The role of miR-370 in fibrosis after myocardial infarction

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Abstract. In the present study, we investigated the expression of miR-370 in the border area of infarction after myocardial infarction and its role in the process of post-infarction fibrosis. A myocardial infarction model in Sprague-Dawley rats was established. After two weeks, the mRNA levels of transforming growth factor-β1 (TGFβ1), TGFβRII, ColIa1, ColIIIa1 and miR-370 and the expression of TGFβ1, TGFβRII and α-smooth muscle actin (α-SMA) proteins in the border area of infarction were detected by real-time fluorescence quantitative polymerase chain reaction (qRT-PCR) and western blot analysis. Cardiac fibroblasts in neonatal rat were isolated and cultured, and the changes in the above indicators were detected after AngII and miR-370 intervention. Luciferase reporter gene assay was conducted to verify whether TGFβRII was a target gene of miR-370. In the border area after myocardial infarction, the expression of miR-370 decreased, while mRNA levels of TGFβ1, TGFβRII, ColIa1 and ColIIIa1 and levels of TGFβ1, TGFβRII and α-SMA proteins were all increased. Luciferase reporter gene assay confirmed that TGFβRII was the target gene of miR-370. miR-370 reduced the expression of TGFβRII and inhibited the increased expression of TGFβRII and collagen protein caused by AngII. As well, its inhibited the differentiation effect of muscle fibroblasts while it did not inhibit the expression of TGFβ1. miR-370 inhibited the expression of TGFβRII protein by combining with TGFβRII mRNA. miR-370 also partially blocked TGFβ1-TGFβRII and induced the downstream signal transduction pathways, thus exerting anti-fibrotic effects.

Introduction

Cardiovascular disease induces the highest incidence, morbidity and mortality worldwide (1). The resulting cardiac remodeling is associated with the underlying pathological changes in most heart diseases (myocardial infarction, heart failure and atrial fibrillation), and myocardial fibrosis is the most important pathological feature of cardiac tissue remodeling (2). Cardiac fibroblasts (CFbs) account for 2/3 of heart cells, which plays an important role in the homeostasis of the cardiac extracellular matrix metabolism and remodeling of cardiac tissue (3). Studies on the biological activity of CFbs (proliferation, differentiation and migration) may provide the basis for exploring the mechanism of cardiac remodeling and developing new therapeutic strategies.

It has been widely confirmed that the transforming growth factor-β1 (TGFβ1) signal transduction pathway plays an important role in the process of cardiac fibrosis (3). TGFβ1 acts on downstream transcription factors and then regulates the expression of target genes and proteins through the binding on their receptors namely TGFβ receptor one and two (TGFβRI/TGFβRII) (4). It can promote the transformation of CFbs into its active myofibroblasts form, which enhances the migration, proliferation and collagen synthesis, and thus, leads to fibrosis (5).

However, microRNAs (miRNAs), a kind of small RNA of 18-24 bp length, can inhibit such a process. Indeed, mature miRNAs combine with 3' untranslated regions (3'-UTR) of the gene and inhibit the target gene transcription and/or degrade the target gene mRNAs, which affects the expression of the target proteins (6). Accordingly, an increasing number of studies have indicated that miRNAs play an important role in many kinds of heart diseases (7).

The present study mainly focused on the role of miR-370 in the myocardial remodeling after myocardial infarction

Materials and methods

Rat myocardial infarction models. Experiments were in compliance with the council of China on Animal Care and were approved by the Animal Ethics Committee of the Medical College of He Xi University. Sprague-Dawley (SD) male rats (180-250 g) were randomly divided into two groups including, the myocardial infarction group (n=3) and sham operation group (n=3). Rats were anesthetized with pentobarbital sodium (0.1%) and were assisted breathing with small animal ventilator. Their electrocardiograms were recorded with II leads and the third, fourth rib gap was open to expose the left atrial appendage. Anterior descending artery was ligated with 7-0 ligation line 2 mm at the lower edge of the right in the left

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atrial appendage. Two weeks later, rats were anesthetized, and the heart was quickly removed. After the residual blood was washed with normal saline, the samples were stored at -80˚C.

Hematoxylin and eosin (H&E) and Masson staining. Rats of both groups were anesthetized, and their heart was taken out. Saline was used to wash residual blood. Following 24 h of fixation with 4% poly-formaldehyde, tissues were embedded with paraffin, sliced and stained using H&E and Masson procedures.

