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MicroRNA-199a regulates myocardial fibrosis in rats by targeting SFRP5

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Abstract. – OBJECTIVE: Myocardial fibrosis seriously affects normal heart function. This study focused on the role of microRNA-199a in regulating rat myocardial fibrosis by targeting secreted frizzled-related protein 5 (SFRP5).

MATERIALS AND METHODS: The *in vitro* myocardial fibrosis model was established by 10 μM isoproterenol (ISO) induction in cardiac fibroblasts (CFs) for 24 h. Expression levels of microRNA-199a, collagen I and α smooth muscle actin (α-SMA) were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Protein levels of SFRP5 and transforming growth factor-β1 (TGF-β1) in CFs were detected by Western blot. The binding condition between microRNA-199a and SFRP5 was verified by luciferase reporter gene assay. After transfection of microRNA-199a inhibitor or SFRP5 overexpression plasmid, proliferative and migratory rates of CFs were determined by cell counting kit-8 (CCK-8) and transwell assay, respectively.

RESULTS: ISO treatment remarkably upregulated microRNA-199a expression in CFs. Transfection of microRNA-199a inhibitor could inhibit proliferation, migration and cardiac fibroblast-to-myofibroblast transformation (CMT) of CFs. Luciferase reporter gene assay confirmed the binding of microRNA-199a to SFRP5 3'UTR. Moreover, SFRP5 overexpression reversed the effects of microRNA-199a inhibitor on proliferation, migration, and CMT of CFs.

CONCLUSIONS: MicroRNA-199a deficiency can inhibit the proliferative and migratory potentials of CFs, as well as CMT by targeting SFRP5, thus exerting the protective effect on myocardial fibrosis.

Key Words:
MicroRNA-199a, Myocardial fibrosis, SFRP5.

Introduction

The main features of myocardial fibrosis are excessive secretion and deposition of extracellular matrix (ECM, mainly collagen fibers) in the myocardial tissues. These pathological changes may result in cardiac dysfunction, metabolic disorder, myocardial stiffness enhancement, cardiac systolic dysfunction, and reduction of coronary blood flow. Uncontrolled cardiac injuries finally lead to malignant arrhythmia and myocardial infarction[1-3].

Cardiac fibroblasts (CFs) are important sources of ECM during the fibrotic progression. The phenotype of activated and proliferated CFs simultaneously transforms into myofibroblasts, which is called CMT. ECM is abundantly secreted and deposited during CMT, in which type I and type III collagen are excessively synthesized but less degraded[4,5]. Massive deposition of ECM ultimately leads to the proliferation of CFs. The main effector cells of myocardial fibrosis are myofibroblasts with a great abundance of α smooth muscle actin (α-SMA), and they also secrete a large amount of collagen, cytokines, growth factors, etc.[6-8]. Therefore, CFs exert a key role in the development of fibrosis.

MicroRNAs are a class of endogenous, non-coding RNAs consisting of approximately 22 bases. They participate in the post-transcriptional regulation of mRNA translation or degradation by binding to the 3'UTR of the target microRNA, thus mediating various biological processes, such as cell proliferation, differentiation, and apoptosis[9-11]. In recent years, microRNA is confirmed to be closely related to fibrosis. Certain microRNAs can regulate most of the fibrosis-related signaling pathways, such as transforming growth factor-β (TGF-β), mitogen-activated protein kinase (MAPK) and epithelial-mesenchymal transition (EMT) pathways[12-14]. Therefore, microRNAs may be utilized as key regulators in the development of cardiac fibrosis-related diseases.

