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MicroRNA-29a suppresses cardiac fibroblasts proliferation via targeting VEGF-A/MAPK signal pathway

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ABSTRACT

Cardiac fibroblasts proliferation is the most important pathophysiological character of cardiac fibrosis while the underlying mechanisms are still incompletely known. MicroRNAs (miRNAs) regulate gene expression by binding to specific sites. Studies have been indicated that miRNA-29a play a key role in cardiac fibrosis. VEGF-A carries out its functions through MAPK signaling pathway in cardiac fibrosis. Existing proofs predict that the VEGF-A is one of the potential targets of miRNA-29a. We therefore probe the role of miRNA-29a and its latent target VEGF-A during cardiac fibrosis. In our study, miRNA-29a was down-regulated while VEGF-A was up-regulated in cardiac fibrosis tissues. The rat cardiac fibroblasts that were transfected with miRNA-29a inhibitor exhibited low-expression of miRNA-29a, enhanced VEGF-A protein and mRNA expression. Nevertheless, the cardiac fibroblasts transfected with miRNA-29a mimics obtained the opposite expression result. Furthermore, over-expression of miRNA-29a suppresses cardiac fibroblasts proliferation. In conclusion, these results suggested that miRNA-29a suppresses cardiac fibrosis and fibroblasts proliferation via targeting VEGF-A/MAPK signal pathway implicating that miRNA-29a might play a role in the treatment of cardiac fibrosis.

1. Introduction

Cardiac fibrosis is an important pathological feature of cardiac remodeling in heart diseases [1,2]. Excessive deposition of collagen and extracellular matrix (ECM) will lead to cardiac fibrosis and eventually causes organ dysfunction while fibroblasts take a predominant part in this process [3,4]. Myofibroblasts, the activation of fibroblasts, are characterized by over-expression of collagen, ECM proteins, α-smooth muscle actin (α-SMA) and other fibrosis related protein [5].

MicroRNA (miRNA) is short, evolutionarily conserved, small noncoding RNAs that exist in mammalian cells of 19–25 nucleotide-long molecules in length [6]. Binding to the 3′ untranslated region (3′-UTR) of the complementary mRNA sequence, miRNA could block translation, silence gene expression or even degrade mRNA which occurs less frequently [7]. Through above ways, miRNA regulates proliferation, epithelial mesenchymal transition (EMT), metastasis and other cellular processes [8,9]. Recent studies have shown that miRNA-29 family involved in heart diseases and these miRNAs are designated cardio-miRNAs [10]. miRNA-29 family that includes miRNA-29a, miRNA-29b and miRNA-29c play a role in cardiac fibrosis [11–13]. However, the molecular mechanisms of miRNA-29a in cardiac fibrosis, is not completely understood.

Vascular endothelial growth factor-A (VEGF-A), considered as the key regulator in angiogenesis and tumor proliferation, could activate fibroblasts to myofibroblats and eventually contribute to cardiac fibrosis [14,15]. Caveolin-1 degrades the internalized VEGF receptor 2 (VEGFR2) which integrate with VEGF-A, lowing the level of caveolin-1 lead to fibrosis via up-regulating VEGF signaling [16]. VEGFR signaling is regulated by FRS2α, which know to be involved in fibroblast growth factor (FGF) receptor signaling, could lead to activation of MAPK signaling [17].

It still remains unclear that what the exact role miRNA-29a plays in cardiac fibrosis. Therefore, we probed into the expression of miRNA-29a in cardiac fibrosis and miRNA-29a's biological behaviors including cell proliferation. The association between miRNA-29a and VEGF-A expression was also detected in this study.

Abbreviation: miRNAs, icroRNAs; PDGF, platelet-derived growth factor; ECM, extracellular matrix; DMSO, dimethyl sulfoxide; OD, optical density; VEGF-A, vascular endothelial growth factor A; 3′-UTR, 3′ untranslated region.

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Supporting our hypothesis, we found that miRNA-29a modulates VEGF-A mediated activation of MAPK signaling in cardiac fibrosis. These results further support miRNA-29a acts as a key regulator of pathological cardiac fibrosis, suppresses cardiac fibroblasts proliferation and fibrosis by targeting VEGF-A/MAPK signal pathway.

2. Materials and methods

2.1. Reagents

Isoprenaline (ISO) was purchased from Shanghai He Feng Chemistry Plant (Shanghai, China). PICP and PIIINP kits were obtained from Huamei Biotechnology Institute (Wuhan, China). Mouse monoclonal antibodies for α-SMA, collagen I were purchased from Boster (Wuhan, China), VEGF-A polyclonal antibody was purchased from Abcam (Cambridge, UK). ERK1/2, p-ERK1/2 antibodies were purchased from Cell Signaling (Beverly, MA, USA.). VEGF-A, α-SMA, collagen I, β-actin primers were produced by the Shanghai San Gong Biological and Technological Company (Shanghai, China). Streptavidin peroxidase (SP) immunohistochemical kit was obtained from the Zhong Shan Biotechnology Corporation (Beijing, China). Reverse transcription reaction system and SYBR Green RealMaster Mix were purchased from MBI Fermentas Corporation (Ontario, Canada). Secondary antibodies for goat anti-rabbit immunoglobulin (Ig) G horse radish peroxidase (HRP), rabbit anti-goat IgG HRP, goat anti-mouse IgG HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA).

