

MicroRNA-93 promotes angiogenesis and attenuates remodeling via inactivation of the Hippo/Yap pathway by targeting Lats2 after myocardial infarction

CHENGJIE MA^{1*}, PEIPEI PENG^{2*}, YAN ZHOU³, TIANYA LIU^{4,5}, LIJUAN WANG⁴ and CHEN LU⁴

¹Clinical and Research Center of Infectious Disease, Beijing Ditan Hospital, Capital Medical University, Beijing 100015; ²Department of Anesthesiology, First Affiliated Hospital with Nanjing Medical University, Nanjing, Jiangsu 210029; ³Department of Neurology, The Affiliated Hospital of Xuzhou Medical University; ⁴Jiangsu Province Key Laboratory of Anesthesiology, School of Anesthesiology; ⁵Department of Pharmacy, The Affiliated Hospital of Xuzhou Medical University, Xuzhou Medical University, Xuzhou, Jiangsu 221002, P.R. China

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Abstract. Inactivation of the Hippo pathway protects the myocardium from cardiac ischemic injury. MicroRNAs (miRs) have been reported to play pivotal roles in the progression of myocardial infarction (MI). The present study examined whether miR-93 could promote angiogenesis and attenuate remodeling after MI via inactivation of the Hippo/Yes-associated protein (Yap) pathway, by targeting large tumor suppressor kinase 2 (Lats2). It was identified that transfection of human umbilical vein endothelial cells with miR-93 mimic significantly decreased Lats2 expression and Yap phosphorylation, increased cell viability and migration, and attenuated cell apoptosis following hypoxia/reoxygenation injury. Moreover, increased expression of miR-93 resulted in an improvement of cardiac function, promotion of angiogenesis and attenuation of remodeling after MI. Additionally, miR-93 overexpression significantly decreased intracellular adhesion molecule 1 and vascular cell adhesion protein 1 expression levels, as well as attenuated the infiltration of neutrophils and macrophages into the myocardium after MI. Furthermore, it was found that miR-93 overexpression significantly suppressed Lats2 expression and decreased the levels of phosphorylated Yap in the myocardium after MI. Collectively, the present results suggested that miR-93 may exert a protective effect

against MI via inactivation of the Hippo/Yap pathway by targeting Lats2.

Introduction

Acute myocardial ischemia leads to an augmentation in the production of reactive oxygen species, and the activation of deleterious cellular signaling that causes damage of cardiomyocytes and endothelial cells (1). Endothelial dysfunction results in the production of proinflammatory cytokines and the upregulation of adhesion molecules, which leads to the infiltration of inflammatory cells to the infarction region (2). Furthermore, the impairment of endothelial cell has been identified as an important factor in aggravating cardiac remodeling (3). Moreover, reduction of capillary densities within infarcted hearts further accelerates the remodeling process, which results in heart failure (4).

The Hippo pathway is an evolutionally conserved pathway that plays an important role in cell proliferation, apoptosis and angiogenesis (5,6). The core components of the Hippo pathway include the large tumor suppressor 2 (Lats2), Yes-associated protein (Yap) and its paralog protein Transcriptional co-activator with PDZ-binding motif (5). Activation of Lats2 induces the phosphorylation of Yap, causing its cytoplasmic retention and functional inactivation (7,8). Previous studies have shown that inactivation of the Hippo pathway is essential for the regulation of cardiac regeneration, function and remodeling after myocardial infarction (MI) (9,10). Furthermore, Lats2 is necessary to regulate the ability of Yap in the epicardium for coronary vascular formation (11). It has been shown that inhibition of endogenous Lats2 results in reduced infarct size and augmented Yap activation after myocardial ischemic injury (8,12). Moreover, loss of Lats2 in the developing heart induces an increase in Yap activation and is sufficient to cause cardiomyocyte proliferation (13). Additionally, Lats2 knockdown in endothelial cells decreases cell apoptosis, and increases migration and endothelial tube formation (6,14,15).

MicroRNAs (miRs) are small, non-coding RNAs that function as post-transcriptional regulators to suppress target

Correspondence to: Dr Chen Lu, Jiangsu Province Key Laboratory of Anesthesiology, School of Anesthesiology, Xuzhou Medical University, 209 Tongshan Road, Xuzhou, Jiangsu 221002, P.R. China
E-mail: lucoct@163.com

*Contributed equally

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gene expression via mRNA degradation or translational inhibition (16). Previous studies, including our own, have demonstrated the involvement of miRs in the regulation of angiogenesis and cardiac remodeling in response to MI (4,17-19). It has been reported that miR-302-367 promotes cardiomyocyte proliferation by repressing Lats2 expression via regulation of the Hippo pathway (20). In addition, a previous study showed that miR-93 is involved in angiogenesis and metastasis by targeting Lats2 (21).

The aim of the present study was to investigate whether miR-93 exerts a protective role in endothelial cells and the myocardium after MI via modulation of the Hippo/Lats2/Yap pathway. The present results suggested that upregulation of miR-93 protected endothelial cells from hypoxia/reoxygenation (H/R) injury. Furthermore, increased expression of miR-93 promoted cardiac angiogenesis in ischemic hearts by regulating the Hippo/Yap pathway and suppressing Lats2 expression.

Materials and methods

Animals. Shanghai populations of Kunming mice (n=220; age, 7-8 weeks; male; weight, 28-30 g) were obtained from the Division of Laboratory Animal Resources at Xuzhou Medical University. The mice were maintained under controlled conditions of temperature at 23-25°C, relative humidity of 50-60% and a 12-h light/dark light cycle with free access to food and water. The experiments were performed in accordance with The Guide for The Care and Use of Laboratory Animals published by The National Institutes of Health (NIH Publication, 8th edition, 2011) (22). All aspects of the animal care and experimental protocols were approved by Xuzhou Medical University Committee on Animal Care.

In vitro experiments. Human umbilical vein endothelial cells (HUVECs; cat. no. 8000; ScienCell Research Laboratories, Inc.) were cultured in EBM-2 (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 11111044) supplemental with EGM-2 SingleQuots kit (Lonza Group Ltd.; cat. no. cc-4147) and 20% FBS (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 10099141) at 37°C and 5% CO₂. Human cardiac microvascular endothelial cells (HCMECs; cat. no. 6000; ScienCell Research Laboratories, Inc.) were seeded onto a fibronectin-precoated culture dishes (2 µg/cm²; ScienCell Research Laboratories, Inc.; cat. no. 8248) and maintained in endothelial cell medium (ScienCell Research Laboratories, Inc.; cat. no. 1001) at 37°C and 5% CO₂. miR-93 mimic (5'-CAAAGUGCUGUUCGUGCAGGUAG-3') and scrambled (scr) miR mimic (5'-ACUACUGAGUGACA GUAGA-3') were purchased from Shanghai GenePharma Co., Ltd. Cells were transfected with miR-93 mimic (40 nmol) or scr-miR mimic (40 nmol) using RNAiFectin (Applied Biological Materials, Inc.; cat. no. G073). After 48 h of transfection, the cells were collected for isolation of miRNAs. miR-93 levels were examined by reverse transcription-quantitative PCR (RT), as described below. After transfection, the cells were incubated with hypoxia buffer containing 118 mM NaCl, 24 mM NaHCO₃, 1.0 mM NaH₂PO₄, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 20 mM sodium lactate, 16 mM KCl, and 10 mM 2-deoxyglucose (pH 6.2) at 37°C with 5% CO₂ and 95% N₂ for 4 h, and subjected to reoxygenation with EBM-2

(cat. no. 11111044; Gibco; Thermo Fisher Scientific, Inc.) supplemental with EGM-2 SingleQuots kit (cat. no. cc-4147; Lonza Group Ltd.) for 24 h at 37°C with 5% CO₂. Cells subjected to H/R without transfection served as positive controls (H/R). Cells that were not exposed to H/R served as the normoxia group.

