

Original Article

MiR-24 can promote the effects of induction of pluripotent stem cells on myocardial infarction fibrosis

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Abstract: Myocardial infarction and fibrosis is one pathological process for heart remodeling after cardiac infarction, and often causes cardiac function impairment or even heart failure. Induced pluripotent stem cells (iPS) provide new insights for treating cardiovascular diseases. MicroRNA (miR)-24 exerts certain roles in cardiac infarction and remodeling. Its potentially synergistic function with iPS in treating myocardial fibrosis after infarction, however, has not been fully illustrated nor did its mechanisms. Healthy male Wistar rats were randomly divided into acute myocardial infarction (AMI) group, which was prepared for AMI model using blockade of left descending branch of coronary artery; iPS group, which received mouse-derived iPS after AMI surgery; miR-24 group, which was transfected with miR-24 lentivirus; and miR-24-iPS combined group. Type M ultrasound was used to evaluate cardiac function. Western blotting was employed to detect myocardial fibrosis and MMP-2 expression. ELISA and test kit were used for measuring serum inflammatory factors IL-6 or TNF- α and caspase 3 activity, respectively. Either single or combined therapy of miR-24 and iPS effectively improved cardiac function indexes, inhibited type I collagen, MMP-2, IL-6, TNF- α or caspase 3 expressions ($P < 0.05$ compared to AMI model group). Combined therapy had more potent improvements on myocardial fibrosis ($P < 0.05$ compared to either iPS or miR-24 group). MiR-24 could facilitate iPS to regulate extracellular matrix and type I collagen, modulate apoptosis and inflammation, thus improving myocardial fibrosis after infarction.

Keywords: MiR-24, induced pluripotent stem cells, acute myocardial infarction, fibrosis

Introduction

The incidence of cardiovascular diseases such as coronary heart disease (CHD) is rapidly increasing due to aging population and life style transition. Acute myocardial infarction (AMI) can be induced by the rupture of atherosclerosis plaque, leading to platelet activation and recruiting, eventually leading to acute blockade of coronary artery [1, 2]. AMI is often accompanied with ventricular remodeling, during which geometric structure, morphology and function of infarcted or non-infarcted ventricular regions are changed [3, 4]. Ventricular remodeling is one chronically progressed process. AMI-related fibrosis is one pathological process during late phase of cardiac remodeling after infarction, and often causes cardiac function impairment or even heart failure, thus severely affecting patient's life quality [5]. Extracellular matrix (ECM) is one important pathogenesis mecha-

nism during ventricular remodeling. The remodeling of ECM forces artery to expand to maintain normal vascular tension, inducing the release of cytokines or growth factors from mononuclear or macrophage, thus affecting production and degradation of ECM, whose degradation further recruits more inflammatory cells toward injury sites for participating myocardial collagen fibrosis [6, 7]. ECM consists of various components such as macro-molecules and structural proteins, including collagen, glycosaminoglycan, fibronectin, proteoglycan, elastin and laminin proteoglycan, among which myocardial collagen is the major component [8, 9]. Therefore the decrease of myocardial infarcted fibrosis is critical for AMI treatment. Classical drugs and intervention therapy, however, obtained unsatisfactory efficacy [10]. The identification of novel strategy for treating myocardial infarction and fibrosis is one research hotspot.

iPS in treating cardiac infarction

Induced pluripotent stem cells (iPS) can be derived from various somatic cells of animals, and are generated by re-programming and transformation, followed by proliferation and specific differentiation into various somatic cells, including myocardial cells with normal constriction and excitability functions [11]. iPS can be derived from autograft cells of patients, thus overcoming immune rejection and ethical issues of traditional embryonic stem cells (ESCs), and has become one hotspot in cardiovascular disease treatment [12, 13]. microRNA (miR) is one small molecule RNA for regulating biological functions and is widely distributed in both animals and plants [14, 15]. Study has reported that miR-24 exerted certain functions in myocardial infarction and heart remodeling [14, 16]. Whether miR-24 exerts synergistic functions with iPS to improve myocardial fibrosis, however, has not been fully illustrated, nor did the related mechanism.

