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Microvesicles containing microRNA-21 induce myocardial fibrosis via AKT pathway

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Abstract. – OBJECTIVE: To explore the role of microvesicles (MVs) containing microRNA-21 in myocardial fibrosis and their underlying mechanism.

MATERIALS AND METHODS: Myocardial ischemia rat model was first constructed for collecting myocardial tissues. Myocardial fibrosis-related indicators and microRNA-21 expression in myocardial tissues of ischemia rats were detected. After in vitro primary rat cardiomyocytes were treated with TGF-β1, myocardial fibrosis-related indicators and microRNA-21 expression in primary rat cardiomyocytes were also detected. Furthermore, AKT (protein kinase B) pathway-related genes were detected by Western blot. Rescue experiments were performed after primary rat cardiomyocytes were transfected with a microRNA-21 inhibitor.

RESULTS: MVs containing microRNA-21 were overexpressed during the process of myocardial fibrosis. AKT pathway was remarkably activated after TGF-β1 treatment in cardiomyocytes. Meanwhile, myocardial fibrosis was significantly alleviated after microRNA-21 expression was inhibited.

CONCLUSIONS: Overexpressed MVs containing microRNA-21 promoted myocardial fibrosis via AKT pathway.

Key Words:
Microvesicles, MicroRNA21, Myocardial fibrosis, AKT.

Introduction

Globally, the mortality and prevalence of cardiovascular diseases have been slowly increased in recent years. In China, the mortality of cardiovascular diseases ranks the first, accounting for over 40% of all causes of death. Cardiovascular diseases not only pose a severe burden on affected patients and families, but also influence society and economic development.

Myocardial fibrosis (MF) is the final pathological result of cardiovascular diseases caused by various pathogenesis. Myocardial ischemia and hypoxia, resulted from moderate and severe coronary atherosclerotic stenosis, can induce the development of MF1. However, the exact pathogenesis of MF has not been fully elucidated. Current papers considered that the renin-angiotensin-aldosterone system, regulatory cytokines, oxidative stress, inflammatory factors and endothelial dysfunction are closely related to MF[2-5]. Microvesicles (MVs), as an intercellular communication mediator, have been well recognized[6]. Almost all types of cells in mammals can secrete MVs containing cellular information, such as proteins, lipids, nucleic acids, etc. MVs could transfer information to target cells via ligand-receptors. Studies have shown that MVs participate in the immune escape of tumor cells[7], renal interstitial fibrosis[8,9] and cardiovascular diseases[10,11].

MicroRNA is a type of endogenous non-coding small RNA12, which is widely presented in eukaryotic organisms. Functionally, microRNAs can regulate apoptosis of tumor cells[13]. MicroRNAs also exert a regulatory role in renal development and homeostasis diseases[14,15]. Recent investigations[16-19] have shown that microRNAs are greatly involved in tissue fibrosis, especially in MF. In the present research, we focused on the specific role of microRNA-21 in MF and its underlying mechanism. We aimed to provide new treatment targets in chronic ischemic heart diseases.

Materials and Methods

Experimental Rats

A total of 24 male Wistar rats weighing from 245-320 g were obtained from Slaccas (Shanghai, China). Rats were fed with standard diet and free water. These rats were randomly assigned into four groups, namely sham operation group, ischemia Day 1 group, ischemia Day 3 group and ischemia Day 7 group. Rats were sacrificed at the postoperative 1st, 3rd and 7th day, respectively. This study was approved by the Animal Ethics Committee of Hanchuan City People's Hospital Animal Center.

