European Review for Medical and Pharmacological Sciences 2020; 24: 2070-2076

MiRNA-1297 inhibits myocardial fibrosis by targeting ULK1

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Abstract. – OBJECTIVE: The aim of this study was to explore the potential effect of miRNA-1297 on myocardial fibrosis (MF) and its underlying mechanism.

MATERIALS AND METHODS: MF model was established by cardiac perfusion of Angiotensin II (Ang-II) in mice. The primary myocardial fibroblasts were extracted from MF mice (Ang-II infusion group) and controls (sham group), respectively. The relative levels of miRNA-1297 and ULK1 in the *in vivo* and *in vitro* MF models were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Meanwhile, the protein expressions of fibrosis-related genes in MF mice and primary myocardial fibroblasts were determined by Western Blot. Subsequently, the Dual-Luciferase Reporter Gene Assay was applied to verify the downstream gene of miRNA-1297. In addition, a series of rescue experiments were conducted to elucidate the role of miRNA-1297/ULK1 in regulating MF.

RESULTS: Masson staining showed plenty of micro-vessels around myocardial tissues and significantly increased contents of intercellular collagen in Ang-II infusion group when compared with those in the sham group. Western blot results revealed that the protein expressions of Col1a1 and α-SMA were significantly upregulated in myocardial tissues of MF mice. QRT-PCR data illustrated that miRNA-1297 was remarkably downregulated in MF model. ULK1 was verified as the target gene of miRNA-1297, which was upregulated in the MF model. The overexpression of miRNA-1297 or the knockdown of ULK1 could downregulate the protein levels of Col1a1 and α-SMA in primary myocardial fibroblasts extracted from MF mice. Notably, ULK1 overexpression could reverse the regulatory effect of miRNA-1297 on MF.

CONCLUSIONS: MiRNA-1297 suppresses myocardial fibrosis *via* down-regulating ULK1.

Key Words:
Myocardial fibrosis, MiRNA-1297, ULK1.

Introduction

Currently, the incidence of cardiovascular diseases is extremely high in China. Therefore, the prevention and treatment of cardiovascular diseases are of great significance in clinical practice. Heart failure (HF), resulted from the impaired diastolic and systolic function of the heart, is the end-stage of multiple cardiovascular diseases. Myocardial fibrosis (MF) is the major cause of changes in the ventricular structure. It is accompanied by the whole process of HF. Previous studies have found that the pathogenesis of MF involves different types of cells, including cardiomyocytes, cardiac fibroblasts, etc. The biological functions of these cells are altered under neurological and humoral factors[1]. Furthermore, the changes at the genetic level can also influence cardiac function[1].

MicroRNAs (miRNAs) are a type of highly conserved RNAs with approximately 22 bases in length. They have been found not to encode proteins. In 1993, Lee et al[2] discovered a small molecule RNA that could regulate the growth and development of *C. elegans*. The synthesis of miRNA is initiated in the nucleus, and the DNA sequences encoding miRNAs are further transcribed into primary miRNAs (pri-miRNAs). Subsequently, pri-miRNA is processed into a precursor containing a stem-loop structure with 70 bases, namely pre-miRNA. By translocation

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into the cytoplasm, pre-miRNA is processed to mature miRNA with 22 bases[3]. Mature miRNA is well concerned due to its post-transcriptional regulation, which mainly influences the stability of transcripts[4]. Zhou et al[5] have demonstrated that miRNA affects cardiac development and remodeling. MiR-18/19 is involved in the regulation of CTGF, TSP-1, type I, and type III collagen, thereby regulating MF progression[6]. A growing number of miRNAs have been identified to participate in the progression of cardiovascular diseases. This may provide novel directions in clinical treatment.

Autophagy is a process of cell self-digestion and recycling. It allows the degradation of organelles and cytoplasm by lysosomes[7]. ULK1 belongs to the class of serine/threonine protein kinases. It phosphorylates Beclin1 and increases the activity of the VPS34 complex, thereby inducing autophagy. McAlpine et al[8] have found that ULK1 and ULK2 exert important effects on autophagy induced by nutrient deficiency. In this investigation, ULK1 was predicted as the direct target of miRNA-1297 through bioinformatics method. The aim of this study was to explore the regulatory effect of miR-1279/ULK1 on MF-induced autophagy and the potential mechanism.

