

MicroRNA-146a attenuates isoproterenol-induced cardiac fibrosis by inhibiting FGF2

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Abstract. Cardiac fibrosis is a key factor of heart failure. Increasing evidence suggests that microRNAs (miRNAs/miRs) serve vital roles in the pathogenesis of cardiac fibrosis. The present study aimed to investigate the role of miR-146a-5p in isoproterenol (ISO)-induced cardiac fibrosis. Reverse transcription-quantitative PCR analysis demonstrated that miR-146a-5p expression was downregulated in ISO-treated rat heart tissue and ISO-induced cardiac fibroblasts (CFs). Conversely, the expression levels of basic fibroblast growth factor 2 (FGF2), collagen I and smooth muscle α -actin (α -SMA) were upregulated in ISO-treated rat cardiac tissue and CFs. Furthermore, viability and differentiation were inhibited in ISO-induced CFs transfected with miR-146a-5p mimics. Dual-luciferase reporter assay confirmed that miR-146a-5p targeted FGF2. Notably, FGF2 expression was suppressed following overexpression of miR-146a-5p, while FGF2 expression increased following miR-146a-5p knockdown. In addition, FGF2 knockdown suppressed the expression levels of FGF2, collagen I and α -SMA levels in CFs. Taken together, the results of the present study suggested that the miR-146a-5p/FGF2 pathway may be a novel therapy for cardiac fibrosis.

Introduction

Cardiac fibrosis is a common pathological process in cardiovascular disease (1,2). It is primarily characterized by fibroblast accumulation and excess deposition of extracellular matrix proteins (3,4). Activation of cardiac fibroblasts (CFs) is a key step in cardiac fibrosis progression; CFs proliferate and differentiate into myofibroblasts (5,6). Myofibroblasts,

the primary effector cells of cardiac fibrosis, contain large amounts of α -smooth muscle actin (α -SMA) and secrete abundant collagen and cytokines (7,8). Isoproterenol (ISO) has been indicated to increase the levels of proinflammatory cytokines (9), which further causes cardiac fibrosis by increasing matrix metalloproteinase expression in CFs (9). Thus, confirming the potential mechanisms by which CF activation is regulated may provide novel insights for the prevention and treatment of heart failure.

MicroRNAs (miRNAs/miRs) are endogenous small non-coding RNAs, 22 nucleotides in length, that are capable of post-transcriptionally modulating gene expression by binding to the 3'-untranslated region (UTR) of their target mRNAs (10). Increasing evidence suggests that miRNAs are key regulators of cell differentiation, cancer and cardiac disease (11,12). In addition, several miRNAs have been reported to participate in cardiac fibrosis development. For example, miR-223 modulates cardiac fibrosis following myocardial infarction by regulating RASA1 expression (13). Furthermore, miR-155 knockdown inhibits high glucose-triggered cardiac fibrosis via transforming growth factor (TGF)- β signaling (14). miR-146a has been reported to exhibit an anti-inflammatory and antifibrosis effect (15,16). For example, miR-146a-5p inhibits fibrosis-associated molecules (such as α -SMA and collagen I) in irradiated and TGF- β 1-induced hepatic stellate cells via protein tyrosine phosphatase receptor type A/SRC signaling (17). Moreover, Pan *et al* (18) indicated that miR-146a serves a role in myocardial protection by regulating early growth response (EGR)1. However, the role of miR-146a-5p in cardiac fibrosis progression remains unknown.

Fibroblast growth factor 2 (FGF2) is an intra-ovarian peptide that belongs to the FGF family. It has been reported that FGF2 mediates various biological processes, such as viability, differentiation and angiogenesis (19). A previous study demonstrated that FGF2 exerts its function in radiation-induced fibrosis by enhancing interstitial cell viability and stimulating fibroblast trans-differentiation into myofibroblasts (20,21). However, the function of FGF2 in cardiac fibrosis remains unclear.

The present study aimed to investigate the biological role of miR-146a-5p in cardiac fibrosis and the results demonstrated that miR-146a-5p suppresses ISO-treated cardiac fibrosis in rats by decreasing FGF2 expression, providing promising therapeutic modality for cardiac fibrosis.