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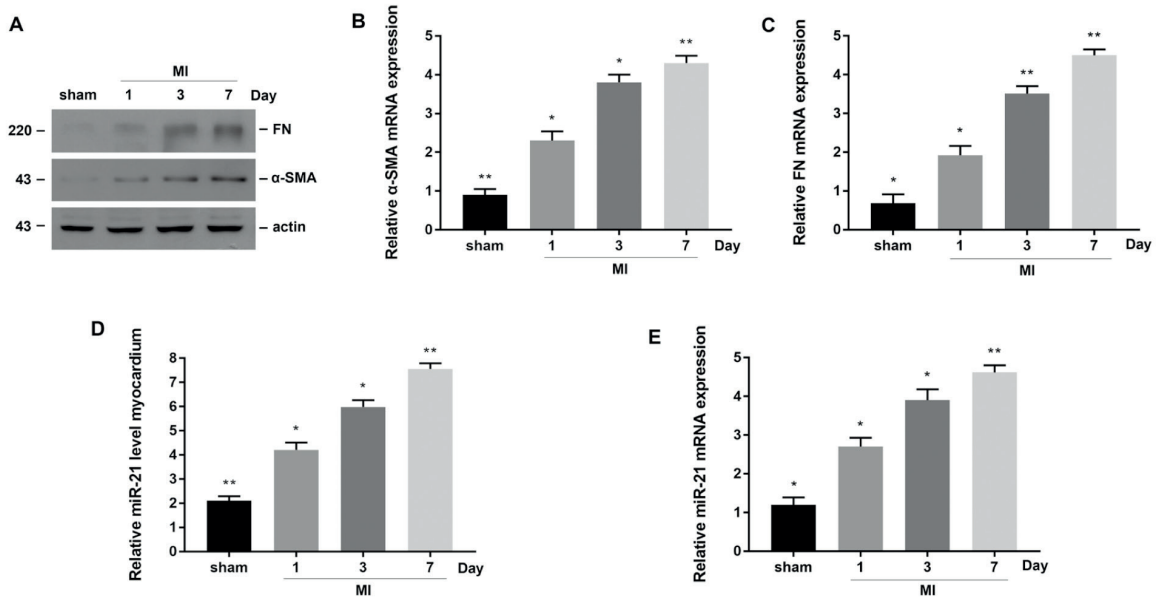

Figure 1. MicroRNA-21 was overexpressed in MF rats. ***A,*** Protein expressions of α-SMA and FN were remarkably increased in MF rats than those of controls in a time-dependent manner. ***B-C,*** The mRNA levels of α-SMA (B) and FN (C) presented a time-dependent elevation. ***D,*** Protein expression of microRNA-21 in myocardial tissues of MF rats was increased in a time-dependent manner compared with those of controls. ***E,*** The mRNA level of microRNA-21 was elevated in myocardial tissues of MF rats.

Myocardial Ischemia Rat Model

Rats were under fasting state 12 h before the surgery. For the myocardial ischemia procedure, rats were injected with 10% chloral hydrate (0.4 mL/100 g) for anesthesia and positioned on the surgery table. A tracheal cannula was inserted into the mouth and connected with an animal breathing apparatus (frequency of 85/min, respiration rate of 1:1 and tidal volume of 18 mL/kg). Subsequently, we performed thoracotomy to expose the heart. The proximal left anterior descending coronary artery was ligated with a 6/0 silk to induce myocardial ischemia. The incision was sutured layer by layer. TTC (triphenyltetrazolium chloride) staining was performed 6 h later. Briefly, rat heart was harvested and washed with saline. The heart was then sliced into 1-mm sections and incubated with 0.1% TTC phosphate buffer (pH 7.4) at 37°C for 7-10 min. Sections were washed with saline for several times and myocardial infarction was observed.

Cell Culture of Primary Rat Cardiomyocytes

Rat hearts of 1-3-day old neonates were collected and washed with 1×PBS (phosphate-buffered saline) containing 1% P/S (pH 7.4). Ventricular muscles were then cut into 1-mm3 tissues and digested according to the instructions of Pri-Cells Isolation of Primary Cell Kit (Thermo Fisher Scientific, Waltham, MA, USA). Cells were collected after centrifugation at 1000 rpm/min for 10 min. After the supernatant was discarded, cells were cultured in pre-coated flask at a density of 5×105/mL and maintained in a 5% CO2 incubator at 37°C. The culture medium was PriCells medium that contained 10% fetal bovine serum (FBS, Rockville, MD, USA), 1% P/S and PriCells Supplement.