Cell culture and treatment. The epicardium of heart of SD neonatal rats (1-3 days) was tore with pincett. The residual blood was washed with 1X PBS. The sample was cut with a pair of scissors (about 1 mm^3), and double enzymes were added (0.25% pancreatin + 0.1% collagenase B) to digest at 37˚C using shock for a total of 10 times, initially for 10 min, and then 6 min each time. The supernatant of each collection was terminated digestion with DMEM culture medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS, Gibco). After centrifugation of cell suspension at 800 x g for 10 min, the cells were cultured in 50 ml culture flask after resuspension with 10% FBS, and then stored at 37˚C in a 5% CO_2 incubator. CFb was obtained after differential for 75 min and discarding the supernatant. Cells were cultured with 10% FBS DMEM and 5% CO_2 at 37˚C. Cell passage occurred in 1-2 days, and cells between passages 2-3 were used for experiments. Serum-free DMEM was used to starve cells for 24 h.

Cell transfection. After washing with serum-free DMEM, the cells were cultured with 2 ml serum-free DMEM for 4-6 h (6-well plate). miR-370 mimics (5'-CAGGUCACG UCUCUGCAGUUACAC-3'), miR-370 antisense inhibitor (5'-GTCCAGTGCAGAGACGTCAATGTG-3'), NC (5'-CAC AUUGTGCUCUCUGCACUGCTC-3') (Guangzhou RioBio Co., Ltd., Guangzhou, China) and transfection reagent lipofectmine®2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) and 500 μl Opti-MEM (Gibco) were mixed, with a standing time of 5 min. Two kinds of mixture mixed and stood for 20 min, and then transfected cells. 6 h later, culture medium was replaced and corresponding stimulation was added.

Measurement of collagen protein. After treatment with the Sircol collagen assay kit (Biocolor), cell samples were stored at 4˚C for 24 h. After centrifugation, 100 μl supernatant was added with 1 ml Sircol dye, mixing, 4˚C, 30 min. After centrifugation, the supernatant was removed and 700 μl Sircol alkali reaction fluid was resuspended. Data were obtained using a spectrophotometer (540 nm; Hitachi, Tokyo, Japan).

Real-time fluorescence quantitative polymerase chain reaction (qRT-PCR). Total RNA in cardiac tissue and CFbs was extracted with TRIzol (Invitrogen Life Technologies). Levels of GAPDH, ColIa1, ColIIIa1, TGFβ1, TGFβRII and miR-370 were detected with qRT-PCR and SYBR-Green (Takara Bio, Dalian, China) (Table I).

Western blotting. Total protein in cardiac tissue and CFbs was extracted with protein lysis buffer (Beyotime Biotech, Jiangsu, China). Proteins were isolated using 60 μg protein system and 10% SDS polyacrylamide gel, and transferred to the polyvinylidene fluoride membrane. After three hours of blocking with 5% skim milk, TGFβ1, TGFβRII, a-SMA (a-smooth muscle actin, Gene Tex) and internal reference APDH (Anti Gene) primary antibody diluent was used to seal the membrane for 16 h. 1X TBST washing membrane for 15 min each time for a total of three times. The secondary antibody (Anti Gene) dilution was incubated for 2 h and then membranes were washed in 1X TBST for 15 min each time for a total of three times. Thereafter, ECL (Thermo Fisher Scientific, Waltham, MA, USA) luminescence was applied. The Bio-Rad Biology system was used to capture images and the results were analyzed.

Target gene prediction of miRNA. We used two databases, TargetScan6.2 (http://www.targetscan.org/ index.html) and PicTar (http://pictar.mdc-berlin.de), to predict the combination of miR-370 and TGFβII mRNA.

Dual luciferase reporter gene assay. 3'-UTR in TGFβRII was sub-cloned based on PCR, and the miRNA binding site in reporter gene vector was constructed. The construct was inserted into multiple cloning sites. After miRNA reporter vector (Ambion Life Technologies, Carlsbad, CA, USA) expressed by pMIR-REPORT™ luciferase (*Hin*dIII and *Spe*I sites) in the serum-free medium for 24 h, human embryonic kidney cell (HEK-293) ($1 \leq 10^5$/well) 1ug PGL3 target DNA (firefly luciferase vector) and 0.1 μg pRL-TK (Ranilla luciferase vector driven by TK) were transfected using liposome (Lipofectamine® 2000, Invitrogen Life Technologies). Forty-eight hours after transfection of dual luciferase, double luciferase activity was detected, strictly according to the method provided by Promega (8).