Materials and Methods

Establishment of MF Model in Mice

Mice in the Ang-II infusion group or sham group received the cardiac perfusion of Angiotensin II or the same volume of saline, respectively. Two weeks later, all mice were sacrificed, and the myocardial tissues harvested. This investigation was approved by the Animal Ethics Committee of Ningxia Medical University Animal Center.

Masson Staining

Paraffin-embedded slices of myocardial tissues in mice were stained with Weigert solution for 5-10 min. After washing, the sections were treated with Ponceau-Fuchsin Acid Solution for 5-10 min, immersed in 2% acetic acid aqueous solution for a moment, and differentiated in 1% phosphomolybdic acid aqueous solution for 3-5 min. Without washing with water, the sections were treated with aniline blue for 5 min, and immersed with 0.2% acetic acid aqueous solution for a moment. Finally, the slices were permeabilized with xylene and mounted with neutral resin.

Cell Culture

The primary myocardial fibroblasts were extracted from myocardial tissues in MF mice and controls, respectively. All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 μg/mL streptomycin, and 0.1 mg/mL streptomycin (Gibco, Grand Island, NY, USA), and maintained in a 37°C, 5% CO_2 incubator.

Cell Transfection

The cells with good viability were pre-seeded into 6-well plates. Then, the cells were transfected with 50-100 nM transfection plasmids provided by GenePharma (Shanghai, China) according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

RNA Extraction

The tissues were fully lysed in 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After maintenance at room temperature for 5 min, 200 μL of chloroform was added, followed by mixing and incubation at room temperature for 5 min. The supernatant was transferred into a new RNase-free centrifuge tube after centrifugation at 4°C, 12,000 rpm for 15 min. Isopropanol with the same volume of the supernatant was added to harvest RNA precipitate by centrifugation. Next, extracted RNA was air dried, quantified and dissolved in diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). RNA samples were preserved at -80°C for use.

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

Extracted RNA was subjected to reverse transcription using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) and amplified by SYBR® Green Master Mix (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: MiRNA-1297: F: 5′-ACACTCCAGCTGGGTCCTTCATTCCA-3′, R: 5′-GTGCAGGGTCCGAGGT-3′; U6: F: 5′-CTCGCTTCGGCAGCACATATA, R:

5′-AAATATGGAACGCTTCACGA-3′; ULK1: F: 5′-ACTTTGGATTCGCTCGGTAC-3′, R: 5′-GGTCAGCCTTTCCATCGTAG-3′; GAPDH: F: 5′-CTCAACTACATGGTCTACATGTTC-3′, R: 5′-ATTTGATGTTAGTGGGGTCTCGCTC-3′.

Western Blot

The total protein in the cells was extracted using radioimmunoprecipitation assay (RIPA). The concentration of the extracted protein was quantified by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The protein samples were electrophoresed on polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After rinsing with the buffer solution (TBST), the membranes were incubated with the corresponding secondary antibody. The immunoreactive bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image J. Software (NIH, Bethesda, MD, USA).

Dual-Luciferase Reporter Gene Assay

Wild-type and mutant-type luciferase vectors of ULK1 were first constructed, namely ULK1 WT and ULK1 MUT, respectively. Then, the cells were co-transfected with ULK1 WT/MUT and miRNA-1297 mimics or NC for 24 h. Subsequently, the cells were fully lysed, followed by centrifugation at 10,000 g for 5 min. 100 μL of the supernatant was harvested for determining the luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 17.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. The quantitative data were represented as mean ± standard deviation ($\bar{x} \pm s$). The *t*-test was used to compare the differences between the two groups. $p<0.05$ was considered statistically significant.

Results

Verification of MF Model in Mice

Masson staining showed plenty of micro vessels around myocardial tissues and significantly increased contents of intercellular collagen in Ang-II infusion group relative to those in the sham group, which were typical manifestations of MF (Figure 1A). The collagen volume fraction (CVF) was remarkably higher in Ang-II infusion group than the control group. This suggested the successful establishment of the MF model in mice (Figure 1B). Subsequently, the protein levels of fibrosis-related genes in mice of Ang-II infusion group and the sham group were determined, respectively. Western blot showed significantly up-regulated Col1a1 and α-SMA in myocardial tissues of MF group than the control group (Figure 1C).

