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MiRNA-182 regulates the cardiomyocyte apoptosis in heart failure

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Abstract. – OBJECTIVE: To investigate the effect of miRNA-182 on cardiomyocyte apoptosis of heart failure (HF).

MATERIALS AND METHODS: HF model in rats was established by rapid ventricular pacing. AAV-miRNA-182 (adeno-associated virus) vector was constructed to upregulate miRNA-182 level and its negative control AAV-NC was prepared. Rats undergoing rapid pacing (pacing group) and sham operation (sham group) were injected with AAV-miRNA-182 or AAV-NC, respectively. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was conducted to determine the miRNA-182 level in rats. Cardiac function test was carried out after the HF model establishment. Western blot was performed to examine the protein levels of human programmed cell death4 (PDCD4) and phosphoacidic cluster sorting protein (PACS2) in rats. Finally, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labeling (TUNEL) assay was performed to evaluate the apoptotic rate of cardiomyocytes.

RESULTS: HF model in rats was successfully established by rapid pacing. Injection of AAV-miRNA-182 markedly upregulated miRNA-182 level in rats. Compared with rats in the sham group, left ventricle ejection fraction (LVEF; 81.8% ± 2.4% *vs.* 64.3% ± 2.2%, $p<0.05$) and left ventricular fractional shortening (LVFS; 44.7% ± 2.4% *vs.* 29.1% ± 0.9%, $p<0.05$) markedly decreased in the pacing + NC group, whereas heart rate (HR) and left ventricular end-diastolic pressure (LVEDP) increased. Compared with rats in the pacing + NC group, HR [(441.6 ± 22) /min *vs.* (368.4 ± 27)/min, $p<0.05$] and LVEDP [(34.8 ± 11.4) mmHg *vs.* (19.4 ± 10.3) mmHg, $p<0.05$] were reduced in the pacing + miRNA-182 group. The protein levels of PDCD4 and PACS2 were downregulated in the pacing + miRNA-182 group relative to the pacing + NC group. Rapid pacing stimulation induced cardiac structural remodeling and cardiomyocyte apoptosis, which were alleviated by injection of AAV-miRNA-182.

CONCLUSIONS: MiRNA-182 inhibits cardiomyocyte apoptosis induced by non-ischemic HF via downregulating PDCD4 and PACS2.

Key Words:
Heart failure, MiRNA-182, PDCD4, PACS2, Apoptosis.

Introduction

Congestive heart failure (HF) is a complex, multifactorial disease, which is the final stage of various heart diseases. HF has become the leading cause of hospitalization and mortality in cardiovascular disease[1,2]. Nowadays, rapid ventricular pacing has been recognized to be an effective method for establishing the HF animal model, mainly applied in dogs, pigs and other large animals[3,4]. The development of gene technology, the discovery of myocardial apoptosis targets and improved success rate of gene transfection allow the gene therapy to be an important approach for refractory HF. In the basic researches, *in vivo* transfection is usually applied in rats or mice due to the high transfection efficacy of lentivirus vectors. Congestive HF model in rats established by rapid pacing is a suitable animal model for exploring gene therapy of HF[5]. Cardiomyocyte apoptosis of HF reduces the amount of working cardiomyocytes and thereafter attenuates myocardial contractility. The process of apoptosis is accompanied by the early onset of HF, which exerts a key role in the progression of HF[6].

In recent years, the role of microRNAs (miRNAs) in cardiovascular diseases has received widespread attention, including myocardial remodeling, myocardial infarction, atherosclerosis, etc[7-9]. The specific effect of miRNA-182 on cardiac function is rarely reported. A relevant study[10] pointed out that miRNA-182 mimics greatly improves cardiac function of diabetic mice by alleviating mitochondrial structural damage, myocardial fibrosis, cardiac hypertrophy and autophagy. Current studies[11,12] demonstrated that upregu-

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lations of PDCD4 and PACS2 induce apoptosis through the mitochondrial pathway. It is unclear whether miRNA-182 could regulate the expressions of PDCD4 and PACS2 to further mediate cardiomyocyte apoptosis of non-ischemic HF. In this study, miRNA-182 level in HF rats was upregulated by injection of AAV-miRNA-182. We subsequently determined the relative levels of miRNA-182, PDCD4 and PACS2, and examined changes of cardiomyocyte apoptosis.

