miR-26b inhibits isoproterenol-induced cardiac fibrosis via the Keap1/Nrf2 signaling pathway

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Abstract. A critical event in cardiac fibrosis is the transformation of cardiac fibroblasts (CFs) into myofibroblasts. MicroRNAs (miRNAs) have been reported to be critical regulators in the development of cardiac fibrosis. However, the molecular mechanisms of action of miRNA (miR)-26b in cardiac fibrosis have not yet been extensively studied. In the present study, the expression levels of miR-26b were downregulated in isoproterenol (ISO)-treated cardiac tissues and CFs. Moreover, miR-26b overexpression inhibited the cell viability of ISO-treated CFs and decreased the protein levels of collagen I and α-smooth muscle actin (α-SMA). Furthermore, bioinformatics analysis and dual luciferase reporter assays indicated that Kelch-like ECH-associated protein 1 (Keap1) was the target of miR-26b, and its expression levels were decreased in miR-26b-treated cells. In addition, Keap1 overexpression reversed the inhibitory effects of miR-26b on ISO-induced cardiac fibrosis, as demonstrated by cell viability, and the upregulation of collagen I and α-SMA expression levels. Furthermore, inhibition of Keap1 expression led to the activation of nuclear factor erythroid 2-related factor 2 (Nrf2), which induced the transcriptional activation of antioxidant-detoxifying proteins in order to protect against cardiac fibrosis. Taken together, the data demonstrated that miR-26b attenuated ISO-induced cardiac fibrosis via the Keap-mediated activation of Nrf2.

Introduction

Cardiac fibrosis is an important contributor to the development of multiple cardiovascular diseases, such as myocardial infarction and hypertension (1). It is primarily characterized by an excessive accumulation of extracellular matrix, including collagen I and collagen III (2-5). Since myofibroblasts exhibit potent proliferative and secretory activities, the transformation of cardiac fibroblasts (CFs) to myofibroblasts is regarded as a crucial step in the development and progression of cardiac fibrosis (6-8). Despite significant advancements in the treatment of cardiac fibrosis, the underlying molecular mechanisms of cardiac fibroblast-to-myofibroblast transformation remain undiscovered.

Accumulating evidence has suggested that miRNAs are highly associated with the pathogenesis of various cardiovascular diseases, including cardiac fibrosis (9,10). Several miRNAs, such as miRNA (miR)-130, miR-328, miR-30a and miR-29a, have been reported to function as potential anti-fibrotic targets in cardiac fibrosis (11-14). Notably, an emerging role has been proposed for miR-26 family members in cardiovascular disease. For example, Wei et al (15) reported that miR-26a expression was markedly decreased in angiotensin II-induced neonatal CFs, and overexpression of miR-26a suppressed the expression of the connective tissue growth factor and collagen I. Although miR-26b has been reported to inhibit the development of hypertrophy (16), the exact role of miR-26b in the pathogenesis of cardiac fibrosis is still unclear.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key regulator of the antioxidative response (17,18). Under normal conditions, Nrf2 remains in an inactive state and is constitutively suppressed by Kelch-like ECH-associated protein 1 (Keap1). In response to stimulation, Nrf2 dissociates from Keap1, translocates to the nucleus and activates the antioxidant response element. Increasing evidence has demonstrated that oxidative stress induces fibroblast activation and increases collagen production in various organs, leading to pathological conditions such as liver, pulmonary and cardiac fibrosis (19-21). This evidence suggests that the Keap1/Nrf2 signaling pathway is a potential candidate for antifibrotic therapy.

The present study demonstrated that miR-26b could protect heart cells from isoproterenol (ISO)-induced cardiac fibrosis via the Keap1/Nrf2 signaling pathway. The findings may provide a novel understanding of the occurrence of cardiac fibrosis and could be used for the development of a promising therapeutic modality for the treatment of this disease.

Materials and methods

Animals and treatments. The experiments involving animals were approved by the Animal Experimentation...
Ethics Committee of the People’s Hospital of Changshou (Chongqing, China). A total of 60 adult male Sprague-Dawley rats (200–220 g) were purchased from the Laboratory of the Animal Center of the Soochow University (Suzhou, China) and randomly divided into the cardiac fibrosis model group (n=30) and the saline group (n=30). The mice were maintained under conditions of 50% relative humidity, a 12-h light/dark cycle and 22°C, and received food and water ad libitum. To establish the cardiac fibrosis group, ISO was injected subcutaneously for 10 days (5 mg/kg/day) (22-24). The Sprague-Dawley rats in the saline group were given equal volumes of saline. The rats were sacrificed by cervical dislocation after deep anesthesia with 2% isoflurane (Baxter International, Inc.) and their hearts were isolated for the subsequent experiments.