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Materials and methods

Animal studies. A total of 32 male Sprague-Dawley (SD) rats (age, 8 weeks; weight, 200–220 g) were obtained from the Laboratory Animal Center of Nanjing the First Hospital, Nanjing Medical University. The rats were maintained under conditions of 50% relative humidity, a 12-h light/dark cycle and 22°C, and received food and water *ad libitum*. All animal experiments were approved by the Animal Care and Use Ethics Committee of Nanjing First Hospital (Nanjing, China; approval no. NFH-081241-2). Rats were randomly divided into two groups (n=16 per group) and intraperitoneally injected with either isoproterenol (ISO; 5 mg/kg/day) for consecutive 10 days or an equal volume of saline. On the 11th day, the rats were sacrificed by cervical dislocation following anesthesia with sodium pentobarbital (50 mg/kg) and hearts were collected. Tissue sections (5 μ m thick) were fixed in 4% formaldehyde solution at 37°C for 24 h and stained with haematoxylin and eosin (H&E) or Masson staining for 10 min at room temperature to observe histological changes in the cardiac tissue. Some samples were immediately frozen and stored at -80°C for RNA and protein analysis.

CF isolation and ISO treatment. CFs were isolated from SD rats as previously described (22). Ventricles were immediately placed in D-Hank's solution, cut into 1-mm³ pieces and subsequently digested using a mixture of 0.2% collagenase and 0.25% trypsin at 37°C for 20 min. Cells were resuspended in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin at 37°C for 2 h to allow fibroblast attachment to the culture plates. The third passage of CFs were used in the present study. Cells (1x10⁴) were seeded into 12-well plates and treated with 10 μ M ISO or saline in serum-free medium for 24 h.

Transfection. Small interfering (si)RNAs targeting FGF2 (siFGF2; 50 nM; 5'-CAGGUGACAGACUUUAUCAA-3'), negative control (siNC; 50 nM; 5'-UUCUCCGAACGUGUCACGUTT-3'), miR-146a-5p mimics (50 nM; 5'-GCCCAA GGUGAAUUUUUGGG-3'), NC mimics (50 nM; 5'-UCA CAACCUCCUAGAAAGAGUAGA-3'), miR-146a-5p inhibitor (50 nM; 5'-CCCAAAAUAUCACCUUUGGGC-3'), NC inhibitor (50 nM; 5'-UUUGUACUACACAAAAGUACUG-3'), pcDNA3.1/FGF2 and pcDNA3.1 were synthesized by Shanghai GenePharma, Co., Ltd. Transfection was performed using Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 20 min and subsequent experiments were performed 48 h post-transfection.

Dual-luciferase reporter assay. The StarBase database (starbase.sysu.edu.cn) revealed that FGF2 is a target gene of miR-146a-5p. The sequences of FGF2 3'-UTR containing putative miR-146a-5p binding sites were inserted into a pmirGLO plasmid (Promega Corporation) to form wild-type (WT) and mutant (MUT) FGF2. Subsequently, miR-146a-5p mimics and vectors were co-transfected into 293 cells using Lipofectamine[®] 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After a 48-h transfection, luciferase activity was detected using a Dual-Luciferase reporter assay system

(Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Blood sample collection and measurement of cardiac markers. After sacrificing the animals, 2 ml blood samples were collected from the carotid artery of rats. Creatine kinase (CK) activity was measured using an auto-analyzer (AU640; Olympus Diagnostics) according to the manufacturer's instructions. The CK isozyme (CK-MB) and cardiac troponin I (cTnI) levels in serum were performed via routine biochemical testing and detected using a 7600 auto-chemistry analyzer (Hitachi Ltd.) according to the manufacturer's instructions.

