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MiR-30b-5p and miR-22-3p restrain the fibrogenesis of post-myocardial infarction in mice *via* targeting PTAFR

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Abstract. – OBJECTIVE: Cardiac fibrosis of post-myocardial infarction (MI) is a precipitating factor of diverse cardiac diseases. MicroRNAs (miRNAs) have been reported to be implicated in the progression of cardiac fibrosis, but the functions and mechanisms of miR-30b-5p and miR-22-3p remain to be investigated.

MATERIALS AND METHODS: Cardiac fibroblasts (CFs) were isolated form mice hearts and treated with Angiotensin II (Ang II) for establishing the cardiac fibrosis model of post-MI. The expression of miRNA and mRNA was examined through quantitative real-time polymerase chain reaction (qRT-PCR). Associated protein levels were measured by Western blot. Cell viability was detected via cell counting kit-8 (CCK-8) assay. Dual-Luciferase reporter assay was administered to analyze the target correlation.

RESULTS: The down-regulation of miR-30b-5p and miR-22-3p while the up-regulation of platelet activating factor receptor (PTAFR) were found in Ang II-treated CFs. Cell proliferation and collagen deposition were refrained by miR-30b-5p and miR-22-3p overexpression and knockdown of PTAFR. MiR-30b-5p and miR-22-3p directly targeted PTAFR. MiR-30b-5p and miR-22-3p inhibitors alleviated the effects on Ang II-treated CFs induced by PTAFR knockdown through promoting PTAFR.

CONCLUSIONS: MiR-30b-5p and miR-22-3p exerted the suppression of fibrogenesis in Ang II-treated CFs via targeting PTAFR, insinuating the indicative roles of miR-30b-5p and miR-22-3p in the fibrogenesis process.

Key Words:
MiR-30b-5p, MiR-22-3p, PTAFR, Cardiac fibroblasts, Myocardial infarction, Fibrogenesis.

Introduction

Cardiac fibrosis is marked by the sedimentation of extracellular matrix (ECM) proteins, mainly including collagen I (col I), collagen III (col III) and alpha-smooth muscle actin (α-SMA), leading to the destruction of myocardium framework to trigger multifarious cardiac diseases of dysfunction, arrhythmia, ventricular sclerosis and even heart failure[1-3]. As cardiomyopathy with ischemia, myocardial infarction (MI) brought about inflammation reaction to expedite the proliferation of cardiac fibroblasts (CFs), which can differentiate into cardiac myofibroblasts (CMs) to generate abundant collagen fibers with the production of cardiac fibrosis and cardiac remodeling[4,5]. Although different therapies for cardiac fibrosis have been developed[6,7], the capture of therapeutic biomarkers is urgently needed due to the complicacy of cardiac fibrosis and unsatisfactory curative effect.

MicroRNAs (miRNAs), a type of small non-coding RNAs (sncRNAs, ~22 nucleotides), can regulate the tumor and disease progression through acting on the 3'-untranslated regions (3'-UTRs) of targets to mediate the levels of downstream genes[8]. MiRNAs promote the development of myocardial fibrosis as therapeutic and prognostic regulators[9]. Importantly, miRNA-221/222 family could resist myocardial fibrosis in the cardiac remodeling induced by excessive pressure[10]. Panizo et al[11] attested that miR-29b and miR-30c regulated by vitamin D receptor activators (VDRAs) were conducive to relieve the uremia-induced cardiac fibrosis. However, the roles and molecular mechanisms of miR-30b-5p and miR-22-3p are both ill-defined.

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Platelet activating factor receptor (PTAFR), located on chromosome 1 and lacking introns[12], was showed to play a significant part in diverse cancers and diseases. Hou et al[13] discovered that the overexpression of PTAFR brought about the promoted possibility of bone metastases of breast cancer. It has been testified that PTAFR was associated with the cardiovascular process of coronary artery disease[14]. The precise function and mechanism of PTAFR in cardiac fibrosis remain to be explored.

Reportedly, Angiotensin II (Ang II) can regulate vasoconstriction and the heart function in cardiac refactoring[15,16]. In our research, CFs were treated with Ang II for the cardiac refactoring of post-MI. The levels and roles of miR-30b-5p, miR-22-3p and PTAFR were investigated. This study aimed at discovering novel regulatory mechanisms and seeking therapeutic targets for cardiac fibrosis.

Materials and Methods

Animal Acquisition and Ethics Statement

Neonatal Sprague-Dawley (SD) mice (1-3 days) were bought from Vital River Laboratory Animal Company (Beijing, China). Standardly, all mice were transitorily fed following the criterion of Animal Care and Use Committee of the National Institutes of Health. Ethically, this research was approved by the Ethics Committee of Animal Experiment of Inner Mongolia People's Hospital.

Isolation, Culture and Treatment of Cardiac Fibroblasts (CFs)

Isolation of CFs was executed after SD mice were anesthetized. In brief, the hearts of mice were excised and minced, followed by the digestion of 0.25% trypsin (Gibco, Rockville, MD, USA). After the centrifugation and resuspension with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) and the addition of 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Gibco, Rockville, MD, USA), the suspension was seeded into the culture flask for 90 min. CFs have preferential proliferation and adherence abilities, compared to CMs. Therefore, the attachment at the bottom of the culture flask was considered as CFs with the discard of the supernatant medium. Isolated CFs were passaged for 3 generations using medium above mentioned in a 37°C incubator with 5% CO_2. CFs at third passage were treated with 0.01 mM Ang II for 24 h to establish the cardiac fibrosis model of post-MI.

Cell Transfection

The mimics of miR-30b-5p and miR-22-3p (miR-30b-5p and miR-22-3p), the inhibitors of miR-30b-5p and miR-22-3p (anti-miR-30b-5p and anti-miR-22-3p), small interfering RNA (siRNA) against PTAFR (si-PTAFR) and relative negative controls (miR-NC, anti-miR-NC and si-NC) were acquired from GENEWIZ (Suzhou, China). Using the Lipofectamine3000 reagent kit (Invitrogen, Carlsbad, CA, USA), oligonucleotides-lipid mixtures were transfected into CFs with 80% confluence in the 6-well plates. CFs were cultivated in the fresh medium until the requirement of experiments.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied for the extraction of total RNA from CFs. Based on the manufacturers' instructions, the PrimeScript™ RT Master Mix (TaKaRa, Dalian, China) was used for transcribing 2 μg RNA into complementary DNA (cDNA), which was amplified by TB Green® Premix Ex Taq™ II Kit (TaKaRa, Dalian, China) through ABI Step One Real-time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The data were analyzed via $2^{-\Delta\Delta Ct}$ approach with the normalization by small nuclear RNA U6 for miRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for mRNA. The murine primers were listed below: miR-30b-5p (forward: 5'-CCAGCAACTGTAAACATCCTACAC-3', reverse: 5'-TATGGTTTTGACGACTGTGTGAT-3'); miR-22-3p (forward: 5'-ACACTCCAGCTGGGAAGCTGCCAGTTGAAG-3', reverse: 5'-GGTGTCGTGGAGTCGGCAA-3'); PTAFR (forward: 5'-GCCCAATAAGGATGGCTCAG-3', reverse: 5'-AAGAAGCAGAAGGCGATGAA-3'); U6 (forward: 5'-GCTTCGGCAGCACATATACTAA-3', reverse: 5'-CGAATTTGCGTGTCATCCTT-3'); GAPDH (forward: 5'-AAGAAGGTGGTGAAGCAGGC-3', reverse: 5'-TCCACCACCCTGTTGCTGTA-3').