Cell culture and treatment. CFs were isolated from neonatal Sprague-Dawley rats. A total of eight neonatal Sprague-Dawley rats (age, 1-3 days; weight, 5-7 g) were obtained from the Laboratory of the Animal Center of the Soochow University. The ventricles of the neonatal rats were minced and digested with a mixed enzyme solution (trypsin: Collagenase I; ratio, 2:1). Subsequently, the cells were plated with DMEM (Sigma-Aldrich; Merck KGaA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) for 60 min at 37°C in humidified air with 5% CO2 until the CFs had adhered to the wall of plate. The culture plates were washed twice with PBS to detach the weakly attached and non-adherent cells and then pure CFs were obtained. The extracted cells were cultured in 90% DMEM (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.) in a humidified incubator at 37°C with 5% CO2. Second- or third-generation CFs were used in these experiments.

Cell transfection. miR-26b mimics (5'-UUCAGUAGUUC AGGAUAAGGUU-3'), miR-26b inhibitor (5'-ACCUAUCUCC AAUAUUAGAA-3') and their respective negative controls miR-NC (5'-UUCUCGAGCUGUCUACGUU-3') and inh-miR-NC (5'-AUCUGAGCAUGCCAGAAU-3') were synthesized by Shanghai GenePharma Co., Ltd. Keap1 cDNA (GenePharma Co., Ltd.) was cloned into pCDNA3.1 vectors synthesized by Shanghai GenePharma Co., Ltd. in order to construct corresponding Keap1 expression vector. The sequence of small interfering RNA (siRNA) against Keap1 (siKeap1, 5'‑GCG CCA AUG UUG ACA CGG A‑3') and the sequence of scrambled RNA (siNC, 5'‑GACUGAGCUGACUGGA‑3') were co‑transfected into 293T cells using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere with 5% CO2. Reverse transcription-quantitative (RT-q)PCR analysis. Total RNA was extracted from cardiac tissues and cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The RNA was reverse transcribed into cDNA using the Takara RT kit (Takara Biotechnology Co., Ltd.) at 37°C for 15 min. The expression levels of miR-26b, collagen I, α-smooth muscle actin (α-SMA), Keap1 and Nrf2 were determined using the SYBR Green PCR kit (Takara Biotechnology Co., Ltd.). G3PDH and U6 were used as controls. The following amplification conditions were used: Pre-denaturation at 95°C for 15 sec, denaturation at 94°C for 30 sec, annealing at 60°C for 20 sec, and extension at 72°C for 40 sec for 40 cycles. Relative gene expression was calculated using the 2^ΔΔCq method (25). The sequences of the primers were as follows: miR-26b forward, 5'-CCCACTTCAAGTAACTCGG-3'; and reverse, 5'-TTTGGCCTCTGGACTTATT-3'; collagen I forward, 5'-CAG AGCACGAGTCTCTGAG-3'; and reverse, 5'-GCCGAAATGGT AGCCTTCTGCG-3'; α-SMA forward, 5'-GGAATGTAAGT GGAATGG-3' and reverse, 5'-ATGAGCCGGTTGTTCTC-3'; Nrf2 forward, 5'-CACATCCAGACAGCAACACAGT-3'; and reverse, 5'-CTA CAAATGGGATGTCTCTGC-3'; GAPDH forward, 5'-CAG TCTACCTTCCTGGATAGC-3' and reverse, 5'-CATGG TGCTTCTCTTCTCTC-3'; U6 forward, 5'-CTGCGTTCGG GCGACACATATACT-3' and reverse, 5'-ACGAAAACTT CGTCGTACCTCTTGGC-3'.

