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MiR–23 enhances cardiac fibroblast proliferation and suppresses fibroblast apoptosis via targeting TGF-β1 in atrial fibrillation

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Abstract. – OBJECTIVE: To investigate the specific role of miR-23 in atrial fibrillation (AF) progression and explore the possible underlying mechanism.

PATIENTS AND METHODS: Right atrial appendage (RAA) tissues were collected from 30 patients with AF and 30 patients with sinus rhythm (SR), respectively. The expression level of miR-23 was detected by quantitative Real time-polymerase chain reaction (qRT-PCR). Moreover, cell counting kit-8 and flow cytometry were performed to detect the proliferation and cell apoptosis of AC16 cells after transfection with miR-23 inhibitor and mimics. Furthermore, luciferase reporter gene assay and RNA immunoprecipitation assay were performed to uncover the possible underlying mechanism.

RESULTS: In the present study, the expression level of miR-23 in RAA tissues of AF patients was significantly higher than that of SR patients. After knockdown of miR-23 in AC16 cells, the proliferation was inhibited and cell apoptosis was induced. However, overexpression of miR-23 significantly promoted cell growth and suppressed cell apoptosis. Further experiments revealed that transforming growth factor-b1 (TGF-β1) was a direct target of miR-23. In addition, TGF-β1 expression was positively correlated with miR-23 expression in AF tissues.

CONCLUSIONS: Our findings indicated that miR-23 could promote the progression of AF via promoting TGF-β1, which might serve as a new direction for interpreting the mechanism of AF development.

Key Words:
Cardiac fibroblast, Atrial fibrillation (AF), MiR-23, TGF-β1.

Introduction

As the most common arrhythmia clinically, atrial fibrillation (AF) is a growing severe problem[1]. In patients with AF, atrial fibrosis is not only associated with the risk of complications, but also with treatment failure[2,3]. Cardiac remodeling, particularly the remodeling of heart structure, is an essential result of AF[4]. Although many researchers have studied the development of AF in the past few decades, the possible underlying mechanism of AF remains unclear. MicroRNAs (miRNAs) are a type of endogenous, small non-coding single-stranded RNA sequences with about 18 to 22 nucleotides in length. They may complementary bind to the untranslated regions (most typically the 30-end) of target mRNAs. Recent studies have shown that miRNA can regulate gene expression and is involved in numerous diseases[5]. Plenty of miRNAs participate in fibrotic signaling by directly targeting the membrane receptor system. For instance, miR-133 and miR-30 are involved in fibrotic signaling by targeting CTGF[6]. Further researches[7] have indicated that both TGF-β and its receptor are directly regulated by miR-133 and miR-30. MiR-29, which directly regulates the development of collagen and fibronectin, has been found to be down-regulated in patients with heart failure[8]. However, the underlying role of miR-23 in cardiac fibrosis remains unclear. Evidence has proved that angiotensin II (AngII) plays an important role in atrial fibrosis by stimulating the endogenous synthesis of TGF-β1[9-11]. Moreover, it has also been found that Ang II-induced TGF-β1 activation is of vital importance in atrial structure remodeling of the human body[12]. In this study, miR-23 was highly expressed in AF patients. Besides, it promoted the proliferation and suppressed the apoptosis of cardiomyocytes *in vitro*. Furthermore, our findings discovered the interaction between miR-23 and TGF-β1 as well as the possible underlying mechanism.

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Patients and Methods

Clinical Samples and Cell Lines

Right atrial appendage (RAA) tissues were obtained from 30 AF patients and 30 SR patients who received cardiac surgery in the Affiliated Hospital of Qingdao University. Informed consent was obtained from each subject before the study. These patients had no other diseases, including pulmonary disease, coronary heart disease, diabetes mellitus infective endocarditis, mellitus, hyperthyroidism, hypertension, active rheumatism, or autoimmune disease. Collected tissues were immediately stored at -80°C for subsequent use. All tissues were confirmed by experienced pathologists. This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. 293T embryonic kidney cell line and AC16 cell line were used in this study. All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, CA, USA), 100 μg/mL streptomycin and 100 IU/mL penicillin in a 37°C, 5% CO_2 incubator.

RNA Extraction And Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA of cells was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) with reverse transcription kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). Thermal cycle was as follows: 95°C for 30 s, 95°C for 5 s, for a total of 40 cycles, and 60°C for 35 s. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method normalized to U6. Primer sequences used in this study were as follows: TGF-β1, F: 5'- CAGATCCTGTCCAAGCTA-3', R: 5'-CCTTGGCGTAGTAGTCG-3'; microRNA-23, F: 5'-CCCTGCAGCTGGAGAGTGTGG-3', R: 5'-TGTGCTCTGCTTGAGAGGTGCT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; β-actin: F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-GCTGATCCACATCTGCTGGAA-3'.