Cell Counting Kit-8 (CCK-8). Cell viability was detected via CCK-8 assay (Dojindo Molecular Technologies, Inc.). Briefly, CFs (1x10⁴ cells/well) were seeded into 96-well plates and transfected with miR-146a-5p mimic, miR-146a-5p inhibitor and NCs. After 36 h, cells were treated with ISO for 24 h and 10 μ l CCK-8 reagent was subsequently added into each well. Following incubation for 4 h at 37°C, absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative (RT-q)PCR. To detect mRNA and miRNA expression levels, total RNA was extracted from cardiac tissue and CFs using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT was performed using a PrimeScript[™] RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocol and qPCR was subsequently performed using a SYBR Green Detection kit (both from Takara Bio, Inc.). The thermocycling conditions were as follows: Pre-denaturation at 95°C for 1 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. mRNA expression levels were normalized to GAPDH, while miR-146a-5p expression levels were normalized to U6. Relative expression levels were calculated using the 2^{- $\Delta\Delta$ C_q} method (23). The primer sequences were as follows: miR-146a-5p forward, 5'-CGAGTCCAGTTTTCCAGGA-3' and reverse, 5'-GTCGTATCCAGTGCAGGG-3'; FGF2 forward, 5'-GCAAAAACGGGGGCTTCTTC-3' and reverse, 5'-AACGGTTAGCACACACTCCT-3'; U6 forward, 5'-CTCGCTTCGGCAGCAC-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; and GAPDH forward, 5'-CAAGCTCATTTCCTGGTATGAC-3' and reverse, 5'-CAGTGAGGGTCTCTCTTCCT-3'.

Western blot assay. Total protein was extracted from CFs using RIPA lysis buffer (Beyotime Institute of Biotechnology) and quantified using the BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins (20 μ g/lane) were separated by 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore). After blocking with 5% non-fat milk for 2 h at room temperature, the membrane was incubated with primary antibodies against FGF2 (1:1,000; cat. no. PB0619; Boster Biological Technology, Ltd.) and GAPDH (1:1,000; cat. no. ab9485; Abcam) overnight at 4°C. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000; cat. no. ab205718; Abcam) for 2 h at room temperature. Finally, protein bands were visualized using an enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology) and band densities were determined using ImageJ software (version 1.48; National Institutes of Health).