Bioinformatics prediction and luciferase reporter assay. The downstream target genes of miR-26b were predicted using the TargetScan database (http://www.targetscan.org). Keap1 was identified as a potential downstream target of miR-26b. 293T cells were obtained from the American Type Culture Collection and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere with 5% CO2. Luciferase reporter assays were used to investigate the regulatory relationship between miR-26b and Keap1. Site-directed mutagenesis was used to create the mutant 3'‑untranslated region (UTR) sequence of Keap1. The wild-type and mutant-type sequences of Keap1 were cloned into the pGL-luciferase plasmid (Promega, Corp.), while the NC and miR-26b mimics sequences were co-transfected into 293T cells using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h incubation at 37°C, firefly and Renilla luciferase activities were evaluated using the Dual-Luciferase Reporter Analysis Kit (Promega Corporation). Normalized relative light units represent firefly luciferase activity/Renilla luciferase activity.

Western blot analysis. Cultured cells were lysed in RIPA buffer (Beyotime Institute of Biotechnology) for total protein extraction. Protein concentration was determined using a BCA assay (Beyotime Institute of Biotechnology). A total of 10 μg protein/lane were separated by SDS-PAGE (10% gel) and subsequently transferred to nitrocellulose membranes. Following blocking with 5% bovine serum albumin (Beijing
Solarbio Science and Technology Co., Ltd.) for 1 h at room temperature, the proteins were incubated with primary antibodies, namely anti-collagen I (cat. no. ab34710), anti-α-SMA (cat. no. ab5694) and anti-β-actin (cat. no. ab8227) (all 1:1,000; all from Abcam), at 4˚C overnight. Following washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse IgG; cat. no. ab205719; and goat anti-rabbit IgG; cat. no. ab205718) (both 1:1,000; both from Abcam) for 2 h at room temperature. The proteins were detected using an enhanced chemiluminescence detection system (ProteinSimple) and analyzed using Image-Pro® Plus software (version 6.0; Media Cybernetics, Inc.).

Statistical analysis. Statistical analyses were performed using SPSS software (version 18.0; SPSS, Inc.). All data are presented as the mean ± standard deviation of at least three independent experiments. The group differences were determined by either Student’s t-test or one-way ANOVA. Multiple comparisons between the groups was performed using the Student-Newman-Keuls method. P<0.05 was considered to indicate a statistically significant difference.

Results

Upregulation of collagen I and α-SMA in ISO-treated cardiac tissues. ISO has been reported to increase the expression of pro-inflammatory cytokines [interleukin (IL)-1, IL-6 and IL-17] (26). The release of cytokines contributes to cardiac fibrosis via upregulation of matrix metalloproteinase expression in CFs (26). In the present study, Sprague-Dawley rats were treated with ISO to induce cardiac fibrosis. Subsequently, RT-qPCR was used to evaluate the mRNA expression levels of collagen I and α-SMA in cardiac tissues. The mRNA expression levels of collagen I and α-SMA were increased in ISO-treated rats compared with those of the untreated rats (Fig. 1A). Furthermore, western blot analysis indicated that the protein expression levels of collagen I and α-SMA were increased in ISO-treated rats compared with those noted in the saline group (Fig. 1B). In addition, ISO was used to treat CFs to induce cardiac fibrosis. RT-qPCR and western blot analysis demonstrated that the expression levels of collagen I and α-SMA were upregulated in ISO-treated CFs compared with those of the control groups (Fig. 1C and D). In summary, the results indicated that
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collagen I and α-SMA were overexpressed in the ISO-treated cardiac fibrosis model.

Expression of miR-26b and Keap1 in ISO-treated cardiac tissue and CFs. The expression of miR-26b and Keap1 in ISO-treated cardiac tissues and normal tissues was determined by RT-qPCR. As shown in Fig. 2A and B, the expression of miR-26b was downregulated in ISO-treated cardiac tissues compared with saline-treated group, while the expression of Keap1 was upregulated in ISO-treated cardiac tissues. Furthermore, the expression of miR-26b and Keap1 was analyzed in vitro (Fig. 2C and D). Consistent with the results in vivo, the expression level of miR-26b was also decreased and the expression level of Keap1 was increased in ISO-treated CFs compared with the saline group.

miR-26b suppresses the cell viability of CFs. To investigate the precise role of miR-26b in ISO-induced cardiac fibrosis, ISO-treated CFs were transfected with miR-26b mimics to overexpress miR-26b, and with the miR-26b inhibitor to decrease miR-26b expression. The transfection efficiency was confirmed by RT-qPCR (Fig. 3A). miR-26b overexpression significantly inhibited the cell viability of ISO-treated CFs, while miR-26b inhibition enhanced the growth of ISO-treated CFs (Fig. 3B). Furthermore, western blot analysis indicated that miR-26b overexpression decreased the protein levels of collagen I and α-SMA compared with those of the miR-NC group. In contrast to these findings, miR-26b inhibition caused an increase in the expression levels of collagen I and α-SMA proteins (Fig. 3C). Taken collectively, the data indicated that miR-26b inhibited the transformation of CFs into myofibroblasts.

