and collagen area were similar to the Model group. However, fibrosis and area were significantly decreased in the NRG-1 overexpression group compared with the NC group, whereas the NRG-1 knockdown group exhibited a significant increase in fibrosis and collagen area compared with the NC group (Fig. 3B, C). Collagen histochemistry (Fig. 3D, E) indicated minimal collagen I expression in the vessel wall of the Sham group. Collagen I expression was significantly up-regulated in the Model and NC groups, with no statistically significant differences between them. However, compared with the NC group, collagen I expression was significantly down-regulated in the NRG-1 overexpression group and further up-regulated in the NRG-1 knockdown group. These findings confirm increased collagen fibre deposition post-AMI and the regulatory role of NRG-1 in collagen fibre deposition post-AMI.

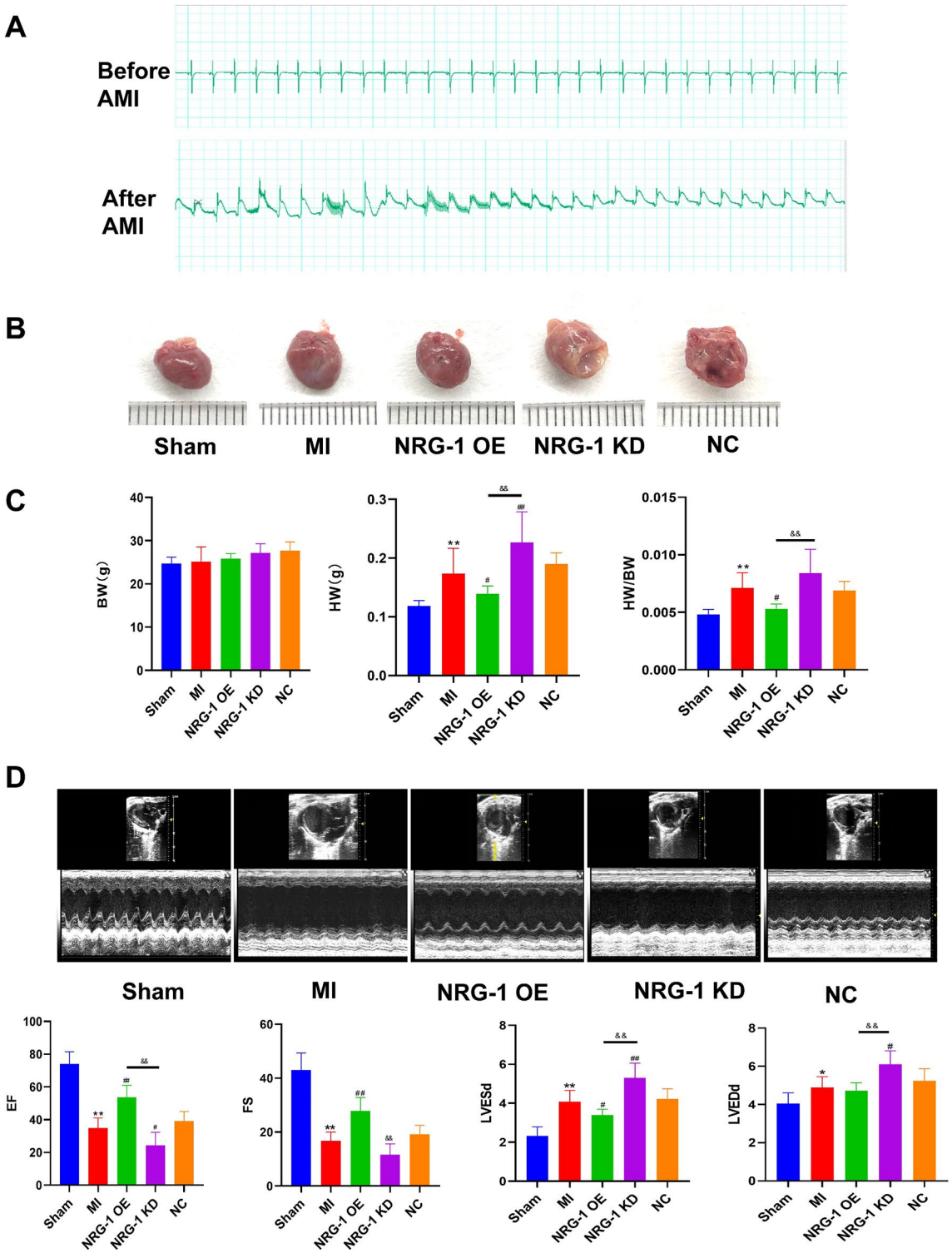


Fig. 2 NRG-1 regulates cardiac function and cardiac morphology after AMI. (**A**) ECG: before ligation: sinus rhythm, normal ECG. After ligation: LAD, ST segment arched dorsal elevation. (**B**) After 4 weeks of modeling, the cardiac morphology in each group. (**C**) Cardiac hypertrophy index of each groups. (**D**) Echocardiography of each groups. Note: $^{*}P<0.05$ vs. Sham group, $^{**}P<0.01$ vs. Sham group; $^{\#}P<0.05$ vs. Model group, $^{\#\#}P<0.01$ vs. Model group; $^{\&}P<0.05$ vs. NRG-1 OE group, $^{\&\&}P<0.01$ vs. NRG-1 OE group