Western Blot

After lysing via radioimmunoprecipitation assay (RIPA) buffer with proteinase inhibitor (Sigma-Aldrich, St. Louis, MO, USA), centrifugation and boiling, total proteins from CFs were

harvested. Then, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was implemented to separate 60 μg proteins, which was promptly transferred onto the polyvinylidene difluoride (PVDF) membranes (Sigma-Aldrich; St. Louis, MO, USA), followed by the blockage with 5% skim milk (Sigma-Aldrich, St. Louis, MO, USA) at 4°C overnight and the incubation of primary antibodies at indoor temperature for 4 h. The primary antibodies of rabbit contained anti-PTAFR (Abcam, Cambridge, UK, ab104162, 1:1000), anti-cyclin D1 (Abcam, Cambridge, UK, ab16663, 1:1000), anti-proliferating cell nuclear antigen (anti-PCNA; Abcam, Cambridge, UK, ab18197, 1:1000), anti-Col I (Abcam, Cambridge, UK, ab34710, 1:1000), anti-Col III (Abcam, Cambridge, UK, ab7778, 1:1000), anti-α-SMA (Abcam, Cambridge, UK, ab5694, 1:1000) or anti-GAPDH (Abcam, Cambridge, UK, ab181602, 1:3000). Next, membranes were incubated by goat anti-rabbit secondary antibody (Abcam, Cambridge, UK ab205718, 1:5000) for about 45 min at indoor temperature. Ultimately, the enhanced chemiluminescence (ECL) reagent (Sigma-Aldrich; St. Louis, MO, USA) was applied for the exposure of the blots.

Cell Counting Kit-8 (CCK-8)

CFs were inoculated into 96-well plates with the 10^3 cells and 200 μL medium per well. After treatment and transfection for 48 h, CFs were incubated with 20 μL CCK-8 reagent (Sigma-Aldrich, St. Louis, MO, USA) for 2 h. Eventually, a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) was utilized for examining the optical density (OD) value at 450 nm.

Dual-Luciferase Reporter Assay

TargetScan and starBase were used for the target prediction of miRNA. The 3'-UTR sequences of wide-type (WT) PTAFR containing the binding points of miR-30b-5p or miR-22-3p were individually cloned into pGL3 vector (Promega, Madison, WI, USA), generating two types of PTAFR 3'UTR WT Luciferase reporter plasmids (PTAFR#1 3'UTR WT and PTAFR#2 3'UTR WT). Likewise, the matched mutant-type (MT) controls (PTAFR#1 3'UTR MUT and PTAFR#2 3'UTR MUT) were obtained. After the co-transfection of these constructs with miRNA (miR-30b-5p or miR-22-3p) or miR-NC into CFs, the relative Luciferase activity was detected via the Dual-Luciferase reporter system (Promega, Madison, WI, USA) following the producer's direction.

Statistical Analysis

After independent repetition of three times, data were revealed as the mean ± standard deviation (SD) and processed though SPSS 19.0 (IBM, Armonk, NY, USA), and GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). The comparisons of groups were administrated by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's test. $p < 0.05$ was considered as significant at the statistical level.

Results

MiR-30b-5p and MiR-22-3p Were Down-Regulated but PTAFR Was Up-Regulated In Ang II-Treated CFs

CFs were treated with 0.01 mM Ang II for 24 h to establish the fibrogenesis model of post-MI. QRT-PCR presented that Ang II triggered a marked decrease of the expression of miR-30b-5p (Figure 1A) and miR-22-3p (Figure 1B) in comparison to the untreated control group. On the contrary, the mRNA (Figure 1C) and protein (Figure 1D) levels of PTAFR were both enhanced after treatment with Ang II. The dysregulation of miR-30b-5p, miR-22-3p and PTAFR in Ang II-treated CFs hinted their critical roles in the process of cardiac fibrosis.

Overexpression of MiR-30b-5p Impeded Cell Proliferation and the Secretion of Collagen Induced by Ang II In CFs

To explore the role of miR-30b-5p in cardiac fibrosis, miR-30b-5p mimic was transfected into Ang II treated CFs. As Figure 2A illustrated, miR-30b-5p mimic transfection and Ang II co-treatment recovered the expression of miR-30b-5p in CFs, suggesting the great overexpression efficiency of miR-30b-5p. CCK-8 demonstrated that Ang II strikingly promoted the cell viability of CFs, which was hindered by transfection of miR-30b-5p (Figure 2B). Also, Western blot revealed the protein levels of cyclin D1 and PCNA (pro-proliferation markers) were lower in the Ang II+miR-30b-5p group than these in the Ang II group (Figure 2C). Comparatively speaking, the protein levels of Col I, Col III and α-SMA (pro-collagen markers) were enhanced after induction of Ang II, whereas the increase was partly alleviated following overexpression of miR-30b-5p (Figure 2D). Taken together, miR-30b-5p suppressed the proliferation and collagen secretion of Ang II-stimulated CFs.