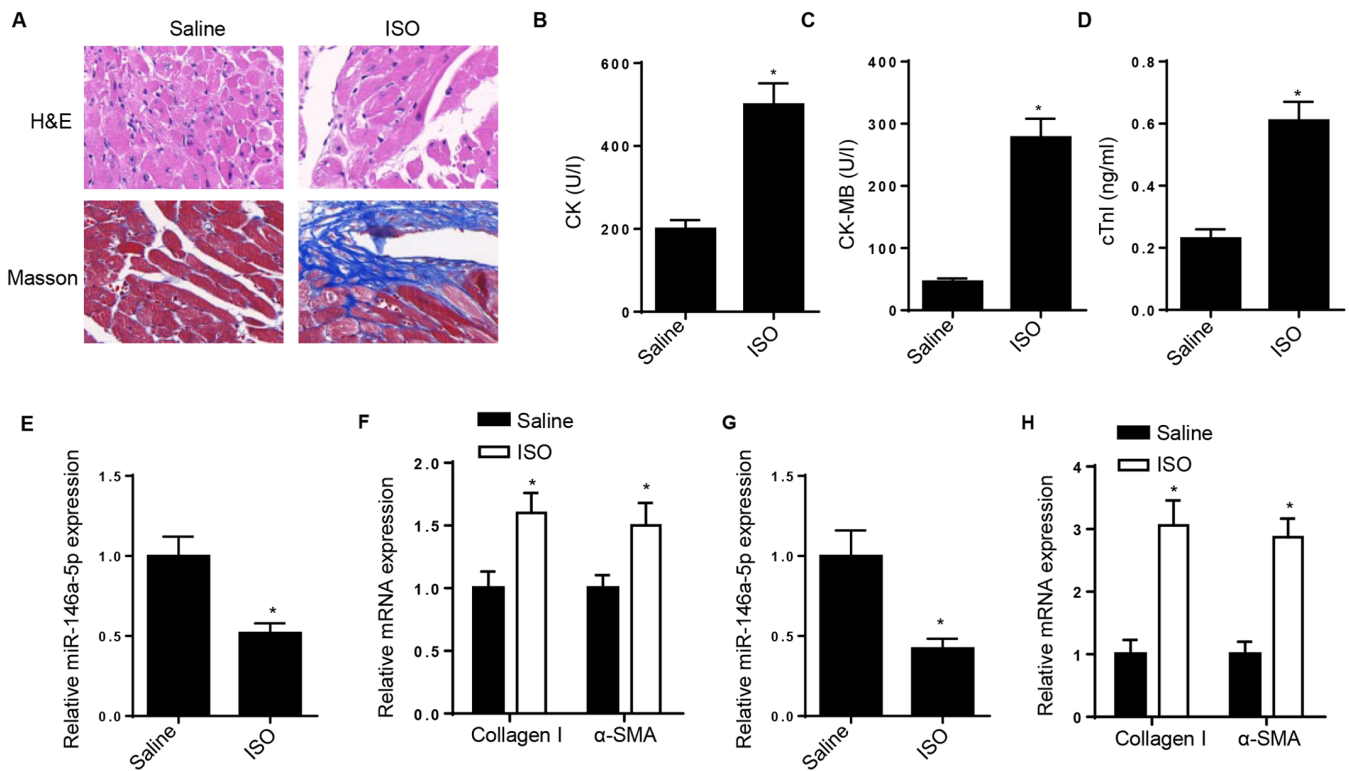


Figure 1. miR-146a-5p expression is downregulated in ISO-induced CFs and rat heart. (A) H&E and Masson staining determined myocardial collagen deposition in myocardial tissue following ISO injection. (B) CK, (C) CK-MB and (D) cTnI concentrations were measured in the serum of ISO-treated and normal control rats. RT-qPCR analysis determined expression levels of (E) miR-146a-5p and (F) collagen I and α -SMA in ISO-treated rat heart tissue. RT-qPCR analysis determined levels of (G) miR-146a-5p and (H) collagen I and α -SMA in ISO-treated CFs. * P <0.05 vs. saline. miR, microRNA; ISO, isoproterenol; CF, cardiac fibroblast; H&E, hematoxylin and eosin; CK, creatine kinase; CK-MB, creatine kinase isozyme; cTnI, cardiac troponin I; RT-q, reverse transcription-quantitative; α -SMA, α -smooth muscle actin.

Statistical analysis. Statistical analysis was performed using SPSS version 23.0 software (IBM Corp.). All experiments were performed in triplicate and data are presented as the mean \pm SD. Unpaired Student's t-test was used to compare differences between two groups, while one-way ANOVA followed by Tukey's post hoc test were used to compare differences between multiple groups. P <0.05 was considered to indicate a statistically significant difference.

Results

miR-146a-5p expression is downregulated in ISO-induced CFs and rat heart. H&E and Masson staining confirmed the cardiac fibrosis model was successfully constructed following treatment with ISO (Fig. 1A). Biomarkers, such as CK, CK-MB and cTnI, are widely applied in diagnosis and monitoring of myocardial lesion (24). Thus, the serum levels of CK, CK-MB and cTnI were detected; the results demonstrated that CK, CK-MB and cTnI levels were upregulated in the serum of ISO-treated rats (Fig. 1B-D). To determine whether miR-146a-5p is implicated in cardiac fibrosis, RT-qPCR analysis was performed to detect changes in miR-146a-5p expression in ISO-treated rat heart. The results demonstrated that miR-146a-5p expression was downregulated in ISO-treated rat heart tissue (Fig. 1E). However, the expression levels of collagen I and α -SMA increased following ISO injection in rat heart tissue (Fig. 1F). *In vitro*, RT-qPCR analysis demonstrated that miR-146a-5p expression was downregulated

in ISO-induced CFs, while collagen I and α -SMA expression levels significantly increased following treatment of CFs with ISO (Fig. 1G and H).