Materials and Methods

AAV Packaging (Adeno-Associated Virus)

Primer sequences were designed based on rat-derived miRNA-182 as follows: Rno-miRNA-182-F: GATCCGCTGTTAAGACTTGCAGTGATGTTTAGCTCCTCTCCATGTGAACATCACAGCAAGTCTGTGCTGCG;

Rno-miRNA-182-R: AATTCGCAGCACAGACTTGCTGTGATGTTCACATGGAGAGGAGCTAAACATCACTGCAAGTCTTAACAGCG. The synthetic deoxyribonucleic acid (DNA) fragment was sufficiently dissolved in 100 μL of annealing buffer. 2 μL of DNA primer fragments were extracted, mixed in 16 μL of annealing buffer and cooled from 100°C to room temperature. Annealed products were diluted 100 times in sterile water. The vector pAAV-ZsGreenmiRNA was digested with restriction endonuclease BamH I + EcoR I. Large fragment was harvested after agarose gel electrophoresis. Recovered large fragment of pAAVZsGreen-miRNA was ligated to the diluted annealing product of rno-miRNA-182, and the plasmid was extracted using a relative plasmid extraction kit (Invitrogen, Carlsbad, CA, USA). Positive colonies were picked for sequencing and the sequencing primer was ZsGreen-F. Lentivirus vector introduced into 293AAV cells using a HET Kit (Invitrogen, Carlsbad, CA, USA). 48 hours later, AAV particles were collected from the filtered supernatant. Unrestricted pAAV-ZsGreenmiRNA (pAAV-NC) was used as negative control.

Animal Procedures

This study was approved by the Animal Ethics Committee of Qiqihar Medical College Animal Center. Rats were randomly assigned into sham group, sham + miRNA-182 group, pacing + NC group and pacing + miRNA-182 group, with 6 rats in each group. Pacemaker and electrode implantation were performed. After suturing the electrode at the apex of the right ventricle, rats in the sham + miRNA-182 group and pacing + miRNA-182 group were injected with 200 μL of AAV-miRNA-182 (1×10^{12} μg/mL) into the left ventricle. Meanwhile, the aorta was clamped for 10 s to allow the lentivirus to be uniformly transfected into the myocardial tissues *via* the coronary artery on the basis of systole. Intraperitoneal injection of 200 000 U penicillin (Sigma-Aldrich, St. Louis, MO, USA) was given after animal procedures for consecutive 7 days. In the pacing + NC group and pacing + miRNA-182 group, the pacemaker was intermittently turned on at postoperative day 5, which was conducted at a frequency of 550/min for 4 weeks from postoperative day 7.

Cardiac Function Test

Cardiac ultrasonography: after termination of rapid pacing, rats were anesthetized by intraperitoneal injection of 3% pentobarbital sodium at a dose of 40 mg/kg. HF (min), left ventricle ejection fraction (LVEF; %) and left ventricular fractional shortening (LVFS; %) were recorded.

Hemodynamic testing was performed using a multi-channel physiological signal acquisition and processing system: under the anesthesia, rat right external carotid artery was intubated to the left ventricle. Left ventricular end-systolic pressure (LVESP; mmHg), left ventricular end-diastolic pressure (LVEDP; mmHg) and LVdP/dtmax (mmMg/s) were recorded.

Pathological Examination

Rat heart was collected after sacrifice and weighed. Myocardial tissues were collected alongside the left ventricular coronal section for hematoxylin-eosin staining (HE) and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick End Labeling (TUNEL; Roche, Basel, Switzerland) staining.