Overexpression of miR-24 in CFs can inhibit expressions of fibrosis-related genes, and CMT[15]. Moreover, miR-214, miR-29, and miR-133a are reported to alleviate myocardial fibrosis by inhibiting the target genes TGFβ1, matrix

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metalloprotein-2 (MMP2), and connective tissue growth factor (CTGF), respectively[16-18]. Celastrol-induced downregulation of miR-21 and phosphorylated extracellular signal-regulated kinase (ERK) inhibits the miR-21/ERK axis, thus preventing myocardial fibrosis[19]. MiR-1 has a high myocardial specificity and shows a highest abundance relative to other microRNAs in the heart, accounting for 40%. A relative study[20] found higher serum level of miR-1 in rats with myocardial fibrosis, suggesting that miR-1 may serve as a novel hallmark for clinical evaluation of acute myocardial infarction.

The pathogenesis of myocardial fibrosis is complicated and has not been completely explained. Effective treatment for myocardial fibrosis is still lacked[21]. Therefore, it is urgent to develop a novel approach for prevention, diagnosis, and treatment of myocardial fibrosis. Current studies on the function of microRNA-199a in myocardial fibrosis are rarely reported. In this paper, we found out that microRNA-199a was highly expressed in rat myocardium with myocardial remodeling. We aim to elucidate the molecular mechanism of microRNA-199a in regulating myocardial fibrosis.

Materials and Methods

Reagents

Isoproterenol (ISO) administration (Southwest Pharmaceutical, Co., LTD, Chongqing, China); Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA); Fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY, USA); Trypsin (Wisent, Canada); Lipofectamine^{TM}2000, TRIzol (Invitrogen, Carlsbad, CA, USA); MicroRNA-199a inhibitor, over-SFRP5 (GenePharma, Shanghai, China).

Isolation and Culture of Primary CFs From Neonatal Rats

Neonatal rats with 6-7 days old were sacrificed and the thoracic cavity were cut open in an inverted T shape. Rat apex was taken and quickly washed in pre-cold phosphate-buffered saline (PBS) for 3-5 times. Apex tissues were cut in 1 mm^3, digested for 6-8 times with 5 min each, and terminated with DMEM containing 10% FBS. The filtered suspension was centrifuged at 150 g for 5 min. The precipitate was cultured in a bottle for 90 min, and those adherent cells were CFs. Cell passage was performed until 80% of confluence. Fourth-generation CFs were harvested for establishing the myocardial fibrosis model with 10 μM ISO treatment for 24 h. This study was approved by the Animal Ethics Committee of Southwest Medical University Animal Center.

Transfection

50 pmol of microRNA-199a inhibitor or 2.5 μL of LipoFiterTM was mixed in 250 μL of serum-free medium. They were mixed together and stand at room temperature for 20 min. The mixture was supplied for cell culture and fresh medium was replaced 4-6 h later.

Cell Counting Kit-8 (CCK-8)

Transfected CFs for 36 h were incubated with 10 μM ISO for another 24 h. These cells were inoculated in the 96-well plate with 1×10^4 cells per well and subjected to viability determination at the appointed time points. Before determination at 450 nm wavelength, 10 μL of CCK-8 (Dojindo, Kumamoto, Japan) was supplied per well for 3 h incubation.

Transwell Assay

Transfected CFs for 36 h were incubated with 10 μM ISO for another 24 h and prepared for suspension. 100 μL of cell suspension was applied in the transwell chamber, which was inserted in a 24-well plate with DMEM containing 20% FBS. At 48 h later, methanol fixation for 30 min and violet crystal dye for 20 min were performed for those penetrating cells, which were finally captured using a microscope for counting.

Luciferase Reporter Gene Assay

HEK293 cells were co-transfected with 20 nmol/L microRNA-199a or control and 600 ng FRP5 3'UTR-pmirGLO for 36 h, respectively. Relative luciferase unit of Firefly (RLU-1) and Renilla (RLU-2) was determined for calculating the luciferase intensity as RLU-1/RLU-2.

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

Total RNA from tissues or cells were extracted by TRIzol, reversely transcribed into complementary deoxyribose nucleic acid (cDNA) and amplified by qRT-PCR. Relative levels of microRNA-199a, SFRP5, Collagen I, Vimentin, DDR2, α-SMA, and Tensin were calculated.