miR-146a-5p suppresses the viability and differentiation of ISO-induced CFs. The effect of miR-146a-5p on cardiac fibrosis was assessed. miR-146a-5p expression increased in rat CFs transfected with miR-146a-5p mimics (Fig. 2A). RT-qPCR analysis demonstrated that overexpression of miR-146a-5p downregulated the expression levels of collagen I and α -SMA (Fig. 2B). In addition, overexpression of miR-146a-5p decreased ISO-induced cell viability (Fig. 2C). Taken together, these results suggest that miR-146a-5p suppressed the viability and differentiation of CFs following treatment with ISO.

miR-146a-5p directly targets FGF2 and FGF2 knockdown decreases collagen I and α -SMA expression levels in CFs. The StarBase database revealed that FGF2 may be a potential target of miR-146a-5p (Fig. 3A). The dual-luciferase reporter assay demonstrated that overexpression of miR-146a-5p repressed the luciferase activity of the 3'-UTR of WT-FGF2, but no changes were observed in the MUT-FGF2 group in 293 cells (Fig. 3B). In addition, RT-qPCR and western blot analysis demonstrated that FGF2 expression decreased following overexpression of miR-146a-5p but increased following miR-146a-5p knockdown in ISO-treated CFs (Fig. 3C and D). Moreover, RT-qPCR and western blot analysis demonstrated

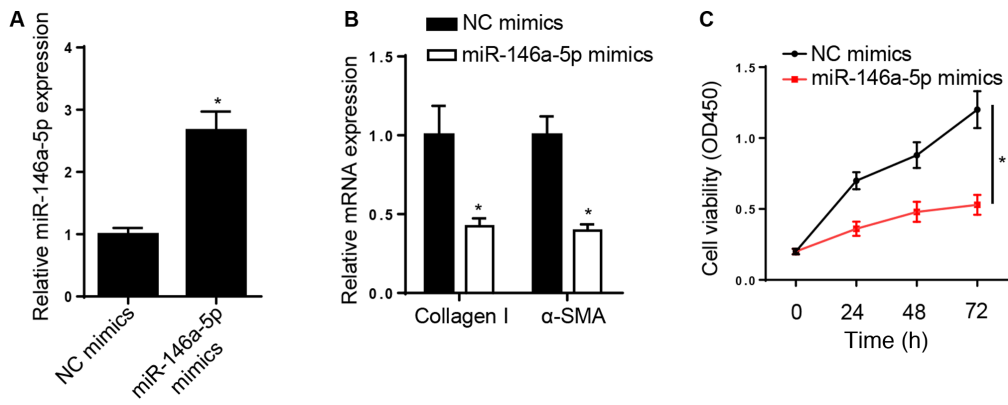


Figure 2. miR-146a-5p suppresses viability and differentiation of ISO-induced CFs. Reverse transcription-quantitative PCR analysis determined (A) miR-146a-5p and (B) collagen I and α -SMA expression levels in ISO-treated CFs transfected with NC and miR-146a-5p mimics. (C) Cell Counting Kit-8 assay showed the viability of ISO-treated CFs transfected with NC and miR-146a-5p mimics. * $P < 0.05$ vs. NC mimics. miR, microRNA; ISO, isoproterenol; CF, cardiac fibroblast; α -SMA, α -smooth muscle actin; NC, negative control; OD, optical density.