Keap1 is a target of miR-26b. The analysis using the bioinformatics prediction database TargetScan demonstrated that Keap1 was a potential target of miR-26b (Fig. 4A). To determine whether miR-26b could directly interact with Keap1, a dual luciferase reporter assay was performed. The results indicated that miR-26b mimics significantly reduced the luciferase activity of wild-type Keap1, whereas no effect was noted in the cells with the mutant 3'UTR of Keap1 (Fig. 4B). Moreover, the data indicated that miR-26b mimics significantly reduced Keap1 expression, while the miR-26b inhibitor increased the expression levels of Keap1 (Fig. 4C and D). These results demonstrated that miR-26b could directly target Keap1 to inhibit its expression.

miR-26b-mediated inhibitory effects on ISO-induced cardiac fibrosis are abrogated by Keap1. To examine whether the effect of miR-26b on ISO-induced cardiac fibrosis is regulated by Keap1, two cell groups of ISO-treated CFs were prepared, one that was transfected with miR-NC, miR-26b
Figure 3. miR-26b suppresses the cell viability of CFs. (A) Reverse transcription-quantitative PCR analysis indicating the relative expression level of miR-26b in ISO-treated CFs transfected with miR-26b mimics and the miR-26b inhibitor. (B) MTT assay results showing the cell viability of ISO-treated CFs transfected with miR-26b mimics and the miR-26b inhibitor. (C) Western blot analysis showing the protein levels of collagen I and α-SMA in ISO-treated CFs transfected with miR-26b mimics and the miR-26b inhibitor. *P<0.05, **P<0.01 vs. respective control. miR, microRNA; inh, inhibitor; ISO, isoproterenol; CF, cardiac fibroblast; NC, negative control; Col1A1, collagen I; α-SMA, α-smooth muscle actin.

Figure 4. Keap1 is one of the targets of miR-26b. (A) Bioinformatics prediction of the binding site for miR-26b in Keap1. (B) The luciferase reporter assay confirmed that miR-26b was able to bind to Wt and not Mut Keap1 in 293T cells. (C) RT-qPCR analysis showed the expression levels of miR-26b and Keap1 in ISO-treated CFs (NC and miR-26b mimics). (D) RT-qPCR analysis showed the expression levels of miR-26b and Keap1 in ISO-treated CFs (NC and miR-26b inhibitor). *P<0.05 vs. respective control. Mut, mutant; Wt, wild-type; NC, negative control; miR, microRNA; Keap1, Kelch-like ECH-associated protein 1; inh, inhibitor; RT-qPCR, reverse transcription-quantitative PCR; CF, cardiac fibroblast; ISO, isoproterenol; NC, negative control.
miR-26b mimics, miR-26b+pcDNA or miR-26b mimics+Keap1, and another that was transfected with inh-miR-NC, miR-26b inhibitor, inh-miR-26b+siNC or inh-miR-26b+siKeap1 (Fig. 5A and B). The data indicated that Keap1 overexpression significantly reduced the inhibitory effect of miR-26b on cell viability (Fig. 5C). Conversely, Keap1 silencing attenuated the cell growth of CFs transfected with the miR-26b inhibitor (Fig. 5D). Moreover, western blot analysis demonstrated that miR-26b mimics decreased the protein levels of collagen I and α-SMA in ISO-treated CFs, indicating that miR-26b inhibited ISO-induced cardiac fibrosis; these effects were abrogated by Keap1 overexpression (Fig. 5E). The opposite effect was noted in the ISO-treated CFs co-transfected with inh-miR-26b and siKeap1 (Fig. 5F).

In summary, these data demonstrated that miR-26b caused an upregulation of Nrf2 expression levels by targeting Keap1 in ISO-treated CFs.