Materials and methods

Experimental animals

Healthy male Wistar rats (2 months old, SPF grade, body weight 250 ± 20 g) were purchased from laboratory animal center of Guangdong Pharmaceutical University and were kept in an SPF grade facility with fixed temperature ($21\pm 1^\circ\text{C}$) and relative humidity (50~70%), with a 12 h/12 h light/dark cycle.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Guangzhou General Hospital of Guangzhou Command.

Major materials and equipment

Mouse-derived iPS was kept in-house. Leukocyte inhibitor factor was purchased from ESGRO (US). DMEM culture medium was purchased from Life (US). Pentobarbital sodium and lidocaine were purchased from Zhaohui (China). PVDF membrane was purchased from Pall Life Sciences (US). Western blotting reagents were purchased from Beyotime (China). ECL reagent was purchased from Amersham Biosciences (US). Rabbit anti-mouse MMP-2 monoclonal antibody, rabbit anti-mouse Col-1 monoclonal antibody and goat anti-rabbit horseradish peroxidase (HRP)-labelled IgG secondary antibody were purchased from Cell Signaling

(US). IL-6 and TNF- α ELISA kit were purchased from R&D (US). RNA extraction kit and reverse transcription kit were purchased from Axygen (US). Microscopic surgical instruments were purchased from Suzhou Medical Instrument (China). Microplate reader was purchased from BD (US). Other common reagents were purchased from Sangon (China).

Animal grouping and treatment

A total of 60 healthy male Wistar rats were randomly divided into 4 groups (N=15 each): (1) AMI group, which received blockade in left descending branch of coronary artery to generate AMI model; (2) iPS group, which received mouse-derived iPS after AMI surgery; (3) miR-24 group, which received miR-24 lentivirus transfection after AMI surgery; and (4) miR-24-iPS combined group, in which miR-24 lentivirus and iPS cells were used for transfection or implantation in AMI rats.

Rat AMI model generation

After general anesthesia, rat was fixed in a supine position. After shaving and sterilization of surgical area, tracheal intubation was performed under visual assistance. The ventilation machine was connected for assisted respiration (tidal volume 4 ml/kg, respiration frequency 80 per min). An incision was made via the fourth rib on left chest; the heart was then exposed by blunting separation of subcutaneous tissues and muscle. The left coronary artery was ligated using 7-0 nylon suture via 2/3 myocardial layer on the bottom edge (1~2 mm) of left ventricular ear. Electrocardiogram was monitored in real time during the surgery. The model was generated when heart tissues turned white accompanied with continuous elevation of ST segment ($>1/2$ R wave) in a single peak. In sham group, the heart was exposed but without ligation.

Preparation and injection of iPS cells

iPS cells were resuscitated and passed in culture. Before transplantation, iPS cells were digested by collagenase, and cultured for 3 days using DMEM medium containing 1000 IU/ml leukocyte inhibitory factor. Rats in iPS cell group were injected with 2×10^7 per ml cell suspension in PBS into the infarcted area during AMI model preparation. Penicillin was applied post-op to prevent infection.

iPS in treating cardiac infarction

Table 1. Effect of miR-24 combined with iPS transplantation on cardiac function of AMI rats

Group	LVEDD (mm)	LVESD (mm)	LVMI
AMI	0.62±0.06	0.44±0.07	3.85±0.17
iPS	0.51±0.02*	0.36±0.05*	3.13±0.18*
MiR-24	0.56±0.05*	0.39±0.03*	3.19±0.03*
MiR-24+iPS	0.47±0.05* ^{#,Δ}	0.31±0.03* ^{#,Δ}	2.51±0.03* ^{#,Δ}

Note: *P<0.05 vs AMI group; #P<0.05 vs iPS group; ΔP<0.05 vs miR-24 group.

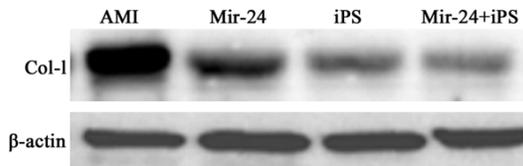


Figure 1. Effect of miR-24 combined with iPS cell transplantation on myocardial type I collagen fiber in AMI rats.