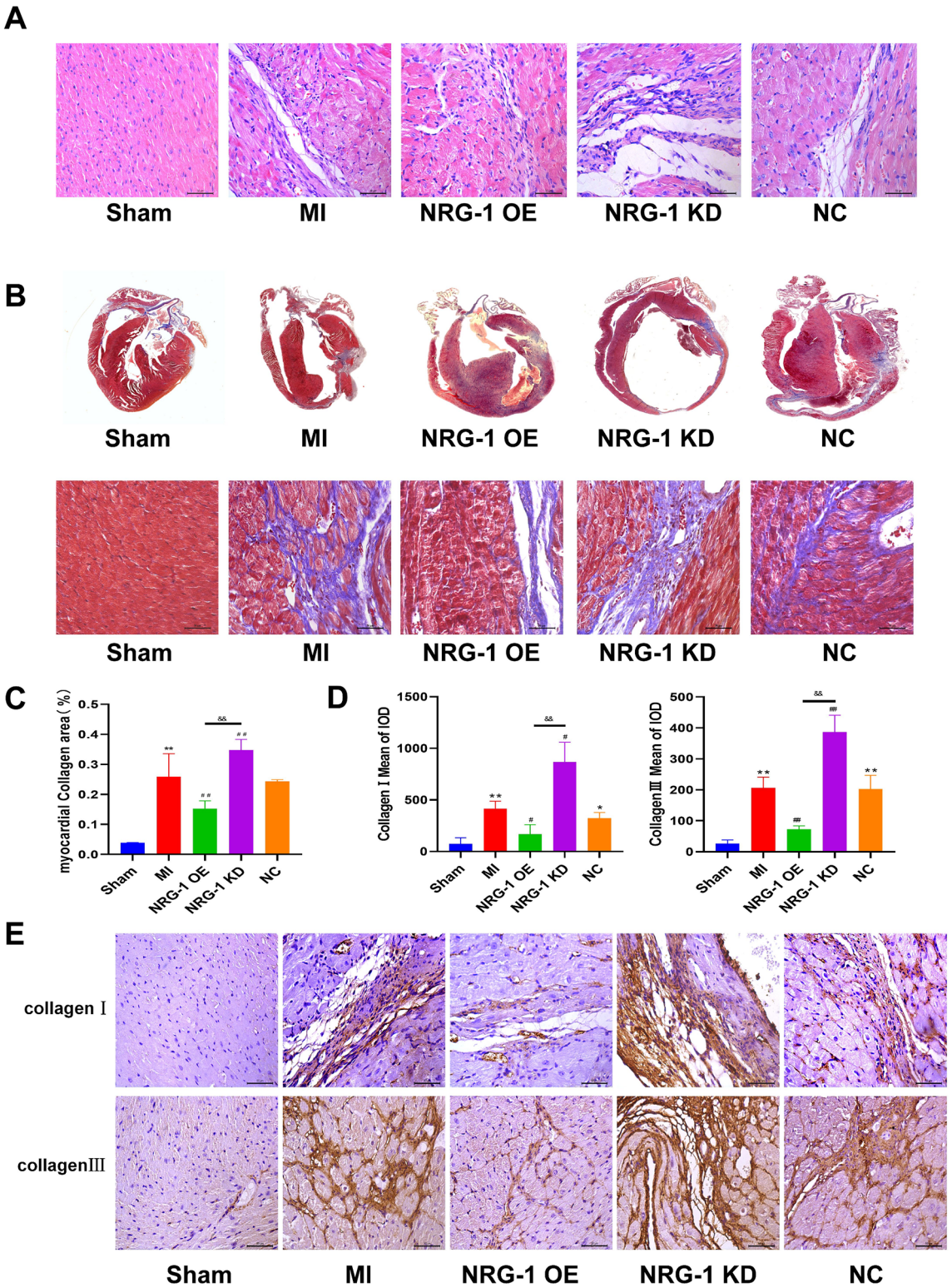


Fig. 3 Cardiac fibrosis after AMI, and regulation of NRG-1 can affect the degree of fibrosis. (**A**) HE staining in each groups. (**B**) and (**C**) Masson staining in each groups. (**D**) and (**E**). Immunohistochemical detection of Collagen I and Collagen III. Note: $^{*}P<0.05$ vs. Sham group, $^{**}P<0.01$ vs. Sham group; $^{\#}P<0.05$ vs. Model group, $^{\#\#}P<0.01$ vs. Model group; $^{\&}P<0.05$ vs. NRG-1 OE group, $^{\&\&}P<0.01$ vs. NRG-1 OE group

Neuregulin-1 inhibited endothelial-mesenchymal transition in mice hearts following acute myocardial infarction via phosphatidylinositol 3-kinase/protein kinase B pathway

Immunohistochemistry results (Fig. 4A, B) demonstrated down-regulated CD31 expression and up-regulated α-SMA expression in the Model and NC groups. Compared with the NC group, the NRG-1 overexpression group exhibited significantly up-regulated CD31 expression and down-regulated α-SMA expression, while the NRG-1 knockdown group displayed the opposite trend, with further down-regulated CD31 expression and up-regulated α-SMA expression. EndMT protein detection (Fig. 4C and D) revealed significantly increased levels of α-SMA and FSP-1 proteins, accompanied by decreased levels of VE-cadherin protein in the Model group post-AMI. Compared with the NC group, the NRG-1 overexpression group showed significantly higher levels of VE-cadherin and CD31 proteins, with lower levels of α-SMA and FSP-1 proteins, indicating inhibition of EndMT. In contrast, the NRG-1 knockdown group showed further decreased VE-cadherin and CD31 protein levels, along with increased α-SMA and FSP-1 proteins, exacerbating EndMT. Evaluation of vascular endothelial secretion function via eNOS detection (Fig. 4E) showed a significant decrease post-AMI, which was inhibited by NRG-1 overexpression and promoted by NRG-1 knockdown. This confirmed that NRG-1 could protect endothelial secretion function during AMI.

NRG-1 and its downstream pathway proteins were analysed via Western blot analysis (Fig. 5). The findings revealed a significant decrease in the protein levels of NRG-1, PI3K, and p-AKT post-AMI. Compared with the NC group, the NRG-1 overexpression group exhibited significantly increased levels of NRG-1, PI3K, and post-AKT proteins, indicating pathway activation. Conversely, the NRG-1 knockdown group showed further reductions in these proteins, confirming suppression of the PI3K/AKT pathway.

Tongxinluo improved acute myocardial infarction-induced cardiac dysfunction and myocardial fibrosis by activating neuregulin-1

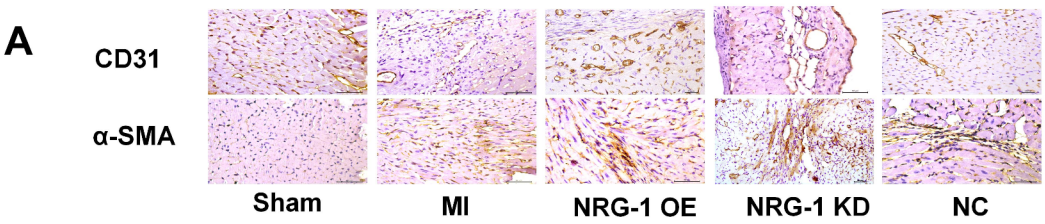
Compared with the Model group, the TXL group exhibited a notable decrease in the HWI, suggesting that TXL mitigates myocardial hypertrophy induced by AMI. Conversely, no significant change was observed in the TXL+NRG-1 knockdown group compared with the NC group (Fig. 6A), indicating that NRG-1 knockdown counteracted the protective effect of TXL. The HWI in the TXL+NRG-1 knockdown group was significantly higher compared with the TXL group, further supporting the role of NRG-1 in mediating the anti-hypertrophic effect of TXL. Additionally, following TXL treatment, there was a significant increase in EF, showing a substantial protective effect on cardiac function. Conversely, NRG-1 knockdown appeared to inhibit the protective effect of TXL (Fig. 6B, C). Moreover, EF levels in the TXL+NRG-1 knockdown group were significantly reduced compared with the TXL group, suggesting that NRG-1 activation is essential for the cardioprotective effects of TXL. Masson's trichrome staining revealed a notable reduction in MF in the TXL group compared with the Model group, which was reversed by NRG-1 knockdown (Fig. 7A). Immunohistochemistry for Collagen I and Collagen III demonstrated downregulation of collagen expression in the TXL group compared with the Model group. However, this effect was countered by NRG-1 knockdown (Fig. 7B). These findings confirm that TXL inhibits collagen fibre deposition following AMI, and that this protective effect can be nullified by NRG-1 knockdown.

