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# The Role of MicroRNA-181a in Myocardial Fibrosis Following Myocardial Infarction in a Rat Model

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Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
Literature Search F  
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**Background:** The role of miR-181a in the development of cardiac disease and in particular, myocardial fibrosis following myocardial infarction (MI) remains unknown. The aim of this study was to explore the role of miR-181a in myocardial fibrosis in a rat model of MI and the expression of TGF- $\beta$  receptor III (T $\beta$ RIII).

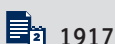
**Material/Methods:** Forty adult male Wistar rats were randomly divided into an MI model group (n=30) and a control group with (n=10). The rat MI model involved ligating the left anterior descending (LAD) coronary artery in the model group; the control group was treated with a sham operation. Cardiac function was assessed using cardiac ultrasound. Myocardial fibroblasts were extracted from the rat hearts and transfected with a miR-mimic or miR-inhibitor, and cell growth was measured using an MTT assay. The level of miR-181a expression was detected using quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blots.

**Results:** miR-181a expression was significantly increased during the progression of MI (P<0.05). Over-expression of miR-181a was associated with increased deposition of extracellular matrix (ECM) components, collagen I and fibronectin. This effect was reversed with the use of a miR-181a inhibitor (P<0.05). Upregulation of miR-181a suppressed the expression of TGF- $\beta$  receptor III (T $\beta$ RIII) by binding with 3'-UTR.

**Conclusions:** In this rat model of MI, the findings were that miR-181a had a role in the progression of myocardial fibrosis. The findings require further studies to determine whether miR-181a might provide a novel therapeutic target to limit myocardial fibrosis following MI.

**MeSH Keywords:** **Endomyocardial Fibrosis • MicroRNAs • Myocardial Infarction**

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## Background

Myocardial infarction (MI) from coronary artery disease results in more than seven million deaths worldwide each year [1]. Major risk factors for MI are hyperlipidemia, diabetes, hypertension, smoking, and age [2]. During MI, the necrotic cardiac myocytes are replaced with scar tissues formed by fibroblasts, and the resulting impaired cardiac function ultimately leads to progressive heart failure [3]. Previous studies have focused on the molecular mechanism of myocardial cells apoptosis and necrosis. However, there are few reports examining the progression of myocardial fibrosis.

MicroRNAs (miRNAs) are a class of non-coding RNAs approximately, 22 nucleotides in length, which play a role in regulating gene expression by binding to 3'-untranslated region (3'-UTR) of target mRNAs [4,5]. There is increasing evidence from published studies that support the importance of miRNAs as regulators in the development of cardiovascular disease. MicroRNA-181a (miR-181a) is known to play an important role in the development of many cancers [6–9]. Previously published studies have shown that miR-181a impedes the terminal differentiation of myoblasts by negatively regulating the homeobox protein Hox-A11 [10]. Yi-Gang Li et al. found that knockdown of microRNA-181a could decrease the arrhythmogenic effect of skeletal myoblast transplantation in a rat model of MI [11].

Transforming growth factor (TGF)- $\beta$  receptor III (T $\beta$ RIII) is a proteoglycan composed of 851 amino acids and is involved in cellular metabolism [12]. As a TGF- $\beta$  superfamily co-receptor, the role of T $\beta$ RIII in regulating TGF- $\beta$  signaling is context-dependent and complex [13,14]. Fei Sun et al. have demonstrated that T $\beta$ RIII was involved in the reduction of myocardial fibrosis following MI, and in the reduction of collagen deposition, and fibroblast activity, that was induced by simvastatin [15].

The aim of this study was to explore the role of miR-181a in myocardial fibrosis in a rat model of MI, including the role of the expression of T $\beta$ RIII.

## Material and Methods

### Experimental animals, and the rat model of myocardial infarction (MI)

Forty male Wistar rats were purchased from the Laboratory Animal Center of Soochow University. The rats had an average age of 2–3 months and weighed between 180–220 gm. The rats in the study were randomly divided into a myocardial infarction (MI) model group (n=30) and a control group (n=10).

The MI model was established by ligating the left anterior descending (LAD) coronary artery in the MI model group; the

control group was treated with a sham operation, as previously described [16]. Because the surgical procedure resulted in some loss of rats, the remaining rats in the MI model were randomly divided into a one-week post-MI group A (n=8), a two-week post-MI group B (n=10), and a four-week post-MI group C (n=10). All animal experiments were performed according to institutional, local and national guidelines on animal research and ethics.