Western Blot

Total protein from cells or tissues was extracted using radioimmunoprecipitation assay (RIPA) (Beyotime, Shanghai, China) and loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) at 300 mA for 100 minutes, it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 hours. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for all statistical analysis, and GraphPadPrism5.0 (La Jolla, CA, USA) was used for figure editing. Data were represented as mean ± SD. The *t*-test and chi-square test were used for analyzing measurement and categorical data, respectively. $p<0.05$ indicated the significant difference.

Results

MicroRNA-199a Expression Increased in ISO-Treated CFs

Here we established the *in vitro* myocardial fibrosis model by ISO treatment in CFs. QRT-PCR was conducted to determine mRNA levels of microRNA-199a, SFRP5, Collagen I, and α-SMA in CFs treated with or without ISO. MicroRNA-199a expression increased, while SFRP5 expression decreased after ISO treatment (Figure 1A, 1B). Moreover, fibrosis-related genes, Collagen I, and α-SMA were upregulated by ISO treatment (Figure 1C). To further explore the biological function of microRNA-199a, we constructed microRNA-199a inhibitor and confirmed its transfection efficacy in CFs (Figure 1D). As a fibrosis-related gene, the protein level of TGF-β1 was markedly upregulated by ISO treatment, but was inhibited by the transfection of microRNA-199a inhibitor (Figure 1E).