Measurement of lactate dehydrogenase (LDH) activity and cell viability. Cell injury was determined by measurement of LDH activity in culture medium using a cytotoxicity detection kit (Beyotime Institute of Biotechnology; cat. no. C0017). Cell viability was examined with 10 µl Cell Counting Kit-8 assay (concentration not commercially available) (Dojindo Laboratories, Inc; cat. no. CK40) at 37°C for 2 h according to the manufacturer's protocol. The optical density was measured at a wavelength of 450 nm.

Migration assay. A previously described method was used to evaluate cell migration (4). Following transfection, the migration of HUVECs or HCMECs was determined using a scratch assay with 10% FBS. HUVECs or HCMECs were scratched with 200 µl tips, and cells were imaged at 0 and 12 h after scratch using a light microscope (magnification, x200). The percentage of the wound area was analyzed with ImageJ software (NIH; version 1.48; <http://rsb.info.nih.gov/ij/>).

Mouse model of MI. MI was induced as previously described (4). Mice (n=160, weight, 28-30 g) were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital (Sigma-Aldrich; Merck KGaA), intubated and ventilated using a rodent ventilator. Body temperature was regulated at 37°C with a heating pad. Following skin incision, hearts were exposed via a left thoracotomy in the fourth intercostal space. The left anterior descending (LAD) coronary artery was permanently ligated with an 8-0 silk suture. Sham-operated mice underwent the same surgical procedure except for ligation of the LAD coronary artery. All animals were recovered in pre-warmed cages.

In vivo transfection of lentivirus expressing miR-93. Mouse hearts were transfected with lentivirus expressing miR-93 or scr-miR via the right common carotid artery, as described previously (4). The recombinant lentiviruses containing miR-93 (1x10⁸ PFU/ml) and lentiviruses containing scrambled miR (1x10⁸ PFU/ml) were obtained from Shanghai GenePharma Co., Ltd. Mice (28-30 g) were anesthetized with an intraperitoneal injection of 60 mg/kg sodium pentobarbital. Following the skin incision, the right common carotid artery was exposed. A micro-catheter was introduced into the right common carotid artery and positioned into the aortic root. 50 µl lentivirus expressing miR-93 or 50 µl scrambled miR was injected via the micro-catheter. The micro-catheter was removed and the right common carotid artery was tightened before the skin was closed. After 7 days of transfection, hearts were harvested for miRNA isolation. miR-93 expression was measured by RT-qPCR for evaluation of transfection efficiency.

RT-qPCR. Ischemic hearts or cultured cells were harvested and isolation of miRNAs was performed using the miR isolation kit (cat. no. RC201; Vazyme Biotech Co., Ltd.). RT-qPCR

was conducted using LightCycler 480 RT-PCR instrument (Roche Diagnostics). According to the manufacturer's protocol, miR-93 expression was quantified using one-step RNA TaqMan qPCR mix kit (Xinhai Gene Testing Co., Ltd.; cat. no. A2302) and TaqMan qPCR mix kit (Xinhai Gene Testing Co., Ltd.; cat. no. A2301). RT conditions were as follows: 16°C for 30 min, 42°C for 30 min and 75°C for 15 min. qPCR amplification conditions were as follows: Initial denaturation at 95°C for 15 min; 40 cycles of 95°C for 10 sec and 60°C for 60 sec. Specific primers were obtained from Applied Biosystems (mmu-miR-93, primer identification no. 001090; miR-93: Forward, 5'-AGGCCCAAAGTGCTGTTCGT-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'; U6 small nucleolar RNA, primer identification no. 001973; U6: Forward, 5'-GCTTC GAGGCAGGTTACATG-3' and reverse, 5'-GCAACACA CAACATCTCCCA-3'). miR-93 levels were quantified using the $2^{-\Delta\Delta C_q}$ method and were normalized to the U6 expression level (23).

Ischemic hearts were collected for isolation of RNA. Total RNA was isolated with total RNA extraction reagent (Vazyme Biotech Co., Ltd.; cat. no. R401-01). According to the manufacturer's instructions, cDNA was synthesized using RT MasterMix (Applied Biological Materials, Inc.; cat. no. G485). The following conditions were used for RT: 25°C for 10 min, followed by 42°C for 15 min. qPCR reactions (10 μ l) consisted of 5 μ l PrimeScript RT master mix (Takara Bio, Inc.), 0.5 μ l primer (final concentration, 10 nM), 2 μ l DEPC water and 2 μ l cDNA. Reactions were run in a LightCycler 480 instrument II (Roche Diagnostics Co., Ltd.). qPCR with SYBR Green detection (Takara Bio, Inc.) was performed. qPCR amplification conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 56°C for 30 sec and 72°C for 30 sec. The following primers were used for cDNA amplification: Lats2: Forward, 5'-GACGATGTTTCAACTGTGCTGTG-3' and reverse, 5'-CAACCAGCATCTCAAAGAGAATCACAC-3'; matrix metalloproteinase (MMP)-2: Forward, 5'-CAAGTCCCCGGCGATGTC-3' and reverse, 5'-TTCTGGTCAAGGTCACCTGTC-3'; and GAPDH: Forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-UUCUCCGAACGUGUCACGUTT-3'. Relative levels were quantified with the $2^{-\Delta\Delta C_q}$ method and were normalized to GAPDH (23).

Echocardiography. Echocardiography was induced as previously described (4,24,25). After 7 days of transfection with lentivirus expressing miR-93 or scr-miR, mice were subjected to MI. Cardiac function was examined by echocardiography prior to MI (baseline), and after MI at days 3, 14 and 21. The percentage of ejection fraction (EF) and fractional shortening (FS) were calculated as follows: EF = (end-diastole volume - end-systole volume)/end-diastole volume x 100; FS = (left ventricular end-diastole diameter - left ventricular end-systole diameter)/left ventricular end-diastole diameter x 100.

Masson's trichrome stain. Cardiac fibrosis was determined using Masson's trichrome staining. Mice were transfected with lentivirus expressing miR-93 or scr-miR 7 days before induction of MI. Subsequently, 3 weeks after MI, hearts were harvested, post-fixed with 4% paraformaldehyde for 12 h at

4°C, and sliced horizontally. Sections (5 μ m) were stained using Masson's trichrome staining kit (cat. no. G1340; Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 1 h according to the manufacturer's protocol (concentrations not commercially available). Stained sections were examined using light microscope (magnification, x12.5) and analyzed using ImageJ software (NIH; version 1.48; <http://rsb.info.nih.gov/ij/>).