### Examination of cardiac function

After the induction of MI, the cardiac function indices of each group was measured using ultrasound examination, as follows: left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), left ventricular end systolic diameter (LVESD), and left ventricular end diastolic diameter (LVEDD).

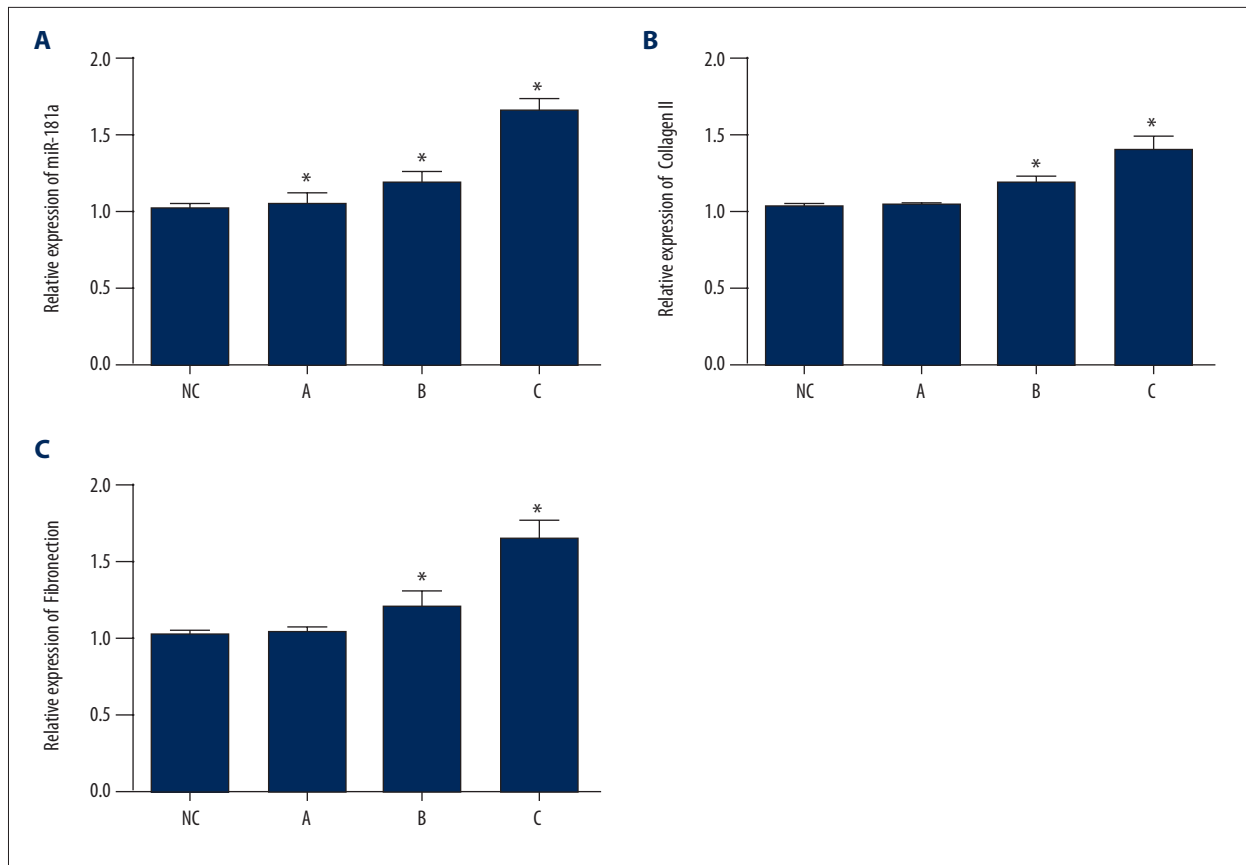
### Cell extraction, culture, and transfection

At the end of the study, the rats were euthanized, and the hearts were removed, placed in culture dishes under aseptic conditions, and rinsed in Hanks' balanced salt solution (HBSS). The heart was cut into small pieces and washed using water containing five times the volume of 0.05% trypsin at 37°C in 15 ml centrifuge tube for 5 minutes. After mixing, the supernatant was discarded. The precipitated component was repeatedly mixed for approximately 10 minutes with approximately five times the volume of 0.08% trypsin and 0.1% collagenase II. After centrifugation, the precipitation was resuspended in 10 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum which was changed 60–90 minutes later and then inoculated into a culture dish. For transfection, myocardial fibroblasts were seeded into plates and transfected with miRNAs or siRNAs (Gene Pharm, Shanghai, China) mixed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, MA, USA), according to the manufacturer's protocols.

### Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells using Trizol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using a reverse transcription kit (Takara Biotechnology, Dalian, China). The relative quantity of cDNA was analyzed by quantitative PCR with SYBR green and the  $\Delta\Delta$ CT method.  $\beta$ -actin was used as the internal control gene to normalize the target genes. All primers were designed and synthesized by GenePharma (Shanghai, China) and shown as follows:

mir-181a mimic, ACCAUCGACCGUUGAUUGUACC;  
miR-181a inhibitor, ACCAUCGACCGUUGAUUGUACC;  
negative controls (NC), UUCUCCGAACGUGUCACGUTT;  
NC inhibitor, CAGUACUUUUGUGUAGUACAA;



**Figure 1.** Expression of miR-181a and the fibrotic index in a rat model of myocardial infarction (MI model group and control group). (A) The expression of miR-181a mRNA is significantly upregulated in the MI group compared with the control group. (B, C) The change in fibrotic index showed a significant increase in the expression of collagen I and fibronectin mRNA. Data are shown as the mean  $\pm$ SD. \* P 0.05 (Student's t-test).

Collagen I, CGAGTATGGAAGCGAAGGT, and CCACAAGCGTGTCTAGGT;  
Fibronectin, CACGGAGGCCACCATTACT, and CTTCAGGGCAATGACGAGAT;  
T $\beta$ RIII, CGTCAGGAGGCACACACTTA, and CACATTTGACAGACAGGGCAAT;  
 $\beta$ -actin, CACGATGGAGGGCCGACTCATC, and TAAAGACCTCTATGCCAACACAGT.

Relative levels of gene expression were expressed relative to  $\beta$ -actin and calculated using the  $2^{-\Delta\Delta Ct}$  method.

#### Cell proliferation assay

An MTT assay was used to assess cell proliferation status. Cells ( $3 \times 10^3$ ) were cultured in 96-well plates and incubated for 24 hours and stained with 0.5 mg/ml MTT for 4 hours. The supernatant was discarded, and 200  $\mu$ l of dimethylsulfoxide (DMSO) was added to dissolve the precipitates. Samples were measured at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

#### Luciferase activity assay

The 3'untranslated region (3'-UTR) of TGF- $\beta$  receptor III (T $\beta$ RIII) containing miR-181a binding sites was amplified and cloned into the psiCHECK-2 luciferase vector (Promega, USA). Similarly, the mutant 3'-UTR of T $\beta$ RIII was cloned into the same vector. Myocardial fibroblasts maintained in 96-well plates were co-transfected with a pGL3-control luciferase reporter, pRL-TK vector, and miR-181a mimic, or miR-Control vector. Transfected cells were detected using the Dual-Luciferase Reporter Assay System (Promega) 48 hours later.