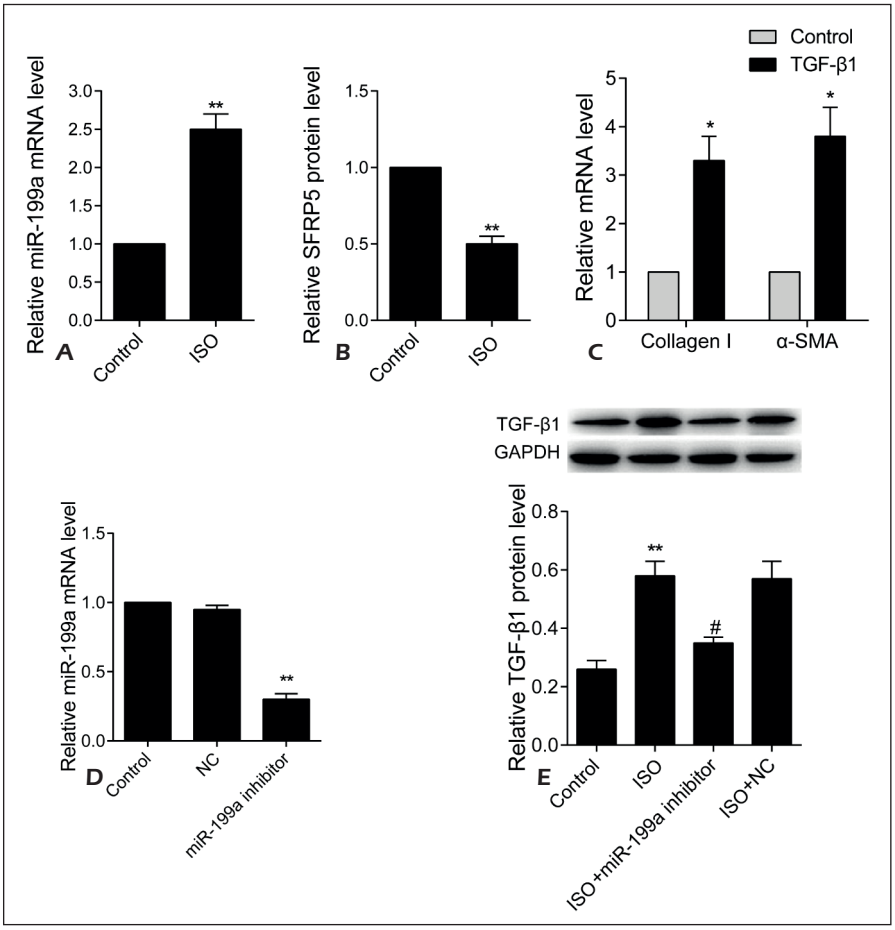

Figure 1. MiR-199a expression increased in ISO-treated CFs. **A**, Relative mRNA level of miR-199a in CFs treated with or without ISO. **B**, Relative mRNA level of SFRP5 in CFs treated with or without ISO. **C**, Relative mRNA level of Collagen I and α-SMA in CFs treated with or without ISO. **D**, Transfection efficacy of miR-199a inhibitor in CFs. **E**, Western blot analyses of TGF-β1 in CFs, ISO-treated CFs, ISO-treated CFs transfected with miR-199a inhibitor, and ISO-treated CFs transfected with negative control. $^{**}p<0.01$ compared with control group; $\#p<0.05$ compared with ISO group. $^{*}p<0.05$; ISO, isoproterenol.

Knockdown of MicroRNA-199a Attenuated Proliferative, Migratory Rates and CMT of CFs

Rat CFs transfected with negative control or microRNA-199a inhibitor were treated with ISO for 0, 24, 48, and 72 h, respectively. Viability curve showed the remarkable proliferative inhibition in CFs transfected with microRNA-199a inhibitor compared with controls (Figure 2A). Subsequently, mRNA levels of fibrotic genes in CFs with ISO treatment for 72 h were determined. As the data revealed, mRNA levels of Vimentin and DDR2 increased, while mRNA levels of α-SMA and Tensin decreased by microRNA-199a knockdown, suggesting a reverse of CMT (Figure 2B). Transwell assay demonstrated that CFs transfected with microRNA-199a inhibitor showed a fewer migratory CFs than controls, indicating the inhibited migratory potential (Figure 2C, 2D).

SFRP5 Was the Target Gene of MicroRNA-199a

Both protein and mRNA levels of SFRP5 were downregulated in CFs transfected with microRNA-199a inhibitor (Figure 3A, 3B). We speculated that SFRP5 may be a potential target gene of microRNA-199a. By constructing the wild-type and mutant-type SFRP5 plasmids, luciferase reporter gene assay showed a remarkable reduction in luciferase intensity of wild-type group, confirming the binding of microRNA-199a to the 3'UTR of SFRP5 (Figure 3C). Hence, we confirmed that SFRP5 was the target gene of microRNA-199a.

SFRP5 Overexpression Reversed the Regulatory Functions of MicroRNA-199a Inhibitor in CFs

To elucidate the possible function of SFRP5 in CFs, overexpression plasmid of SFRP5 (over-SFRP5) was constructed. The CCK-8 assay indicated no significant difference in cell viability between CFs co-transfected with over-SFRP5 and microRNA-199a inhibitor with those of controls (Figure 4A). Moreover, mRNA levels of Vimentin, DDR2, α-SMA, and Tensin did not markedly change after co-transfection of over-SFRP5 and microRNA-199a inhibitor in CFs (Figure 4B). Transwell assay failed to reveal the difference in the migratory rate of CFs co-transfected with over-SFRP5 and microRNA-199a inhibitor with those of controls (Figure 4C, 4D).