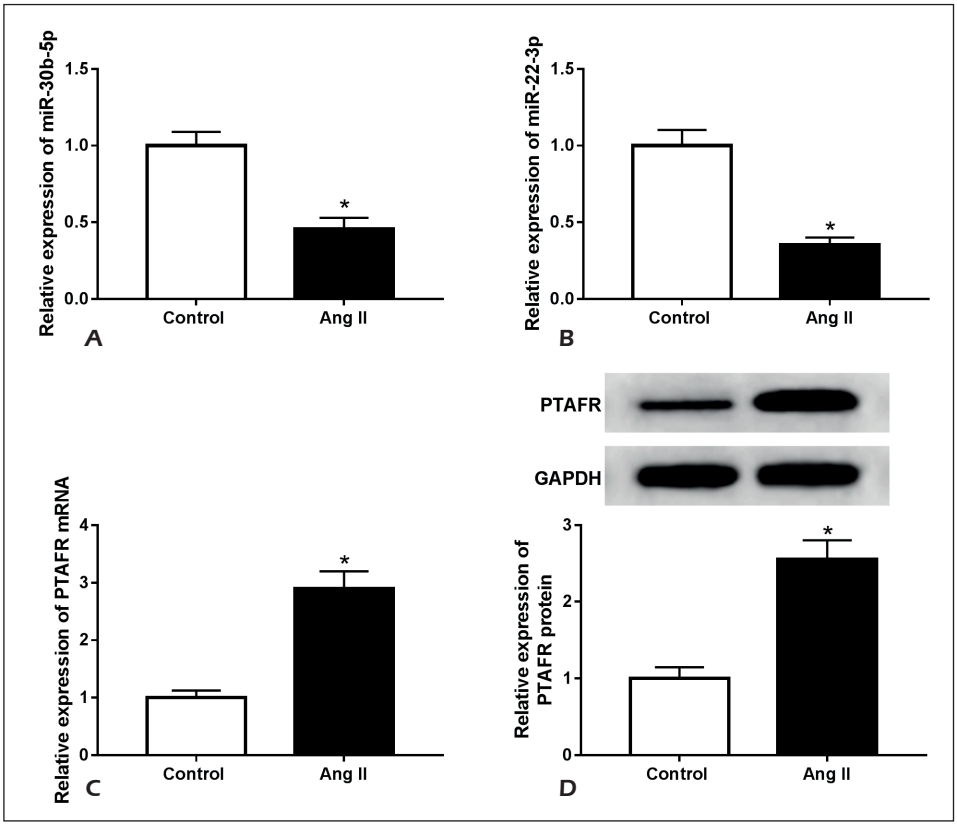

Figure 1. MiR-30b-5p and miR-22-3p were down-regulated but PTAFR was up-regulated in Ang II-treated CFs. **A-B**, The levels of miR-30b-5p (**A**) and miR-22-3p (**B**) were measured by qRT-PCR in CFs treated with Ang II. C, D, QRT-PCR and Western blot were used for detecting the mRNA (**C**) and protein (**D**) levels of PTAFR in Ang II-treated CFs (1.5×). *$p<0.05$.

Up-regulation of MiR-22-3p Generated the Inhibitory Effects on Cell Proliferation and the Deposition of Collagen In CFs Treated With Ang II

Similarly, Ang II treated CFs were transfected with miR-22-3p for investigating the function of miR-22-3p in cardiac fibrosis. First, we determined the overexpression efficiency of miR-22-3p mimic in CFs treated with Ang II, and the transfection of miR-22-3p mimic prominently enhanced the level of miR-22-3p in Ang II-treated CFs (Figure 3A). In CCK-8 assay, the rising of OD value in Ang II-treated CFs was signally ameliorated by miR-22-3p transfection, demonstrating that miR-22-3p inhibited Ang II-stimulated proliferation of CFs cells (Figure 3B). Besides, miR-22-3p transfection counteracted the up-regulation of pro-proliferation markers (cyclin D1 and PCNA) caused by Ang II treatment in CFs, which was consistent with the above findings (Figure 3C), and the same mitigation of Col I, Col III and α-SMA expression was observed in CFs treated with Ang II (Figure 3D). Taken together, miR-22-3p suppressed the proliferation and collagen deposition in the process of cardiac fibrosis.

Knockdown of PTAFR Suppressed Cardiac Fibrosis In Ang II-Treated CFs

After treating with Ang II, CFs were transfected with si-PTAFR and the interference efficiency of si-PTAFR was conspicuous due to the lessening of PTAFR mRNA (Figure 4A) and protein (Figure 4B) levels contrasted to the Ang II group. Notably, Ang II-induced cell viability was restrained by transfection with si-PTAFR (Figure 4C). In addition, the expression of proliferation-promoted and collagen-motivated proteins was examined via Western blot, in which the remarkable relief of protein levels induced by Ang II was found not only in cyclin D1 and PCNA (Figure 4D) but also in Col I, Col III and α-SMA (Figure 4E). The inhibition of proliferation and collagen production by PTAFR knockdown in Ang II-treated CFs implied that PTAFR knockdown might inhibit the cardiac fibrosis.

MiR-30b-5p Targetedly Modulated the Expression of PTAFR

The relation between miR-30b-5p and PTAFR was firstly researched. In the Targetscan software, PTAFR was predicted as a target of miR-30b-5p because of the mutual binding sites between the