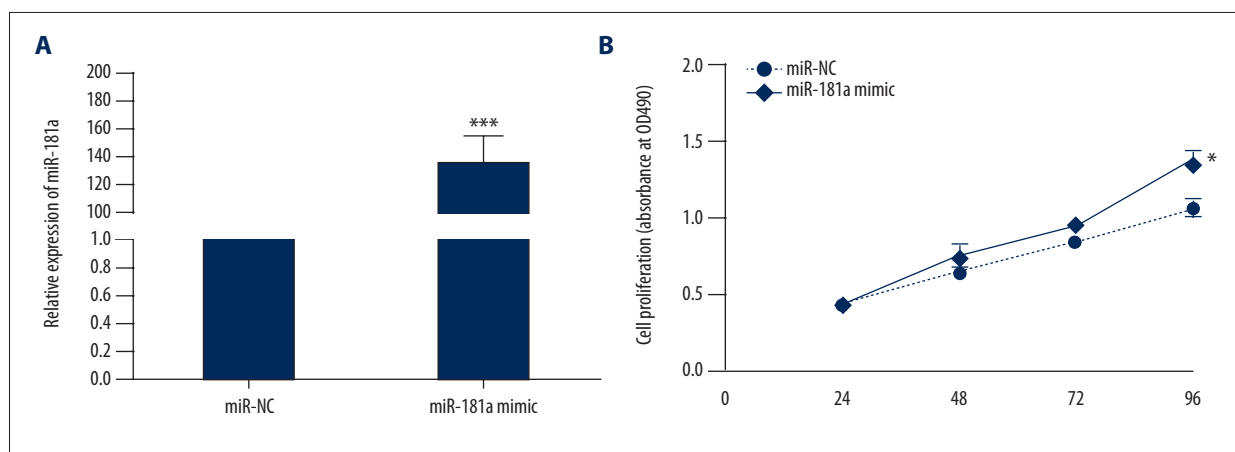
#### Western blot analysis

The cultured cells were washed with ice-cold PBS and lysed in RIPA buffer supplemented with protease inhibitor mixture. After electrophoresis, the protein samples were incubated with the primary antibodies as follows: anti-T $\beta$ RIII, anti-Collagen I, and anti-fibronectin (1: 1000 dilution). Samples were incubated with secondary antibodies conjugated with horseradish peroxidase (HRP). Bands were quantified using ImageJ software.

**Table 1.** Cardiac indexes of MI.

Group	LVESD (mm)	LVEDD (mm)	LVEF (%)	LVFS (%)
NC	2.46±0.30	4.81±0.11	75.38±4.57	48.70±6.45
A	3.26±0.16 <sup>#**</sup>	5.29±0.17 <sup>#**</sup>	58.89±2.13 <sup>#**</sup>	38.50±1.83 <sup>#**</sup>
B	3.69±0.12 <sup>#</sup>	6.51±0.21 <sup>#</sup>	47.58±2.28 <sup>#</sup>	43.29±2.82 <sup>#</sup>
C	4.59±0.21 <sup>#*</sup>	7.51±0.23 <sup>#</sup>	37.88±2.05 <sup>#</sup>	38.89±2.57 <sup>#</sup>

Compared with NC group <sup>#</sup>  $P < 0.05$ ; compared with the B group <sup>\*</sup>  $P < 0.05$ . Values are Mean  $\pm$  SD. LVESD – left ventricular end systolic dimension; LVEDD – left ventricular end diastolic dimension; LVEF – left ventricular ejection fraction; LVFS – fractional shortening. A – MI after one week; B – MI after two weeks; C – MI after four weeks.



**Figure 2.** Effect of miR-181a on cell proliferation detected by the MMT assay. (A) The expression of miR-181a in myocardial fibroblasts treated with miR-Control or the miR-181a (mimic) was detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR). (B) The MMT assay for the proliferation of myocardial fibroblasts transfected, as in A. Data are shown as the mean  $\pm$  SD. \*  $P < 0.05$  and \*\*\*  $P < 0.001$ . (Student's t-test).

### Statistical analysis

Results were expressed as the mean  $\pm$  standard error. The Student's t-test and ANOVA were performed between different groups. All calculations were performed using SPSS version 17.0 software (IBM Software, Chicago, IL, USA) and GraphPad (version 6.0, USA). A value of  $P < 0.05$  indicated a statistically significant difference.

## Results

### Expression of miR-181a and fibrotic index in the rat model of myocardial infarction (MI) of each group

To investigate the effect of miR-181a on the development of myocardial infarction (MI) in a rat model, the expression of miR-181a in cardiac tissues was analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Figure 1A shows that the level of miR-181a mRNA expression was significantly elevated in MI, and that that miR-181a increased