MV Extraction

Cell suspension was collected and centrifuged at 300 g/min for 5 min, 1200 g/min for 20 min and 10,000 g/min for 30 min at 4°C, sequentially. Supernatant was collected and centrifuged again at 110,000 g/min for 60 min at 4°C. Precipitates were collected, MVs were resuspended with an appropriate amount of PBS and mRNAs in MVs were extracted with TRIzol method (Invitrogen, Carlsbad, CA, USA).

Western Blot

The radioimmunoprecipitation assay (RIPA) protein lysate (Beyotime, Shanghai, China) was

used to extract the total protein in each group of tissues. The BCA (bicinchoninic acid) method was used to quantitate the protein concentration. Protein samples were electrophoresed on polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck, Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibodies (FN, α-SMA and CD63, Abcam, Cambridge, MA, USA; PTEN, p-AKT and AKT, Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membrane was incubated with the secondary antibody after rinsing with Tris-buffered saline and Tween-20 (TBST). Chemiluminescence was used to expose the protein bands on the membrane.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the tissues and cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), respectively, followed by measurement of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The cDNA was synthesized according to the instructions of the miScript RT II Kit (15596-026, Life Technology, Gaithersburg, MD, USA). QRT-PCR was then performed based on the instructions of miScript SYBR Green PCR Kit (28073, Qiagen, Duesseldorf, Germany). The relative expression level of the target gene was expressed by 2-ΔΔCt. The primer sequences were as the follows: microRNA-21 (F: 5'- TCAACATCAGTCTGATAAGCTA -3', R: 5'- CATTAATGTCGGACAACTCAAT-3'), α-SMA (F: 5›-CCATGACATGAACCGACCCT-3›, R: 5›- CCGGGTTGTGTTGGTTGTAG-3›), FN (F: 5›- AGCCACATCGCTCAGACAC -3›, R: 5›-GCCCAATACGACCAAATCC-3›), PTEN (F: 5›- CCTGTGAAATAATACTGGTATG-3', R: 5'-CTCCCAATGAAAGTAAAGTACA-3'). AKT (forward: 5'-CTTGCTTTCAGGGCTGCTCA-3', reverse: 5'-TACACGTGCTGCCACACGATAC-3'). CD63 (forward: 5'-AATGGCACGGAGCACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3').