MiR-24 lentivirus construct and transfection

Lentivirus plasmid and miR-25 plasmid were co-transfected into 293T cell line. Viral particles were packaged, harvested and concentrated. MiR-24 lentivirus was injected into local myocardial tissues (4×10^7 TU).

Post-op evaluation of cardiac function

VEVO2100M ultrasound (Visual Sonic, Canada) was used to describe the change of cardiac function at 28 days post-op, including left ventricular quality index, ventricular systolic and diastolic diameter. In brief, rats were fixed in a supine position. Ultrasound probe (model 15L8) was placed near the chest for a horizontal section of left ventricular short axis mammillary muscle. After obtaining clear 2D image, M-type ultrasound cardiac graphic was applied to measure left ventricular end-stage diastolic diameter (LVEDD) and left ventricular end-stage systolic diameter (LVESD). The left ventricular quality index was calculated based on the formula.

Sample collection

Blood sample was collected from abdominal aorta from all rats using vacuum tubes. Blood samples were placed at room temperature for 30min. After blood clotting, 10-min centrifugation was performed under 4°C for 3600 rpm.

The supernatant was saved and stored at -20°C for further use. Myocardial tissues were collected from rats and stored at -80°C.

ELISA for serum IL-6 and TNF-α levels

Rat serum samples were tested for serum IL6 and TNF-α level following the manual instruction of ELISA kit. A linear regression function was generated based on concentrations and respective OD values of standard samples. The concentration in serum sample was then deduced on the regression model from OD values.

Western blotting for type I collagen and MMP-2 protein expressions

Total tissue proteins were extracted from myocardial tissues. In brief, cells were lysed on ice for 15~30 min, with ultrasound treatment (5 s, 4 times). After centrifugation at 10 000 g for 15 min (4°C), the supernatant was saved, quantified and stored at -20°C for Western blot assay. Proteins were separated in 10% SDS-PAGE, and were transferred to PVDF membrane by semi-dry method under an electrical field (110 mA, 1.5 h). Non-specific binding sites were removed by 5% defatted milk powder for 2 h. Anti-Col-1 (1:1000) or anti-MMP-2 monoclonal antibody (1:2000) was added for 4°C overnight incubation. After PBST washing, goat anti-rabbit secondary antibody (1:2000) was added for 30 min incubation at room temperature. ECL reagent was then added for developing the membrane for 1 min, followed by X-ray exposure. The result was obtained by protein imaging system and Quantity One software (Gene, US) for measuring band density. Each experiment was replicated for four times (N=4) for statistical analysis.

Caspase 3 activity assay

Caspase 3 activity in myocardial tissues was evaluated using test kit from all groups. In brief, cells were digested by trypsin, and were centrifuged at 600 g for 5 min under 4°C. The supernatant was discarded, followed by the addition of cell lysis buffer and iced incubation for 15 min. The mixture was then centrifuged at 20 000 g for 5 min under 4°C, followed by the addition of 2 mM Ac-DECD-pNA. OD values at 450 nm wavelength were measured to reflect caspase 3 activity.

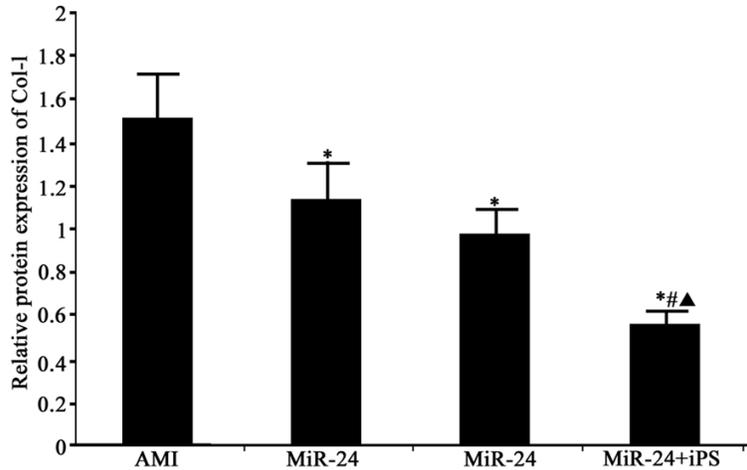


Figure 2. Analysis of miR-24 plus iPS transplantation on type I collagen of AMI rats. *, P<0.05 vs AMI group; #, P<0.05 vs iPS group; Δ, P<0.05 vs miR-24 group.