Immunohistochemistry staining. Immunohistochemical staining was performed as described previously (26). Mice were scarified after MI and perfused with 4% buffered paraformaldehyde via the ascending aorta. Heart tissues were post-fixed with 4% paraformaldehyde for 12 h at 4°C and embedded in paraffin. Sections (5 μ m) were blocked with 4% donkey serum (cat. no. 136110; Jackson ImmunoResearch, Inc.) for 1 h at room temperature and incubated with anti-CD31 (1:50; cat. no. ab28364; Abcam), anti-intracellular adhesion molecule 1 (ICAM-1; 1:50; cat. no. sc8439; Santa Cruz Biotechnology, Inc.) and anti-vascular cell adhesion molecule 1 (1:50; cat. no. sc8304; VCAM-1; Santa Cruz Biotechnology, Inc.) at 4°C overnight. Subsequently, sections were incubated with 50 μ l IHC detection reagent at 25°C (cat. nos. 8114 and 8125, respectively; Cell Signaling Technology, Inc.) according to the manufacturer's protocol (concentrations not commercially available). Slides were stained with DAB Substrate kit (cat. no. 8059; Cell Signaling Technology, Inc.) and then counterstained with 100 μ l hematoxylin (concentration not commercially available) (cat. no. C0107; Beyotime Biotechnology, Inc.) at 25°C for 10 sec. Images were captured using light microscopy (magnification, x400).

Infiltration of neutrophils and macrophages. At 3 days post-MI, hearts were harvested, post-fixed with 4% paraformaldehyde for 12 h at 4°C and sliced horizontally. According to the manufacturer's protocol, infiltrated neutrophils in heart sections (5 μ m) were stained with naphthol AS-D chloroacetate esterase kit (cat. no. 90C2; Sigma-Aldrich; Merck KGaA) followed by hematoxylin (concentrations not commercially available) at 25°C for 10 sec. Accumulation of macrophages in the ischemic hearts was evaluated using the macrophage specific antibody F4/80 (1:50; cat. no. 70076; Cell Signaling Technology, Inc.), followed by counterstaining with 100 μ l hematoxylin (concentration not commercially available) (cat. no. C0107; Beyotime Biotechnology, Inc.) at 25°C for 10 sec. A total of four independent areas of each section were examined using light microscopy at a magnification of x400.

Luciferase reporter assay. Lats2 was predicted as a target of miR-93 by the targetScan (<http://www.targetscan.org/>), miRanda (<http://www.microrna.org/>), and PicTar (<http://pictar.mdc-berlin.de/>) database. Partial fragment of Lats2 wild-type 3'-untranslated regions (UTR; Lats2-3'-UTR-WT) containing the putative miR-93 binding sites or mutant sequence (Lats2-3'-UTR-MUT) were cloned into psiCHECK-2 vectors (Promega Corporation; cat. no. C8021). HUVECs were co-transfected with Lats2-3'-UTR-WT (1 μ g) or Lats2-3'-UTR-MUT (1 μ g) reporter, and miR-93 mimics (50 nM) or scr-miR mimics (50 nM) using Lipofectamine 2000 (cat. no. 11668027; Thermo Fisher Scientific, Inc.) following

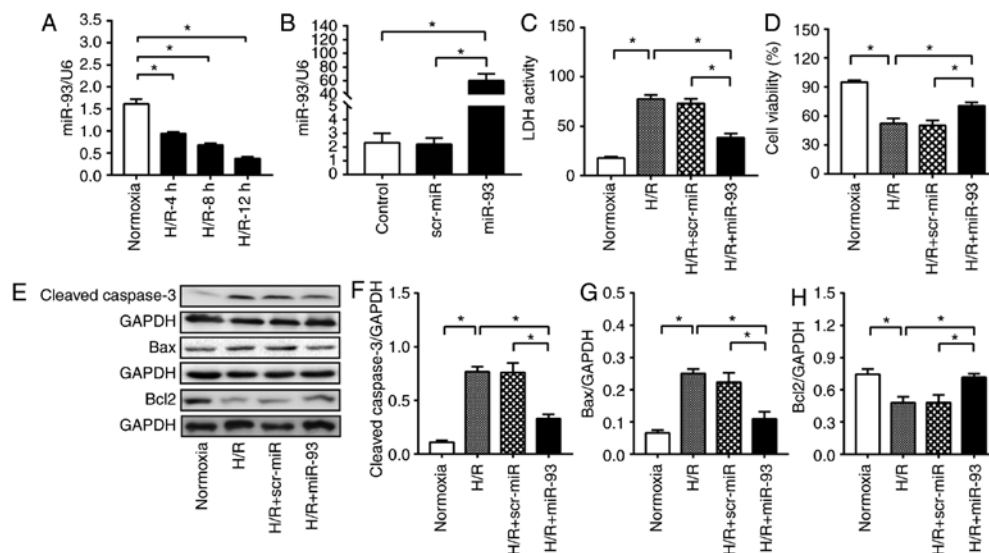


Figure 1. miR-93 attenuates H/R-induced cell injury and cell apoptosis in HUVECs. (A) Time course of miR-93 expression in HUVECs before and after H/R was examined using RT-qPCR. $n=4-5$ replicates per group. (B) RT-qPCR analysis revealed an increased expression of miR-93 in HUVECs transfected with miR-93 mimic. $n=4-5$ replicates per group. After 48 h of transfection, HUVECs were exposed to 4 h hypoxia, followed by 24 h reoxygenation. (C) Transfection of HUVECs with miR-93 mimic decreased LDH activity and (D) increased cell viability following H/R injury. $n=4$ replicates per group. (E) Representative bands of (F) cleaved caspase-3, (G) Bax and (H) Bcl2. Transfection of HUVECs with miR-93 mimic decreased the expression levels of cleaved caspase-3 and Bax, and increased the expression of Bcl2 after H/R injury. $n=4-5$ replicates per group. Data are presented as the mean \pm SEM. Each experiment was repeated three times. * $P<0.05$ vs. the indicated group. H/R, hypoxia/reoxygenation; HUVECs, human umbilical vein endothelial cells; scr, scrambled; miR, microRNA; LDH, lactate dehydrogenase; RT-qPCR, reverse transcription-quantitative PCR.

the manufacturer's instructions. At 48 h of transfection, luciferase activities were examined via comparison with Renilla luciferase activity using the Dual Luciferase reporter assay system (Promega Corporation; cat. no. E1910).

Western blotting. Western blotting was performed as described previously (4). Mice were transfected with lentivirus expressing miR-93 or scr-miR 7 days before induction of MI. Subsequently, 3 days after MI, ischemic hearts were harvested for isolation of cellular proteins. In addition, HUVECs were transfected with miR-93 mimic (40 nmol) or scr-miR mimic (40 nmol) prior to H/R injury, and the cell lysates were prepared using RIPA lysis buffer (Beyotime Biotechnology, Inc.; cat. no. P0013B). The protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.; cat. no. 23227). A total of 100 μ g cellular proteins were separated by 12% SDS-PAGE and then transferred to PVDF membranes (EMD Millipore). PVDF membranes were incubated with following primary antibodies at 4°C overnight: Anti-phosphorylated (p)-Yap (Cell Signaling Technology, Inc.; cat. no. 13008; 1:500), anti-cleaved caspase-3 (Cell Signaling Technology, Inc.; cat. no. 9661; 1:500), anti-Lats2 (ProteinTech Group, Inc.; cat. no. 20276-1-AP; 1:100), anti-Yap (Santa Cruz Biotechnology, Inc.; cat. no. sc101199; 1:100), anti-Bax (Cell Signaling Technology, Inc.; cat. no. 2744; 1:500), anti-Bcl2 (Santa Cruz Biotechnology, Inc.; cat. no. sc56015; 1:100), anti-ICAM-1 (Santa Cruz Biotechnology, Inc.; cat. no. sc8439; 1:100), anti-VCAM-1 (Santa Cruz Biotechnology, Inc.; cat. no. sc8304; 1:100), anti-vascular endothelial growth factor (VEGF; Santa Cruz Biotechnology, Inc.; cat. no. sc7269; 1:100) and anti-GAPDH (ProteinTech Group, Inc.; cat. no. 60004-1-1g; 1:4,000). The membranes were then incubated with peroxidase-conjugated secondary antibodies at 25°C for

2 h (Cell Signaling Technology, Inc.; cat. no. 7074, 1:4000; cat. no. 7076; 1:4,000) and analyzed with an ECL system (Thermo Fisher Scientific, Inc.; cat. no. 32106). The signals were quantified using ImageJ software (NIH; version 1.48; <http://rsb.info.nih.gov/ij/>).