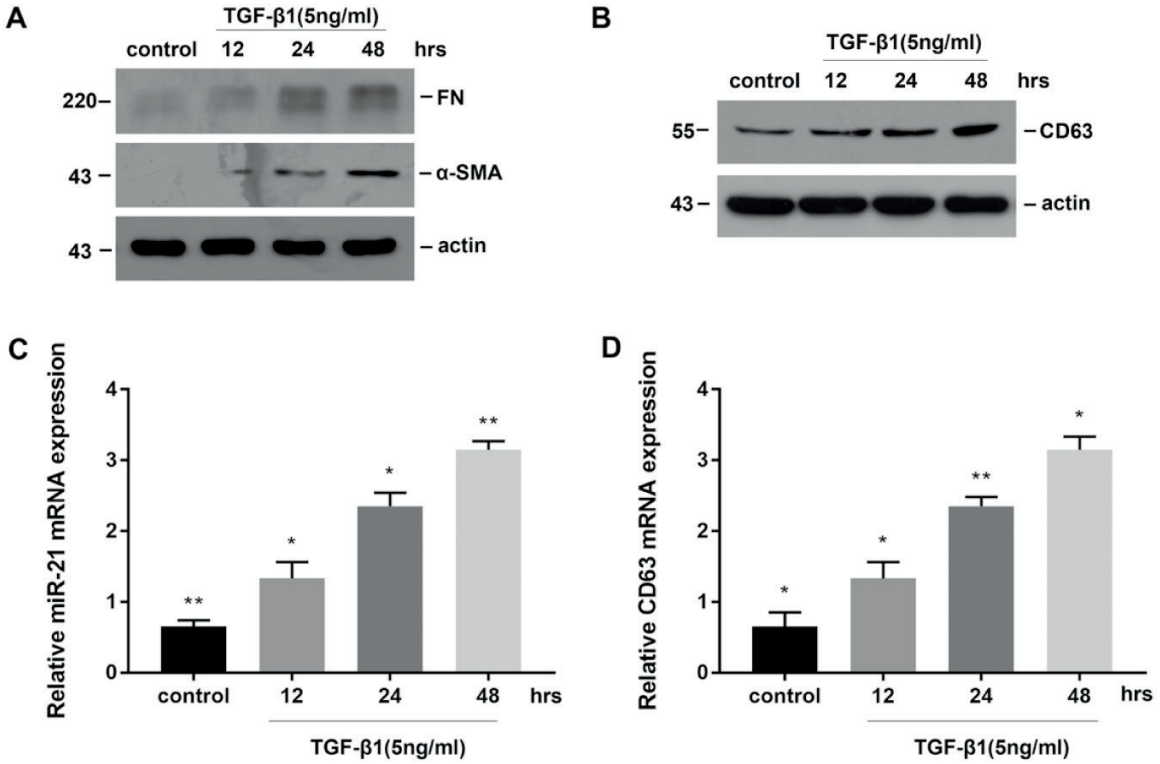

Figure 2. MVs containing microRNA-21 were upregulated in fibrotic primary rat cardiomyocytes induced by TGF-β1. ***A,*** Protein expressions of α-SMA and FN were remarkably increased in cardiomyocytes in a time-dependent manner. ***B-C,*** Both protein ***(B)*** and mRNA ***(C)*** levels of CD63 were elevated in fibrotic cardiomyocytes. ***D,*** Expression level of microRNA-21 showed a time-dependent elevation in fibrotic cardiomyocytes.

Cell Transfection

Cells were seeded into the 6-well plates. After cell confluence was up to 90-95%, 1.5 mL of serum-free medium were replaced in each well. For cell transfection, 2.5 μg of plasmid DNA or 5 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were diluted in 250 μL of Opti-MEM, respectively. After incubation at room temperature for 5 min, the two solutions were mixed and incubated for another 20 min at room temperature. The mixture was then added in each well and cells were incubated for 48 h.

Statistical Analysis

Statistical Product and Service Solutions (SPSS 16.0 Inc., Chicago, IL, USA) statistical software package (SPSS Inc., Chicago, IL, USA) was used for data analysis. Data were expressed as mean±SEM. The t-test was used to analyze the difference between two groups. The one-way ANOVA was used to analyze the data among different groups, followed by LSD (Least Significant Difference) Post-Hoc Test. $p<0.05$ was considered statistically significant.

Results

MicroRNA-21 was Overexpressed in MF Rats

We first constructed MF rat models via induction of myocardial ischemia in left ventricle. Rats were sacrificed and myocardial tissues were collected at the postoperative 1st, 3rd and 7th day, respectively. Protein expressions of MF-related indicators, including α-SMA and FN, were remarkably increased in MF rats than those of controls in a time-dependent manner (Figure 1A). The mRNA levels of α-SMA and FN also presented a time-dependent elevation (Figure 1B and 1C). We speculated that microRNA-21 may participate in MF through literature reviews. Western blot results showed that microRNA-21 in myocardial tissues of MF rats were increased in a time-dependent manner compared with those of controls (Figure 1D). The mRNA level of microRNA-21 showed the same elevated trend (Figure 1E). The above data indicated that microRNA-21 is overexpressed during the process of MF.