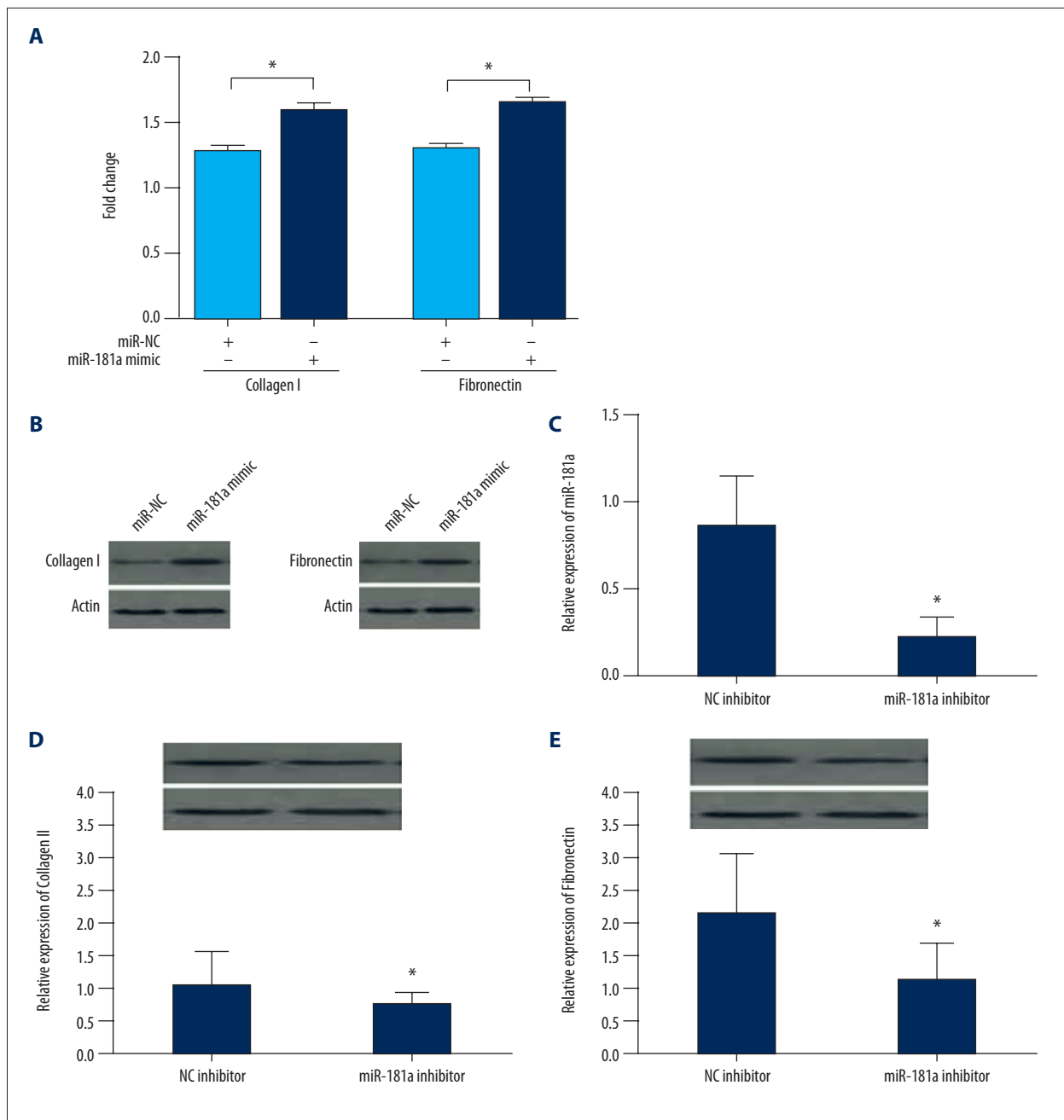
over time during the progression of MI. Also, there was a significant increase in the expression of Collagen I and fibronectin mRNA (Figure 1B, 1C).

### Cardiac index of MI in rats of each group

Cardiac function was examined with echocardiography after induction of MI (Table 1). The left ventricular end systolic diameter (LVESD) and left ventricular end diastolic diameter (LVEDD) levels increased significantly over time ( $P < 0.05$ ) and were significantly increased in the MI group compared with the control group ( $P < 0.05$ ). The left ventricular ejection fraction (LVEF) and the left ventricular fractional shortening (LVFS) were significantly reduced over time ( $P < 0.05$ ) and were significantly reduced in the MI group compared with the control group.