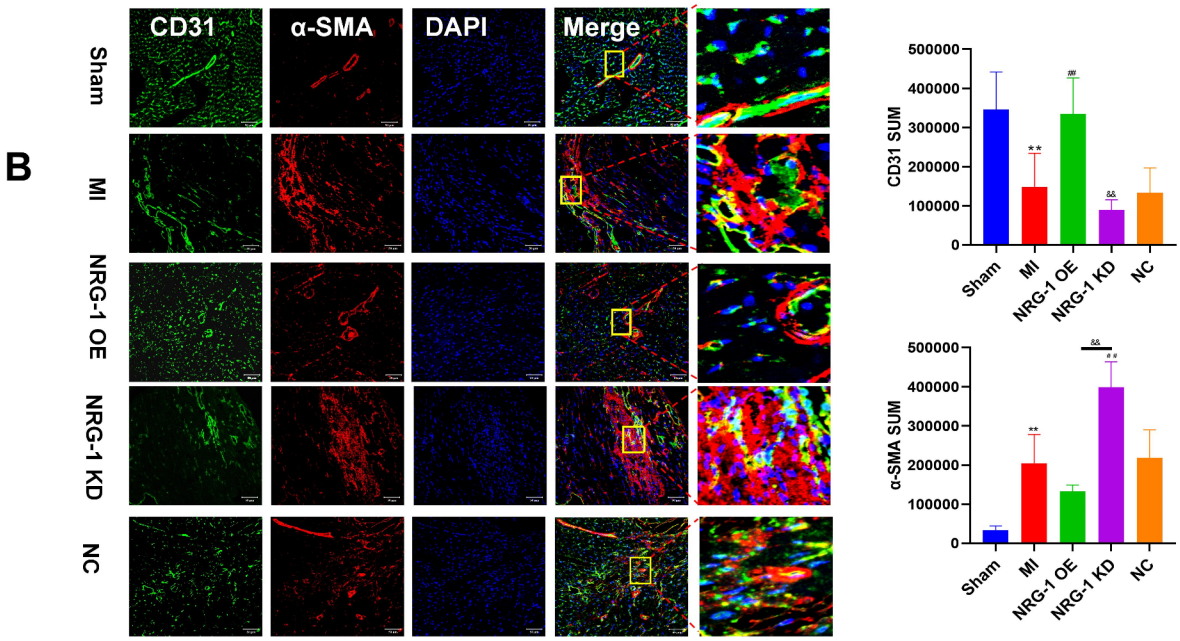
Fig. 4 Endothelial cells transform to mesenchymal cells after AMI, and regulation of NRG-1 can affect this transformation. (**A**) Immunohistochemical detection of endothelial cell marker CD31 and mesenchymal cell marker α-SMA in mouse heart. (**B**) Immunofluorescence CD31 (green), α-SMA (red) and DAPI (blue) stained apical images (original magnification, ×400, scale bar, 50 μm). (**C**) Western blot analysis to detect the protein expression levels of endothelial. (**D**) Western blot analysis to detect the protein expression levels of mesenchymal markers. (**E**) Western blot detects endothelial secretory function eNOs. Note: $^{*}P<0.05$ vs. Sham group, $^{**}P<0.01$ vs. Sham group; $^{\#}P<0.05$ vs. Model group, $^{\#\#}P<0.01$ vs. Model group; $^{\&}P<0.05$ vs. NRG-1 OE group, $^{\&\&}P<0.01$ vs. NRG-1 OE group

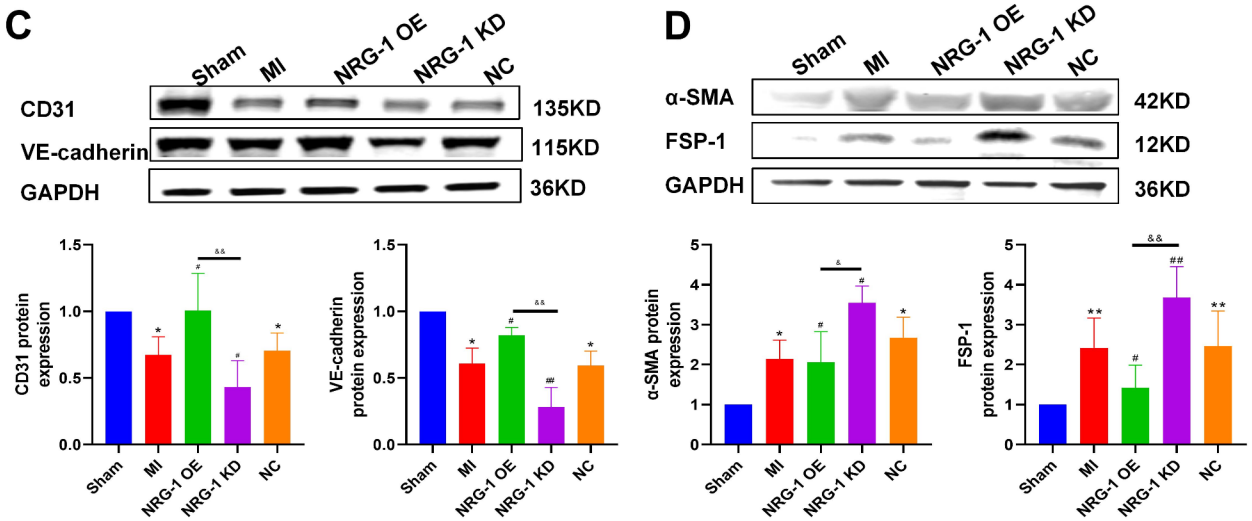
Tongxinluo inhibited endothelial-mesenchymal transition following acute myocardial infarction by activating neuregulin-1/phosphatidylinositol 3-kinase/protein kinase B pathway

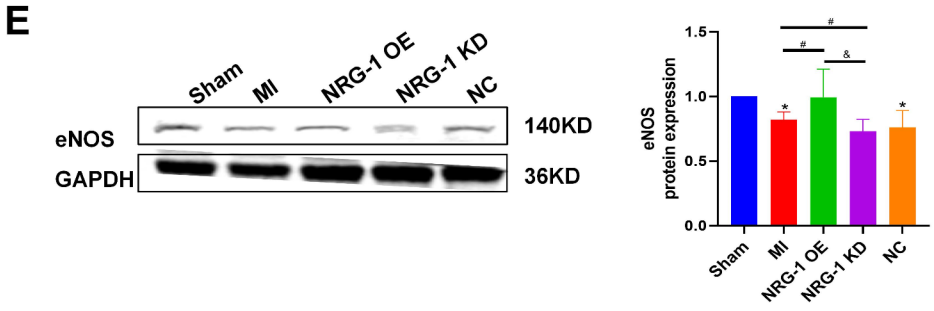
The immunofluorescence findings revealed elevated CD31 expression and reduced α-SMA expression in the TXL group, which was reversed by NRG-1 knockdown (Fig. 8A). Moreover, the TXL+NRG-1 knockdown group showed significantly lower CD31 expression and higher α-SMA expression compared with the TXL group, indicating that NRG-1 is essential for TXL's inhibitory effect on EndMT. Furthermore, compared with the Model group, the TXL group exhibited significantly increased CD31 protein expression and decreased α-SMA protein expression, with NRG-1 knockdown mitigating the effects of TXL (Fig. 8B).

These results validate that TXL ameliorates AMI-induced EndMT, but its efficacy is counteracted by NRG-1 knockdown. Western blot analysis demonstrated that TXL markedly enhanced the expression levels of NRG-1, along with the downstream PI3K and p-AKT proteins. However, this effect was nullified by NRG-1 knockdown (Fig. 9). These findings confirm that TXL inhibits EndMT following AMI


A
CD31
α-SMA
Sham
MI
NRG-1 OE
NRG-1 KD
NC


B
CD31
α-SMA
DAPI
Merge
Sham
MI
NRG-1 OE
NRG-1 KD
NC
CD31 SUM
α-SMA SUM


C
CD31
VE-cadherin
GAPDH
135KD
115KD
36KD
CD31 protein expression
VE-cadherin protein expression
D
α-SMA
FSP-1
GAPDH
42KD
12KD
36KD
α-SMA protein expression
FSP-1 protein expression