MVs Containing microRNA-21 were Upregulated in Fibrotic Primary Rat Cardiomyocytes Induced by TGF-Ð1

Here, we induced the fibrosis of primary rat cardiomyocytes using 5 ng/mL TGF-β1. Protein expressions of α-SMA and FN were remarkably increased in cardiomyocytes in a time-dependent manner (Figure 2A). We further speculated that MVs could exert their regulatory roles in the process of MF. Both protein and mRNA levels of CD63, the hallmark of MVs, were elevated in fibrotic cardiomyocytes (Figure 2B and 2C), indicating MVs were overexpressed in the process of MF. Previous experiments have already confirmed that microRNA-21 was overexpressed in MF rats. In vitro expression level of microRNA-21 also showed a time-dependent elevation in fibrotic cardiomyocytes (Figure 2D).

MVs Containing microRNA-21 Promoted MF

Previous results have already confirmed that MVs containing microRNA-21 were upregulated in fibrotic primary rat cardiomyocytes induced by TGF-β1. We, therefore, speculated whether microRNA-21 may promote MF. Culture medium was collected after cardiomyocytes were treated with TGF-β1 for 48 h. MVs containing microRNA-21 were collected by ultracentrifugation. Subsequently, primary rat cardiomyocytes were treated with MVs containing microRNA-21 for different time points. Both protein and mRNA levels of α-SMA were elevated in a time-dependent manner (Figure 3A and 3B). For comparison, primary rat cardiomyocytes were treated with TGF-β1 and microRNA-21 mimics to observe the fibrosis condition. Surprisingly, α-SMA expression was further elevated in fibrotic cardiomyocytes (Figure 3C and 3D), indicating that microRNA-21 promoted MF.

AKT Pathway was Activated During the Process of MF

We next explored the regulatory mechanism of microRNA-21 in MF. It is reported that PTEN is the target gene of microRNA-2120-22. PTEN activates AKT pathway by dephosphorylation of PIP3 to PIP2. In the present work, PTEN expression was decreased in myocardial tissues of MF rats, whereas p-AKT expression was increased (Figure 4A). The mRNA levels of PTEN and p-AKT presented the similar trend as those of protein expressions (Figure 4B and 4C). Primary rat cardiomyocytes induced by TGF-β1 were collected to detect expression levels of PTEN and p-AKT, which showed the same results as the *in vivo* expressions (Figure 4D-4F).