Statistical analysis. Statistical analysis was performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism (version 6.0; GraphPad Software, Inc, La Jolla, CA, USA). All data were expressed as mean ± standard error. Analysis of variance and Dunnett's test were conducted in comparisons among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-370 expression decreases after myocardial infarction, whereas the expression of TGFβ1 and TGFβRII increases. MASSON staining and qRT-PCR results showed that 2 weeks after myocardial infarction, the collagen deposition in the infarct border area was significantly increased in the myocardial infarction group when compared with the sham operation group (Fig. 1). The expression of TGFβ1 and TGFβRII was significantly increased and miR-370 expression decreased in the border area of the heart infarction. Western blotting results showed that the expression of α-SMA was also significantly increased in the border area, which suggested that CFbs in the infarct border area partially differentiated into myofibroblasts (Figs. 2 and 3).

AngII promotes the differentiation of CFbs and collagen secretion and inhibits the expression of miR-370. When SD rat CFbs were passaged for the third generation and cells were fused

Table I. Primer sequence of miR-370 and mRNAs of TGFβ1, TGFβRII, ColIa1, ColIIIa1 and GAPDH.[a]

Genes	Primers	Sequence
mir-370	RT primers	5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGTAA-3'
	Sense	5'-AGACCAGGTCACGTCTCTG-3'
	Antisense	5'-GACAGACAAACCAGGTTCCA-3'
U6	RT primers	5'-CGCTTCACGAATTTGCGTGTCAT-3'
	Sense	5'-GCTTCGGCAGCACATATACTAAAAT-3'
	Antisense	5'-CGCTTCACGAATTTGCGTGTCAT-3'
TGFβ1	Sense	5'-GCGCCTGCAGAGATTCAAGTCAAC-3'
	Antisense	5'-GTATCAGTGGGGGTCAGCAGCC-3'
TGFβRII	Sense	5'-TCACTAGGCACGTCATCAGC-3'
	Antisense	5'-AGGACAACCCGAAGTCACAC-3'
ColIa1	Sense	5'-TTCACCTACAGCACGCTTGT-3'
	Antisense	5'-TTGGGATGGAGGGAGTTTAC-3'
ColIIIa1	Sense	5'-TTGAATATCAAACCGCAAGGC-3'
	Antisense	5'-GGTCACTTTCACTGGTTGACGA-3'
GAPDH	Sense	5'-AGACAGCCGCATCTTCTTGT-3'
	Antisense	5'-TGATGGCAACAATGTCCACT-3'

[a]U6 was a reference to detect the expression of miR-370, and GAPDH was a reference to detect the expression of mRNAs of TGFβ1, TGFβRII, ColIa1 and ColIIIa1. TGFβ1, transforming growth factor-β1.