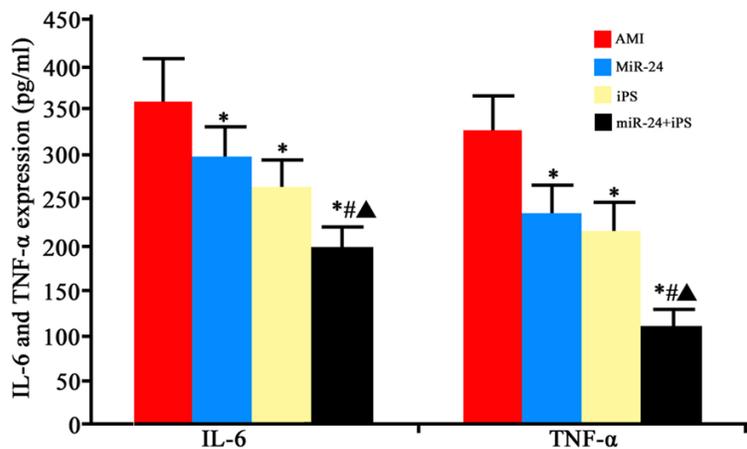


Figure 3. Effect of miR-24 and/or iPS transplantation on serum IL-6 and TNF-α levels in AMI rats. *, P<0.05 vs AMI group; #, P<0.05 vs iPS group; Δ, P<0.05 vs miR-24 group.

and iPS cell transplant on the cardiac function change in AMI model. Results showed significantly smaller LVESD and LVEDD in either single or combined treatment group (P<0.05 compared to AMI group). Left ventricular mass index (LVMI) showed similar pattern as those for LVESD and LVEDD. Combined therapy including miR-24 and iPS transplantation obtained more significant improvements on myocardial functions (P<0.05 compared to iPS or miR-24 group). These results suggested that miR-24 combined with iPS transplantation significantly improved cardiac function of AMI rats (Table 1).

Expressions of Bcl-2 and Bax proteins and effects of iPS cell transplantation

Western blotting was employed to describe the effect of either single or combined treatment using miR-24 or iPS transplantation on type I collagen in myocardial tissues from all groups of rats. Results showed lower myocardial type I collagen expression after either single or combined treatment (P<0.05 compared to AMI group). Combined treatment of miR-24 and iPS obtained more significantly inhibitory effects on type I collagen (P<0.05 compared to either of miR-24 or iPS group, Figures 1 and 2). Therefore the combined treatment of miR-24 and iPS transplantation significantly inhibited type I collagen expression.

Western blotting was employed to describe the effect of either single or combined treatment using miR-24 or iPS transplantation on type I collagen in myocardial tissues from all groups of rats. Results showed lower myocardial type I collagen expression after either single or combined treatment (P<0.05 compared to AMI group). Combined treatment of miR-24 and iPS obtained more significantly inhibitory effects on type I collagen (P<0.05 compared to either of miR-24 or iPS group, Figures 1 and 2). Therefore the combined treatment of miR-24 and iPS transplantation significantly inhibited type I collagen expression.

Serum IL-6 and TNF-α levels in AMI rats after miR-24 and/or iPS transplantation

ELISA approach was employed to detect serum IL6 and TNF-α level after either single or combined treatment using miR-24 or iPS transplantation. Results showed inhibition on serum IL6 and TNF-α secretion in AMI rats after either single or combined treatment (P<0.05 compared to AMI group). Combined treatment of

Statistical analysis

SPSS 19.0 software was used to process all data, of which measurement data were expressed as mean ± standard deviation (SD). The comparison of means among multiple groups was performed using one-way analysis of variance (ANOVA). A statistical significance was defined as P<0.05.