MiRNA-1297 was Downregulated in MF Model

QRT-PCR data revealed that miRNA-1297 was significantly downregulated in mice of Ang-II infusion group relative to the sham group (Figure 2A). The primary myocardial fibroblasts were extracted from myocardial tissues of MF mice and controls to establish the MF model *in vitro*. The third-generation primary myocardial fibroblasts extracted from MF mice were examined to verify their purity. Immunofluorescence examination showed that over 98% of cardiomyocytes were labeled with α-SMA, indicating the successful establishment of the MF model *in vitro* (Figure 2B). The primary myocardial fibroblasts extracted from myocardial tissues of Ang-II infusion group presented significantly higher protein levels of Col1a1 and α-SMA (Figure 2C). Besides, miRNA-1297 was lowly expressed in primary myocardial fibroblasts of MF mice (Figure 2D).

ULK1 was the Target Gene of MiRNA-1297

The potential binding sequences of ULK1 and miRNA-1297 were identified through online prediction (Figure 3A). The relative Luciferase activity was markedly reduced in primary myocardial fibroblasts co-transfected with ULK1 WT and miRNA-1297 mimics. This confirmed the binding relationship between ULK1 and miRNA-1297 (Figure 3B). Both the mRNA and protein levels of ULK1 were downregulated in the primary myocardial fibroblasts extracted from MF mice after the transfection of miRNA-1297 mimics (Figures 3C, 3D). Subsequently, ULK1 was observed remarkably upregulated in the *in vivo* and *in vitro* MF models (Figures 3E, 3F).