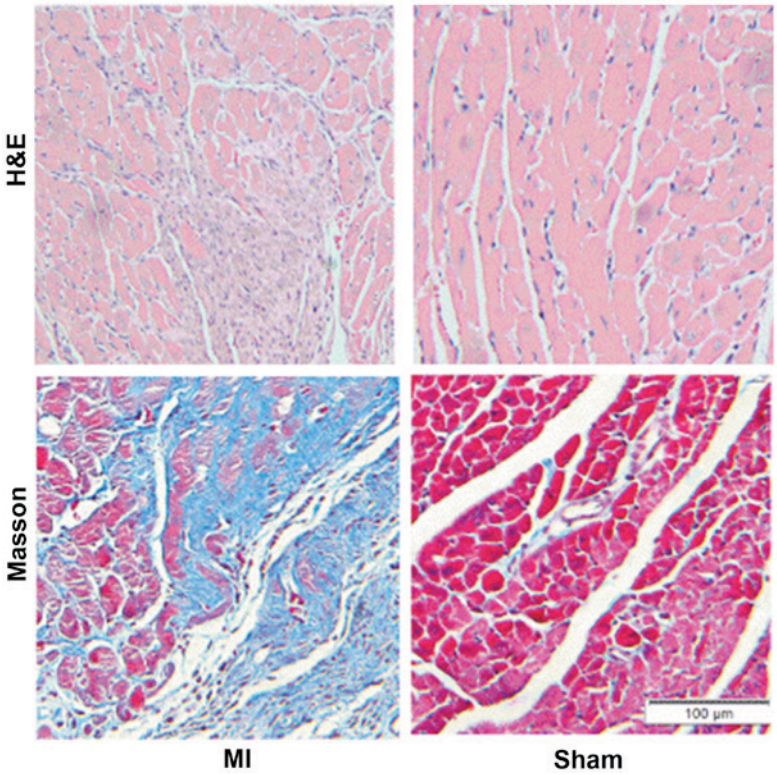

Figure 1. Myocardial infarction model in rats was established, and cardiac tissue was sliced and stained with Masson and H&E. H&E and Masson staining showed that 2 weeks after myocardial infarction, the collagen deposition in the infarct border area was significantly increased (x200 magnification). N=3 in each group, and all data were expressed as mean ± SEM. Sham, sham operation group; MI, myocardial infarction group, H&E, hematoxylin and eosin, SEM, standard error of the mean.

to 85%, the cells were synchronized with serum-free DMEM for 24 h and stimulated with AngII for 24 h, and then the indicators were detected. It showed that TGFβ1, and TGFβRII mRNA and protein levels were increased in fibroblasts under

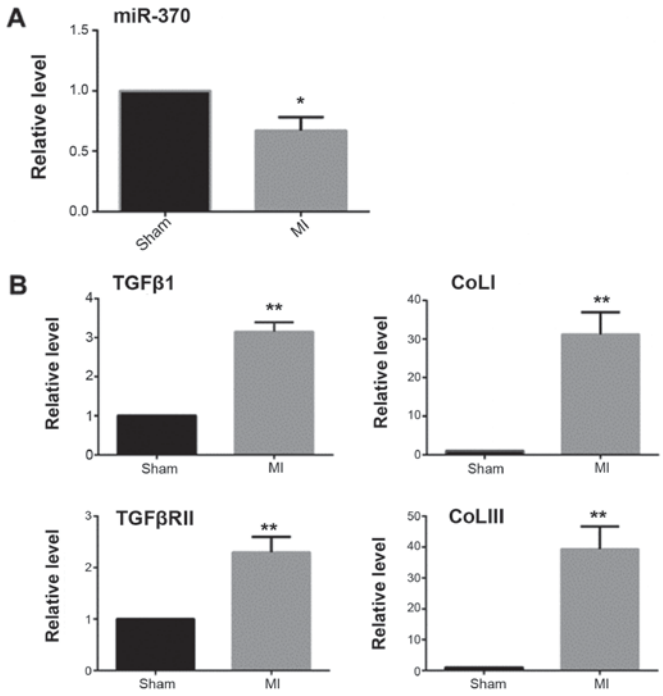

Figure 2. qRT-PCR results. (A) miR-370 expression decreased in the border area when compared with the sham operation group (*P<0.05 vs. sham group). (B) The expression of ColIa1, ColIIIa1, TGFβ1, and TGFβRII mRNAs were significantly increased when compared with the sham operation group (**P<0.01 vs. sham group). N=3 in each group, and data were expressed as mean ± SEM. Sham, sham operation group; MI, myocardial infarction group; PCR, polymerase chain reaction; SEM, standard error of the mean; TGFβ1, transforming growth factor-β1.