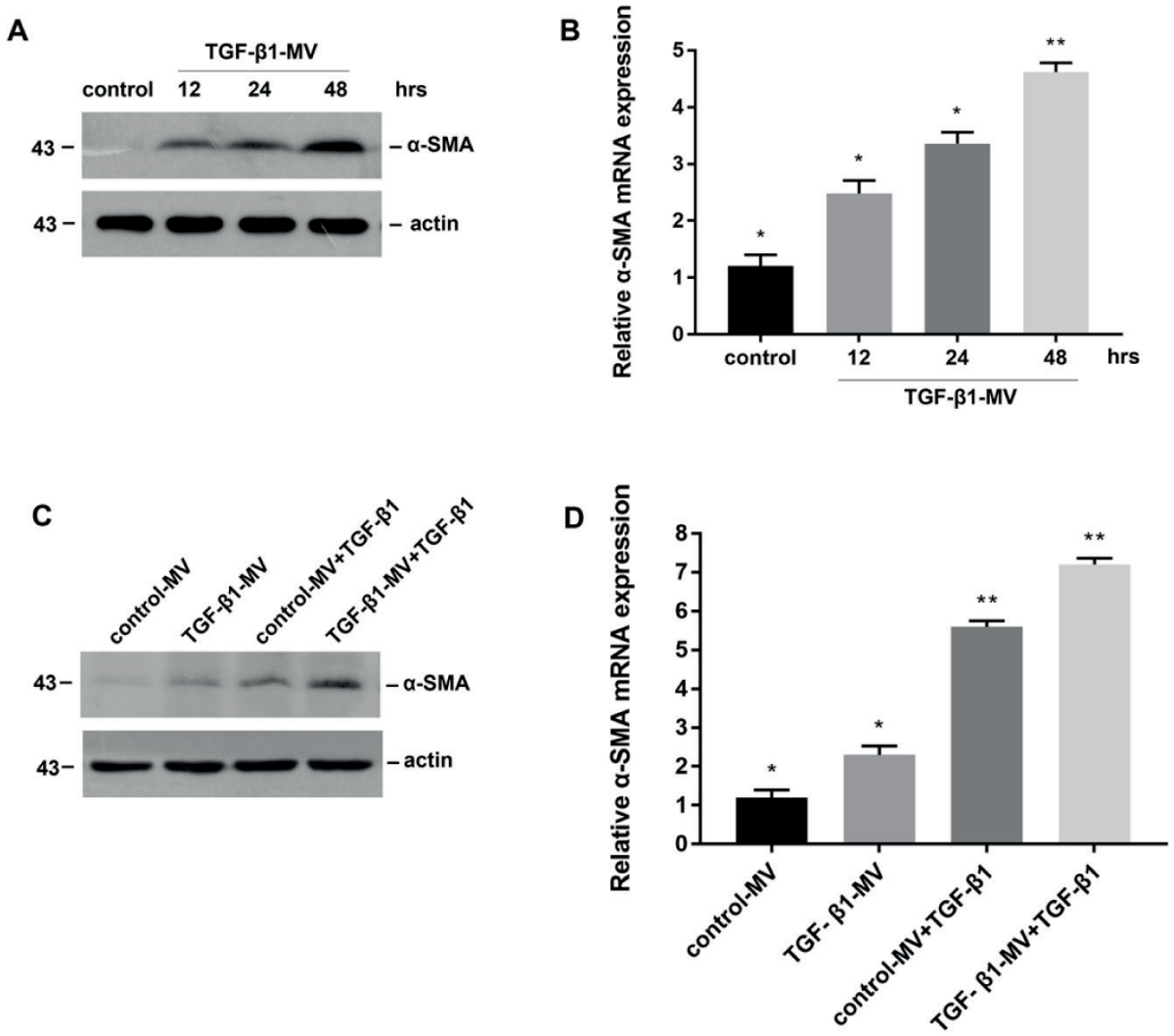

Figure 3. MVs containing microRNA-21 promoted MF. ***A-B,*** Both protein (A) and mRNA (B) levels of α-SMA were elevated in a time-dependent manner in primary rat cardiomyocytes treated with MVs containing microRNA-21. ***C-D***, Both protein ***(C)*** and mRNA ***(D)*** levels of α-SMA were elevated in a time-dependent manner in primary rat cardiomyocytes treated with TGF-β1 and microRNA-21.

MicroRNA-21 Inhibition Attenuated MF via Inhibiting AKT Pathway

Since the effect of microRNA-21 on promoting MF has been demonstrated, we next constructed microRNA-21 inhibitor to determine whether microRNA-21 knockdown could attenuate MF. Transfection efficacy of microRNA-21 inhibitor was shown in Figure 5A. Downregulated α-SMA was observed in fibrotic cardiomyocytes transfected with microRNA-21 inhibitor (Figure 5B). MicroRNA-21 knockdown also led to upregulated PTEN and downregulated p-AKT (Figure 5C and 5D), indicating that microRNA-21 inhibition attenuates MF via inhibiting AKT pathway.