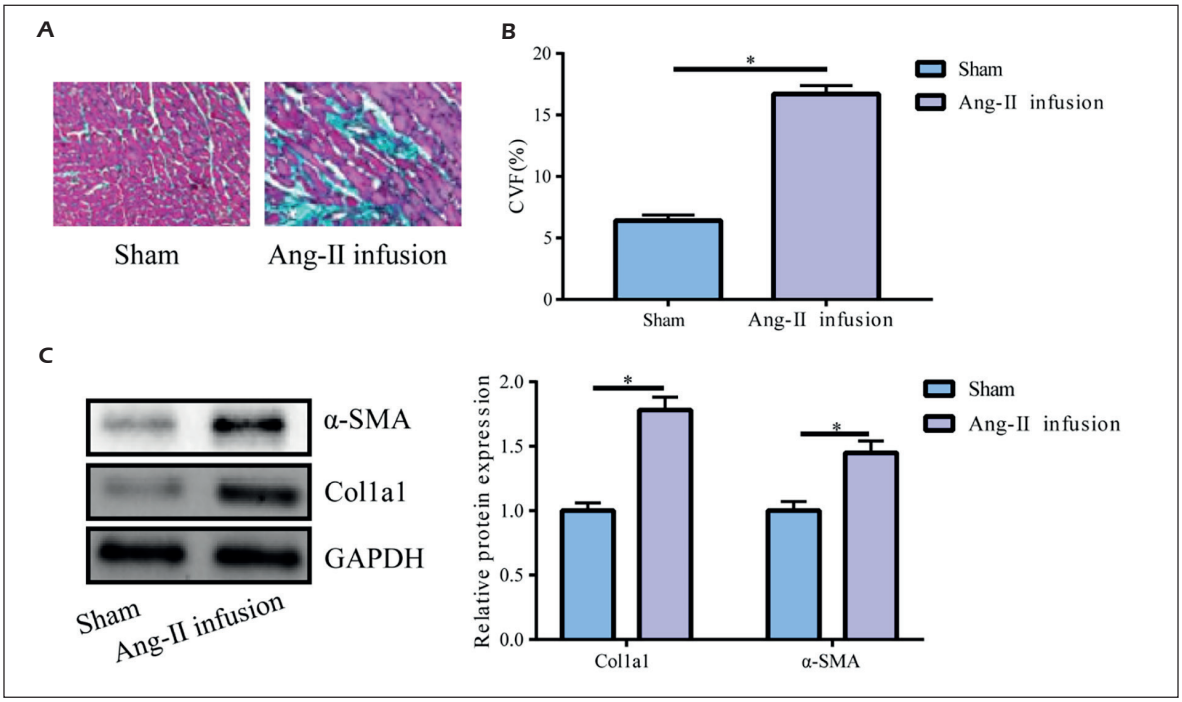

Figure 1. Verification of MF model in mice. **A,** Masson staining showed micro-vessels intercellular collagen around myocardial tissues in mice of Ang-II infusion group and sham group (magnification × 40). **B,** Collagen volume fraction (CVF) in Ang-II infusion group and sham group. **C,** Protein levels of Col1a1 and α-SMA in Ang-II infusion group and sham group.

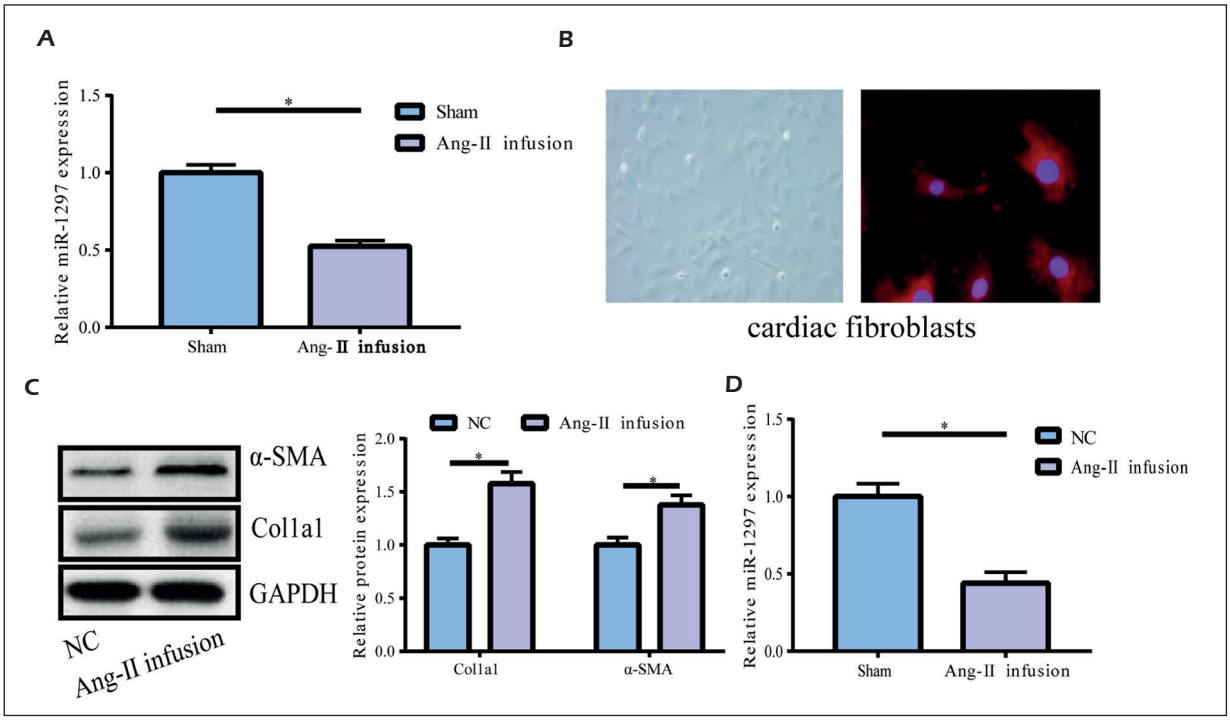

Figure 2. MiR-1297 was downregulated in MF model. **A,** Relative level of miR-1297 in Ang-II infusion group and sham group. **B,** Immunofluorescence showed primary myocardial fibroblasts extracted from myocardial tissues of MF mice (magnification × 40). **C,** Protein levels of Col1a1 and α-SMA in primary myocardial fibroblasts of Ang-II infusion group and sham group. **D,** Relative level of miR-1297 in primary myocardial fibroblasts of Ang-II infusion group and sham group.