Statistical analysis. Data are presented as the mean \pm SEM. Comparisons of data between groups were performed using one ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-93 attenuates H/R-induced cell injury and cell apoptosis in HUVECs. As endothelial cells are critical for angiogenesis in response to MI (4), the present study investigated the effect of H/R on miR-93 expression in HUVECs. It was identified that H/R induced a time-dependent reduction in the expression of miR-93 (Fig. 1A).

To examine the effect of miR-93 on H/R-induced cell injury in HUVECs, HUVECs were transfected with miR-93 mimic or scrambled miR mimic for 48 h. It was found that transfection with miR-93 mimic induced a significant increase in miR-93 expression by 25.1-fold compared with the control (Fig. 1B). Moreover, transfection with scr-miR mimic did not increase miR-93 expression. The present results suggested that H/R resulted in an increase in LDH activity (Fig. 1C). However, transfection with miR-93 mimic significantly decreased LDH activity compared with H/R (38.3 ± 4.3 vs. 77.5 ± 4.3). However, transfection with scr-miR mimic did not affect LDH activity in HUVECs. The results of the CCK-8 assay further demonstrated that H/R significantly decreased cell viability (by 44.9%) compared with normoxia (Fig. 1D). However,

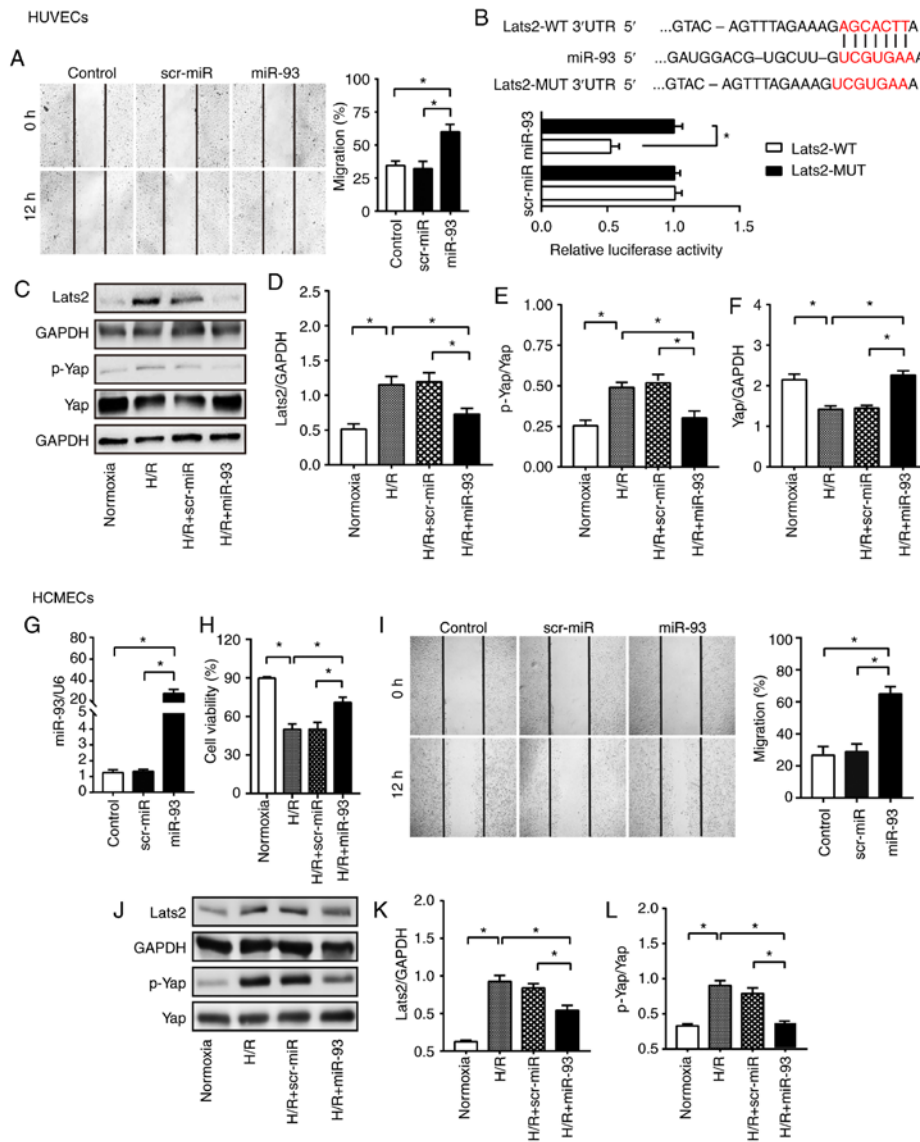


Figure 2. miR-93 enhances endothelial cell migration, and suppresses Lats2 expression and Yap phosphorylation in endothelial cells. (A) Representative images of scratch-wound assay before and 12 h after scratch formation in HUVECs (magnification, x200). The line indicates the width of the gap. Transfection with miR-93 mimic promoted HUVEC migration into the wound area. n=4 replicates per group. (B) Predicted binding sites between miR-93 and Lats2 3'UTR. miR-93 mimics decreased luciferase activity. n=4 replicates per group. (C) Representative bands of Lats2, p-Yap and Yap in HUVECs. Expression of (D) Lats2 was suppressed in miR-93 mimic-transfected HUVECs. Transfection with miR-93 mimic (E) decreased Yap phosphorylation and (F) increased Yap expression in the cytosol after HUVECs H/R. n=4 replicates per group. HCMECs were transfected with miR-93 mimics or scr-miR mimics for 48 h. (G) Reverse transcription-quantitative PCR showed the upregulation of miR-93 in HCMECs by miR-93 mimics transfection. n=4 replicates per group. (H) Transfection of HCMECs with miR-93 mimic increased cell viability following H/R injury. n=4 replicates per group. (I) Representative images of scratch-wound assay at 0 and 12 h after scratch formation in HCMECs (magnification, x100). The line indicates the width of the gap. Transfection with miR-93 mimic promoted HCMEC migration into the wound area. n=4 replicates per group. (J) Representative bands of Lats2 and p-Yap in HCMECs. Western blotting results identified the attenuation of (K) Lats2 and (L) p-Yap expression levels by miR-93 transfection after H/R in HCMECs. Data are presented as the mean \pm SEM. Each experiment was repeated three times. * P <0.05 vs. the indicated group. Lats2, large tumor suppressor 2; Yap, Yes-associated protein; HUVECs, Human umbilical vein endothelial cells; HCMECs, human cardiac microvascular endothelial cells; scr, scrambled; miR, microRNA; H/R, hypoxia/reoxygenation; p, phosphorylated; 3'UTR, 3' untranslated region; WT, wild-type; MUT, mutant.

it was found that the H/R-induced decrease in cell viability was significantly reversed by miR-93 mimic. Moreover, transfection with scr-miR mimic did not affect the H/R-induced decrease in cell proliferation and viability.