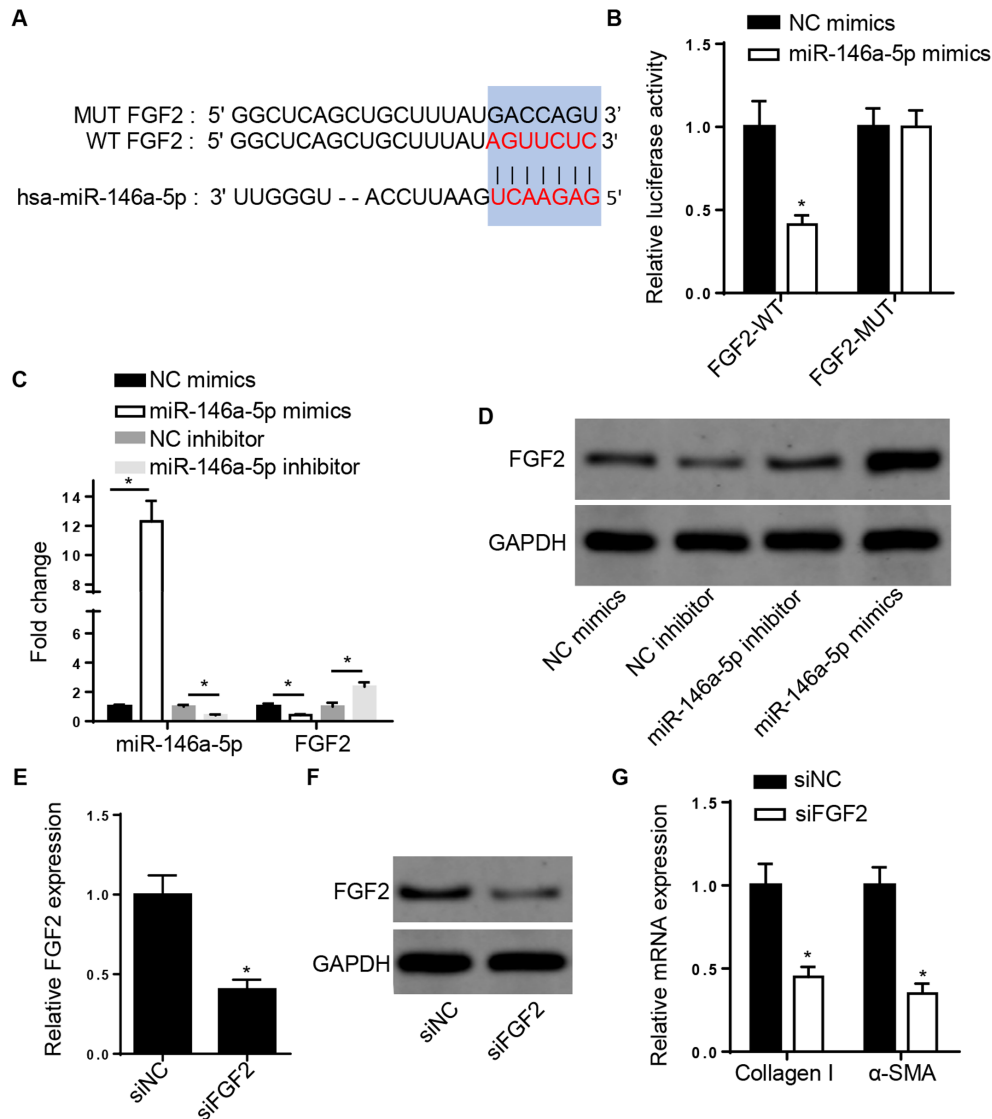


Figure 3. miR-146a-5p directly targets FGF2 and FGF2 knockdown decreases collagen I and α -SMA expression levels in CFs. (A) StarBase database showed the potential binding sites between FGF2 and miR-146a-5p. (B) Luciferase reporter assay was used to determine the binding ability between FGF2 and miR-146a-5p in 293 cells. * $P < 0.05$ vs. NC mimics. (C) RT-qPCR and (D) western blotting were used to assess the mRNA and protein levels of FGF2 in ISO-treated CFs transfected with NC or miR-146a-5p mimics or inhibitor or inhibitor. (E) RT-qPCR and (F) western blotting showed mRNA and protein levels of FGF2 in CFs transfected with siNC or siFGF2. (G) RT-qPCR analysis showed collagen I and α -SMA mRNA levels in ISO-treated CFs transfected with siNC or siFGF2. * $P < 0.05$ vs. siNC. miR, microRNA; FGF2, fibroblast growth factor 2; α -SMA, α -smooth muscle actin; NC, negative control; CF, cardiac fibroblast; RT-q, reverse transcription-quantitative; ISO, isoproterenol; si, small interfering; WT, wild-type; MUT, mutant.