### miR-181a promoted the proliferation of myocardial fibroblasts *in vitro*

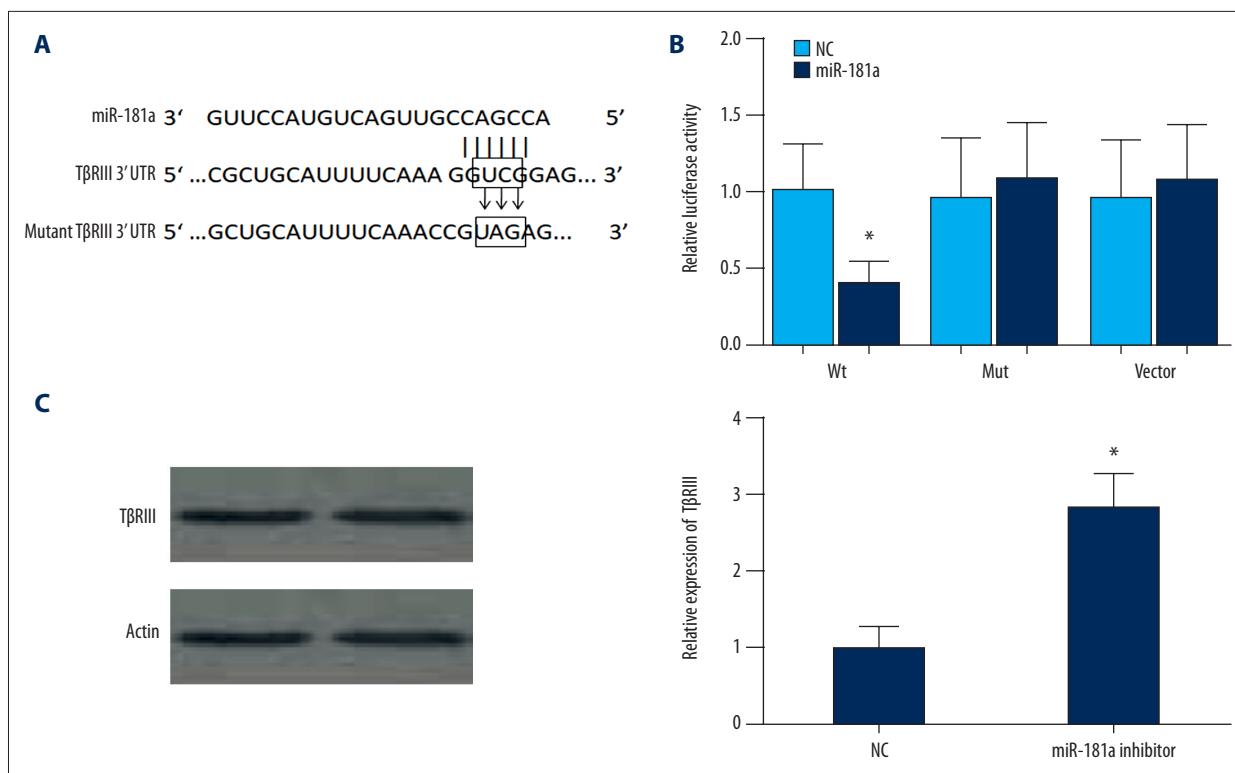
To explore the role of miR-181a in the progression of MI, miR-181a overexpression in myocardial fibroblasts was generated



**Figure 3.** Expression of collagen I and fibronectin, the fibrosis index, in myocardial fibroblasts. **(A)** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) detects the mRNA levels of collagen I and fibronectin in cells treated with miR-Control or the miR-181a (mimic). **(B)** Western blot measurements of the expression of collagen I and fibronectin in cells transfected, as in **A**. **(C)** The expression of miR-181a in cells treated with control-inhibitor and miR-181a inhibitor detected by qRT-PCR. **(D, E)** Western blots and qRT-PCR for the expression of collagen I and fibronectin in cells treated, as in **C**. Data are shown as the mean  $\pm$ SD. \*  $P < 0.05$  and \*\*\*  $P < 0.001$ . (Student's t-test).

via retrovirus infection. The results of qRT-PCR showed that miR-181a was significantly upregulated in myocardial fibroblasts transfected with a miR-181a mimic when compared with miR-Control (Figure 2A). To explore the role of miR-181a in cell proliferation, an MMT assay was performed to assess

the viability of cardiac fibroblasts in MI. The results showed that the growth rate of myocardial fibroblasts transfected with the miR-181a mimic was significantly increased (Figure 2B).



**Figure 4.** Upregulation of miR-181a suppresses the expression of TGF- $\beta$  receptor III ( $T\beta RIII$ ) by binding with 3'-UTR. Using bioinformatics analysis, 3' UTR of TGF- $\beta$  receptor III ( $T\beta RIII$ ) is shown to be highly conserved and to bind to miR-181a. The 3'-UTR binding sites are shown as follows: **(A)** Luciferase reporter assay shows that transfection of miR-181a significantly restricts the relative luciferase activity in myocardial fibroblasts. **(B)** There appear to be few differences in luciferase activities between the control group and normal cells containing miR-21 in the control group and PTEN mutation 3'-UTR group (\*  $P < 0.5$ ). **(C)** After cells were transfected with miR-181a inhibitor, TGF- $\beta$  receptor III ( $T\beta RIII$ ) expression levels are significantly upregulated. These findings show that miR-181a suppresses the expression of  $T\beta RIII$  by binding with the 3'-UTR.