To assess the effect of miR-93 on the apoptosis of HUVECs, the present study examined the expression levels of cleaved caspase-3, Bax and Bcl2. Cleaved caspase-3 and Bax are important pro-apoptotic proteins, while Bcl2 is an important anti-apoptotic protein (27). The present results indicated that the miR-93 mimic significantly attenuated the expression levels

of cleaved caspase-3 by 56.8% and Bax by 56.1% compared with H/R (Fig. 1F-H). However, H/R-decreased Bcl2 expression was significantly higher in miR-93-transfected HUVECs (0.7 ± 0.03) compared with H/R group (0.5 ± 0.06). Furthermore, scr-miR mimic did not affect the H/R-induced increase in Bax and cleaved caspase-3 expression levels, nor the decreased in Bcl2 expression.

miR-93 promotes HUVEC migration. Subsequently, the effect of miR-93 on HUVEC migration was determined by

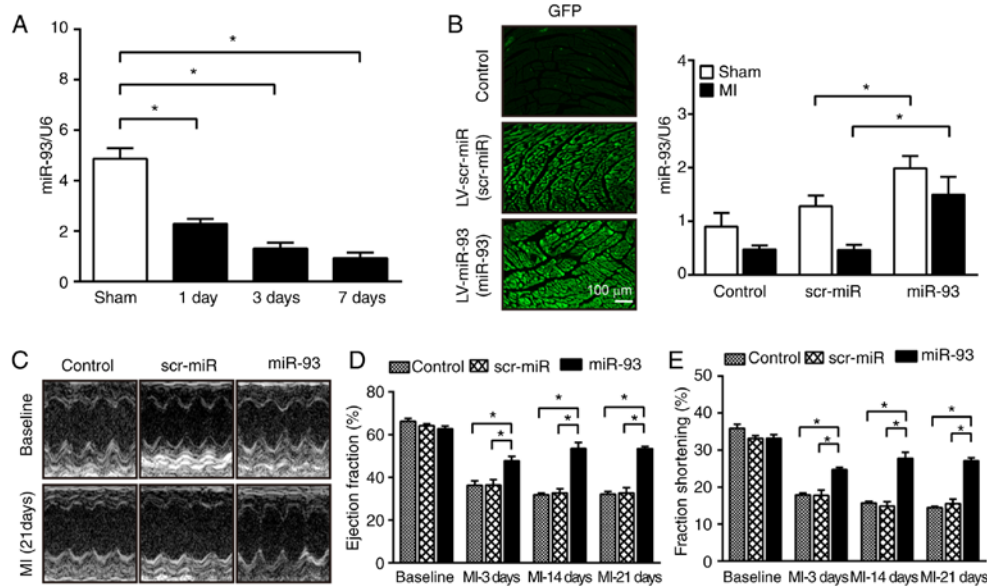


Figure 3. Increased expression of miR-93 attenuates cardiac dysfunction after MI. (A) miRNAs were isolated from sham-operated and infarcted hearts at 1, 3 and 7 days after MI. RT-qPCR analysis revealed a downregulation of miR-93 expression in the myocardium after MI. $n=4-5$ mice per group. (B) Representative images of heart sections at 7 days after transfection with lentiviral vectors (magnification, $\times 200$). RT-qPCR revealed the expression level of miR-93 in the myocardium after transfection. $n=4-5$ mice per group. (C) Representative images of M-mode echocardiography at baseline and 21 days after MI. Echocardiography parameters were measured at baseline and at 3, 14 and 21 days after MI. Increased expression of miR-93 reversed MI-induced decreases in (D) EF% and (E) FS%. $n=5-7$ mice per group. Data are presented as the mean \pm SEM. * $P < 0.05$ vs. the indicated group. scr, scrambled; miR/miRNA, microRNA; MI, myocardial infarction; LV, lentivirus; EF, ejection fraction; FS, fraction shortening; RT-qPCR, reverse transcription-quantitative PCR.

scratch assay. It was demonstrated that the control group cells migrated by 34.6% at 12 h. However, the miR-93 mimic-transfected HUVECs migrated into the wound area by 60.0% at 12 h post-scratch (Fig. 2A). In line with this observation, scr-miR mimic-transfected HUVECs exhibited a lower capacity to migrate compared with the miR-93 mimic group. miR-93 suppresses Lats2 expression and inactivates the Hippo pathway in HUVECs. Lats2, as a core kinase in the Hippo signaling pathway, was predicted to be a potential target of miR-93 using a miRNA database. It has been reported that the Hippo pathway regulates cell proliferation and apoptosis, as well as angiogenesis (5,6). Therefore, the present study examined whether miR-93 could suppress Lats2 expression in HUVECs, thus leading to inactivation of the Hippo/Yap pathway. To assess this hypothesis, Lats2-3'-UTR-WT or Lats2-3'-UTR-MUT vectors containing WT or MUT miR-93 binding sites were constructed, respectively. It was demonstrated that co-transfection of the reporters with miR-93 mimic induced a significant reduction in luciferase activity compared with scrambled miR (Fig. 2B). Furthermore, it was identified that the miR-93 mimic induced a significant reduction in Lats2 protein expression by 36.7% compared with H/R group (Fig. 2C and D). In addition, Lats2 expression was significantly lower in miR-93-transfected HUVECs (0.7 ± 0.1) compared with scr-miR mimic-transfected HUVECs (1.2 ± 0.1) after H/R injury. Subsequently, the protein expression levels of major downstream modulators in the Hippo pathway were examined. It was found that transfection with miR-93 mimic significantly attenuated Yap phosphorylation, thus increasing the expression of Yap in the cytosol of HUVECs (Fig. 2E and F). However, transfection with scr-miR mimic did not alter the expression levels of p-Yap and Yap after H/R injury in HUVECs.

miR-93 enhances HCMEC viability and migration. To further investigate the effect of miR-93 on the viability and migration of endothelial cells, HCMECs were transfected with miR-93 mimic or scr-miR mimic for 48 h. The present results suggested that transfection of miR-93 mimic induced a significant upregulation of miR-93 expression in HCMECs (Fig. 2G). Furthermore, transfection of miR-93 mimics significantly increased cell viability (Fig. 2H) and the capacity to stimulate endothelial cell migration (Fig. 2I) after H/R injury. In addition, it was found that miR-93 mimic-transfected HCMECs exhibited a downregulation in Lats2 expression (Fig. 2J and K) and Yap phosphorylation (Fig. 2J and L) following H/R injury, when compared with HCMECs transfected with scr-miR mimic after H/R injury.

miR-93 results in an improvement of cardiac function after MI. To evaluate the effect of miR-93 on MI *in vivo*, the present study examined whether miR-93 expression in the myocardium could be altered after MI. The present results indicated that the expression of miR-93 was significantly decreased by 53.0% on day 1, 73.1% on day 3 and 80.9% on day 7 after MI compared with sham-operated mice (Fig. 3A), thus suggesting that increased expression of miR-93 may serve a protective role against MI. To further investigate the role of miR-93 in the development of MI, lentivirus vectors expressing miR-93 mimic were used to increase miR-93 expression in the myocardium. Hearts that were not subjected to transfection served as the controls. RT-qPCR results identified that miR-93-transfection significantly increased miR-93 expression by 2.2-fold in the myocardium after MI compared with scr-miR transfection (Fig. 3B). Moreover, miR-93 expression was also significantly elevated in the myocardium in sham-operated mice transfected with miR-93 compared with the scr-miR group (Fig. 3B).