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

Rat heart was harvested and washed with 0.9% saline. Myocardial tissues of the left ventricular free wall were lysed in 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) for 5 min, mixed in 200 μL of chloroform and centrifuged at 4°C, 14 000 rpm/min for 15 min. The upper aqueous phase was transferred to a new RNase-free Eppendorf tube (EP; Eppendorf, Hamburg, Germany). Isodose isopropanol was applied for centrif-

ugation at 4°C, 14 000 rpm/min for 10 min. The precipitated RNA was washed with 75% ethanol, air dried and dissolved in 15-60 μL of diethyl pyrocarbonate (DEPC; Beyotime, Shanghai, China) water. Concentration and purity of extracted water were determined, and qualified ones were subjected to reverse transcription. Primer sequences were searched from GeneBank, and constructed using Premier Primer Software as follows: MiRNA-182, F: UUUGGCAUGGUAGAACUCACACCG, R: AGUGUGAGUUCUACCAUUGCCAAA; U6, F: CTCGCTTCGGCAGCACA, R: AACGCTTCACGAATTTGCGT. The constructed complementary deoxyribonucleic acid (cDNA) was amplified for quantitative Real Time-Polymerase Chain Reaction qRT-PCR.

Western Blot

Total protein was extracted from rat myocardium, quantified and electrophoresed. After transferring on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), they were incubated with primary antibodies at 4°C. At the other day, membranes were incubated with horseradish peroxidase (HRP)-labeled IgG (1:5000) for 2 h. Bands were exposed with the enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA), and integral optical density was analyzed by gel imaging analysis system with the internal reference of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

TUNEL

0.5 cm^3 of left ventricular tissues were paraffin-embedded and sliced into 54 μm sections for TUNEL assay. Slices were placed into 200 ml of 0.01 mol/L citrate buffer (pH 6.0) and heated to 90-95°C. 80 ml of distillation-distillation H_2O (20-25°C) was quickly added for cooling. Slides were washed 3 times with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) and incubated in 20% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) for 30 min. Subsequently, the TUNEL reaction mixture was applied on the slides for 90-min incubation at 37°C, blocked in 3% H_2O_2 methanol solution for 10 min and incubated in POD transformant for 30 min. Color development was performed by DAB/H_2O_2 (three times, 5 min each time) and hematoxylin staining. Slides were dehydrated, sealed and observed under an optical microscope.

Statistical Analysis

Data were analyzed by Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) statistical software. Quantitative data were expressed as mean ± standard deviation ($\bar{x}\pm s$). The comparison between groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Cardiac Function Determination

Compared with rats in the sham group, LVEF (81.8% ± 2.4% *vs.* 64.3% ± 2.2%, $p<0.05$) and LVFS (44.7% ± 2.4% *vs.* 29.1% ± 0.9%, $p<0.05$) markedly decreased in the pacing + NC group, whereas HR and LVEDP increased. Compared with rats in the pacing + NC group, HR [(441.6 ± 22)/min *vs.* (368.4 ± 27)/min, $p<0.05$] and LVEDP [(34.8 ± 11.4) mmHg *vs.* (19.4±10.3) mmHg, $p<0.05$] were reduced in the pacing + miRNA-182 group (Table I).

Table I. The results of cardiac ultrasonography and hemodynamic testing.

Parameters	Sham	Sham + miR-182	Pacing + NC	Pacing + miR-182	p
HR (/min)	369.8±28	382.6±15	441.6±22[ab]	368.4±27[c]	0.0001
LVEF (%)	81.8±2.4	81.4±2.3	64.3±2.2[ab]	65.4±2.2	0.0001
LVFS (%)	44.7±2.4	44.4±1.3	29.1±0.9[ab]	29.6±1.4	0.0001
LVESP (mmHg)	119.7±18.7	122.0±17.4	101.7±10.7	89.7±11.7	0.0001
LVEDP (mmHg)	3.0±2.2	2.9±0.5	34.8±11.4[ab]	19.4±10.3[c]	0.0001
LVdP/dtmax (mmHg/s)	4403±1438	5514±1949	1326±245[ab]	1979±680[c]	0.0001

a: $p<0.05$, *vs.* sham group;
b: $p<0.05$, *vs.* sham + miR-182 group;
c: $p<0.05$, *vs.* pacing + NC group. HR: heart rate; LVEF: left ventricle ejection fraction; LVFS: left ventricular fractional shortening; LVESP: left ventricular end-systolic pressure; LVEDP: left ventricular end-diastolic pressure; LVd P/dt: rate of change of left ventricular pressure.