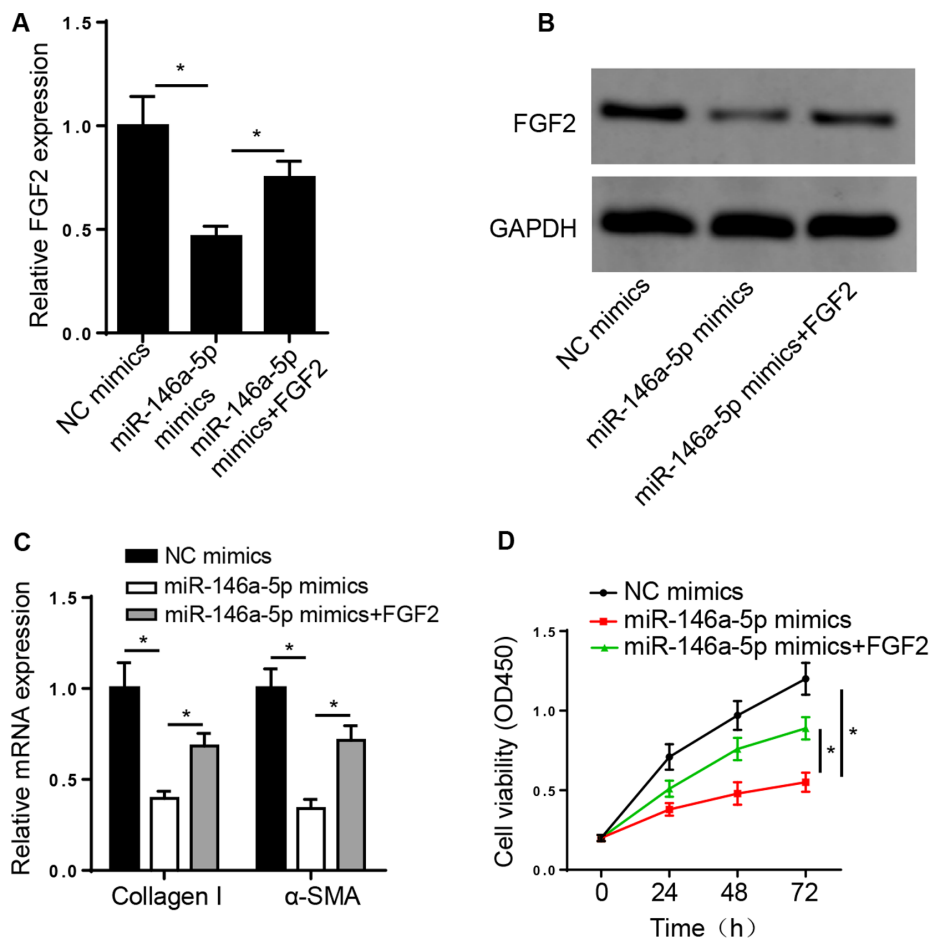


Figure 4. miR-146a-5p suppresses ISO-treated cardiac fibrosis by targeting FGF2. (A) RT-qPCR and (B) western blotting were performed to determine the mRNA and protein levels of FGF2 in ISO-treated CFs transfected with NC or miR-146a-5p mimics or miR-146a-5p mimics + pcDNA3.1/FGF2. (C) RT-qPCR and (D) Cell Counting Kit-8 assay determined collagen I and α -SMA expression levels, as well as viability, in ISO-treated CFs transfected with NC or miR-146a-5p mimics or miR-146a-5p mimics + pcDNA3.1/FGF2. * P <0.05. miR, microRNA; ISO, isoproterenol; FGF2, fibroblast growth factor 2; RT-q, reverse transcription-quantitative; CF, cardiac fibroblast; NC, negative control; OD, optical density.

that FGF2 expression decreased following FGF2 knock-down in rat CFs (Fig. 3E and F). The expression levels of fibrosis-associated collagen I and α -SMA were also inhibited following FGF2 knockdown (Fig. 3G).