Cell Transfection

AC16 cells were transfected with miR-23 mimics and inhibitor (Genepharma Co., Ltd. Shanghai, China) in strict accordance with the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Non-specific siRNA was used as a negative control.

Cell Counting Kit-8 (CCK-8) Assay

Transfected cells were seeded into 96-well plates at a density of 4 × 10^3 cells per well. After culture for 0, 24, 48 and 72 h, 10 μL CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added in each well, followed by incubation at 37°C for 2 h in dark. Optical density (OD) value at the wavelength of 450 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Apoptosis Analysis

Apoptosis of transfected cells was evaluated by the Annexin V-APC/7-AAD Apoptosis Detection Kit II (KeyGEN BioTESCH Co., Ltd, Nanjing, China). Flow cytometry (FACScan, BD Biosciences, Franklin Lakes, NJ, USA), programmed with CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA), was utilized to distinguish viable, dead, early apoptotic and late apoptotic cells. The percentage of each type of cells was compared between the control group and the experimental group.

Luciferase Reporter Gene Assay

In our study, wild-type (WT) 3′-UTR and TGF-β1 3′-UTR were cloned with pGL3 vector (Promega, Madison, WI, USA). Site-directed mutagenesis of TGF-β1 binding site in mutant (MUT) 3′-UTR and miR-23 3′-UTR was performed with quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). MUT-3′-UTR/WT-3′-UTR and miR-control/mimics were used for cell transfection. 48 h later, luciferase activity was detected by the dual luciferase reporter assay system (Promega, Madison, WI, USA).

RNA immunoprecipitation assay (RIP)

RIP assay was performed in strict accordance with the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Expression of co-precipitated RNAs was detected by qRT-PCR.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 Software (SPSS, Chicago, IL, USA) was used for all statistical analysis. Experimental date were expressed as mean ± standard deviation ($\bar{x}$±s). x^2-test, Student t-test and Kaplan-Meier method were selected when appropriate. $p<0.05$ was considered statistically significant.

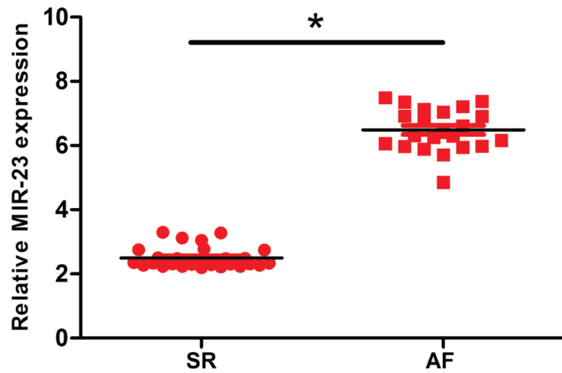

Figure 1. Expression level of miR-23 in RAA tissues. MiR-23 expression was significantly increased in AF patients when compared with SR patients. Data were expressed as mean ± standard error of mean. *$p<0.05$.

Results

MiR-23 Level in AF and SR Patients

We first detected miR-23 expression in RAA tissues of 30 AF patients and 30 SR patients by qRT-PCR, respectively. Results showed that the expression of miR-23 in AF patients was significantly higher than that of SR patients (Figure 1).

MiR-23 Promoted the Proliferation and Suppressed Cell Apoptosis of AC16 Cells

AC16 cells were then transfected with miR-23 inhibitor or mimics. Transfection efficiency was detected by qRT-PCR (Figure 2A). CCK-8 assay indicated that the proliferation of AC16 cells was significantly promoted after miR-23 overexpression (Figure 2B). However, the proliferation of AC16 cells was remarkably inhibited after miR-23 knockdown (Figure 2C). Moreover, flow cytometry revealed that the apoptosis of AC16 cells was significantly inhibited after miR-23 overexpression (Figure 2D). Similarly, the apoptosis of AC16 cells was significantly promoted after miR-23 knockdown (Figure 2E).

Expression of TGF-β1 in AF Patients

TargetScan was used to search mRNAs containing miR-23 complementary bases. It was predicted that miR-23 harbored TGF-β1 binding sites (Figure 3A). Subsequently, we detected TGF-β1 expression in RAA tissues. Results demonstrated that TGF-β1 expression in AF patients was significantly higher than that of SR patients (Figure 3B).