Results

Effect of miR-24 combined with iPS cell transplantation on myocardial function in AMI rats

We used M-type ultrasound to compare the effect of either single or combined of miR-24

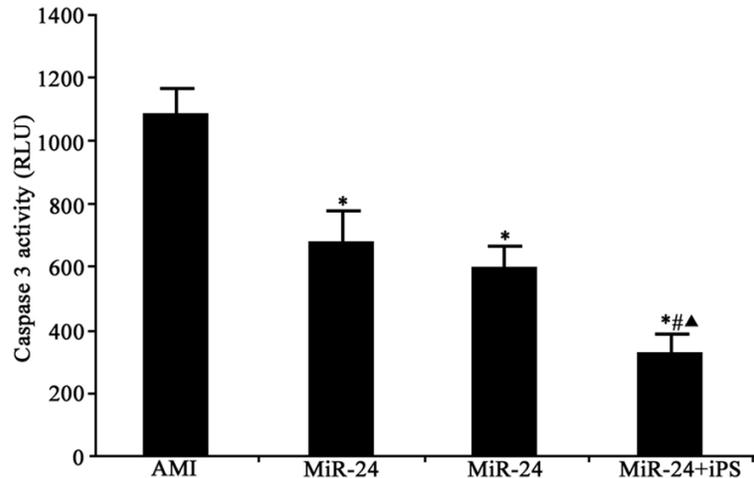


Figure 4. Effects of miR-24 plus iPS transplantation on caspase 3 activity in AMI rats. *, $P < 0.05$ vs AMI group; #, $P < 0.05$ vs iPS group; Δ, $P < 0.05$ vs miR-24 group.

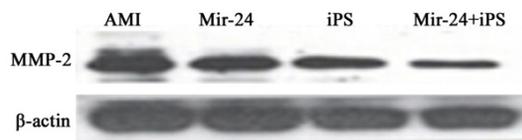


Figure 5. Effect of miR-24 combined with iPS cell transplantation on myocardial MMP-2 expression in AMI rats.

miR-24 and iPS obtained more significantly inhibitory effects on IL6 and TNF- α levels ($P < 0.05$ compared to either of miR-24 or iPS group, **Figure 3**). These results suggested that the combined treatment of miR-24 and iPS transplantation significantly inhibited serum IL6 and TNF- α expression in AMI rats.

Effects of miR-24 combined with iPS cell transplantation on caspase 3 activity

Caspase 3 activity assay kit was employed to detect the effect of either single or combined treatment using miR-24 or iPS transplantation on caspase 3 activity in myocardial tissues of AMI rats. Results showed inhibition on myocardial caspase 3 activity in AMI rats after either single or combined treatment ($P < 0.05$ compared to AMI group). Combined treatment of miR-24 and iPS obtained more significantly inhibitory effects on caspase 3 activity ($P < 0.05$ compared to either of miR-24 or iPS group, **Figure 4**). These results suggested that the combined treatment of miR-24 and iPS transplantation significantly inhibited caspase 3 ac-

tivity in myocardial tissues in AMI rats, further modulating apoptotic homeostasis.

Effect of miR-24 combined with iPS transplantation on MMP-2 expression in myocardial tissues of AMI rats

Western blotting was used to detect the effect of either single or combined treatment using miR-24 or iPS transplantation on MMP-2 expression in myocardial tissues of AMI rats. Results showed decreased myocardial MMP-2 expression in AMI rats after either single or combined treatment ($P < 0.05$ compared to AMI group). Combined treatment of miR-24 and iPS obtained more significantly inhibitory effects on myocardial MMP-2 expression ($P < 0.05$ compared to either of miR-24 or iPS group, **Figures 5 and 6**). Therefore the combined treatment of miR-24 and iPS transplantation significantly inhibited MMP-2 in myocardial tissues in AMI rats.