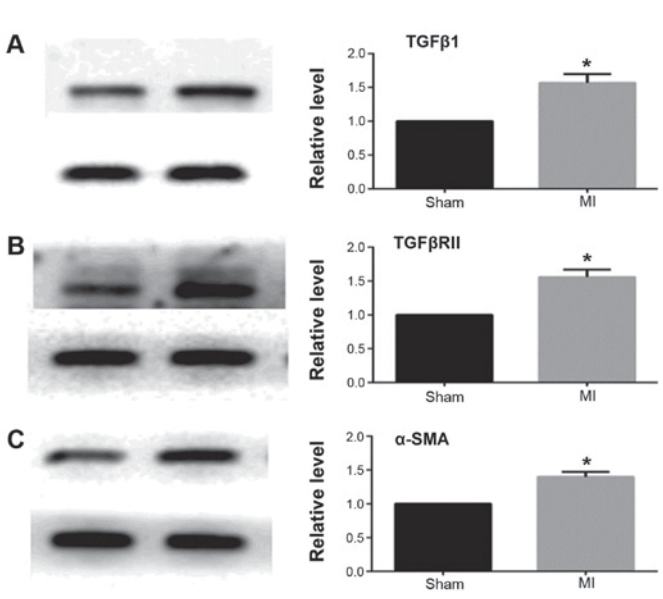

Figure 3. Western blotting results. (A) The expression of TGFβ1, (B) TGFβRII and (C) α-SMA was significantly increased in the border area when compared with the sham operation group. N=3 in each group; all data were expressed as mean ± SEM; Sham (sham operation group), MI (myocardial infarction group). *P<0.05 vs. sham group. SEM, standard error of the mean; TGFβ1, transforming growth factor-β1; a-SMA, a-smooth muscle actin.

the induction of AngII. The increase of α-SMA promoted the transformation of fibroblasts into myofibroblasts and increased the expression of collagen. The results also showed that AngII significantly inhibited the expression of CFb miR-370, which

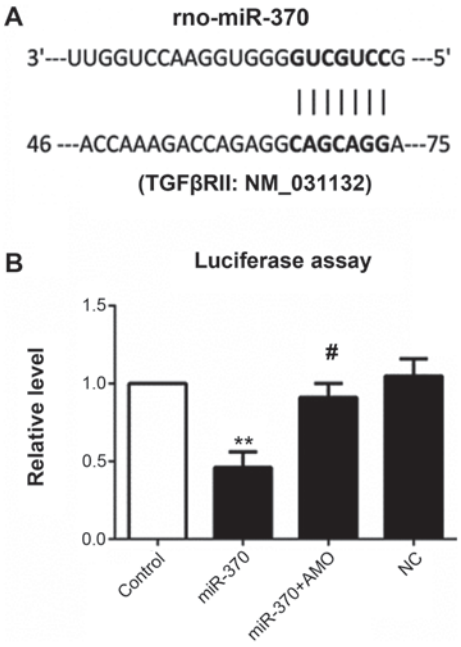

Figure 4. Validation of TGFβRII as a target gene of miR-370. (A) Predicted binding site of 3'UTR in TGFβRII mRNA and miR-370. (B) Luciferase construct transfected with miR-370 and 3'UTR in TGFβRII significantly reduced the activity of luciferase. The co-transfection of miR-370 and AMO-370 reduced the activity of luciferase. While the luciferase activity had no change in NC transfection group. All data were expressed as mean ± SEM. AMO-370, AMO miR-370 inhibitor; miR-370, miR-370 mimics; NC, negative control. **P<0.01 vs. control; #P<0.05 vs. miR-370. SEM, standard error of the mean.

indicated that the decrease of miR-370 may be related to the increase of TGFβRII.

miR-370 inhibits TGFBRII expression and inhibits myocardial fibrosis induced by AngII. Dual luciferase reporter gene assay showed that luciferase activity was significantly decreased in the plasmid group co-transfected with miR-370 mimics and contained the 3'UTR gene sequence of TGFβRII when compared with the control group. miR-370 mimics and inhibitor co-transfection significantly inhibited decreased luciferase activity induced by miR-370 mimics, while there were no effects in the NC transfection group (Fig. 4).

qRT-PCR results showed that the levels of TGFβRII, ColI1a and ColIII1a mRNAs in the miR-370 mimics + AngII group were significantly decreased compared with the AngII group. There were no significant differences in the levels of the above indexes in the miR-370 inhibitor + AngII, NC + AngII and AngII groups. Level of TGFβ1 mRNA did not differ in the above four groups. Western blot analysis revealed that, compared with the other three groups, TGFβRII and α-SMA protein levels and collagen secretion decreased in the miR-370 mimics + AngII group, however, miR-370 mimics did not inhibit the increase in TGFβ1 induced by AngII (Figs. 5 and 6).