Discussion

Nowadays, the incidence of cardiovascular diseases has been increased annually because of lifestyle changes, genetic inheritance and other factors. Cardiomyocytes are stable cells, the structure and function of which could be severely affected by external stimuli. Dysfunctional cardiomyocytes eventually lead to MF in the absence of efficient treatment. MF is mainly characterized by hypertrophy of myocardial interstitial fibroblasts, excessive collagen deposition and abnormal distribution. MF is closely related to various cardiovascular diseases, including hypertension,

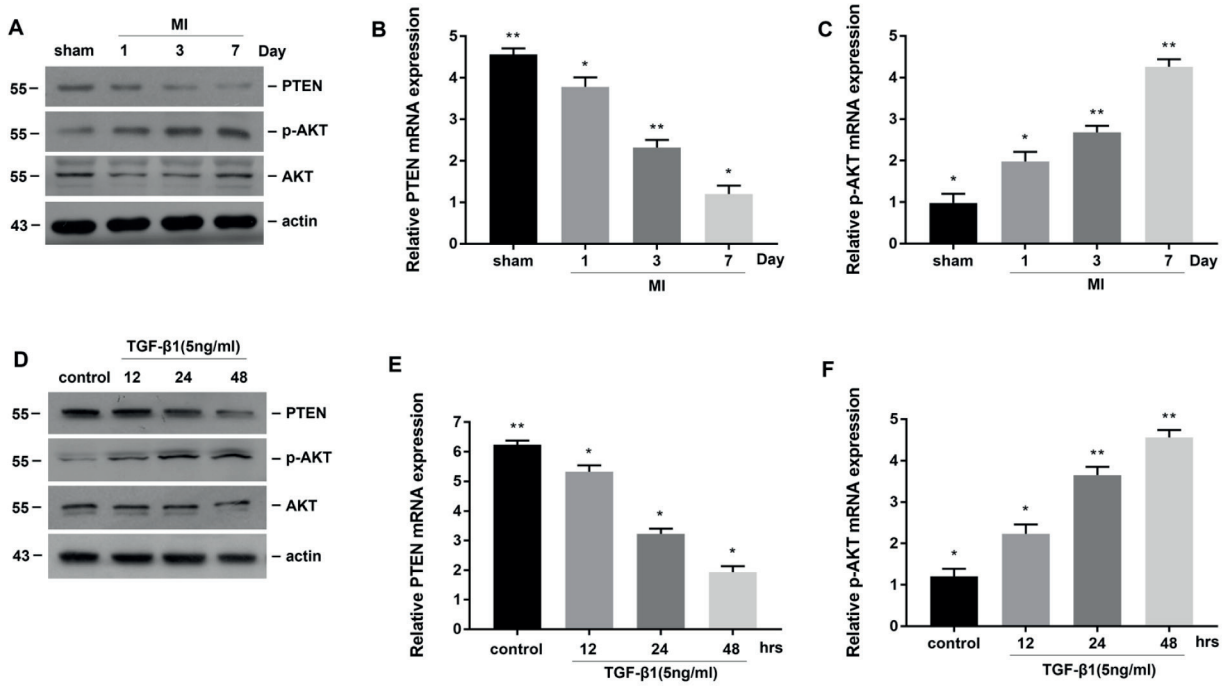

Figure 4. AKT pathway was activated during the process of MF. ***A,*** PTEN and p-AKT expressions in myocardial tissues. ***B-C,*** The mRNA levels of PTEN (B) and p-AKT (C) in myocardial tissues. ***D,*** Protein expressions of PTEN and p-AKT in fibrotic cardiomyocytes. E-F, The mRNA levels of PTEN (E) and p-AKT (F) in fibrotic cardiomyocytes.