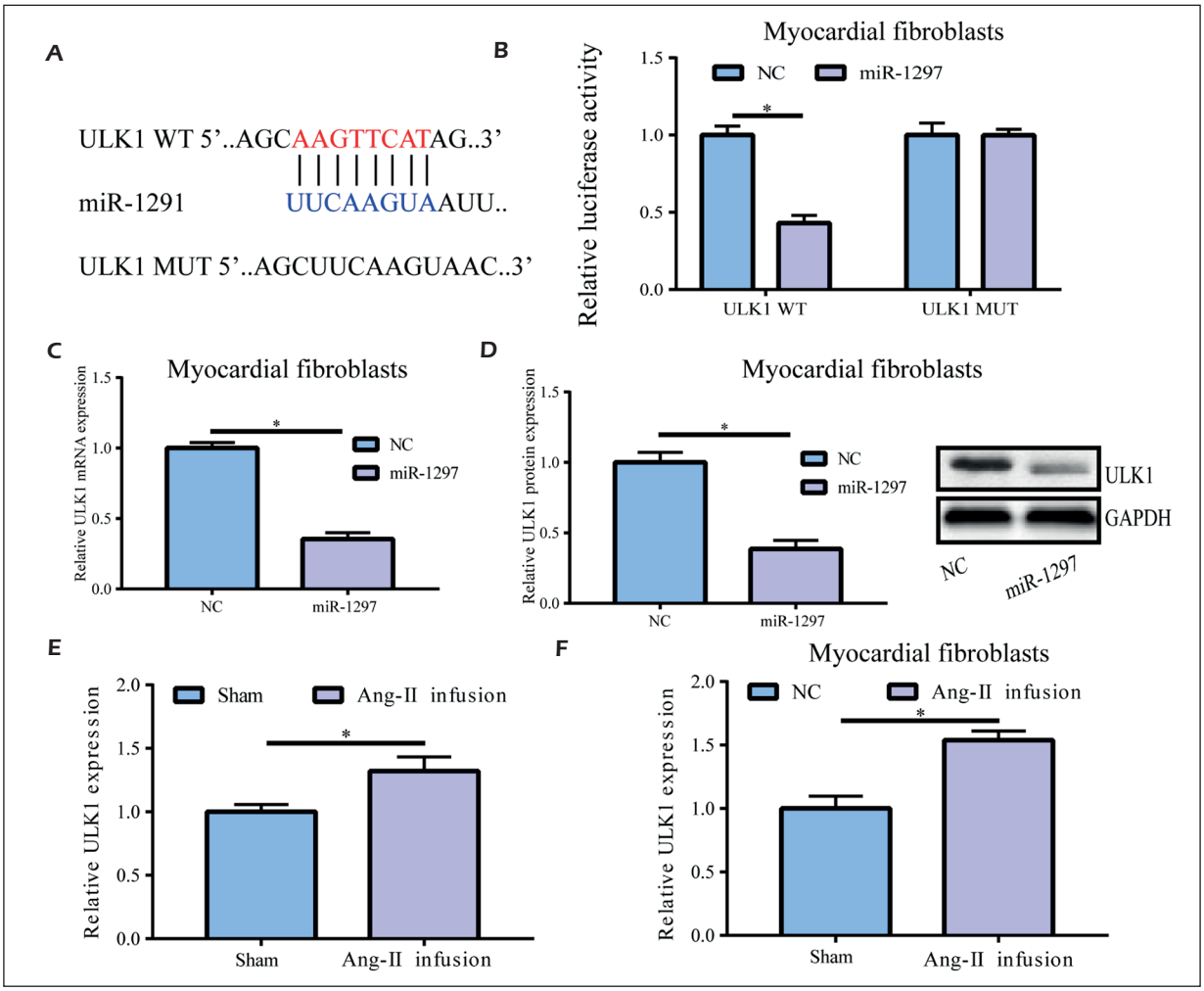

Figure 3. ULK1 was the target gene of miR-1297. **A,** Potential binding sequences of ULK1 and miR-1297. **B,** Relative luciferase activity primary myocardial fibroblasts co-transfected with ULK1 WT/MUT and miR-1297 mimics/NC. **C,** The mRNA level of ULK1 in primary myocardial fibroblasts of Ang-II infusion group and sham group. **D,** The protein level of ULK1 in primary myocardial fibroblasts of Ang-II infusion group and sham group. **E,** Relative level of ULK1 in mice of Ang-II infusion group and sham group. **F,** Relative level of ULK1 in primary myocardial fibroblasts of Ang-II infusion group and sham group.