Relative Level of MiRNA-182 in Rats

Injection of AAV-miRNA-182 significantly upregulated miRNA-182 level in rats of the sham + miRNA-182 group. Identically, AAV-miRNA-182 administration remarkably upregulated miRNA-182 level in rats of the pacing + miRNA-182 group relative to the pacing + NC group (Figure 1). The above qRT-PCR data revealed sufficient efficacy of AAV-miRNA-182.

Western Blot Analyses of PDCD4 and PACS2 in Rat Myocardium

Compared with the sham group, the protein levels of PDCD4 and PACS2 were upregulated in rat myocardium of the pacing + NC group. However, the protein levels of PDCD4 and PACS2 decreased in rat myocardium of the pacing + miRNA-182 group relative to the pacing + NC group (Figure 2). Semi-quantitative analyses of PDCD4 and PACS2 were depicted in Figure 3. It is suggested that miRNA-182 overexpression downregulated PDCD4 and PACS2 in HF rats undergoing rapid pacing.

MiRNA-182 Influenced Cardiomyocyte Apoptosis of HF Rats

Rat heart was quickly harvested after sacrifice and myocardial tissues were collected alongside the left ventricular coronal section. HE staining revealed normal morphology, clear structure and striation, and ordered fiber arrangement in rat myocardium of the sham group and sham + miRNA-182 group. Nevertheless, cardiomyocyte edema, fatty degeneration, interstitial hyperemia, inflammatory cell infiltration, myocardial fiber arrangement disorder was observed in rat myocardium of the pacing + NC group. These pathological lesions were alleviated in the pacing + miRNA-182 group (Figure 4). 0.5 cm^3 of left ventricular tissues were paraffin-embedded and sliced into 54 μm sections for TUNEL assay. A great number of stained TUNEL-positive cardiomyocytes were seen in the pacing + NC group, whereas these stained cells were barely seen in the sham group and sham + miRNA-182 group. In the pacing + miRNA-182 group, TUNEL-positive cardiomyocytes were fewer relative to the pacing + NC group (Figure 5). Semi-quantitative analysis of cardiomyocyte apoptosis was illustrated in Figure 6.

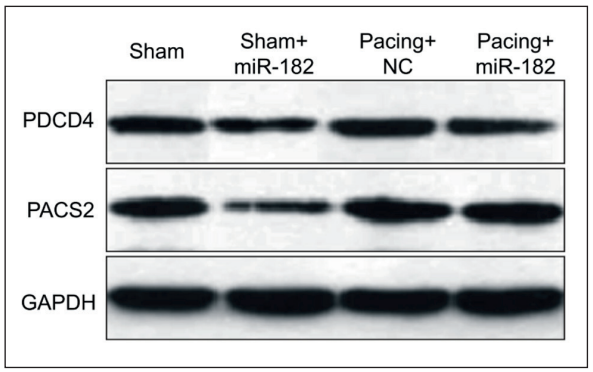

Figure 2. Western blot analyses of PDCD4 and PACS2 in rat myocardium.