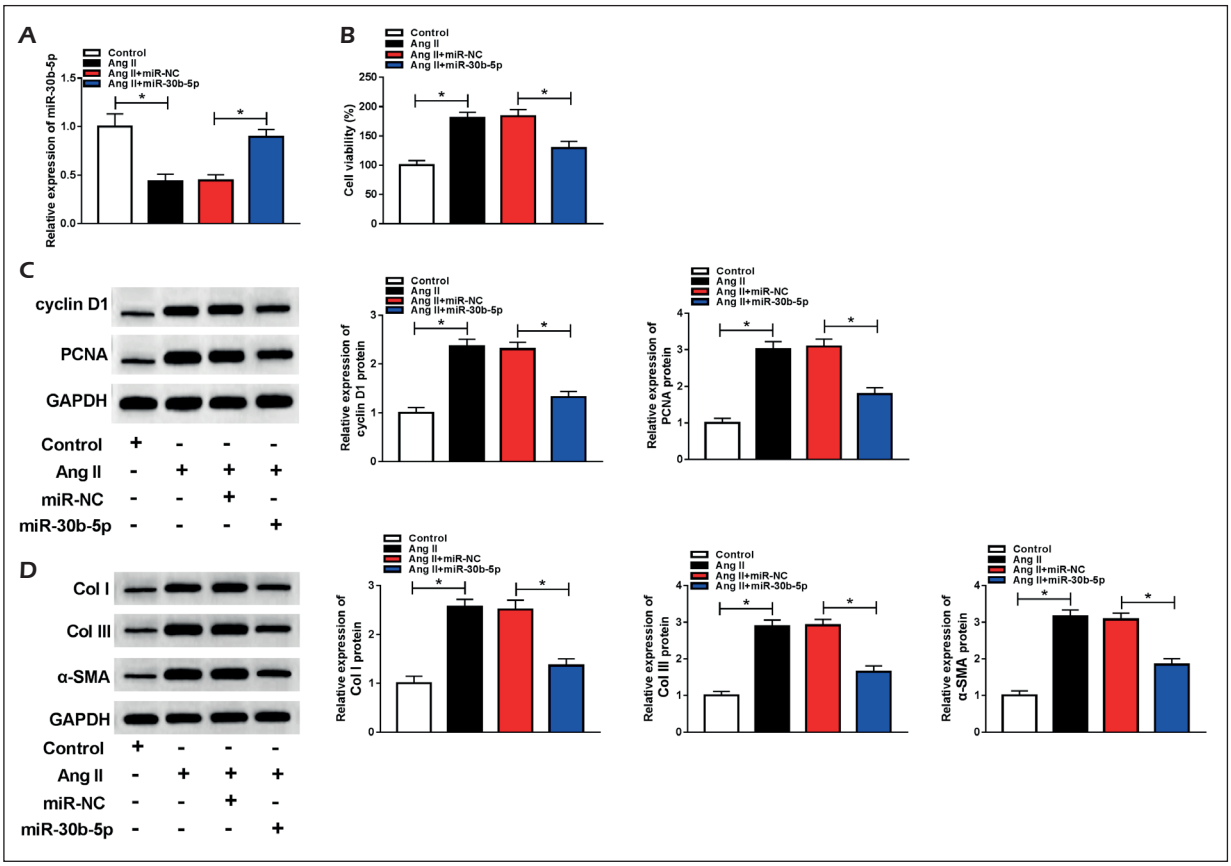

Figure 2. Overexpression of miR-30b-5p impeded cell proliferation and the secretion of collagen in Ang II-treated CFs. **A**, The overexpression efficiency of miR-30b-5p was evaluated by qRT-PCR in Ang II-treated CFs. **B**, CCK-8 was applied for detecting cell viability after treatment with Ang II and transfection with miR-30b-5p for CFs. **C-D**, Western blot was utilized for measuring the protein levels of pro-proliferation markers (**C**) and pro-collagen markers (D) (1×). *p<0.05.

PTAFR#1 3'UTR WT and miR-30b-5p (Figure 5A). Furthermore, Dual-Luciferase reporter assay testified that the co-transfection of miR-30b-5p and PTAFR#1 3'UTR WT evidently weakened the Luciferase activity of CFs, but the expurgation of the binding sites for miR-30b-5p eliminated the restraining impact (Figure 5B). Then, we detected the PTAFR expression levels by qRT-PCR and Western blot in CFs transfected with miR-30b-5p or anti-miR-30b-5p or matched controls. The transfection of miR-30b-5p down-regulated the mRNA and protein expression of PTAFR, while the depletion of miR-30b-5p revealed an opposite phenomenon (Figure 5C and D). Thus, miR-30b-5p directly modulated the expression of target gene PTAFR.

PTAFR Was a Direct Downstream Target Gene of MiR-22-3p

The bioinformatic analysis of starBase software exhibited the presumptive conjunct sites between the PTAFR#2 3'UTR WT and miR-22-3p (Figure 6A). The further affirmation of this target relation was implemented through Dual-Luciferase reporter assay, in which the Luciferase activity in PTAFR#2 3'UTR WT group was significantly refrained by miR-22-3p mimic transfection, whereas it remained unchanged in PTAFR#2 3'UTR MUT+miR-22-3p co-transfected group (Figure 6B). Besides, qRT-PCR and Western blot showed that miR-22-3p transfection repressed the mRNA and protein levels of PTAFR but miR-22-3p inhibitor had the opposite effect on PTAFR expression (Figure 6C and D). Verifiably, PTAFR was a downstream target of miR-22-3p, and it was directly regulated by miR-22-3p.

Inhibition of MiR-30b-5p Ameliorated the Repression of Cardiac Fibrosis Caused by si-PTAFR in Ang II-Treated CFs

To ascertain whether the certain regulatory mechanism between miR-30b-5p and PTA-

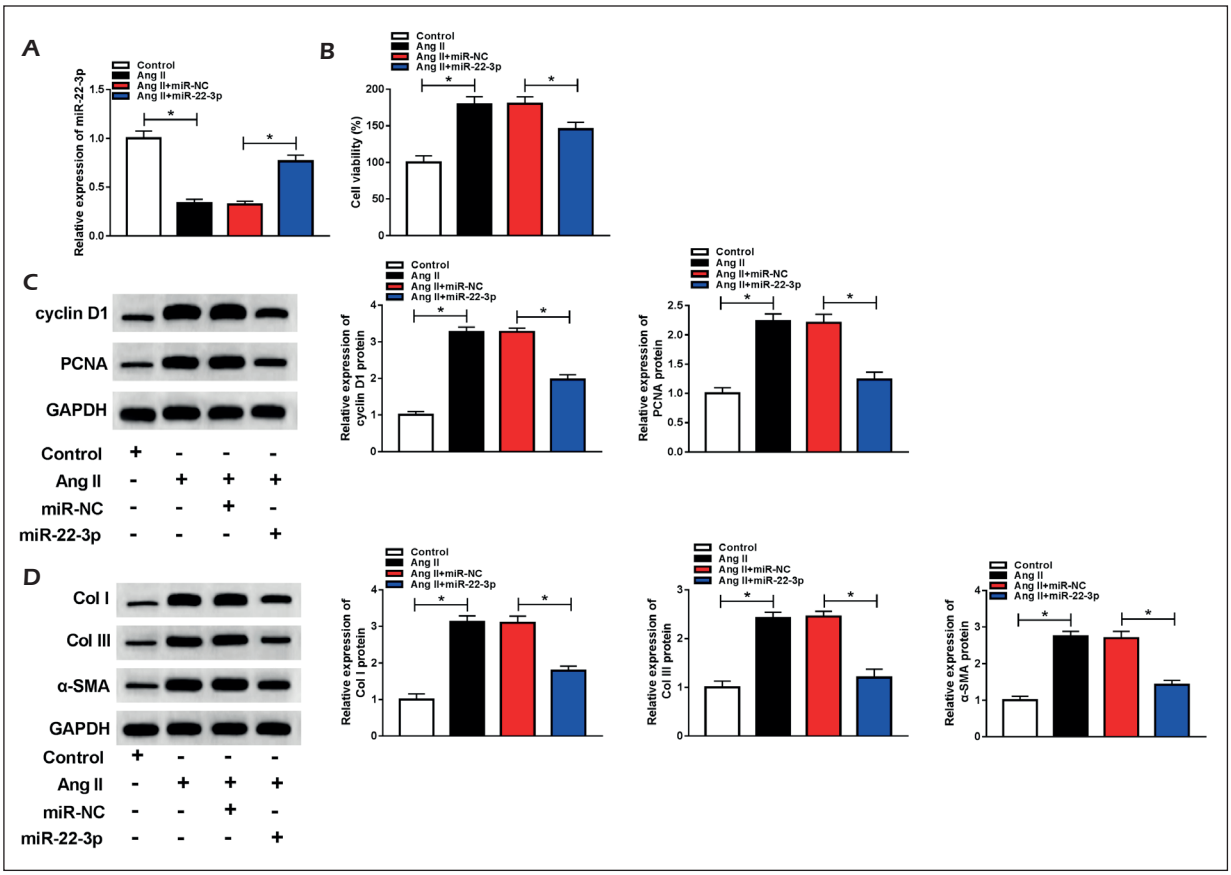

Figure 3. Up-regulation of miR-22-3p generated the inhibitory effects on cell proliferation and the deposition of collagen in CFs treated with Ang II. **A**, QRT-PCR was used to assess the overexpression efficiency of miR-22-3p. **B**, Cell viability was examined via CCK-8 in CFs treated with Ang II and transfected with miR-22-3p or relative controls. **C**-**D**, The protein levels of proliferation-promoted markers (**C**) and collagen-promoted markers (**D**) were determined through Western blot (1×). *p<0.05.