MiRNA-1297 Mediated MF Via Down-regulating ULK1

After overexpression of miRNA-1297 in primary myocardial fibroblasts, the protein levels of Col1a1 and α-SMA were markedly downregulated (Figure 4A). Similar trends were observed at protein levels of Col1a1 and α-SMA after transfection of si-ULK1 (Figure 4B). Therefore, we speculated about the involvement of ULK1 in miRNA-1297-mediated MF. Of note, the downregulated levels of Col1a1 and α-SMA in the cells overexpressing miRNA-1297 were upregulated to some extent by the overexpression of ULK1 (Figure 4C). Hence, these findings concluded that miRNA-1297 alleviated MF *via* downregulating ULK1.

Discussion

MiRNA-1297 is reported to be an anti-tumor gene. Zhang et al[9] have found that miRNA-1297 is downregulated in human lung adenocarcinoma, which also suppresses tumor cell proliferation by directly targeting the oncogene TRIB2. In colorectal cancer, miRNA-1297 inhibits tumor growth by targeting Cox-2[10]. However, Li et al[11] have suggested the carcinogenic role of miRNA-1297. In human laryngeal squamous cell carcinoma, miR-1279 is upregulated and accelerates the malignant phenotypes of tumor cells by regulating PTEN. MiRNA-1297 is capable of accelerating the proliferative ability of NSCLC[12]. In testicular germ cell tumors, upregulated miRNA-1297 mediates