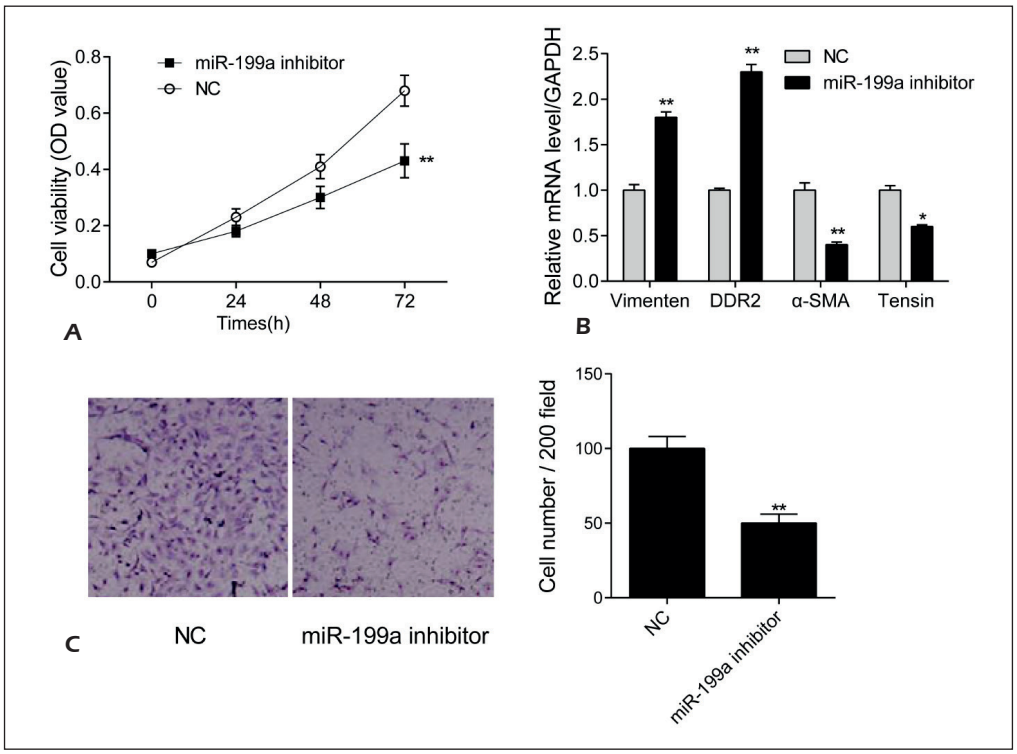

Figure 2. Knockdown of miR-199a attenuated proliferative, migratory rates and CMT of CFs. **A**, Transfection of miR-199a inhibitor inhibited ISO-induced proliferation in CFs. **B**, Transfection of miR-199a inhibitor increased mRNA levels of Vimentin and DDR2, while decreased mRNA levels of α-SMA and Tensin in CFs. **C**, Transfection of miR-199a inhibitor inhibited ISO-induced migration in CFs. n=3, $^{**}p<0.01$ compared with NC group.