E
eNOS
GAPDH
140KD
36KD
eNOS protein expression

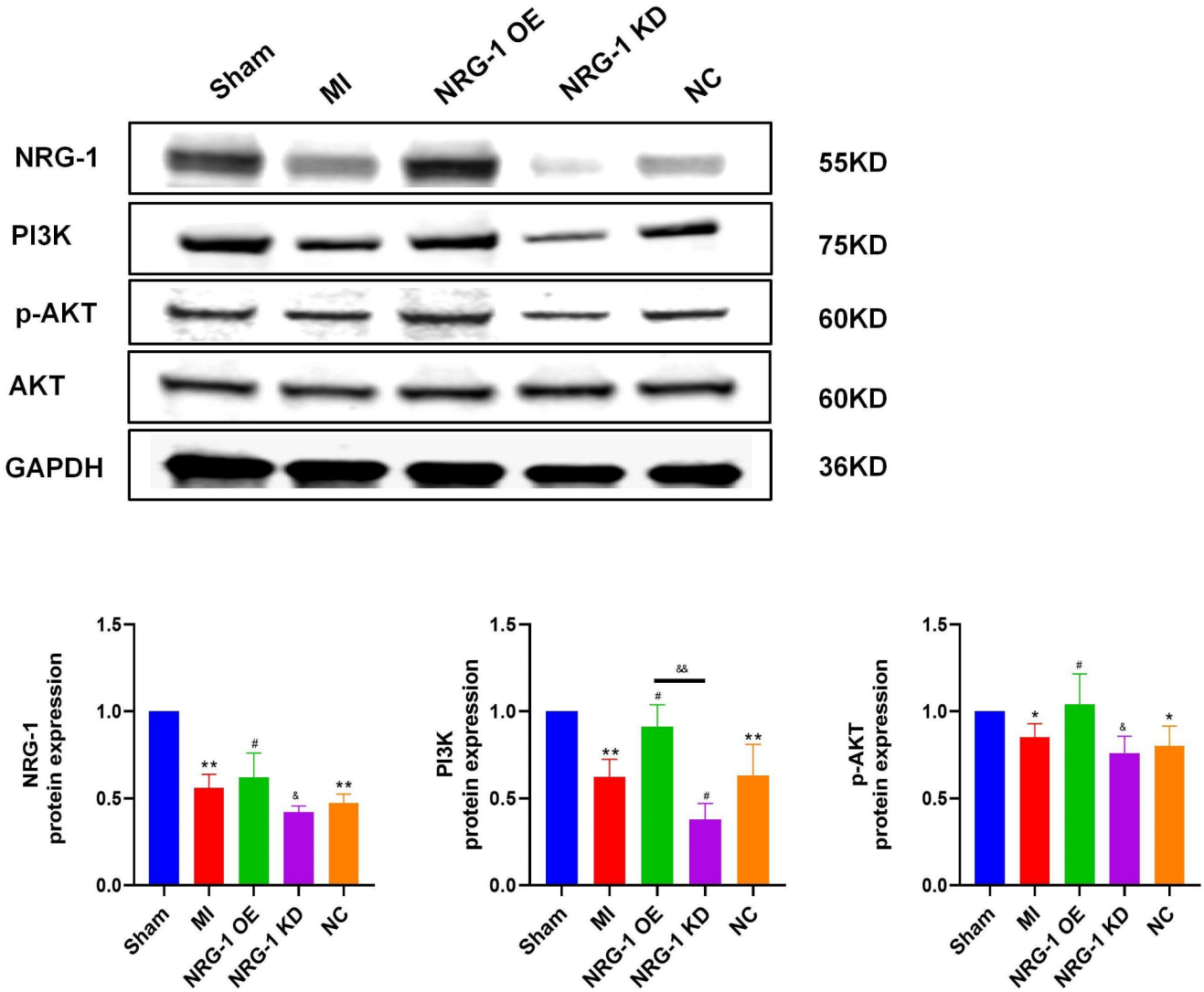


Fig. 5 Regulation of NRG-1 could affect downstream signaling pathway proteins. The expression of NRG-1, PI3K, p-AKT and AKT protein in different groups. Note: $^{*}P<0.05$ vs. Sham group, $^{**}P<0.01$ vs. Sham group; $^{\#}P<0.05$ vs. Model group, $^{\#\#}P<0.01$ vs. Model group; $^{\&}P<0.05$ vs. NRG-1 OE group, $^{\&\&}P<0.01$ vs. NRG-1 OE group

by activating the NRG-1 and its downstream PI3K/AKT signalling pathway.

Discussion

Acute myocardial infarction poses a serious threat to life, often resulting in MF, a pivotal pathological event that significantly impacts prognosis. Myocardial fibrosis manifests as the excessive accumulation of collagen fibres within the myocardium, leading to cardiac stiffening and compromised contractility (Ogiso et al. 2019). Despite its significant impact on patient outcomes, there are currently no effective treatments available for post-AMI MF. The mechanism underlying MF following AMI is not fully understood (Vas 2020). Acute myocardial infarction initiates a complex cascade of events that activate fibroblasts and promote collagen fibre production, driven by processes such as inflammation, oxidative stress and cardiomyocyte apoptosis. Recently, EndMT has emerged as a critical process in cardiac repair post-AMI, especially in pathological cardiac remodelling and subsequent cardiac fibrosis (Travers et al. 2016). The excessive deposition of extracellular matrix, facilitated by activated fibroblasts, is pivotal in driving MF. In this study, we observed that AMI induces EndMT in mice, as evidenced by the upregulation of mesenchymal cell markers and downregulation of endothelial cell markers. These findings underscore the significant role of EndMT in AMI-induced MF.

Neuregulin-1 plays a crucial role in cardiac development and functional regulation by interacting with tyrosine kinase receptors in the heart (e.g. HER3 and HER4).