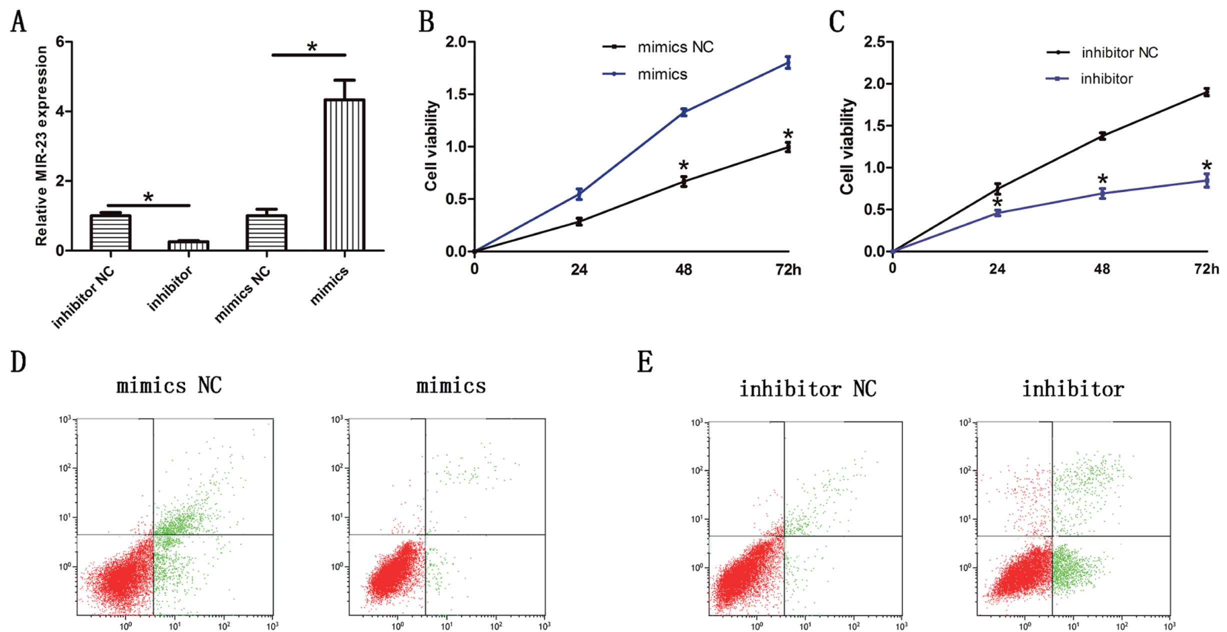

Figure 2. MiR-23 promoted the proliferation of AC16 cells. ***A,*** MiR-23 expression in AC16 cells transfected with miR-23 control, inhibitor or mimics was detected by qRT-PCR. U6 was used as an internal control. ***B,*** CCK-8 assay showed that overexpression of miR-23 significantly increased the proliferation of AC16 cells. C, CCK-8 assay showed that knockdown of miR-23 significantly inhibited the proliferation of AC16 cells. ***D,*** Apoptosis of AC16 cells was significantly inhibited after miR-23 overexpression. ***E,*** Apoptosis of AC16 cells was remarkably promoted after miR-23 knockdown. *$p<0.05$, as compared with control cells.