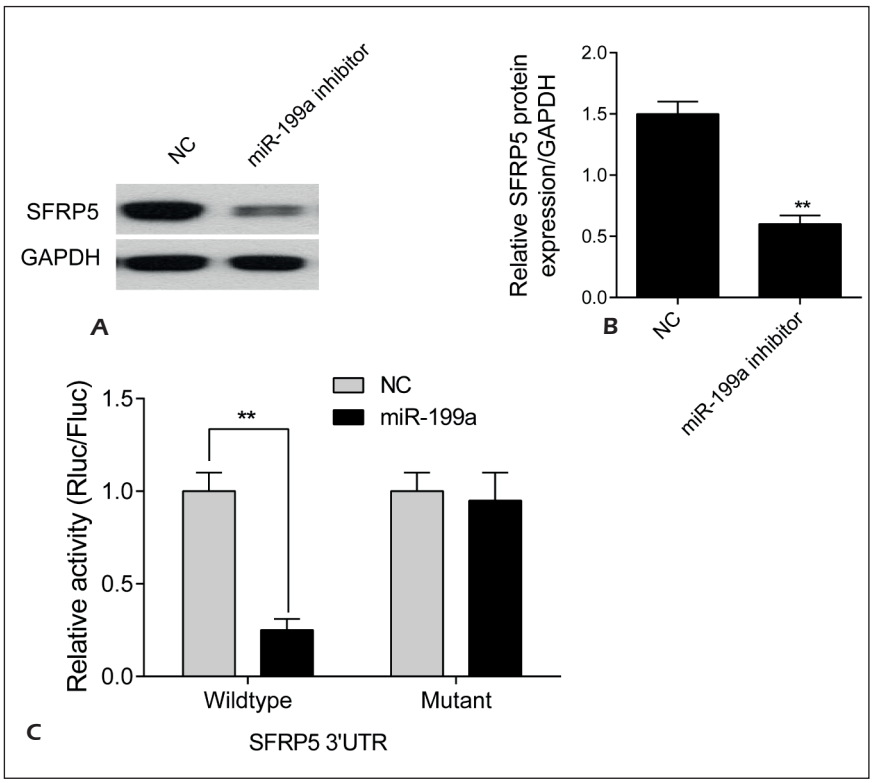

Figure 3. SFRP5 was the target gene of miR-199a. **A**, Transfection of miR-199a inhibitor downregulated protein level of SFRP5 in ISO-induced CFs. **B**, Transfection of miR-199a inhibitor downregulated mRNA level of SFRP5 in ISO-induced CFs. **C**, Luciferase intensity decreased in the wild-type group, while the mutant-type group did not significantly change. **$p<0.01$ compared with NC group.

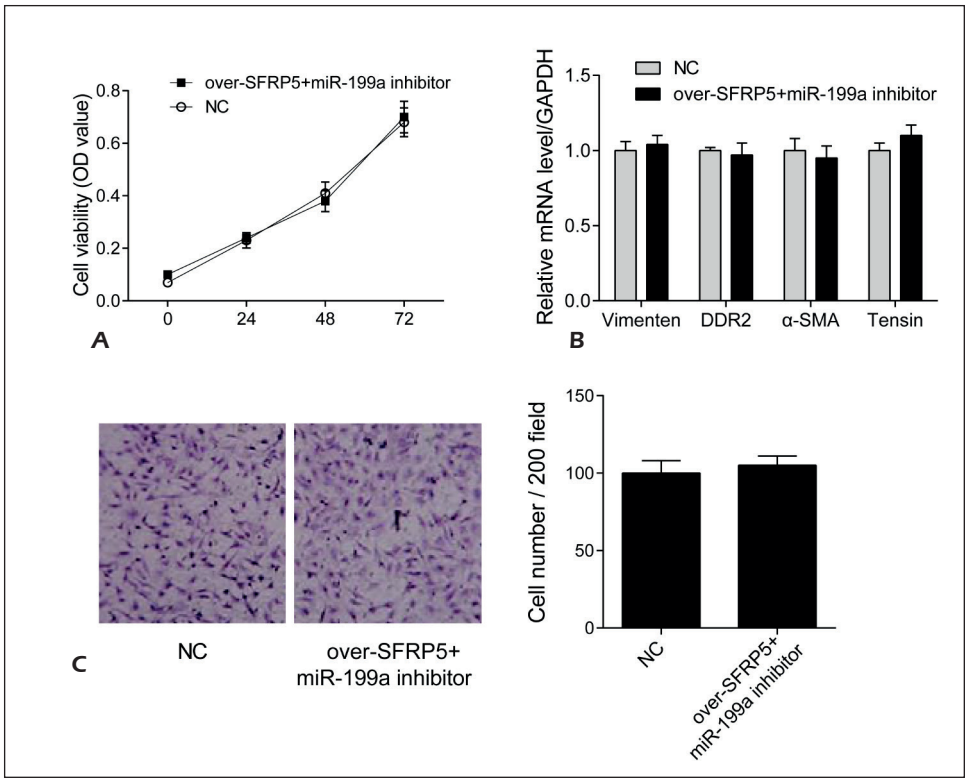

Figure 4. SFRP5 overexpression reversed the regulatory functions of miR-199a inhibitor in CFs. **A**, CCK-8 assay showed viability in CFs co-transfected with over-SFRP5+miR-199a inhibitor and controls. **B**, The mRNA levels of Vimentin, DDR2, α-SMA, and Tensin in CFs co-transfected with over-SFRP5+miR-199a inhibitor and controls. **C**, Transwell assay showed migration in CFs co-transfected with over-SFRP5+miR-199a inhibitor and controls. n=3, **$p<0.01$ compared with NC group.