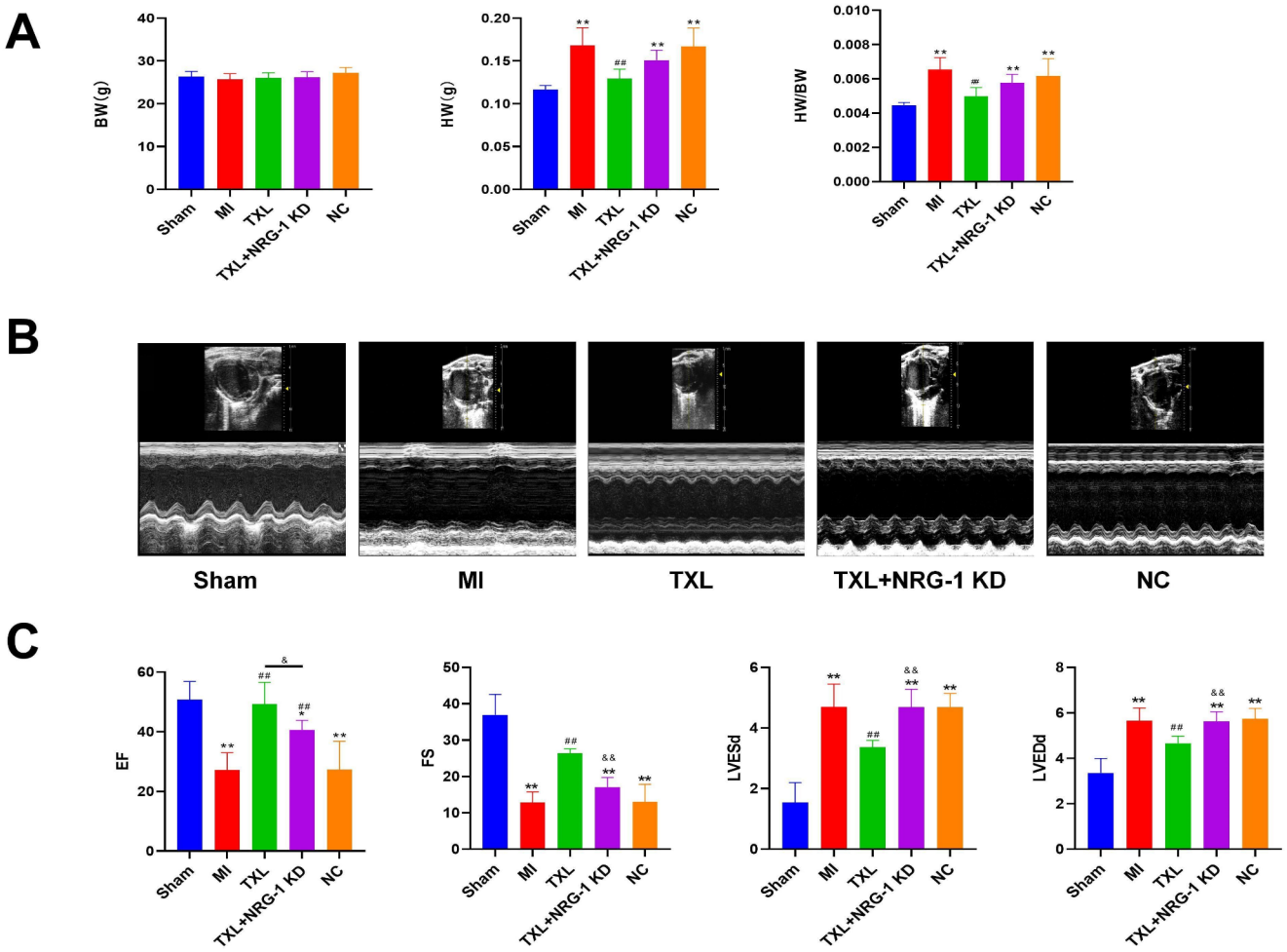


Fig. 6 TXL can improve the cardiac function of AMI mice, and knocking down the expression of NRG-1 can inhibit this effect. (**A**) Cardiac hypertrophy index of each groups; (**B**) Echocardiography of each groups. (**C**) Cardiac ejection fraction (EF), shortening fraction (FS), left ventricular end-systolic internal diameter (LVESD), and left ventricular end-diastolic internal diameter (LVEDD) in each group. Note: $^{**}P<0.01$ vs. Sham group; $^{\#}P<0.05$ vs. Model group, $^{\#\#}P<0.01$ vs. Model group

This interaction contributes to the formation of cardiomyocytes and inhibits MF (Gordon et al. 2022). A recent study showed that NRG-1 promotes angiogenesis, inhibits fibrosis and improves cardiac function (Chang et al. 2022), findings that are consistent with the results of the present study. Our results confirm that the inhibition of EndMT could mitigate cardiac remodelling and improve cardiac function following AMI, with NRG-1 identified as a key regulator of EndMT. Moreover, our previous studies have demonstrated that NRG-1 exerts anti-fibrotic effects by inhibiting EndMT– a process involved in the formation of fibroblasts and extracellular matrix–mediated through the activation of the NRG-1 signalling pathway (Qin et al. 2021). Consistent with previous studies, NRG-1 overexpression in our study suppressed cardiac fibrosis and alleviated the ventricular remodelling, whereas NRG-1 knockdown exacerbated the fibrosis deposition and compromised cardiac function.

However, it is important to acknowledge that in this study, NRG-1 knockdown/overexpression (KD/OE) was carried out via an adeno-associated virus (AAV) system, which is not cell-specific. This raises the question of whether the TXL-mediated upregulation of NRG-1 acts predominantly in cardiomyocytes, fibroblasts, or both. While the broad activation of NRG-1 may reflect systemic effects, the precise cellular targets remain unclear. This ambiguity should be addressed in future studies, potentially using more cell-specific approaches to better elucidate the direct role of NRG-1 in these cell types.

The PI3K/AKT signalling pathway is involved in various cellular processes (e.g. cell proliferation, differentiation, migration and transcription). It also regulates MF by inhibiting reactive oxygen species generation, apoptosis and autophagy (Ma et al. 2021). Considering its role in angiogenesis and inflammatory factor recruitment, this pathway may indirectly affect NO production in endothelial cells

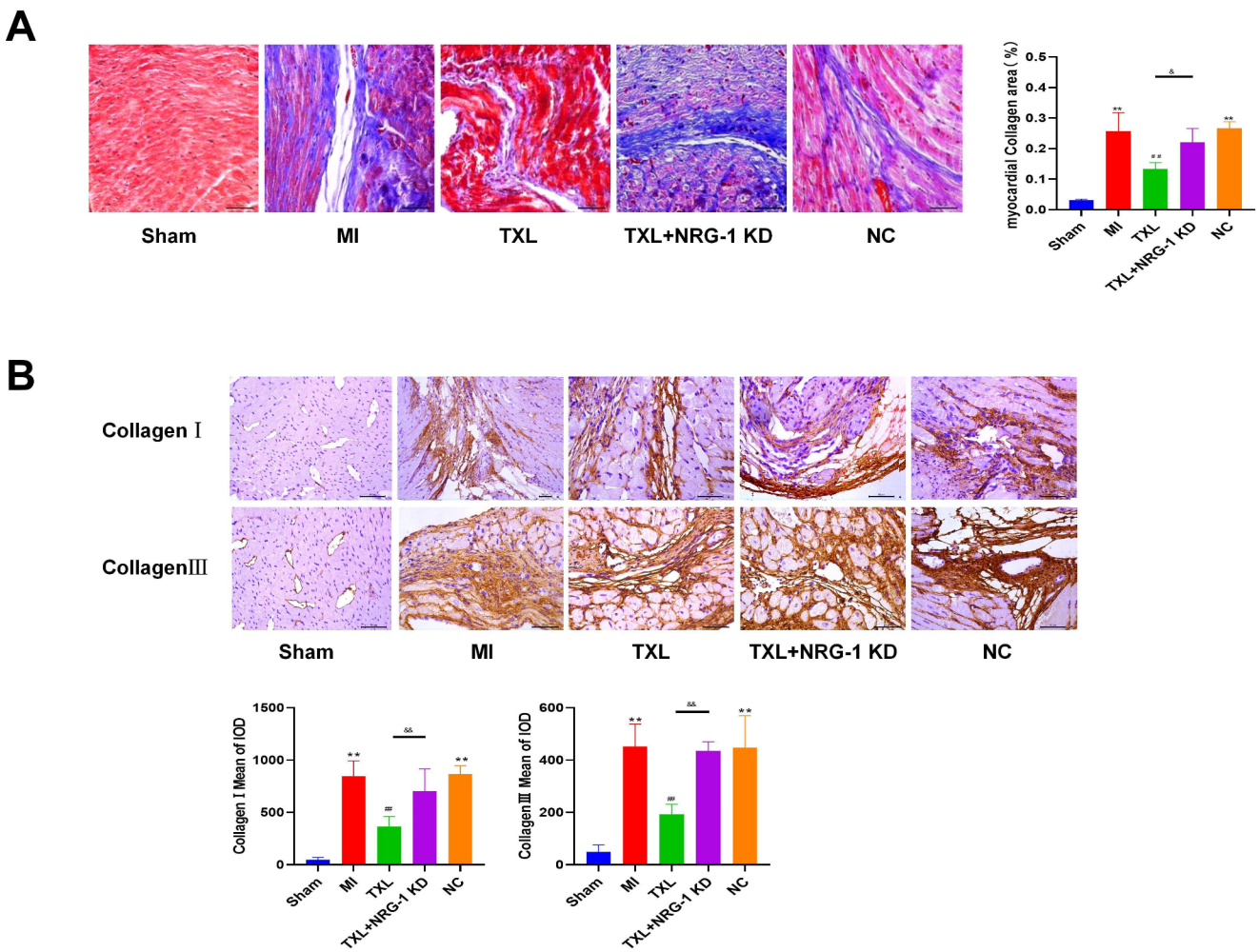


Fig. 7 TXL can inhibit myocardial fibrosis in AMI mice, and knocking down the expression of NRG-1 can inhibit this protective effect. (**A**) Myocardial fibrosis of mice in each group (Masson staining)(×400). (**B**) Collage I and Collagen III expressions were detected by immunohistochemistry (×400). Note: $^{**}P<0.01$ vs. Sham group;$^{\#}P<0.05$ vs. Model group, $^{\#\#}P<0.01$ vs. Model group; $^{\&}P<0.05$ vs. TXL group. $^{**}P<0.01$ vs. Sham group; $^{\#\#}P<0.01$ vs. Model group; $^{\&}P<0.05$ vs. TXL group