Discussion

The present study showed that the expression of miR-370 in the myocardium was decreased after myocardial infarction. It can inhibit the myocardial remodeling induced by AngII after myocardial infarction. The underlying mechanism is

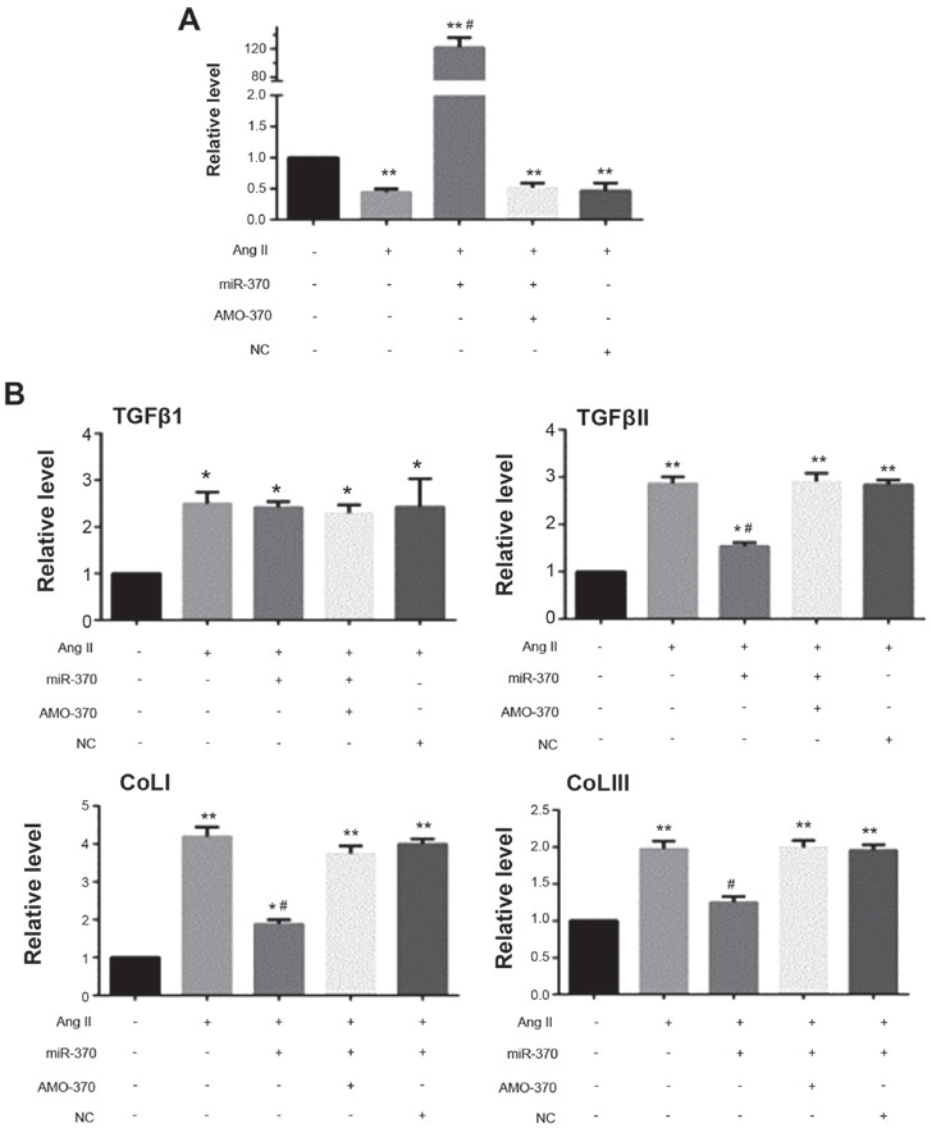

Figure 5. qRT-PCR results. (A) Detection of AngII (100 nM); transfection of miR mimics (50 nM) + AngII; transfection of miR inhibitor (100 nM) + AngII/transfection of miR NC (50 nM) + AngII stimulating CFbs for 24 h, the level of miR-370 was detected. (B) Detection of AngII (50 nM); transfection of miR mimics (50 nM) + AngII; transfection of miR inhibitor (100 nM) + AngII/transfection of miR NC (50 nM) + AngII stimulating CFbs for 24 h, levels of TGFβ1, TGFβRII, ColIa1, ColIIIa1, GAPDH mRNAs were detected (*P<0.05 vs. Sham group; **P<0.01 vs. sham group; $^{\#}$P<0.05 vs. AngII group). N=3 in each group; The data are expressed as mean ± SEM. AMO-370 AMO, miR-370 inhibitor; miR-370, miR-370 mimics; NC, negative control; CFbs, cardiac fibroblasts.

that miR-370 reduces the expression of TGFβRII, inhibits the TGFβ1-SMAD signal transduction pathway so as to exert the effects of inhibiting myocardial fibrosis.