Discussion

In this study, we successfully established the HF model in rats by rapid ventricular pacing. In the murine model, sustained rapid ventricular pacing can lead to ventricular remodeling and increased wall stress. We obtained the conclusions

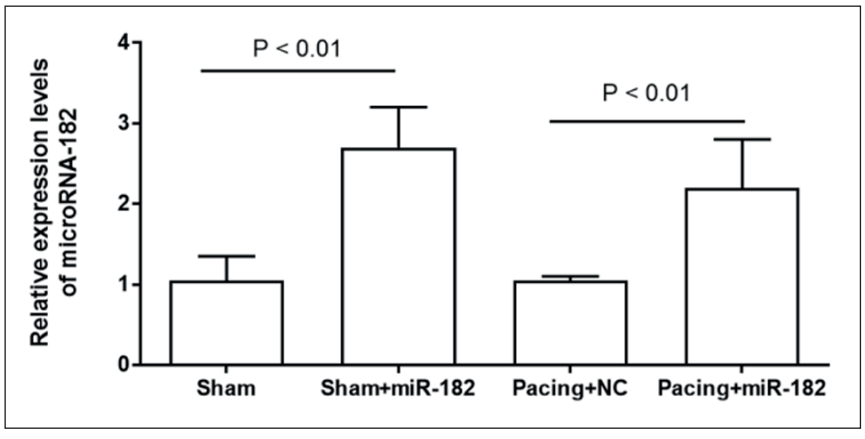

Figure 1. Relative level of miR-182 in rat myocardium.

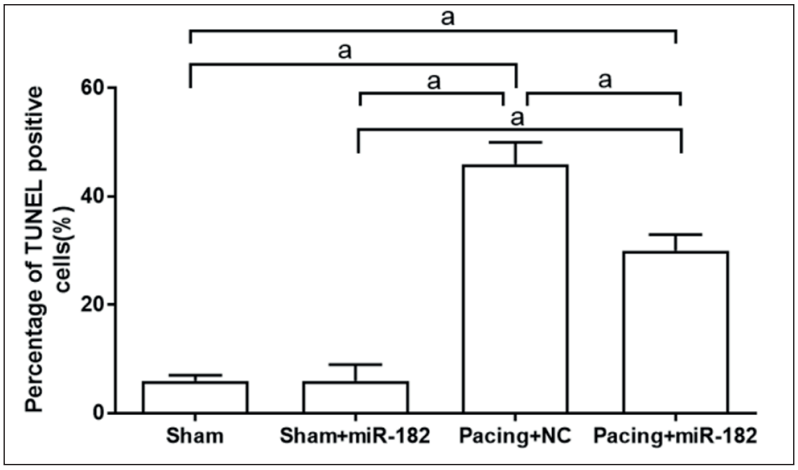

Figure 3. Semi-quantitative analyses of the protein levels of PDCD4 and PACS2 in rat myocardium (a: $p<0.01$, b: $p<0.05$).

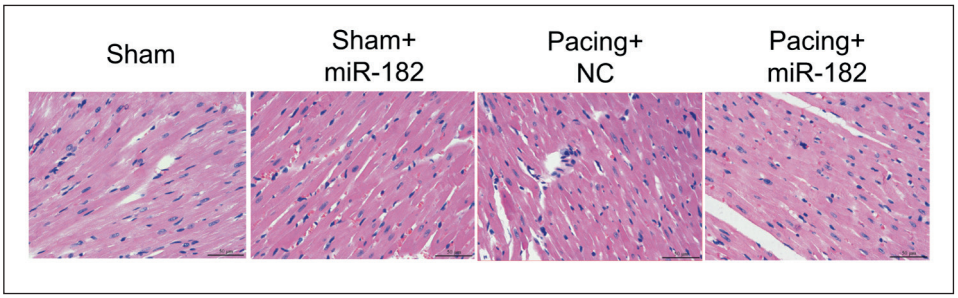

Figure 4. HE staining of rat myocardium (magnification × 400).

in the present experiment as follows: (1) Rapid ventricular pacing downregulated miRNA-182 level, induced cardiomyocyte apoptosis and HF in rats; (2) Overexpression of miRNA-182 inhibited cardiomyocyte apoptosis, exerting the anti-apoptotic ability in HF.