by influencing NOS activity or expression. Activating this pathway can promote the apoptosis of AMI endothelial cells and inhibit their proliferation (Yin et al. 2019). Moreover, the PI3K/AKT pathway plays an important role in EndMT, contributing to the formation of fibroblasts and extracellular matrix. A previous study has shown that NRG-1 can activate the PI3K/AKT pathway following hypoxia injury (Wang et al. 2017). To investigate the influence of NRG-1 on EndMT regulation, we analysed the expression of PI3K/AKT pathway-related proteins. To explore the influence of NRG-1 on EndMT regulation, we analysed the expression of PI3K/AKT pathway-related proteins. Tongxinluo administration activated the NRG-1/PI3K/AKT signalling pathway, which was essential for inhibiting EndMT and reducing MF. Activation of the PI3K/AKT signalling pathway may enhance the expression or function of NRG-1, thereby amplifying its cardioprotective effects.

Tongxinluo is a traditional Chinese medicine used for various cardiovascular diseases (e.g. atherosclerosis, angina pectoris and ischemia-reperfusion injury) (Chen et al. 2018). It has antioxidant, anti-inflammatory and anti-fibrotic properties, which have cardioprotective effects (Ma et al. 2009). Furthermore, TXL has been shown to attenuate MF following AMI by inhibiting EndMT and activating the NRG-1 signalling pathway (Yin et al. 2019). In our investigation, TXL demonstrated protective effects against MF post-AMI by inhibiting EndMT. We observed that TXL effectively activated NRG-1, whereas NRG-1 knockdown substantially compromised its therapeutic efficacy against MF. Additionally, our study corroborated the critical role of NRG-1/PI3K/AKT pathway activation in mitigating MF. These findings suggest that NRG-1 activation is a crucial mechanism by which TXL exerts its anti-fibrotic effects. Furthermore, our study corroborates the importance of the

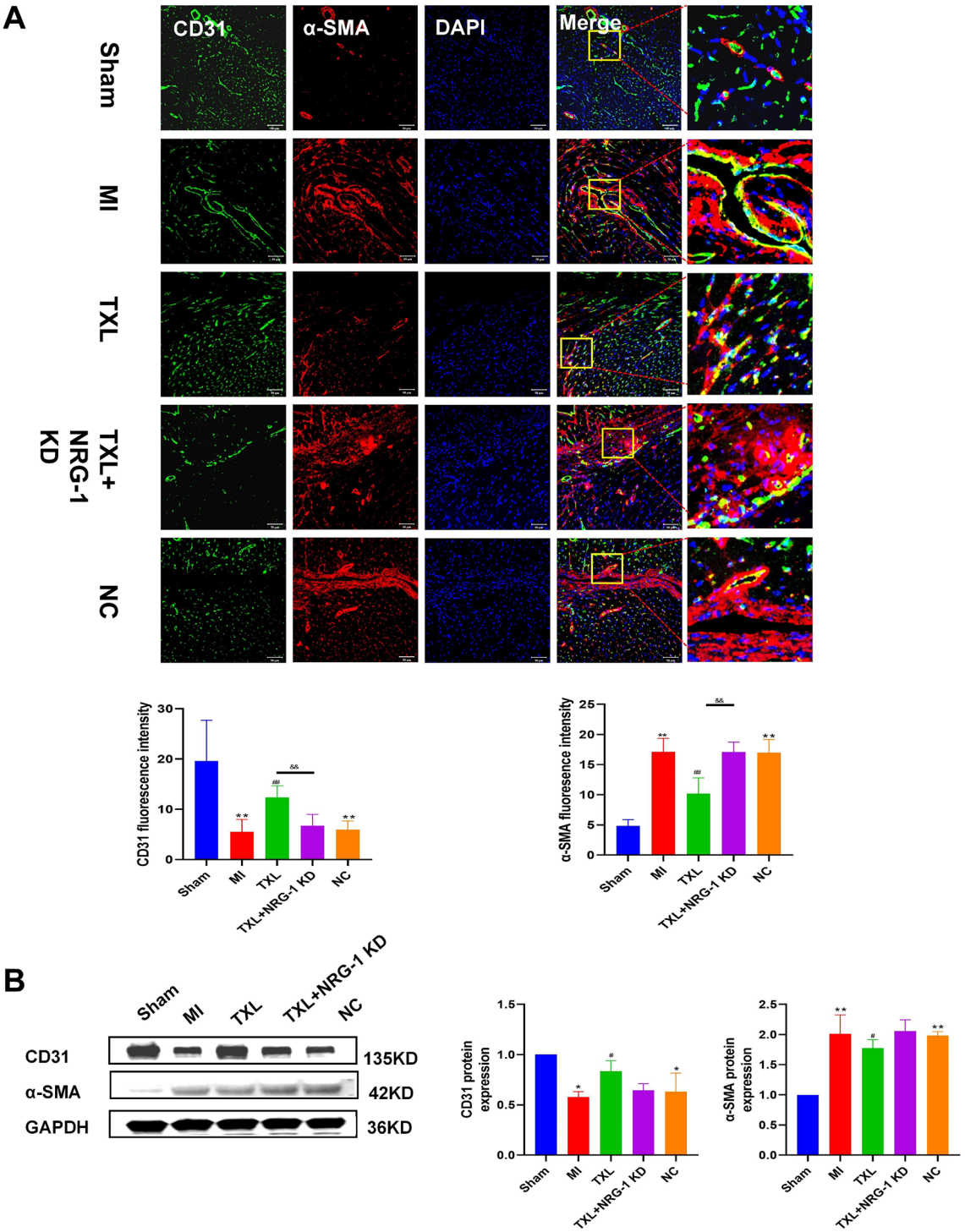


Fig. 8 TXL can inhibit endothelial mesenchymal transdifferentiation in AMI mice, and knocking down the expression of NRG-1 can inhibit this protective effect. (**A**) The expressions of CD31 and α-SMA were detected by immunofluorescence CD31 (green), α-SMA (red), DAPI (blue) (×400,50 μm). (**B**) The expression of CD31 and α-SMA protein in different groups. Note: $^{*}P<0.05$ vs. Sham group, $^{**}P<0.01$ vs. Sham group; $^{\#}P<0.05$ vs. Model group, $^{\#\#}P<0.01$ vs. Model group; $^{\&}P<0.05$ vs. TXL group

NRG-1/PI3K/AKT pathway in alleviating MF, reinforcing its potential as a therapeutic target.