### miR-181a promoted extracellular matrix formation *in vitro*

The expression of fibronectin and collagen I was detected using qRT-PCR and Western blot to assess the accumulation of extracellular matrix. The results showed that the over-expression of miR-181a increased the levels of fibronectin and collagen I (Figure 3A). Furthermore, the production of fibronectin and Collagen I was significantly increased in myocardial fibroblasts (Figure 3B). This finding indicated that over-expression of miR-181a increased fibrosis (collagen formation) in myocardial fibroblasts. A small interfering RNA (si-RNA) against miR-181a was successfully designed and assessed using qRT-PCR (Figure 3C). The results of qRT-PCR confirmed the expression of collagen I and fibronectin were significantly reduced in cardiac fibroblasts (Figure 3D, 3E), indicating that miR-181a regulated myocardial fibrosis *in vitro*.

### miR-181a targets with 3'-UTR of $T\beta RIII$

Bioinformatics analysis was used to screen the potential targeting miRNAs binding with miR-181a. The results showed that the 3'-UTR of  $T\beta RIII$  was highly conserved and bound with

miR-181a. The 3'-UTR binding sites are shown in Figure 4A. The luciferase reporter assay showed transfection of miR-181a could significantly restrict the relative luciferase activity in myocardial fibroblasts (Figure 4B) ( $P < 0.5$ ), indicating a direct interaction between miR-181a and  $T\beta RIII$ . Furthermore, after cells were transfected with miR-181a inhibitor,  $T\beta RIII$  expression levels were significantly upregulated (Figure 4C). Overall, these results showed that miR-181a suppressed the expression of  $T\beta RIII$  by binding with the 3'-UTR.

## Discussion

MicroRNAs (miRNAs) are single-stranded, non-coding RNAs, first identified in the nematode, *Caenorhabditis elegans* in 1993 [17,18]. There is growing evidence that miRNAs play an important role in myocardial cell death and regeneration [19]. miR-181a participates in several gene regulatory processes such as development, differentiation, hematopoiesis, and immune modulation [20,21]. Previous studies have shown miR-181a has varied effects in different types of tumors [22,23]. In cardiac disease, Jianbing Zhu



et al. reported that circulating miR-181a levels in patients with myocardial infarction (MI) were significantly changed in a time-dependent manner, indicating the potential value of plasma miR-181a as a novel biomarker in MI [24]. However, the molecular mechanism of miR-181a in MI remains poorly understood.

This current study focused on the role of miR-181a in myocardial fibrosis following MI in a rat model that used coronary artery ligation. The results of this study showed a significant increase in the expression of miR-181a in rats following MI that was time-dependent. Also, the fibrosis indices measured in this study (fibronectin, Collagen I) increased following MI. An MMT assay showed that over-expression of miR-181a accelerated the proliferation of myocardial fibroblasts, and that upregulation of miR-181a promoted the expression of mRNA and the production of the related protein of these indices of fibrosis. These findings were reversed by using knockdown of miR-181a. The data support the role of miR-181a in the promotion of cardiac fibrosis following MI., in this rat model

TGF- $\beta$  receptor III (T $\beta$ RIII) is a transmembrane proteoglycan, which acts as a regulator in the TGF- $\beta$  signaling pathway. In

cardiac disease, recent studies have shown that T $\beta$ RIII plays an important role in reducing myocardial fibrosis by reducing collagen production [16,25]. In this study, the use of bioinformatics analysis showed that miR-181a closely targeted 3'-UTR of T $\beta$ RIII. These results support that over-expression of miR-181a could lower T $\beta$ RIII mRNA expression and related protein production, and also support the view that miR-181a may induce myocardial fibrosis through the modulation of T $\beta$ RIII.

## Conclusions

This study investigated the role of miR-181a in the pathogenesis of myocardial infarction (MI) and the development of myocardial fibrosis in a rat model. The findings require further study to determine whether miR-181a might provide a novel therapeutic target to limit myocardial fibrosis following MI.

## Conflict of interest

All authors declare no conflicts of interest.

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