2.2. Animals and induction of model

Forty adult male Sprague-Dawley (SD) rats weighing 200–220 g were purchased from the Experimental Animal Center of Anhui Medical University. SD rats were randomly divided into two groups (twenty rats per group). All animal experiments were performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the NIH guidelines (Guide for the care and use of laboratory animals). The research protocol is approved by the Anhui Medical University Institutional Animal Care and Use Committee.

Cardiac fibrosis was produced by common ISO as previously described [18]. SD rats were injected subcutaneously with ISO (15 mg (kg body mass)$^{-1}$, once a day) to induce experimental cardiac fibrosis (ISO group) or the same volume of normal saline (Saline group). Three weeks later, after heparin injection (625U/100 g), deep anesthesia was induced with pentobarbital (50 mg/100 g body weight). The heart was removed and washed in PBS medium. Heart tissue specimens were fixed in 4% phosphate-buffered paraformaldehyde. Other specimens were snap-frozen in liquid nitrogen and stored at −80 °C for RNA and protein analysis.

2.3. Determination of cardiac weight index (CWI)

The hearts were then weighed, and the CWI was calculated as heart mass (mg) divided by body mass (g).The ratio of left ventricular weight to body weight (LVW/BW) was calculated.

2.4. Histological analysis

Hearts were isolated, fixed, dehydrated, paraffin-embedded, and sectioned as previously described. Tissue sections (5 μm thick) were stained with H&E and Masson's trichrome stain. They were mountedon normal glass slides and stained with Masson trichrome forhistological examination. For the collagen volume fraction (CVF) analysis, ×400 magnification images (15–20 from 3 to 5 sections) was performed with Image-Pro 6.2 software and assessment of CVF used the following formula: CVF = collagen area/total area.

2.5. Immunohistochemistry

The tissue samples were embedded in paraffin and cut into 4 μm thick sections. Sections (4 μm thick) mounted on poly-L-lysine-coated glass slides, and used for immunohistochemistry. The sections were dewaxed in xylene and dehydrated in alcohol and antigen retrieval was achieved by microwaving in citric saline for 15 min. Endogenous peroxidase activity was blocked by 3% H_2O_2 at 37 °C for 20 min. Then sections were incubated in 10% normal goat serum at 37 °C for 30 min to block nonspecific protein-binding sites. Sections were then incubated in primary antibodies against VEGF-A (1:200) and α-SMA (1:50). Primary antibodies were detected by rabbit anti-mouse and goat anti-rabbit non-biotinylated regents (Zhong Shan, Beijing, China), according to the instructions of the manufacturer. The sections were incubated with biotinylated secondary antibody for 60 min at room temperature, washed in PBS, and stained with DAB (3,3′-diaminobenzidine). Slides were counter stained with hematoxylin before dehydration and mounting. At least five random fields of each section were examined, and semi-quantitative evaluations were analyzed with a Photo and Image Auto analysis System (Image-pro-plus, China).

2.6. Immunofluorescence

To detect the expression of the VEGF-A protein, immunofluorescence was also carried out on cardiac fibroblasts induced with PDGF-BB. Cells were plated on glass coverslips and fixed with 70% ethanol for 20 min at −20 °C for VEGF-A immunostaining or 4% paraformaldehyde for 15 min at room temperature for other proteins immunostaining. In short, cells were first incubated for 30 min at room temperature with 2% bovine serum albumin (BSA) to reduce nonspecific binding. Then followed by overnight incubation at 4 °C with antibodies for rabbit polyclonal VEGF-A antibody (1:50) and mouse anti-α-SMA (1:100). After washing in PBS, sections were incubated in the dark for 3 h at 37 °C with fluorescein isothiocyanate (FITC) -conjugated goat anti-rabbit IgG (1:50; Boster, Wuhan, China) and tetramethylrhodamineisothiocyanate (TRITC)-conjugated goat anti-mouse IgG (1:50, Boster). After washing with PBS, the slies were mounted with 50% glycerol and 50% PBS. Coverslips were mounted on to microscope slides using fluorescence mounting medium (Dako) and observed under an inverted fluorescence microscope (Olympus).

2.7. ELISA

Collagen levels in serum were measured using ELISA kits (CUSABIO, Hua Mei Biotech CO., LTD. Wuhan, China) according to the manufacturer's instructions. OD values were determined at 450 nm on a micro-plate reader (KHB ST-360, Shanghai, China). Collagen concentrations were quantified by comparison to the standard curves. Samples were analyzed in triplicate.

2.8. Cell culture and treatment

Cardiac fibroblasts were extracted from neonatal rats purchased from animal center of Anhui Medical University.The fibroblasts were seeded at a concentration of 2×10^4 cells/cm^2, and cultured with Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich Corp., St.Louis, MO, USA) containing 10% (v/v) fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT, USA) in a CO_2 incubator (Thermo Electronic, USA) with a humidified atmosphere of 5% CO_2 at 37 °C. Cells were passaged every 3 days. 24 h before transfection