FR, CFs were treated and transfected as groups of Ang II, Ang II+ si-PTAFR, Ang II+si-PTAFR+anti-miR-30b-5p or relative controls. As shown in Figure 7A and B, knockdown of PTAFR reduced the mRNA and protein levels of PTAFR in CFs up-regulated by Ang II, while miR-30b-5p inhibitor partly abated this reduction. The alleviation of cell viability inhibited by si-PTAFR in Ang II-treated CFs was also definite (Figure 7C). Western blot suggested that the decrease of cyclin D1 and PCNA protein levels caused by si-PTAFR in Ang II-treated CFs was lightened through the repression of miR-30b-5p (Figure 7D), and the same phenomenon was found in Col I, Col III and α-SMA levels (Figure 7E). All results indicated the si-PTAFR-induced suppression of cardiac fibrosis in Ang II-treated CFs was weakened by the depletion of miR-30b-5p.

Down-Regulation of MiR-22-3p Relieved the si-PTAFR-Induced Impacts on CFs Treated With Ang II

After the treatment and transfection with Ang II, Ang II+si-PTAFR, Ang II+si-PTAFR+anti-miR-22-3p or respective controls into CFs, the modulatory relation between miR-22-3p and PTAFR was explored. First, qRT-PCR and Western blot displayed that miR-22-3p inhibitor attenuated the inhibitory effects of PTAFR intervention on the abundance of PTAFR mRNA and protein in Ang II stimulated CFs (Figure 8A and B). The depression of PTAFR resulted in a decrease of cell viability in Ang II-treated CFs, which was abrogated by miR-22-3p inhibitor (Figure 8C). Also, transfection of si-PTAFR triggered the restraint of cyclin D1 and PCNA protein levels (Figure 8D) and Col I, Col III and α-SMA levels (Figure 8E)

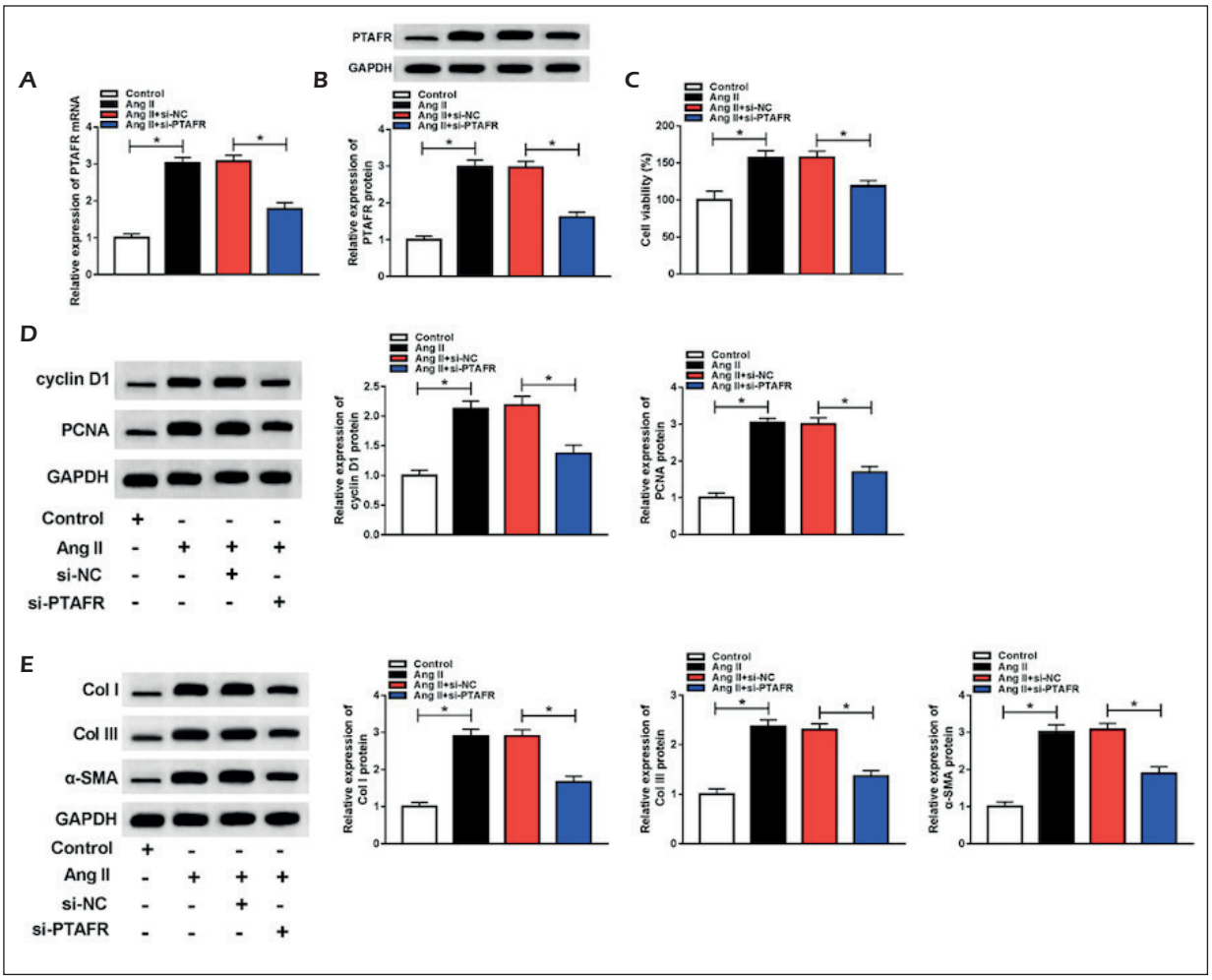

Figure 4. Knockdown of PTAFR suppressed cardiac fibrosis in Ang II-treated CFs. ***A***-***B***, QRT-PCR and Western blot were conducted to analyze the interference effectiveness of si-PTAFR in Ang II-treated CFs (1×). C, The detection of cell viability was executed by CCK-8 in Ang II-treated CFs transfection with si-PTAFR or si-NC. ***D***-***E***, The examination of pro-proliferation markers (***D***) and pro-collagen markers (***E***) levels was administered by Western blot (1×). *$p<0.05$.

in Ang II-treated CFs, but transfection of anti-miR-22-3p attenuated the inhibition. In part, the suppressive impacts caused by si-PTAFR on Ang II-treated CFs were eliminated after miR-22-3p down-regulation.