miR-146a-5p suppresses ISO-treated cardiac fibrosis by targeting FGF2. To determine whether miR-146a-5p exerts its biological role in fibrogenesis via FGF2, ISO-induced CFs were transfected with NC or miR-146a-5p mimics and miR-146a-5p mimics + pcDNA3.1/FGF2. RT-qPCR and western blot analysis demonstrated that pcDNA3.1/FGF2 transfection rescued the suppressive effect of miR-146a-5p overexpression on FGF2 mRNA and protein expression levels in CFs (Fig. 4A and B). Furthermore, overexpression of miR-146a-5p suppressed the expression levels of collagen I and α -SMA and inhibited cell viability; these effects were reversed following overexpression of FGF2 (Fig. 4C and D). Taken together, these results suggest that FGF2 was involved in cardiac fibrosis progression in ISO-induced CFs.

Discussion

The results of the present study demonstrated that miR-146a-5p served a key role in ISO-treated cardiac fibrosis: miR-146a-5p

suppressed cardiac fibrosis and downregulated the expression levels of collagen I and α -SMA by modulating FGF2.

Increasing evidence suggests that miRNAs are implicated in cardiac fibrosis development. For example, Yuan *et al* (25) demonstrated that miR-21 accelerates cardiac fibrosis following myocardial infarction by regulating Smad7. Wei *et al* (26) indicated that downregulation of miR-155 suppresses cardiac fibrosis during angiotensin II-induced cardiac remodeling. Zhang *et al* (27) reported that miR-323a-3p facilitates pressure overload-induced cardiac fibrosis via TIMP3. A previous study reported that miR-146a-5p expression is downregulated in the liver of CCl₄-treated rats and that miR-146a-5p attenuates liver fibrosis by suppressing the profibrogenic effects of TGF- β 1 and lipopolysaccharide (28). Combination of miR-21 and miR-146a attenuates cardiac dysfunction and apoptosis during acute myocardial infarction in mice (29). The present study investigated the role of miR-146a-5p in cardiac fibrosis. H&E and Masson staining confirmed that the model of cardiac fibrosis was successfully induced by ISO treatment of CFs. Furthermore, miR-146a-5p expression was downregulated in ISO-induced rat heart tissue and CFs, whereas expression levels of collagen I and α -SMA increased following treatment with ISO. Overexpression of miR-146a-5p suppressed collagen I and α -SMA expression levels and attenuated viability of

ISO-treated CFs. Consistently, transfection with miR-146a-5p mimics decreased the viability of ISO-induced CFs.

FGF2 is a pro-angiogenic factor involved in wound repair (30) and mediates several biological functions, such as viability, differentiation and angiogenesis (19). A recent study demonstrated that miR-203 attenuates keloid fibroblast viability and invasion by regulating early growth response 1 and FGF2 (31). FGF2 has been reported to mediate ISO-treated cardiac hypertrophy by activating ERK signaling (32). In the present study, StarBase database and dual-luciferase reporter assay demonstrated that miR-146a-5p targeted FGF2. In addition, RT-qPCR analysis demonstrated that overexpression of miR-146a-5p suppressed FGF2 expression. Notably, miR-146a-5p overexpression decreased the expression levels of collagen I and α -SMA in CFs; these effects were reversed following overexpression of FGF2 in ISO-treated CFs. Taken together, these results suggest that miR-146a-5p suppressed ISO-treated cardiac fibrosis by decreasing FGF2 expression.

In conclusion, the results of the present study suggest that miR-146a-5p served a vital role in the development of ISO-treated cardiac fibrosis by regulating FGF2. Thus, the miR-146a-5p/FGF2 axis may be a potential novel target for the treatment of cardiac fibrosis.

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Availability of data and materials

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

Authors' contributions

HZ and YH designed the present study. HZ, HW and YH performed the experiments, analyzed the data and prepared the figures. HZ and YH drafted the initial manuscript. HZ and YH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Care and Use Ethics Committee of Nanjing First Hospital (Nanjing, China; approval no. NFH-081241-2).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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