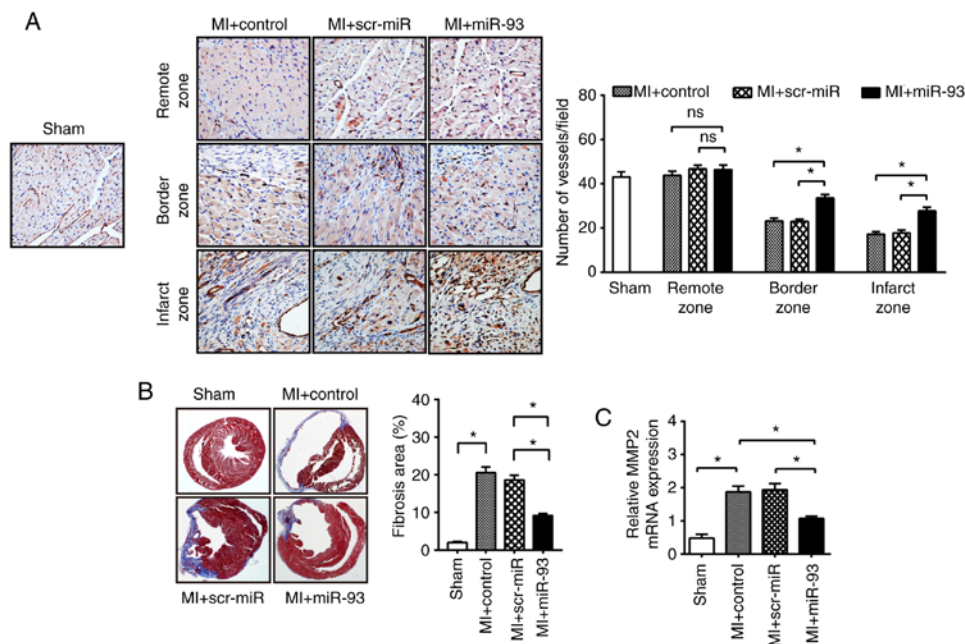


Figure 4. Increased expression of miR-93 promotes angiogenesis and attenuates collagen deposition after MI. (A) Representative immunohistochemical images of CD31 in the heart sections (magnification, x200). Quantitative analyses revealed the enhancement of microvascular density in miR-93-transfected hearts after MI. n=3 mice per group. (B) Representative images of collagen deposition in the heart sections (magnification, x12.5). Quantitative analyses demonstrated the attenuation of cardiac fibrosis by transfected with lentivirus expressing miR-93 after MI. n=3-5 mice per group. (C) Reverse transcription-quantitative PCR analysis demonstrated the downregulation of MMP-2 mRNA expression in miR-93-transfected hearts after MI. n=4-5 mice per group. Data are presented as the mean \pm SEM. *P<0.05 vs. the indicated group. MI, myocardial infarction; scr, scrambled; miR, microRNA; MMP-2, matrix metalloproteinase 2; ns, not significant.

The present study then evaluated the effect of miR-93 on cardiac function after MI. It was demonstrated that MI induced significant decreases in EF% and FS% values in the control and scr-miR-transfected mice from at days 3-21 compared with the respective baseline (Fig. 3D and E). However, the values of EF% and FS% in miR-93-transfected mice were significantly higher compared with scr-miR-transfected mice at all the time points measured after MI. Furthermore, there were no significant differences in the baseline of cardiac function among the three groups.

miR-93 enhances angiogenesis and attenuates cardiac fibrosis after MI. The present *in vitro* data indicated that miR-93 promoted endothelial cell proliferation and migration. Angiogenesis is an important factor in the restoration of cardiac function after MI (28). Therefore, the present study examined whether upregulation of miR-93 could enhance angiogenesis in an ischemic heart. It was identified that the microvascular density in the border area and infarcted area at 21 days after MI was greatly decreased by 46.1 and 60.2%, respectively, compared with the sham control group (Fig. 4A). Furthermore, miR-93 overexpression significantly promoted microvascular density in the infarcted myocardium compared with the MI control (27.7 ± 1.7 vs. 17.2 ± 1.1). However, transfection with scr-miR did not inhibit the MI-induced decrease in microvascular density.

Reduction in the microvascular density in myocardium accelerates the cardiac remodeling process (29). Cardiac fibrosis further contributes to cardiac dysfunction after MI (30). Thus, the present study examined cardiac fibrosis using Masson's Trichrome staining of collagen deposition at

day 21 after MI. No difference in the deposition of MI-induced cardiac fibrosis was observed between the scr-miR-transfected group and the control. However, the percentage of fibrotic area in miR-93-transfected hearts was significantly reduced by 35.7% compared with scr-miR-transfected hearts after MI (Fig. 4B). MMP-2 is involved in collagen deposition after MI (31). Moreover, it was identified that MI-induced MMP-2 mRNA expression was significantly decreased in miR-93-transfected hearts (1.1 ± 0.1) compared with the control hearts (1.9 ± 0.2 ; Fig. 4C). However, transfection with scr-miR did not alter MMP-2 expression in the myocardium after MI (Fig. 4C).

miR-93 attenuates the expression of adhesion molecules in the myocardium after MI. MI-induced endothelial activation is an important determinant that causes cardiac dysfunction (32). Hence, the present study investigated the effect of miR-93 on the expression levels of ICAM-1 and VCAM-1, which are two important adhesion molecules that are highly expressed in activated endothelial cells (26). It was identified that control and scr-miR transfection groups had a higher level of positive staining of ICAM-1 and VCAM-1 in the myocardium after MI (Fig. 5A and B). However, overexpression of miR-93 resulted in a decreased amount of positive staining of ICAM-1 and VCAM-1 in the ischemic myocardium. Furthermore, western blotting results demonstrated that transfection with miR-93 mimic significantly reduced the protein expression levels of ICAM-1 by 54.5% and VCAM-1 by 46.4%, compared with the MI control (Fig. 5C and D). However, transfection with scr-miR did not attenuate the protein expression levels of ICAM-1 and VCAM-1 in the myocardium after MI (Fig. 5C and D).