Discussion

In view of the aggressive injuries for heart evoked by cardiac fibrosis of post-MI, ameliorating cardiac fibrosis is of the essence in advancing the prognosis of MI and preventing the cardiac diseases from happening. Here, our study revealed that miR-30b-5p and miR-22-3p were both down-regulated in the mice model of post-MI (CFs treated with Ang II), accompanied by the accumulation of collagen fibers. However, overexpression of miR-30b-5p and miR-22-3p effectively restrained the fibrogenesis in Ang II-treated CFs via regulating the expression of PTAFR. All discoveries suggested that miR-30b-5p and miR-22-3p were concerned in the etiopathogenesis of cardiac fibrosis, as innovative indicators for the therapy of diseases caused by cardiac fibrosis.

Proverbially, miRNAs including miR-30 and miR-22, could participate in the progression of cardiac fibrosis[17-19]. Nagpal et al[20] asserted that the up-regulation of miR-125b contributed to the proliferation of CFs and the transition from CFs to CMs to induce cardiac fibrosis. In a model of acute cardiac allograft transplantation, suppres-

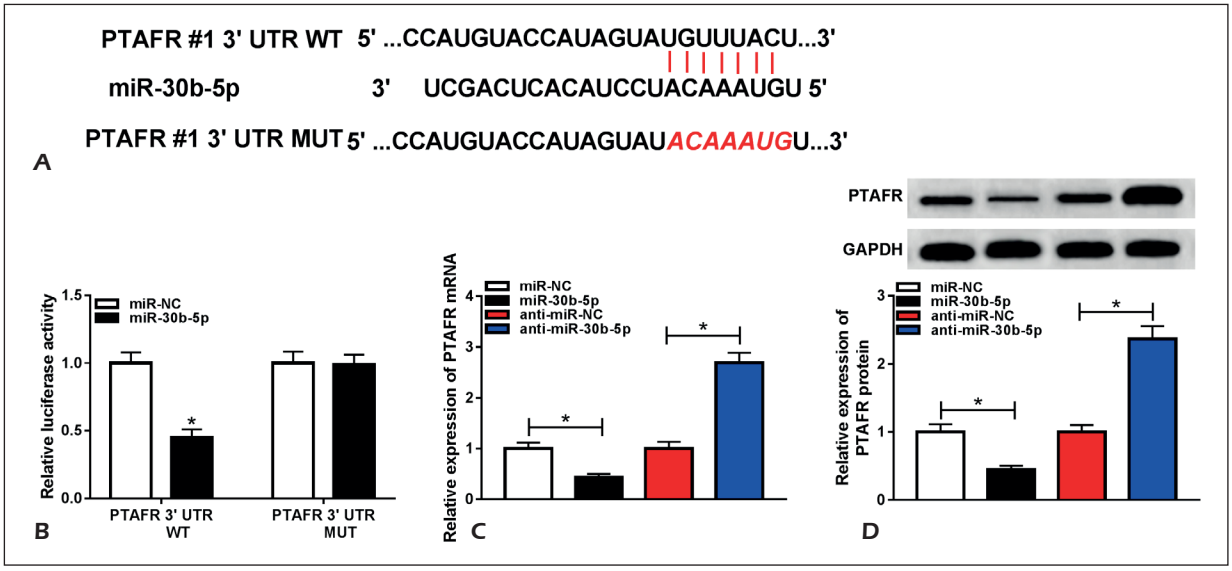

Figure 5. MiR-30b-5p targetedly modulated the expression of PTAFR. **A**, Targetscan was used for the prediction of the binding sites between miR-30b-5p and PTAFR. B, The Luciferase activity of CFs co-transfected with PTAFR#1 3'UTR WT or PTAFR#1 3'UTR MUT and miR-30b-5p or miR-NC was determined by Dual-Luciferase reporter system. **C-D**, The mRNA (**C**) and protein (**D**) levels of PTAFR were measured through qRT-PCR and Western blot after transfection with miR-30b-5p, anti-miR-30b-5p or relative controls (1×). *p<0.05.

sion of miR-21 inhibited the fibrocyte cumulation and fibrosis formation[21]. Also, miR-327 motivated the expression of fibrosis-related gene and brought about the prominent converting of CFs to CMs, but the silence of miR-327 abrogated this effect to reduce the cardiac fibrosis in the Ang II-induced cardiac restructuring[22]. Inversely, Sang et al[23] claimed that miR-133a was expressed at a low level and repressed the cardiac fibrosis through targeting protein kinase B (AKT) in the rats with