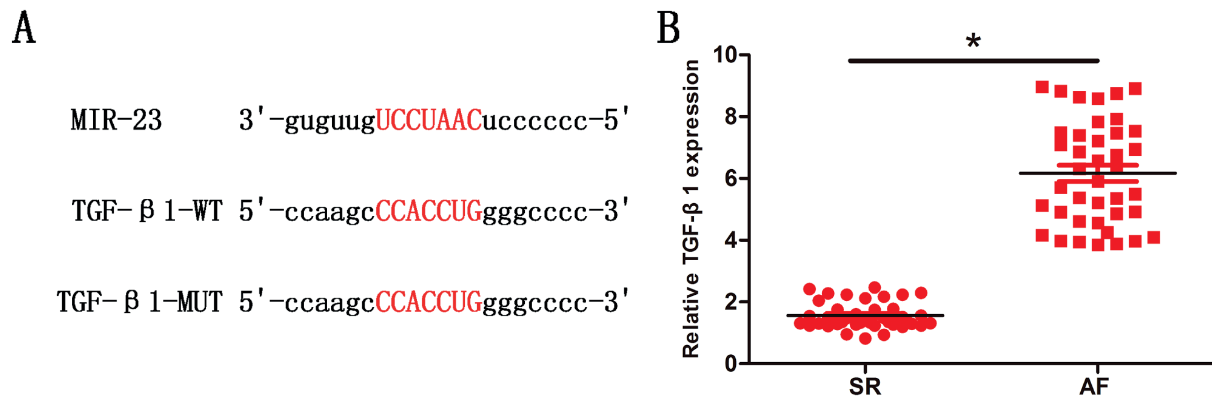

Figure 3. Expression level of TGF-β1 in AF patients. ***A,*** The binding sites of TGF-β1 on miR-23. ***B,*** TGF-β1 expression in RAA tissues of AF patients was significantly higher than that of SR patients.

The Interaction Between TGF-β1 and miR-23

QRT-PCR results showed that the expression of TGF-β1 in the miR-23 mimics group was significantly higher than that of the control group (Figure 4A). However, the level of TGF-β1 in AC16 cells transfected with miR-23 inhibitor was significantly down-regulated (Figure 4B). Meanwhile, luciferase reporter gene assay revealed that the luciferase activity of miR-23-WT cells transfected with TGF-β1 mimics was remarkably reduced, whereas the luciferase activity of miR-23-MUT cells transfected with TGF-β1 was not significantly changed (Figure 4C). RIP assay demonstrated that TGF-β1 was remarkably enriched in the miR-23 group when compared with the control group, suggesting that miR-23 might serve as a TGF-β1 sponge (Figure 4D). Furthermore, expression of miR-23 was positively correlated with TGF-β1 expression in AF tissues (Figure 4E).

Discussion

Previous researches has proved that miRNAs participate in the development of multiple diseases. In the present study, the expression of miR-23 in RAA tissues of AF patients was significantly upregulated than that of SR patients. Furthermore, after miR-23 overexpression, the proliferation of AC16 cells was significantly promoted and cell apoptosis was suppressed. However, knockdown of miR-23 could significantly inhibit the growth and induce the apoptosis of AC16 cells. These data indicated that miR-23 promoted the proliferation and suppressed the apoptosis of cardiomyocytes. Latest studies have shown that miRNAs play an important role in disease progression by targeting related genes. Previous works have proved that several miRNAs participate in the proliferation of cardiac AF and fibroblasts. For example, highly expressed miR-21 promotes cardiac fibrosis through the CADM1/STAT3 pathway, which may be a potential therapeutic target[13]. MiRNA-29a suppresses fibroblasts proliferation and cardiac fibrosis *via* targeting the VEGF-A/MAPK signaling pathway[14]. In addition, miR-378 secreted by cardiomyocytes after mechanical stress plays a suppressive role in excessive cardiac fibrosis[15]. Meanwhile, miR-101a acts an anti-fibrotic role in cardiac fibrosis by modulating the TGF-β signaling pathway[16]. Furthermore, through the miR-135a-TRPM7-collagen pathway, miR-135a participates in the inhibition of cardiac fibrosis[17]. The above studies all indicate that miRNAs are of vital importance in AF and fibroblast proliferation. MiR-23, as a member of the miR-23-27-24 gene cluster, has been found to be involved in the development of many diseases. For instance, researches[18] have demonstrated that miR-23 participates in endothelial apoptosis and angiogenesis of cardiac ischemia and retinal vascular. Moreover, miR-23 regulates myelin formation and oligodendroglia development by downregulating lamin B1[19]. In addition, inhibition of miR-23 may protect myocardial function from ischemia-reperfusion injury *via* the restoration of glutamine metabolism[20]. Our findings revealed that compared with SR patients, the expression of TGF-β1 in AF patients was significantly upregulated. Luciferase reporter gene assay demonstrated that TGF-β1 could directly bind to miR-23. Meanwhile, RIP assay indicated that TGF-β1 was significantly en-

riched by miR-23. In addition, TGF-β1 expression was significantly upregulated after miR-23 overexpression. Expression of miR-23 was positively associated with TGF-β1 expression in AF tissues. All these above findings suggested that miR-23 promoted the proliferation of cardiomyocytes *via* targeting TGF-β1.

Conclusions

We identified that miR-23 could induce AF through upregulating TGF-β1. This suggested that miR-23 might serve as a candidate therapeutic target for AF.