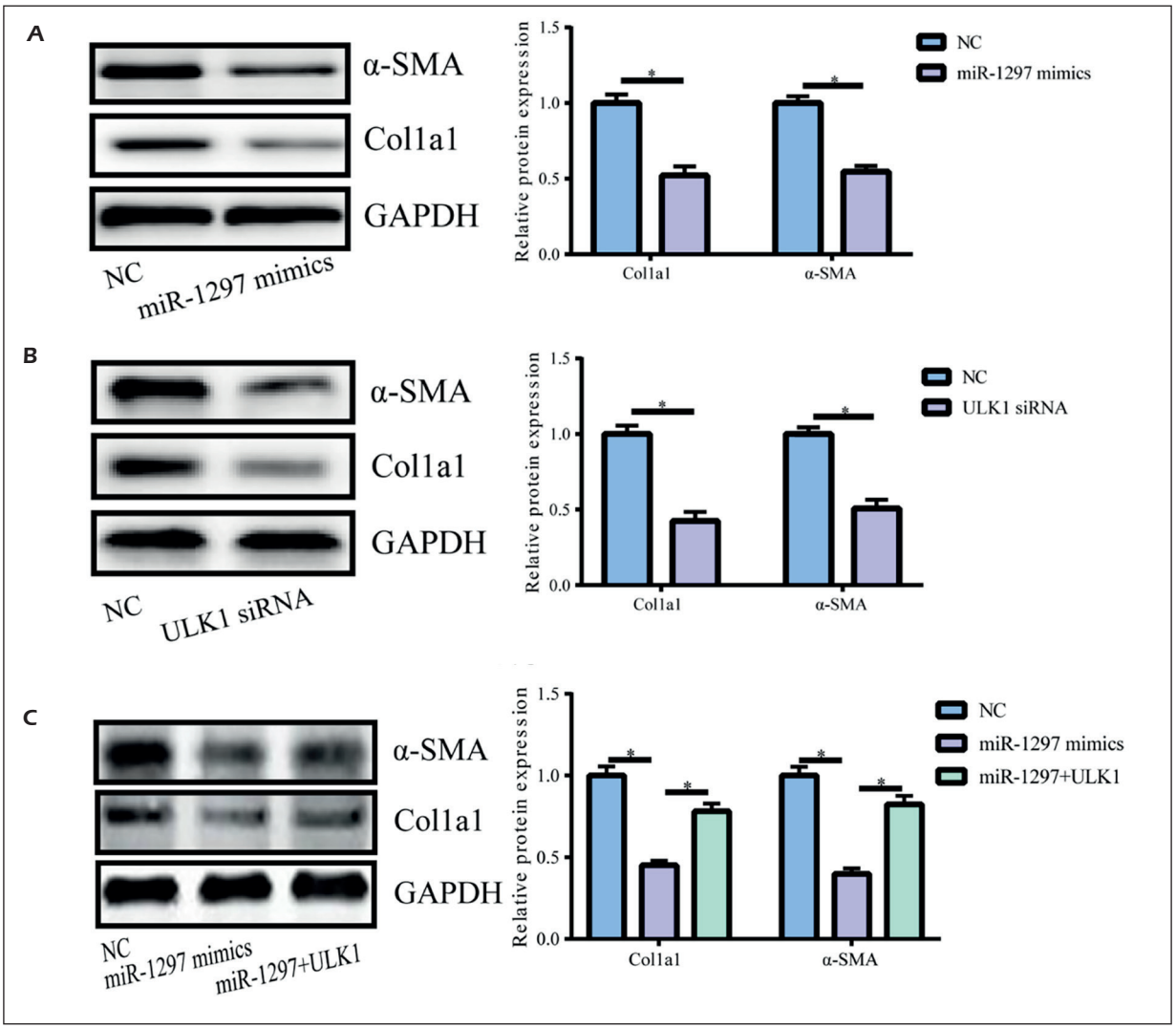

Figure 4. MiR-1297 mediated MF *via* down-regulating ULK1. **A,** Protein levels of Col1a1 and α-SMA in primary myocardial fibroblasts transfected with NC or miR-1297 mimics. **B,** Protein levels of Col1a1 and α-SMA in primary myocardial fibroblasts transfected with NC or si-ULK1. **C,** Protein levels of Col1a1 and α-SMA in primary myocardial fibroblasts transfected with NC, miR-1297 mimics or miR-1297 mimics+pcDNA-ULK1.

tumor progression by regulating lncRNA Meg3 and PTEN-PI3K/AKT pathway[13]. Furthermore, miRNA-1297 exerts a key role in suppressing liver cancer proliferation and inducing apoptosis *via* EZH2[14]. In this study, miRNA-1297 was found significantly downregulated in *in vivo* and *in vitro* MF models. These findings indicated that miRNA-1297 might participate in the progression of MF. After transfection of miRNA-1297 mimics, the protein levels of Col1a1 and α-SMA were markedly downregulated.

ULK1 is critical in the initial phase of autophagy[15]; however, its role in malignancies remains unclear. ULK1 is activated by hypoxia, and the silence of ULK1 accelerates tumor cells death under normoxia and hypoxia conditions[16]. The up-regulation of ULK1 is associated with the prognosis of certain malignancies, leading to cell apoptosis in tumors[17-19]. In addition, ULK1 interacts with Vab-8, which is a protein mediating cell surface expression of Sax-3/Robo in the tactile neuron growth cone[20]. Tomoda et al[21] have shown that SynGAP and syntenin are regulators of the Rab5-dependent endocytic pathway in mice. They have also been identified to be the binding partner of ULK1, demonstrating the relationship between endocytosis and axonal growth. Through online prediction, ULK1 was predicted

as the target of miRNA-1297 in this work. Subsequent Dual-Luciferase Reporter Gene Assay confirmed their binding relationship. Furthermore, the transfection of miRNA-1297 mimics could down-regulate ULK1 level in primary myocardial fibroblasts. In the MF model, ULK1 presented significantly higher abundance.

Our results indicated that the protein levels of Col1a1 and α-SMA were remarkably down-regulated after miRNA-1297 overexpression or ULK1 knockdown. Therefore, we speculated that miRNA-1297 regulated MF-induced autophagy in cardiomyocytes *via* targeting ULK1. Rescue experiments illustrated that the downregulated levels of Col1a1 and α-SMA in cardiomyocytes overexpressing miRNA-1297 were partially reversed by ULK1 overexpression, which proved our speculation.

Conclusions

In this report it is demonstrated that miRNA-1297 suppresses MF *via* down-regulating ULK1. These results provide a potential therapeutic target for MF.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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