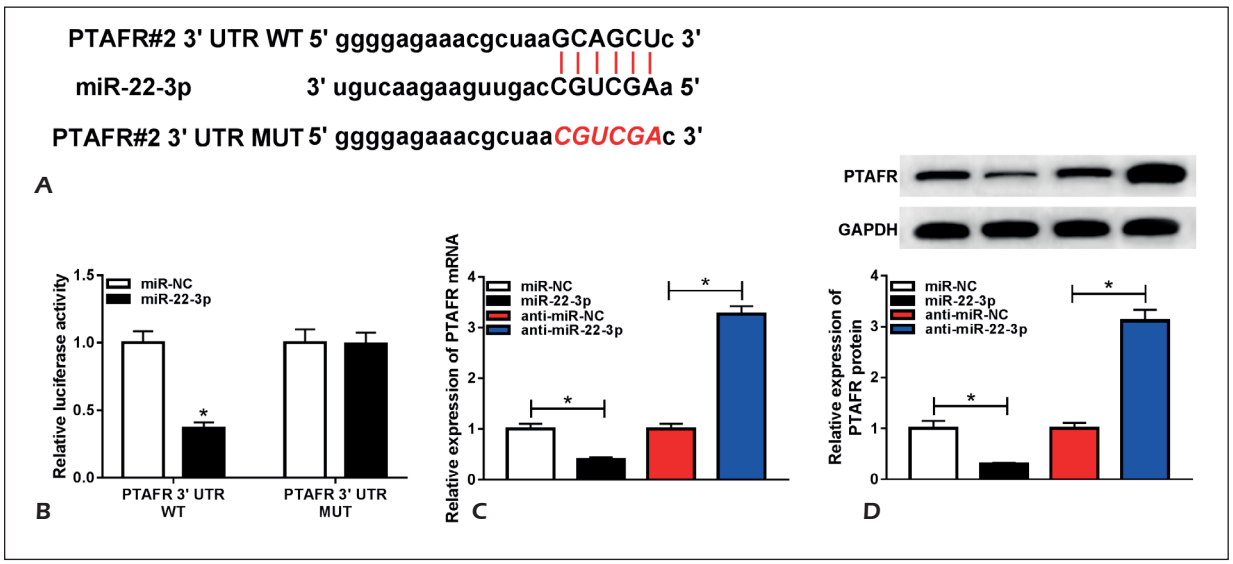

Figure 6. PTAFR was a direct downstream target gene of miR-22-3p. **A**, Bioinformatic analysis between PTAFR#2 3'UTR WT and miR-22-3p was carried out by starBase. **B**, Dual-Luciferase reporter assay was performed to confirm the relationship between PTAFR and miR-22-3p. **C-D**, After transfection with miR-22-3p, anti-miR-22-3p or corresponding controls into CFs, qRT-PCR and Western blot were used for detecting the mRNA (**C**) and protein (**D**) levels of PTAFR (1×). *p<0.05.

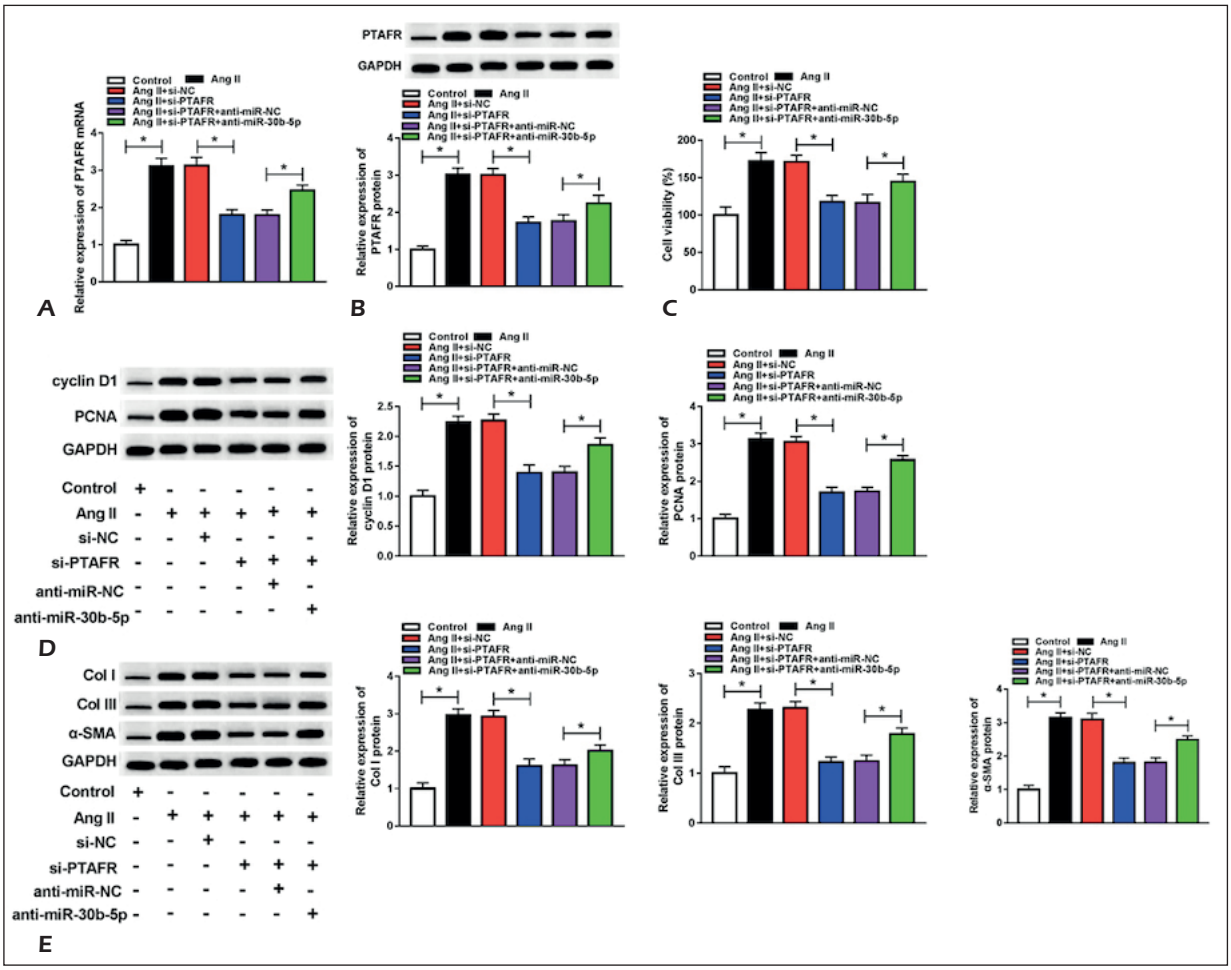

Figure 7. Inhibition of miR-30b-5p ameliorated the repression of cardiac fibrosis caused by si-PTAFR in Ang II-treated CFs. ***A***-***B***, QRT-PCR and Western blot were implemented to examine the expression of PTAFR after treatment with Ang II, Ang II+ si-PTAFR, Ang II+si-PTAFR+anti-miR-30b-5p or matched controls (1×). C, Cell viability was assessed by CCK-8. **D**-**E**, Western blot was used to detect the protein expression of proliferation-promoted markers (**D**) and collagen-promoted markers (**E**) (1×). **p*<0.05.

chronic heart failure. Tao et al[24] declared that miR-29a was down-regulated in CFs and fibrosis tissue, and reduced CFs proliferation of neonatal rats through targeting specific signaling pathway. Taking into consideration the dissimilar roles of miRNAs in cardiac fibrosis, we designed experiments to investigate the explicit functions of miR-30b-5p and miR-22-3p. First, the decrease of miR-30b-5p and miR-22-3p expression was found in Ang II-treated CFs. Besides, the overexpression of miR-30b-5p and miR-22-3p both led to the decrease of cell proliferation and collagen deposition in Ang II-treated CFs, indicating the inactivation of the differentiation from CFs to CMs and ultimate inhibition of fibrogenesis. Wang et al[25] found that miR-30 expression was descended in CFs of post-MI and miR-30 inhibitor abolished the repressive effect on collagen production caused by long non-coding RNA (lncRNA) n379519. The collagen synthesis stimulated by Ang II was relieved by the promotion of miR-22 in CFs[26]. The inhibitory roles of miR-30b-5p and miR-22-3p in cardiac fibrosis during our report were coincident with the above findings of their parental miRNAs.