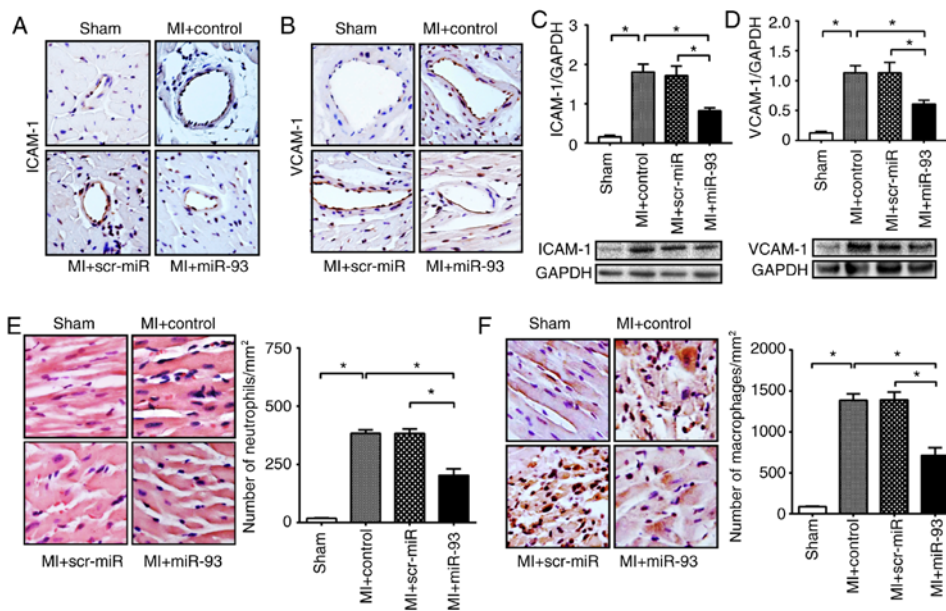


Figure 5. Increased expression of miR-93 attenuates the infiltration of neutrophils and macrophages into the myocardium after MI. After 7 days of transfection, mice were subjected to MI for 3 days. Representative immunohistochemical images of ICAM-1 and VCAM-1 in the heart sections (magnification, x400). Immunohistochemistry found a decrease in the positive staining of (A) ICAM-1 and (B) VCAM-1 in the ischemic myocardium, following transfected with lentivirus expressing miR-93 after MI. The dark brown color indicates positive staining. Western blot analysis showed the attenuation of (C) ICAM-1 and (D) VCAM-1 protein expression levels in miR-93 transfected hearts after MI. $n=4-5$ mice per group. Representative images of infiltrated neutrophils and macrophages in heart sections (magnification, x400). Increased expression levels of miR-93 attenuated MI-induced infiltration of (E) neutrophils and (F) macrophages into the myocardium. The pink color indicates positive neutrophils. The dark brown color indicates positive macrophages. $n=3$ mice per group. Data are presented as the mean \pm SEM. * $P<0.05$ vs. the indicated group. MI, myocardial infarction; scr, scrambled; miR, microRNA; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

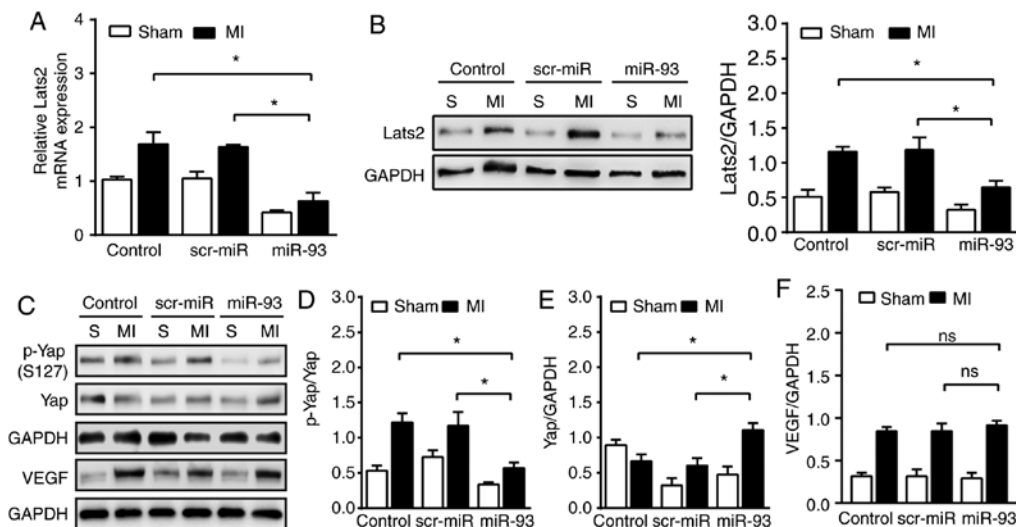


Figure 6. Increased expression levels of miR-93 suppress Lats2 expression levels and inactivates the Hippo/Yap pathway after MI. (A) Reverse transcription-quantitative PCR analysis identified the downregulation of Lats2 mRNA expression by transfection of lentivirus expressing miR-93 on day 3 of MI. $n=4$ mice per group. (B) Western blot analysis revealed the suppression of Lats2 expression by lentivirus transfection expressing miR-93 after MI. $n=4-5$ mice per group. (C) Representative bands of p-Yap, Yap and VEGF on day 3 of MI. Increased expression of miR-93 induced a (D) decrease in the phosphorylation levels of Yap and an (E) increase in total Yap expression after MI. $n=4-6$ mice per group. (F) Increased expression of miR-93 in the myocardium had no effect on the expression of VEGF after MI. $n=4$ mice per group. Data are presented as the mean \pm SEM. * $P<0.05$ vs. the indicated group. ns, not significant. MI, myocardial infarction; scr, scrambled; miR, microRNA; Lats2, large tumor suppressor 2; Yap, Yes-associated protein; p, phosphorylated; VEGF, vascular endothelial growth factor; S, sham.

miR-93 attenuates the infiltration of neutrophils and macrophages into the myocardium after MI. In the early inflammatory response to MI, circulating neutrophils and macrophages infiltrate the infarcted area via the endothelial barrier, thus exacerbating cardiac dysfunction (33). Therefore, the present

study examined the effect of miR-93 on the infiltration of neutrophils and macrophages into the myocardium after MI. The present results suggested that the number of neutrophils and macrophages was significantly increased following MI compared with the respective sham control (Fig. 5E and F).

Furthermore, miR-93 overexpression significantly attenuated MI-induced infiltration of neutrophils by 40.7% and macrophages by 47.3% compared with the respective MI control (Fig. 5E and F). However, transfection with scr-miR did not prevent MI-induced accumulation of neutrophils and macrophages in the myocardium.

miR-93 suppresses Lats2 expression and inactivates the Hippo/Yap pathway after MI. The present *in vitro* data indicated that miR-93 attenuated endothelial injury by targeting Lats2. Thus, the present study subsequently investigated whether increased expression of miR-93 could suppress Lats2 expression in the myocardium following MI. Abundant mRNA expression levels of Lats2 were observed in sham and MI control hearts compared with the respective miR-93-transfected hearts (Fig. 6A). Moreover, western blot analysis results revealed that miR-93 overexpression significantly downregulated the expression of Lats2 by 44.4% in the sham group and by 45.4% in MI hearts, compared with the scr-miR transfected group (Fig. 6B). It was found that increased expression of miR-93 significantly attenuated Yap phosphorylation (S127) by 53.1% after MI compared with the MI control (Fig. 6D). However, transfection with scr-miR did not prevent the downregulation of p-Yap after MI. The present results indicated that overexpression of miR-93 significantly increased total Yap expression by 65.6 and 83.6% after MI compared with the control and scr-miR-transfected group, respectively (Fig. 6E). The present study also examined the effect of miR-93 on VEGF expression after MI, and it was identified that increased expression of miR-93 in the myocardium had no effect on the expression of VEGF after MI (Fig. 6F).