Discussion

Although multiple treatment approaches including drug, surgery and intervention exist for AMI, the clinical treatment efficacy is still unsatisfactory with high recurrent rate, poor prognosis, both of which cause shorter survival span of AMI patients and lower life quality, bringing heavy burdens for the public health. Therefore the establishment of effective stem cell treatment is of critical importance for improving AMI treatment efficacy [17]. microRNA is one small molecule RNA consists of 19~25 nucleic acids with similar molecular biological features, and participates in regulating various body biological functions such as growth/development and environmental acclimation. Due to its various existing forms, miR can be modulated by physiological and developmental signals [18, 19]. Each miR can regulate more than 200 target genes, suggesting that at least 1/3 of functional coding genes in human are modulated by miR. The role of miR in cardiovascular diseases has become one research hotspot [20]. miR-24 has been found to participate in the regulation of cardiovascular diseases and can facilitate existence of myocardial cells [21, 22]. iPS has

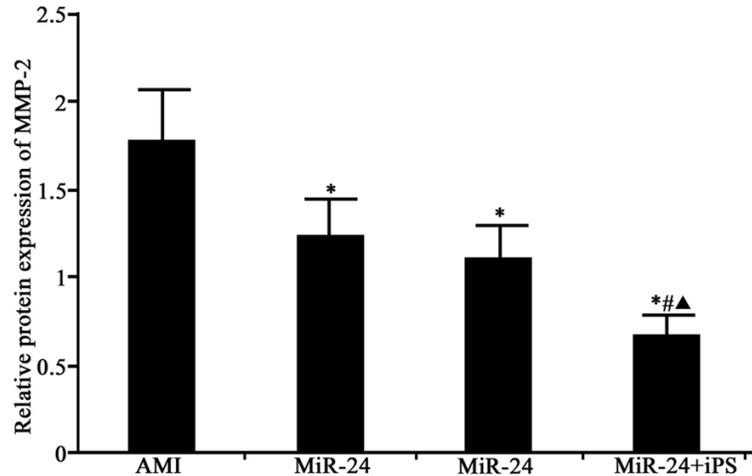


Figure 6. Analysis of miR-24 plus iPS transplantation on myocardial MMP-2 expression of AMI rats. *, $P < 0.05$ vs AMI group; #, $P < 0.05$ vs iPS group; Δ, $P < 0.05$ vs miR-24 group.

features with ESCs, and has pluripotent differentiation potency, and can be obtained from somatic cells of various species including human, pig and monkey [23]. In this study, mouse-derived iPS was transplanted into AMI model rats, whose cardiac function was later found to be significantly improved, along with inhibition of myocardial fibrosis and inflammation. Whether the combined therapy using miR-24 and iPS have synergistic effects on myocardial infarction-fibrosis, however, has not been reported, nor did related mechanism. This study demonstrated that the transfection of miR-24 lentivirus into myocardial tissues of AMI rats exerted the improvement of cardiac function and inhibition on fibrosis post-myocardial infarction, and decrease of MMP-2 or inflammation. In combined treatment group, improvements of cardiac function and myocardial fibrosis post-infarction were more potent. Therefore the combined approach decreases MMP-2 or inflammation, with more potent inhibition on myocardial fibrosis. Type I collagen fiber consists of more than 90% of all myocardial collagens. Due to its relatively smaller elasticity and extensivity, plus rigidity, type I collagen fiber plays an important role in formation and maintenance of tension in ventricular wall. The elevation of type I collagen fiber leads to higher rigidity [24]. During AMI onset, it is commonly accompanied with sharp elevation of MMP-2 which affects ECM, followed by further elevation during fibrosis or ventricular remodeling process [6]. Inflammation is one of important

pathological mechanism underlying myocardial fibrosis and infarction. The elevated secretion of inflammatory factors leads to elevated myocardial infarction-fibrosis [25]. As one of the most potent member of apoptotic family, caspase 3 activity induces cell apoptosis [26]. Therefore, miR-24 plus iPS decreased caspase 3 activity, regulated apoptosis, inhibited MMP functions or inflammation, thus improving cardiac function and fibrosis post-AMI.

Conclusion

MiR-24 can facilitate iPS-modulation on ECM and type I collagen, regulate apoptosis and inflammation, and improve myocardial fibrosis after infarction. Such combined plan provides new insights for treating AMI, and might decrease morbidity or mortality of cardiac infarction.

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Disclosure of conflict of interest

None.

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