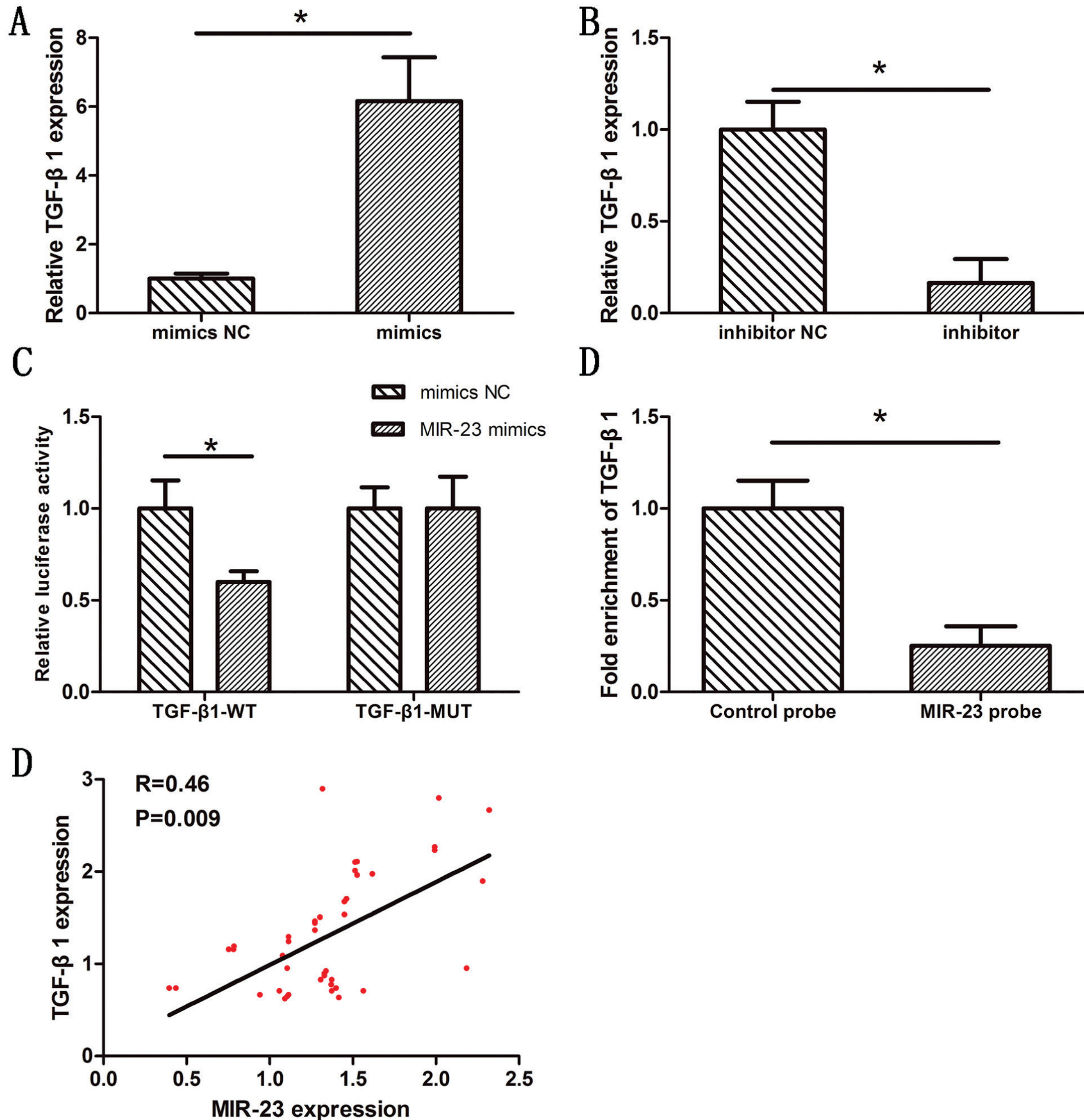

Figure 4. Interaction between miR-23 and TGF-β1. ***A,*** TGF-β1 expression was significantly increased in the miR-23 mimics group when compared with the control group. ***B,*** TGF-β1 expression was significantly decreased in the miR-23 inhibitor group when compared with the control group. ***C,*** Co-transfection of TGF-β1 and miR-23-WT in AC16 cells strongly decreased the luciferase activity, while co-transfection of TGF-β1 and miR-23-MUT did not change the luciferase activity. ***D,*** RIP assay demonstrated that TGF-β1 was significantly enriched in the miR-23 group when compared with the control group. ***E,*** Expression of miR-23 was positively associated with TGF-β1 expression in AF tissues. *p <0.05.

Conflict of interest

The authors declare no conflicts of interest.

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