It is reported that PDCD4 can promote tumor cell apoptosis by affecting the translation of eukaryotic initiation factor-4A (eIF4A) and eIF4G[13]. An *in vitro* study[14] illustrated the downregulated pro-apoptotic genes and upregulated anti-apoptotic genes in PDCD4$^{-/-}$ insulin-secreting β cells. PACS2 is an initiator of cell apoptosis, which accelerates the displacement of mitochondrial death pathway agonists[15,16]. In our work, cardiomyocyte apoptosis was induced by rapid pacing in HF rats, which was consistent with other animal models[17]. After overexpression of miRNA-182 by injecting AAV-miRNA-182, the expressions of PDCD4 and PACS2 were downregulated, and the apoptotic rate of cardiomyocytes decreased in HF rats. Due to the limitations in the present study, we did not explore the role of miRNA-182 knockdown in cardiomyocyte apoptosis of HF. Besides, changes in the neurohumoral factor at post-HF require to be further investigated.

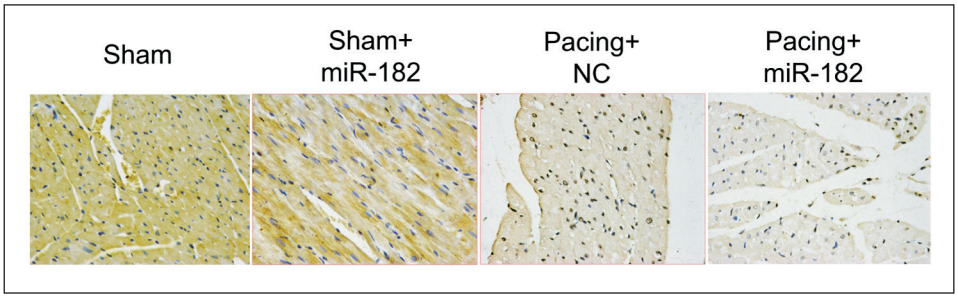

Figure 5. TUNEL staining of rat myocardium (magnification × 400).

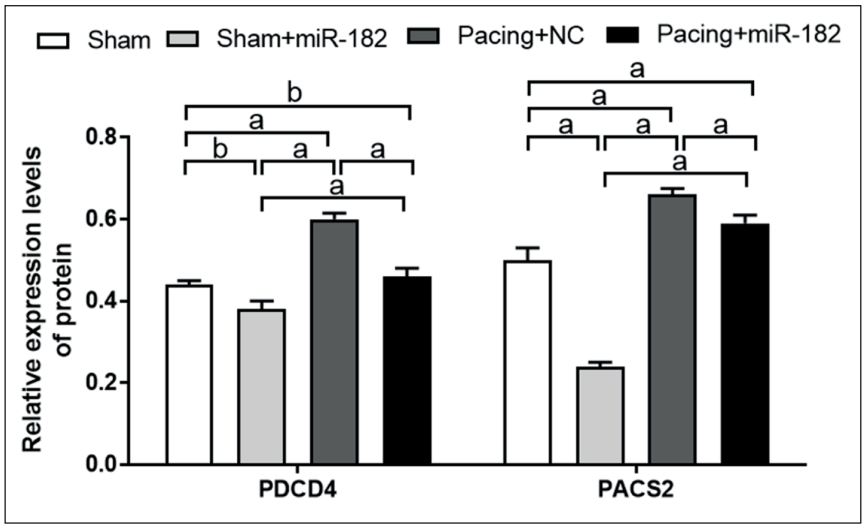

Figure 6. Semi-quantitative analysis of TUNEL-positive cells.

The upregulation of miRNA-182 remarkably inhibited cardiomyocyte apoptosis in HF rats undergoing rapid pacing. Meanwhile, we found that PDCD4 and PACS2 were important factors closely related to apoptosis, which may be involved in the remodeling of the myocardial structure during the process of HF. Our results strongly suggested that miRNA-182 could affect cardiomyocyte apoptosis by regulating the expressions of PDCD4 and PACS2.

Conclusions

We demonstrated that miRNA-182 inhibits cardiomyocyte apoptosis induced by non-ischemic HF *via* downregulating PDCD4 and PACS2.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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