The above data suggested that the inhibitory effects of microRNA-199a knockdown on proliferative, migratory rates and CMT of CFs were reversed by SFRP5 overexpression.

Discussion

Previous studies[22,23] have shown that microRNA-199a is downregulated in myocardial tissue of hypoxic preconditioning rats. Mice overexpressing microRNA-199a present significant cardiac hypertrophy and inhibition of cardiomyocyte autophagy. Scholars[24-26] have reported that microRNA-199a exerts its biological function by targeting the GSK3β/mTOR pathway. Srf-induced microRNA-199a knockdown suppresses phenotypic transformation and migratory potential in high-glucose treated cells[27]. Fornari et al[28] found that microRNA-199a promotes cardiomyocyte proliferation in rats with myocardial infarction. In the myocardial fibrosis, however, the specific mechanism of microRNA-199a has been rarely reported.

This work indicated that microRNA-199a was highly expressed in ISO-induced CFs, the *in vitro* myocardial fibrosis model. We speculated that microRNA-199a may be involved in the development of myocardial fibrosis. Subsequently, we found that TGF-β1 expression was remarkably downregulated after microRNA-199a knockdown in CFs, suggesting that microRNA-199a may promote myocardial fibrosis. Luciferase reporter gene assay further verified the binding of microRNA-199a to SFRP5 3'UTR, suggesting that SFRP5 was a target gene for microRNA-199a. Knockdown of microRNA-199a could upregulate myocardial fibroblast markers Vimentin and DDR2, but downregulated fibroblast markers α-SMA and Tensin. Moreover, microRNA-199a knockdown suppressed CMT, proliferative and migratory potentials of CFs, thus protecting myocardial fibrosis.

SFRPs are antagonists of the Wnt pathway, including 8 members in three subgroups based on the sequence homology. In the SFRPs family, SFRP1, SFRP2, and SFRP5 belong to the first subgroup, SRP3 and SFRP4 are the second subgroup, and Sizzled, Sizzled2, and Crescent are the third subgroup[29-31]. Researches[32,33] have shown that SFRP2 regulates the Wnt pathway by competing with Frizzled, the specific receptor of the Wnt pathway, through the homologous CRD (cysteine rich domain). Chatani et al[34] found that Wnt5a enhances the proliferative and migratory rates of hepatic stellate cells, which are inhibited by SFRP5. In addition, CCL4 decreases liver expression and plasma level of Wnt5a, while SFRP5 knockdown greatly enhances the degree of CCL4-induced liver fibrosis. We may conclude that SFRP5 improves fibrosis in the liver by inhibiting the Wnt5a/Fz2 axis. In this study, microRNA-199a inhibitor markedly decreased SFRP5 expression in CFs. More importantly, SFRP5 overexpression reversed the inhibitory effects of microRNA-199a inhibitor on proliferative, migratory potentials and CMT of CFs.

To sum up, our results indicated that microRNA-199a could directly bind to SFRP5 3'UTR. Besides, microRNA-199a knockdown suppressed SFRP5 expression, reduced proliferative and migratory rates, and inhibited CMT of CFs, thereafter alleviating the secretion and deposition of ECM. It is believed that microRNA-199a inhibition exerted anti-fibrosis role by targeting SFRP5.

Conclusions

We found that microRNA-199a deficiency can inhibit the proliferative and migratory potentials of CFs, as well as CMT by targeting SFRP5, thus exerting a protective effect on the myocardial fibrosis.

Conflict of Interests

The authors declare that they have no conflict of interest.

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