Discussion

The present results suggested that miR-93 was involved in the pathogenesis of MI. It was identified that transfection of HUVECs with miR-93 mimic resulted in a cytoprotective effect, along with the enhancement of cell proliferation and viability, and the attenuation of cell apoptosis after H/R injury. Moreover, an increased expression of miR-93 led to the improvement of cardiac function, promotion of angiogenesis and reduction of cardiac remodeling, as well as attenuation of the infiltration of neutrophils and macrophages into the myocardium following MI. Furthermore, the suppression of Lats2 expression and inactivation of the Hippo/Yap pathway were involved in the underlying mechanisms of the protective role of miR-93 against MI, both *in vitro* and *in vivo*. Collectively, the present results indicated that there may be an association between miR-93 and the development of MI, and thus may provide a promising therapeutic target.

Previous findings have shown that miRNAs, which are small non-coding RNAs, play critical roles in MI (17-19). A recent study showed that adipose-derived stromal cells-derived miR-93-5p-containing exosomes prevents MI-induced cardiac injury by inhibiting autophagy and the inflammatory response (34). In line with this, the present study identified that increased expression of miR-93 reversed MI-induced cardiac dysfunction and remodeling. However, the protective mechanism of miR-93 after MI has not been fully elucidated. It has been revealed that angiogenesis is a critical step for heart

repair in response to MI (4). Previous studies, including our own, have demonstrated that the promotion of angiogenesis by miRNAs induces functional recovery of ischemic hearts after MI (4,17-19). Moreover, previous studies have reported the effect of miR-93 on cardiomyocytes after H/R injury but not on endothelial cells (34,35). Endothelial cells are the most abundant non-cardiomyocytes in the heart of adult mammals (32). Therefore, in the present study, the effect of miR-93 on the endothelial cells were evaluated. Previous findings have shown that miR-93 enhances endothelial cell viability and inhibits endothelial cell apoptosis under normoxia (36). However, the cell function of miR-93 in endothelial cells under hypoxia remains to be investigated. The present results indicated that transfection with miR-93 mimic significantly increased endothelial cell viability in response to H/R injury. Furthermore, H/R-induced endothelial apoptosis was significantly inhibited by transfected with miR-93 mimic. Cleaved capase-3 and Bax are two important pro-apoptotic proteins, while Bcl2 is an anti-apoptotic protein (27). In the present study, it was found that transfection with miR-93 decreased in Bax expression and increased Bcl2 expression after H/R. In addition, transfection with miR-93 mimic led to an enhancement in the migratory capacity of endothelial cells. Therefore, the present *in vitro* data suggested that miR-93 may exhibit a cytoprotective effect against H/R injury in endothelial cells.

The Hippo pathway is an evolutionally conserved pathway that plays an important role in the regulation of cell proliferation, differentiation and apoptosis (5,6). Activation of Lats1/2, a core kinase of the Hippo pathway, results in the phosphorylation of Yap, which leads to its cytoplasmic retention and functional inactivation (5). Fang *et al* (21) reported that miR-93 could suppress the transcription of the Lats2 3'-UTR and inhibit Lats2 expression. Consistent with Fang *et al* (21), the present study identified that transfection with a miR-93 mimic suppressed Lats2 expression levels in endothelial cells after H/R injury. Furthermore, it was observed that the mRNA and protein expression levels of Lats2 in the myocardium were significantly downregulated by lentivirus expressing miR-93. Thus, the present results suggested that miR-93 may interact with the 3'-UTR of Lats2, which results in Lats2 mRNA degradation and translational repression of Lats2. Previous studies have shown that inactivation of the Hippo pathway decreases cardiac remodeling and preserves cardiac function after MI (9,10). In the heart, Lats1 and Lats2 regulate cardiomyocyte renewal (37). Moreover, Lats2, but not Lats1, plays an important role in mediating cardiomyocytes apoptosis (38). Lats2 is highly increased in the myocardium after myocardial I/R injury and MI, and depletion of Lats2 reduces the infarct size after myocardial I/R injury (8). However, overexpression of Lats2 leads to a reduction of left ventricle systolic function (38). In the present study, it was found that increased expression of miR-93 significantly downregulated Lats2 expression in the myocardium after MI. Moreover, suppression of Lats2 expression by miR-93 attenuated the infarct size and cardiac dysfunction after MI. Collectively, the present results indicated that miR-93 plays a protective role against H/R injury in endothelial cells via the suppression of Lats2 expression.

Induction of angiogenesis plays a key role in the infarct healing process, such as improvement of cardiac function and

attenuation of cardiac remodeling (39). The Hippo/Lats2/Yap pathway is an important determinant of endothelial proliferation and migration (6,14). Lats2-knockdown decreases endothelial cell apoptosis, and increases migration and endothelial tube formation (6). However, overexpression of Yap induces an enhancement of the angiogenic activity of endothelial cells (14,40). The present results identified that transfection with miR-93 mimic significantly decreased Lats2 expression and increased Yap expression in HUVECs. Therefore, it was speculated miR-93 may promote angiogenesis via the modulation of the Hippo pathway. Moreover, the present *in vivo* results demonstrated that miR-93 overexpression significantly increased the number of small vessels in the myocardium after MI. In addition, it was found that increased expression of miR-93 significantly attenuated cardiac fibrosis after MI. Western blotting results demonstrated that miR-93 overexpression decreased the phosphorylation of Yap, and increased Yap expression in ischemic myocardium by targeting Lats2. Therefore, it is speculated that miR-93 enhanced angiogenesis by suppressing Lats2 expression, resulting in inactivation of the Hippo/Yap pathway, which is positively associated with the improvement of cardiac function and attenuation of remodeling after MI.

VEGF is a critical pro-angiogenic factor (41). The present results suggested that increased expression of miR-93 did not alter VEGF expression in the myocardium after MI. Long *et al* (42) reported that miR-93 reduces VEGF-A expression in cultured podocytes and glomeruli, while another previous study showed that VEGF was not affected by upregulation of miR-93 in both cultured HUVECs and in ischemic gastrocnemius muscle from BALB/cJ mice (43). Thus, these results suggest that miR-93 regulation of its target gene may be dependent on context and cell type.

ICAM-1 and VCAM-1, two important adhesion molecules that are highly expressed in activated endothelial cells, play an important role in promoting the infiltration of inflammatory cells across the endothelium and into the myocardium (44,45). The accumulation of neutrophils and macrophages in ischemic myocardium contributes to cardiac dysfunction following MI (46,47). Therefore, preservation of endothelial function is an important factor for the attenuation of MI-induced cardiac dysfunction. The present study found that miR-93 overexpression significantly prevented MI-induced ICAM-1 and VCAM-1 expression, and significantly attenuated MI-induced infiltration of neutrophils and macrophages into the myocardium. Moreover, Yap is involved in the regulation of endothelial inflammation and activation (48). Thus, it is possible that miR-93 attenuates adhesion molecules and the infiltration of inflammatory cells by targeting the Hippo/Lats2/Yap pathway after MI.

In conclusion, to the best of our knowledge, the present study is the first to demonstrate that miR-93 may be an important regulator of cardiac angiogenesis in ischemic hearts via the suppression of Lats2 and inactivation of the Hippo/Yap pathway. The present results help to further the understanding of the interaction between miR-93 and endothelial cells, and its role in MI. Thus, miR-93 may be a potential therapeutic target for MI.

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Availability and data and materials

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

Authors' contributions

CM and PP carried out cell experiments, animal surgery and molecular biology studies. YZ and TL analyzed the data. LW performed western blotting and Masson's trichrome staining. CL designed and supervised the study, and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All aspects of the animal care and experimental protocols were approved by Xuzhou Medical University Committee on Animal Care.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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