It has been confirmed that the expression of miR-370 in peripheral blood leukocytes was higher in patients with acute coronary syndrome than that in normal patients (9). But the expression of miR-370 in the myocardium after myocardial infarction and the corresponding mechanisms have not been studied. The present study showed that in myocardial remodeling, the expression of miR-370 in the infarct border area was decreased after myocardial infarction. Fibroblasts differentiated into myofibroblasts and collagen secretion was increased. AngII promoted the expression of TGFβ1 and TGFβRII in CFbs and inhibited the expression of miR-370 at the same time. miR-370 can be combined with the 3-UTR in TGFβRII mRNA and inhibit the expression of TGFβRII, which result in the inhibition of the biological effects of AngII on CFbs, including differentiation of CFbs and the increase of collagen expression. However, miR-370 did not block the increase in TGFβ1 induced by AngII. The results suggested that the biological effects of miR-370 on AngII may be mediated by inhibiting TGFβRII expression and blocking downstream signal transduction pathways, but not inhibiting the generation of TGFβ1.

Myocardial remodeling in myocardial infarction is divided into two stages. The first stage is the physiological repair after infarction, which belongs to the protective repair. The second stage is the pathological remodeling caused by the continuous accumulation of extracellular matrix (9). In pathological remodeling after myocardial infarction, CFbs transform into α-SMA positive myofibroblasts under the continuous action of multiple biological effector molecules, such as AngII, TNF-α and TGF-β. The ability of myofibroblasts to migrate, proliferate and secrete collagen is obviously stronger. Hence, the transformation into myofibroblasts is the key link of pathological remodeling after infarction (10).