It is important to acknowledge that the cell-specific effects of TXL-mediated NRG-1 activation remain unclear due to the non-cell-specific nature of the AAV system employed in this study. While the systemic upregulation of NRG-1 may yield beneficial therapeutic effects, the precise cellular mechanisms—whether NRG-1 acts primarily

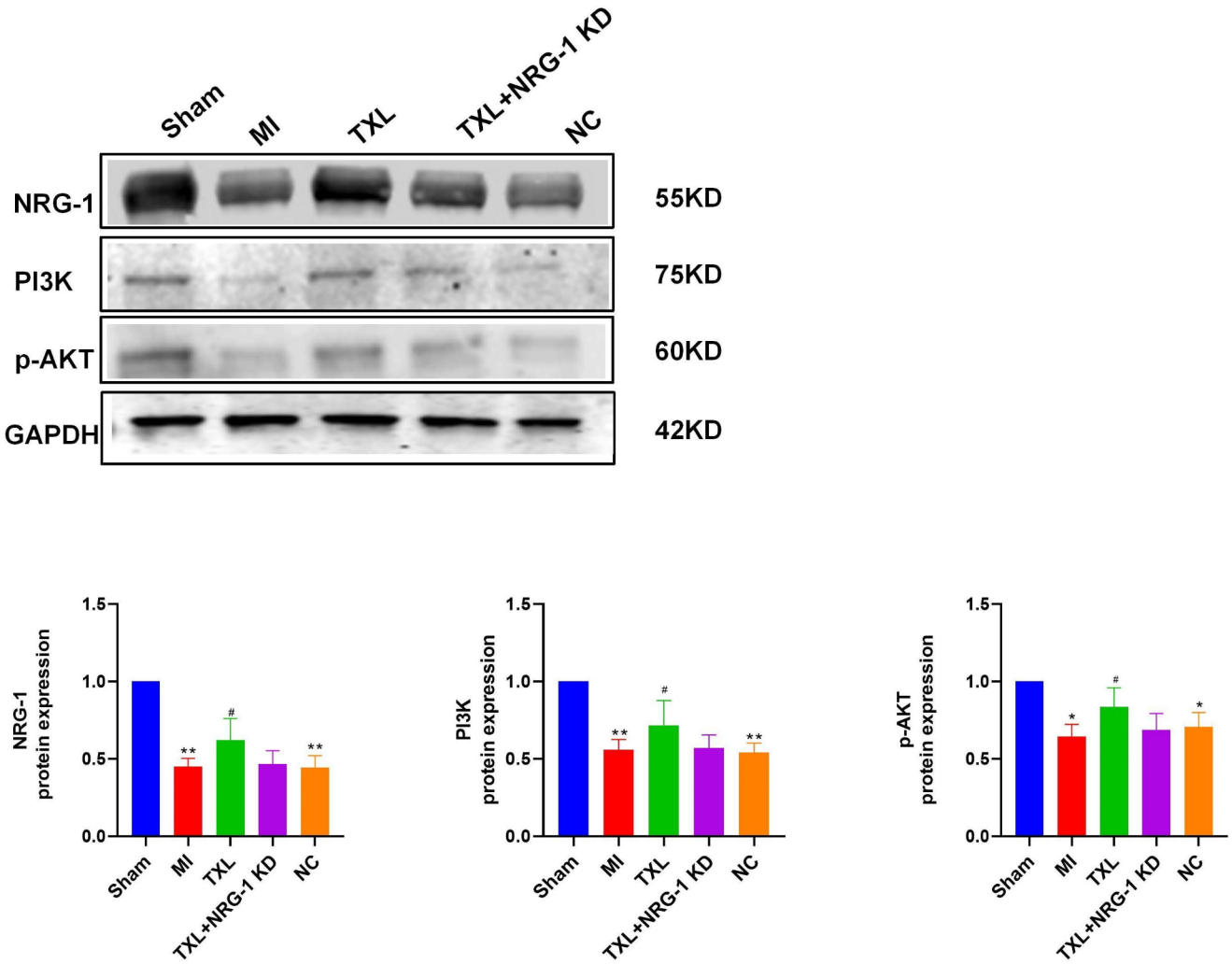


Fig. 9 Tongxinluo can inhibit the expression of NRG-1 and downstream pathway protein in AMI mice, and knocking down the expression of NRG-1 can inhibit this protective effect. The expression of NRG-1 PI3K and p-AKT protein in different groups. Note: $^{*}P<0.05$ vs. Sham group, $^{**}P<0.01$ vs. Sham group; $^{\#}P<0.05$ vs. Model group, $^{\#\#}P<0.01$ vs. Model group

on cardiomyocytes, fibroblasts, or both cell types—remain to be determined. To address this limitation, future studies should utilize more targeted approaches, such as tissue-specific Cre-Lox systems or cell-type-specific promoters, to selectively modulate NRG-1 expression in either cardiomyocytes or fibroblasts. Such approaches would enable a clearer understanding of the specific cellular contributions of NRG-1 in mitigating myocardial fibrosis (MF) and in modulating cardiac remodelling post-AMI.

Additionally, TXL has been shown to influence immune responses by modulating the expression of pro-inflammatory cytokines such as INF-γ and MMP-9, and by regulating the balance of T-cell subsets, including CD4+CD28-T cells and CD4+CD25+Treg cells (Su et al. 2010), thus exerting an immunomodulatory effect and providing a better immune environment for NRG-1. This enhances the neuroprotective and immunomodulatory effects of NRG-1. Furthermore, NRG-1 may further support the immunomodulatory effects of TXL by promoting the survival and proliferation of immune cells.

In this study, although a reduction in α-SMA-positive myofibroblasts was observed, which may affect scar formation, surviving mice showed good recovery after AMI. Masson staining showed marked fibrosis and ventricular thinning, but the remaining ventricular wall maintained contractile function. A small number of mouse deaths were attributed to the AMI modeling process rather than TXL treatment. However, a 4-week observation period may not fully capture long-term outcomes such as progressive ventricular thinning or rupture.

These findings emphasize the need for long-term studies to assess the long-term effects of TXL on ventricular remodeling and survival. Further studies should explore the balance between reducing pathological fibrosis and

maintaining scar integrity to inform optimal AMI treatment strategies.

Conclusions

In conclusion, this study demonstrated that NRG-1 played a crucial role in regulating EndMT and MF after AMI, mediated by the PI3K/AKT pathway. We also showed that TXL had anti-fibrotic effects by activating NRG-1 and inhibiting EndMT. These findings suggest that NRG-1 might be a potential therapeutic target for preventing cardiac remodelling and improving cardiac function following AMI. However, further studies are needed to identify the effective components of TXL and the molecular mechanisms of NRG-1 activation.

Acknowledgements We are grateful to Mr.Stephen S for his invaluable help in the linguistic embellishment of the thesis.

Author contributions Li Z conceived of the study, and Yin YJ, Wei YR, Liu Y, Han NX and Wang XQ participated in its design and data analysis and statistics and Hao YJ, Wang YF, Hou YL and Jia ZH helped to draft the manuscript. All authors read and approved the final manuscript.

Funding This research was funded by National Natural Science Foundation of China (No. 81973692).

Data availability The data that support the findings of this study are available from the corresponding author JLM upon reasonable request.

Declarations

Ethics approval and consent to participate The study was conducted in accordance with the Declaration of Helsinki, and all protocols and procedures for handling animals in accordance with the ethical guidelines of the Ethics Committee of Hebei Academy of Integrated Traditional Chinese and Western Medicine (No: N2021065).

Consent for publication Not applicable.

Conflict of interest Prof. Zhenhua Jia reports affiliated to Hebei Yiling Hospital, a non-profit medical institution, which is two completely independent legal entities with Shijiazhuang Yiling Pharmaceutical Co., LTD. Prof Jia is the spouse of Ms. Rui Wu, who holds shares and serves as a director of Shijiazhuang Yiling Pharmaceutical Co., LTD. He has fully disclosed these interests to the research committee and have developed an approved plan to manage any potential conflicts that may arise from such an arrangement and ensured the scientificity, objectivity and authority of the research results.