Concerning the role of PTAFR in cardiac fibrosis, PTAFR was showed to be up-regulated and this elevation conduced to the collagen production and cardiac remodeling in mice[27]. Liang et al[28] proved that down-regulation of PTAFR ameliorated the influences on cell proliferation and collagen secretion induced by pro-fibrotic lncRNA (PFL). In conformity with these discoveries, Ang II triggered the up-regulation of PTAFR in CFs of mice, and knockdown of PTAFR

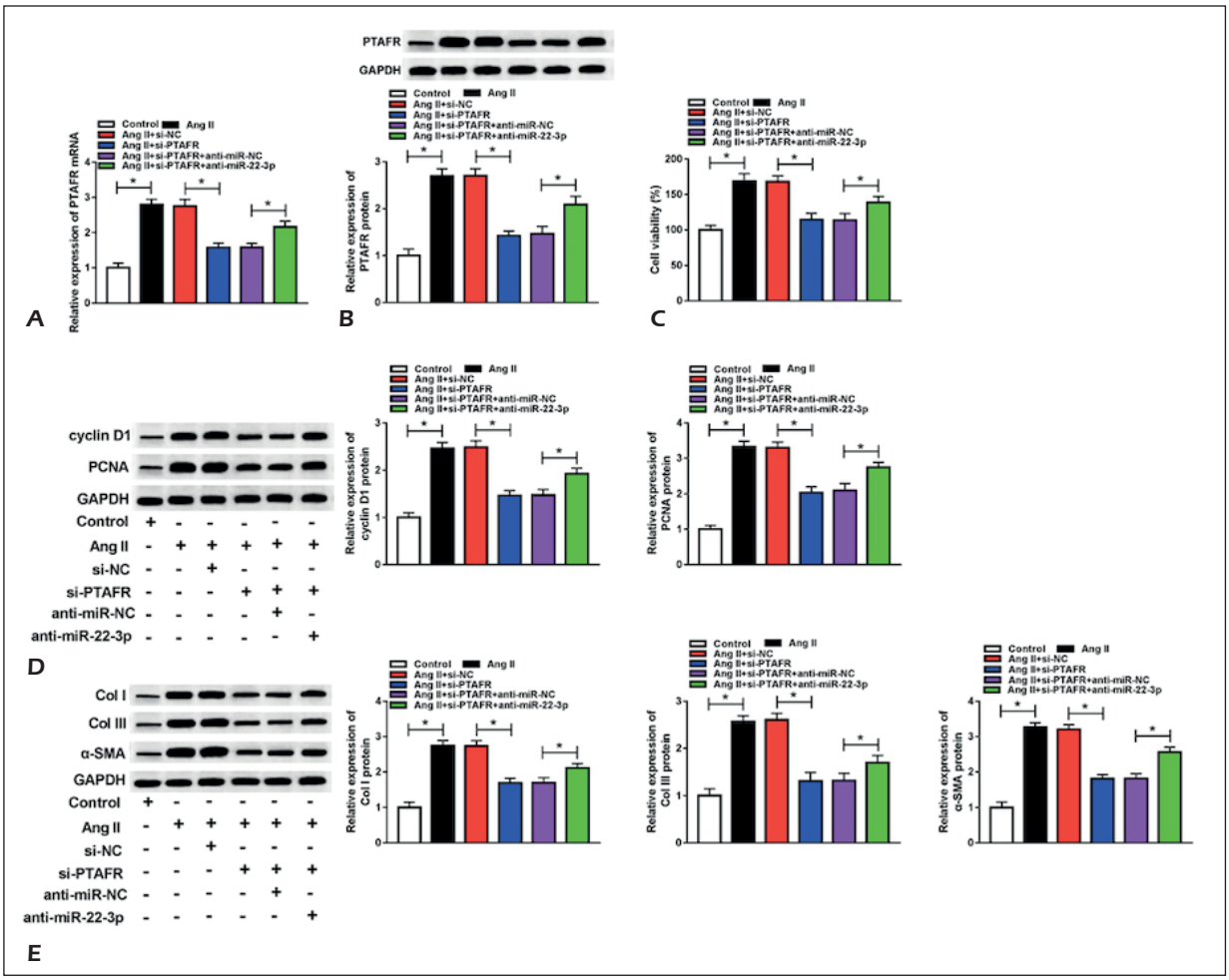

Figure 8. Down-regulation of miR-22-3p relieved the si-PTAFR-induced impacts on CFs treated with Ang II. **A-B**, QRT-PCR and Western blot were used for the detection of PTAFR mRNA and protein expression in CFs treated with Ang II, Ang II+ si-PTAFR, Ang II+si-PTAFR+anti-miR-22-3p or relative controls (1×). **C**, The viable CFs were measured by CCK-8. **D-E**, Cell proliferation and collagen formation were evaluated by the levels of pro-proliferation markers (**D**) and pro-collagen markers (**E**) via Western blot (1×). *$p<0.05$.

reversed the promotion of cell proliferation and collagen aggregation induced by Ang II, exerting the anti-fibrosis effect for Ang II-treated CFs. Pronouncedly, the roles of miR-30b-5p/miR-22-3p overexpression and PTAFR knockdown in cardiac fibrosis were identical, insinuating the potential correlation between miR-30b-5p/miR-22-3p and PTAFR. The miRNA-mRNA modulatory axis was reported in cardiac pathology of Na/H exchanger isoform 1 (NHE1) mice[29]. Through the bioinformatics analysis and experiments verification, PTAFR was identified as the target of both miR-30b-5p and miR-22-3p during this report. Moreover, miR-30b-5p and miR-22-3p inhibitors could regulate cell proliferation and the deposition of collagen via targeting PTAFR.

Conclusions

Our results shed light on the roles and mechanisms of miR-30b-5p and miR-22-3p in the cardiac fibrosis of post-MI in mice. MiR-30b-5p and miR-22-3p generated the anti-fibrosis effects and provided the protective functions for Ang II-treated CFs through directly reducing PTAFR. As the first exploration of the roles of miR-30b-5p/miR-22-3p and confirmation of miR-30b-5p/miR-22-3p-PTAFR regulatory axis in cardiac fibrosis, this study afforded the foundation and brand-new thought for the treatment of cardiac fibrosis-related diseases. MiR-30b-5p and miR-22-3p might be promising biomarkers; however, further researches of clinic analysis are required to conduct the

evaluation for the regulatory roles of miR-30b-5p and miR-22-3p in cardiac fibrosis.

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Conflict of Interests

The authors declare that they have no financial conflict of interests.

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