**Discussion**

Abnormal activation and cell viability of CFs is a pivotal step in the occurrence and development of cardiac fibrosis. Therefore, identifying approaches that inhibit CF activation may be an effective therapeutic strategy to prevent cardiac fibrosis. The present study demonstrated that miR-26b inhibits ISO-induced cardiac fibrosis via the Keap1/Nrf2 signaling pathway. Previous studies have shown that Nrf2 plays an important role in myofibroblast dedifferentiation and its activity is under the control of Keap1 (27). Therefore, it was assumed that miR-26b and Keap1 may exert their roles in cardiac fibrosis via activation of Nrf2. The data demonstrated that the upregulation of miR-26b increased the expression levels of Nrf2 in ISO-treated CFs, while this effect was abolished by Keap1 overexpression (Fig. 6A). In contrast to these findings, treatment of the cells with the miR-26b inhibitor caused a downregulation in the expression levels of Nrf2, and Keap1 silencing alleviated the inhibitory effect of miR-26b inhibitor on the expression of Nrf2 (Fig. 6B). In summary, these data demonstrated that miR-26b caused an upregulation of Nrf2 expression levels by targeting Keap1 in ISO-treated CFs.
suppressed the activation and cell viability of ISO-treated CFs by upregulating Nrf2 expression and targeting Keap1.

It has been thoroughly documented that miRNAs are involved in the development and progression of cardiac fibrosis. For example, Zhou et al (28) reported that miR-21 could promote cardiac fibroblast-to-myofibroblast transition by downregulating the expression of Jagged1. Moreover, several studies have indicated that the miR-26 family plays a central role in the development of cardiovascular disease by controlling critical signaling pathways or downstream gene targets. For example, miR-26a inhibits pathological and physiological angiogenesis by targeting bone morpho-
genetic protein/SMAD1 signaling in endothelial cells (29). Han et al (16) reported that miR-26b inhibited the development of cardiac hypertrophy by inhibiting the expression of GATA binding protein 4. However, the functional role of miR-26b, which is a member of the miR-26 family, has not been extensively studied. The present study demonstrated that miR-26b was significantly downregulated in ISO-treated cardiac tissues and CFs. Furthermore, we investigated the effects of miR-26b on ISO-treated CFs. The results indicated that miR-26b mimics inhibited cell viability and decreased the protein levels of collagen I and α-SMA, while the miR-26b inhibitor increased the cell growth rate and the expression levels of collagen I and the α-SMA proteins. The data suggested that miR-26b exerts a suppressive role on ISO-induced cardiac fibrosis.

The induction of oxidative stress contributes to the occurrence and development of cardiac fibrosis. The Nrf2-antioxidant response element signaling pathway acts as a critical cellular defense mechanism that antagonizes oxidative stress (30,31). Keap1 is an important regulator and repressor of this signaling pathway (30,31). For example, Civantos et al (32) reported that sitagliptin ameliorated oxidative stress in diabetic kidney rat tissues through downregulation of miR-200a, which further regulated the Keap1/Nrf2 signaling pathway. Moreover, Yang et al (33) reported that Nrf2 could protect against hepatic stellate fibrosis by functionally activating the transcription of antioxidant response genes. In the present study, bioinformatics analysis and luciferase reporter assays demonstrated that Keap1 could directly interact with miR-26b, and that its overexpression abrogated the inhibitory effect of miR-26b on ISO-treated CFs, as determined by increased cell viability and expression of fibrosis-related indices in ISO-treated CFs. Moreover, it was demonstrated that the targeting of Keap1 by miR-26b caused an upregulation of Nrf2 expression. Therefore, the data suggested that miR-26b could regulate the Keap1/Nrf2 signaling pathway to inhibit ISO-induced cardiac fibrosis.

Overall, the present study demonstrated for the first time, to the best of our knowledge, that miR-26b could attenuate ISO-induced cardiac fibrosis by activating Nrf2 and by interacting with Keap1. These findings may provide additional evidence for the development of a novel therapeutic strategy for cardiac fibrosis and for understanding the pathogenesis of this disease.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions
SX and ZZ designed the present study. ZZ collected the samples, and ZZ and JL performed all the experiments. SX and JL analyzed the data and prepared the figures. SX drafted the initial manuscript. ZZ reviewed and revised the initial manuscript. All authors approved the final published version of this manuscript.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of the People's Hospital of Changshou. The experiments involving animals were approved by the Animal Experimentation Ethics Committee of the People's Hospital of Changshou.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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