References

Berezin AE, Berezin AA (2020) Adverse cardiac remodelling after acute myocardial infarction: old and new biomarkers. DIS MARKERS 2020:1215802

Carter HE, Schofield D, Shrestha R (2019) Productivity costs of cardiovascular disease mortality across disease types and socioeconomic groups. Open Heart 6:e939

Chaitman BR, Alexander KP, Cyr DD et al (2021) Myocardial infarction in the ISCHEMIA trial: impact of different definitions on incidence, prognosis, and treatment comparisons. Circulation 143:790–804

Chang T, Liu C, Yang H et al (2022) Fibrin-based cardiac patch containing neuregulin-1 for heart repair after myocardial infarction. Colloids Surf B Biointerfaces 220:112936

Chen Y, Li M, Zhang Y et al (2018) Traditional Chinese medication Tongxinluo attenuates apoptosis in ox-LDL-stimulated macrophages by enhancing Beclin-1-induced autophagy. Biochem Biophys Res Commun 501(2):336–342. https://doi.org/10.1016/j.bbrc.2018.03.094

Cui H, Li N, Li X et al (2016) Tongxinluo modulates cytokine secretion by cardiac microvascular endothelial cells in ischemia/reperfusion injury. AM J TRANSL RES 8:4370–4381

Fan D, Takawale A, Lee J et al (2012) Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. Fibrogenesis Tissue Repair 5:15

Gardarsdottir HR, Sigurdsson MI, Andersen K et al (2022) Long-term survival of Icelandic women following acute myocardial infarction. SCAND CARDIOVASC J 56:114–120

Geissler A, Ryzhov S, Sawyer DB (2020) Neuregulins: protective and reparative growth factors in multiple forms of cardiovascular disease. Clin Sci (Lond) 134:2623–2643

Gordon DM, Cunningham D, Zender G et al (2022) Exome sequencing in multiplex families with left-sided cardiac defects has high yield for disease gene discovery. PLOS GENET 18:e1010236

Gulati A, Japp AG, Raza S et al (2018) Absence of myocardial fibrosis predicts favorable Long-Term survival in New-Onset heart failure. Circ Cardiovasc Imaging 11:e7722

Jakob L, Bedulina DS, Axenov-Gribanov DV et al (2017) Uptake kinetics and subcellular compartmentalization explain lethal but not sublethal effects of cadmium in two closely related amphipod species. ENVIRON SCI TECHNOL 51:7208–7218

Jordan NP, Tingle SJ, Shuttleworth VG et al (2021) MiR-126-3p is dynamically regulated in Endothelial-to-Mesenchymal transition during fibrosis. INT J MOL SCI;22

Kang W, Cheng Y, Zhou F et al (2019) Neuregulin–1 protects cardiac function in septic rats through multiple targets based on endothelial cells. Int J Mol Med 44(4):1255–1266

Kuramochi Y, Guo X, Sawyer DB (2006) Neuregulin activates erbB2-dependent Src/FAK signaling and cytoskeletal remodeling in isolated adult rat cardiac myocytes. J MOL CELL CARDIOL 41:228–235

Liu S, Zhang Z, He Y et al (2023) Inhibiting leukocyte-endothelial cell interactions by Chinese medicine Tongxinluo capsule alleviates

no-reflow after arterial recanalization in ischemic stroke. CNS NEUROSCI THER 29:3014–3030
Ma Q, Zhang S, Ning Y et al (2009) Effect of Tongxinluo on endothelial function and hypersensitive C-reactive protein in acute coronary syndrome patients undergoing percutaneous coronary intervention. Zhong Nan Da Xue Xue Bao Yi Xue Ban 34(6):550–554
Ma W, Zhang X, Liu Y (2021) miR-124 promotes apoptosis and inhibits the proliferation of vessel endothelial cells through P38/MAPK and PI3K/AKT pathways, making it a potential mechanism of vessel endothelial injury in acute myocardial infarction. EXP THER MED 22:1383
Ndrepepa G, Tiroch K, Fusaro M et al (2010) 5-year prognostic value of no-reflow phenomenon after percutaneous coronary intervention in patients with acute myocardial infarction. J AM COLL CARDIOL 55:2383–2389
Noll JM, Sherafat AA, Ford GD et al (2024) The case for neuregulin-1 as a clinical treatment for stroke. Front Cell Neurosci 18:1325630. https://doi.org/10.3389/fncel.2024.1325630
Ogiso K, Koriyama N, Obo T et al (2019) Performance of a 2-step insulin infusion protocol with adjustment of insulin doses for Asians in the medical intensive care unit following cardiothoracic surgery. Diabetol Int 10:12–23
Parameswarappa C, Agarwal D, Jalali K (2019) Role of complete laser photocoagulation and wide-field imaging in aggressive posterior retinopathy of prematurity. BMJ Case Rep 12(5):e230031
Qin W, Cao L, Massey IY (2021) Role of PI3K/Akt signaling pathway in cardiac fibrosis. MOL CELL BIOCHEM 476:4045–4059
Shahriary GM, Kataria H, Karimi-Abdolrezaee S (2019) Neuregulin-1 fosters supportive interactions between microglia and neural stem/progenitor cells. STEM CELLS INT 2019:8397158
Su W, Sun A, Xu D et al (2010) Tongxinluo inhibits oxidized low-density lipoprotein-induced maturation of human dendritic cells via activating peroxisome proliferator-activated receptor gamma pathway. J Cardiovasc Pharmacol 56(2):177–183
Travers JG, Kamal FA, Robbins J et al (2016) Cardiac fibrosis: the fibroblast awakens. CIRC RES 118:1021–1040
Vas Z (2020) Contributions to the taxonomy and biogeography of nemeritis Holmgren (Hymenoptera: Ichneumonidae: Campopleginae). ZOOTAXA 4758:4753–4758
Virani SS, Alonso A, Benjamin EJ et al (2020) Heart disease and stroke Statistics-2020 update: A report from the American heart association. Circulation 141:e139–e596
Wang J, Zhou J, Wang Y et al (2017) Qiliqiangxin protects against anoxic injury in cardiac microvascular endothelial cells via NRG-1/ErbB-PI3K/Akt/mTOR pathway. J CELL MOL MED 21:1905–1914
Wang X, Zhuo X, Gao J et al (2019) Neuregulin-1beta partially improves cardiac function in Volume-Overload heart failure through regulation of abnormal calcium handling. FRONT PHARMACOL 10:616
Wang Y, Wei J, Zhang P et al (2022) Neuregulin-1, a potential therapeutic target for cardiac repair. FRONT PHARMACOL 13:945206
Wei WX, Jiang YH (2023) To investigate the clinical efficacy and potential mechanism of Tongxinluo capsules in preventing coronary restenosis based on Meta-Analysis and network Pharmacology analysis. Evid Based Complement Alternat Med 2023:7985459
Wu L, Islam MR, Lee J et al (2021) ErbB3 is a critical regulator of cytoskeletal dynamics in brain microvascular endothelial cells: implications for vascular remodeling and blood brain barrier modulation. J Cereb Blood Flow Metab 41:2242–2255
Yin Y, Zhang Q, Zhao Q et al (2019) Tongxinluo attenuates myocardiac fibrosis after acute myocardial infarction in rats via Inhibition of Endothelial-to-Mesenchymal transition. BIOMED RES INT 2019:6595437
Zeisberg EM, Tarnavski O, Zeisberg M et al (2007) Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. NAT MED 13:952–961