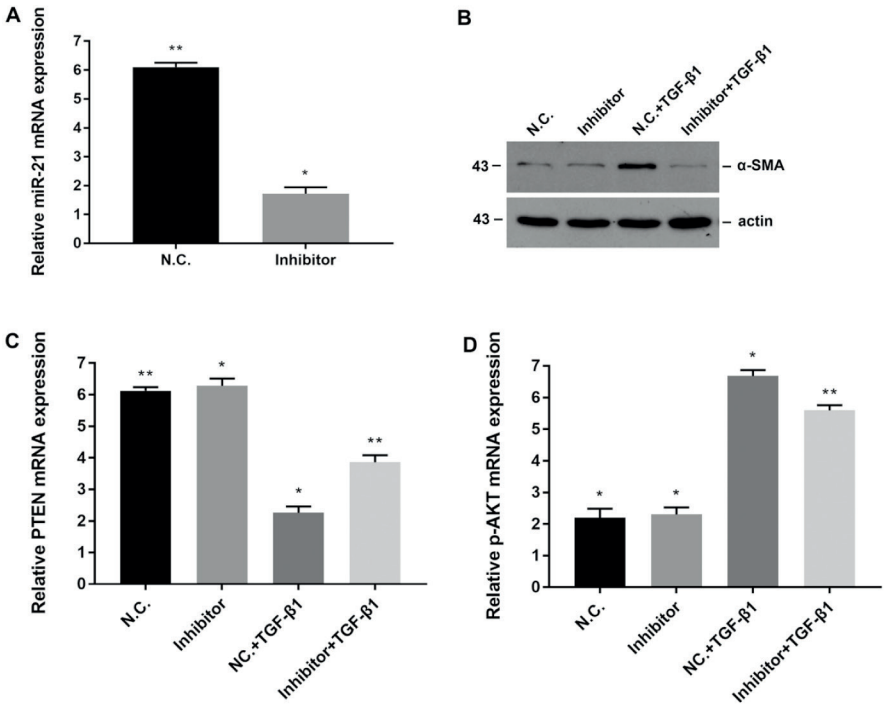

Figure 5. MicroRNA-21 inhibition attenuated MF via inhibiting AKT pathway. A, Transfection efficacy of microRNA-21 inhibitor. B, Protein expression of α-SMA in fibrotic cardiomyocytes transfected with microRNA-21 inhibitor. C-D, The mRNA levels of PTEN (C) and p-AKT (D) in fibrotic cardiomyocytes transfected with microRNA-21 inhibitor.

chronic heart failure, hypertrophic cardiomyopathy, dilated cardiomyopathy and viral myocarditis. It is reported that MF is a potential risk factor for sudden cardiac death.

Previous studies have shown that MF has important significance in the occurrence and development of cardiovascular diseases. The specific mechanism, however, is not yet clear. In recent years, MVs have received extensive attention as a novel signaling molecular delivery mediator. MVs are widely presented in biological organisms that transmit biological molecules and affect the cellular functions. Therefore, we hypothesized that myocardial cells could secrete MVs containing certain signaling molecules so as to transmit information that could affect MF.

MVs contain a variety of biologically active molecules, including microRNAs. Previous studies have shown that microRNA-21 is involved in the tissue fibrosis, such as renal interstitial fibrosis, pulmonary interstitial fibrosis, and hepatic tissue fibrosis. Therefore, we considered that MVs containing microRNA-21 may exert a crucial role in MF.

In the present study, MF rat models were established via induction of myocardial ischemia. We found that MF-related indicators, α-SMA and FN, were significantly increased. Then, we confirmed that microRNA-21 was upregulated during the process of MF at a time-dependent manner. Next, TGF-β1-induced fibrosis was observed in primary rat cardiomyocytes. We found that MVs containing microRNA-21 were overexpressed in these primary rat cardiomyocytes and the level of microRNA-21 also showed a time-dependent elevation in fibrotic cardiomyocytes. After primary rat cardiomyocytes were treated with MVs containing microRNA-21 for different time points, the results showed that expression of α-SMA was significantly increased at a time-dependent manner. These findings verified that MVs containing microRNA-21 could remarkably promote MF. Next, we attempted to explore the molecular mechanism. Results demonstrated that PTEN expression was reduced while p-AKT expression was enhanced in myocardial tissues of MF rats, indicating that AKT pathway was activated during the process of MF. At last, we performed the rescue experiments to verify our suggestion. The rescue results showed that microRNA-21 knockdown increased PTEN and decreased p-AKT, suggesting that microRNA-21 inhibition attenuates MF via inhibiting AKT pathway.

Conclusions

We showed that overexpressed MVs containing microRNA-21 promotes myocardial fibrosis via AKT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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