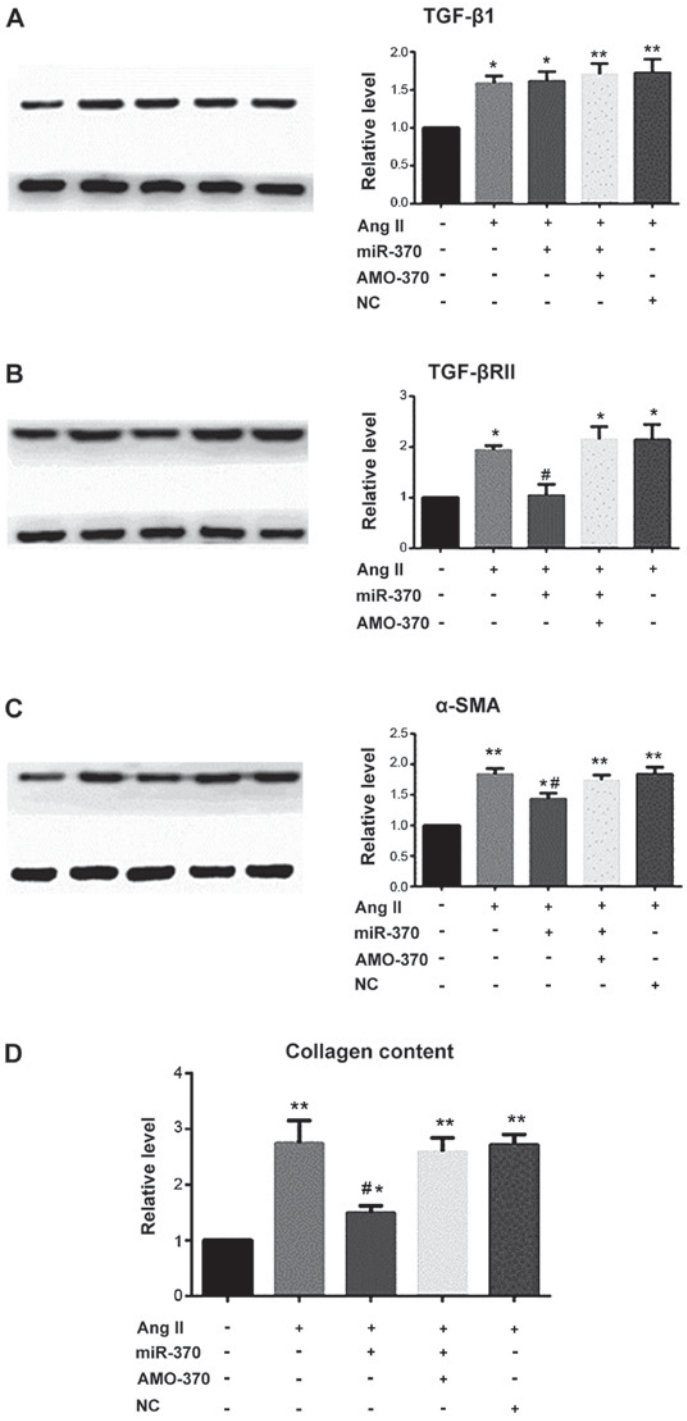

Figure 6. Western blotting results and detection of collagen content. Detection of AngII (100 nM); transfection of miR mimics (50 nM) + AngII; transfection of miR inhibitor (100 nM) + AngII/transfection of miR NC (50 nM) + AngII stimulating CFbs for 24 h, (A) levels of TGFβ1, (B) TGFβRII and (C) a-SMA were detected. (D) Detection of AngII (50 nM); transfection of miR mimics (50 nM) + AngII; transfection of miR inhibitor (100 nM) + AngII/transfection of miR NC (50 nM) + AngII stimulating CFbs for 24 h, collagen content was detected (*P<0.05 vs. sham group; **P<0.01 vs. sham group; $^{\#}$P<0.05 vs. AngII group). N=3 in each group; all data were expressed as mean ± SEM; AMO-370, AMO miR-370 inhibitor; miR-370, miR-370 mimics; NC, negative control. Polymerase chain reaction, PCR; SEM, standard error of the mean; TGFβ1, transforming growth factor-β1.

TGFβ1-SMAD signal transduction pathway plays an important role in the process of cardiac remodeling. Previous studies have indicated that many induced fibrosis factors, such as AngII and ET1, take effect by promoting the expression of TGFβ1 (11). Indeed, TGFβ1 is the key factor of fibrogenic effects. TGFβ1 has an effect on TGFβ receptor complex on the cell surface and leads to the phosphorylation of downstream transcription factor SMAD2/3, which is further combined with SMAD4 to form the complex. The complex enters into the cell and combines with cellular DNA, or it can form a special transcription factor to regulate the target genes and target proteins (4). Previous findings have shown that nicotine can lead to atrial fibrosis in dogs by increasing the expression of TGFβRII receptor in the atrial fibroblasts, enhancing the downstream signal transduction and increasing the expression of collagen (12).

An increasing number of microRNAs including Let-7, mir-133 and -30c have been shown to exert an inhibitory effect on organ fibrosis by blocking the TGFβ1-TGFβRI/TGFβRII-SMAD signal transduction pathway (13,14). Of these, Let-7 has been shown to inhibit the expression of TGFβRI and thus, play a protective role in renal fibrosis (15). miR-24 was demonstrated to exert anti-fibrotic effects by inhibiting the expression of TGFβ1 in CFbs (15). miR-21, a small RNA that has been widely confirmed to have fibrogenic effects, has also been shown that it leads to fibrosis by inhibiting the expression of TGFβRIII, seen that TGFβRIII can inhibit TGFβRI/II, thereby promoting TGFβRI/II-induced downstream signal transduction (16,17). A recent study has showed that nicotine in tobacco reduced mir-590 in dog atrial fibroblasts, increased the expression of TGFβRII and then enhanced the secretion of collagen and proliferation (12). This suggests that it is feasible to affect the expression of TGFβR and to inhibit myocardial fibrosis through the intervention of miRNAs levels.

In total, our results suggest that miR-370 exerts anti-fibrotic effects by decreasing the expression level of TGFβRII and inhibiting TGFβ1-TGFβRI/II-SMAD signal transduction pathway. However, a large number of studies are needed to validate the specific mechanisms of miRNAs in myocardial remodeling after myocardial infarction and the application of miRNAs in the treatment of cardiovascular diseases.

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