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Decreased miR-208 induced ischemia myocardial and reperfusion injury by targeting p21

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Aberrant expression of miR-208 was previously reported in cardiomyocytes after cardiac ischemia reperfusion (CIR) injury. However, the underlying mechanism has never been elucidated. In the current study, the relative level of miR-208 was determined in the hearts of CIR injury mice models using real time PCR. The effect of miR-208 on cardiomyocytes apoptosis was determined by Hoechst staining and annexin V-PI staining. Meanwhile, caspase3 activity was explored using an assay kit. To identify left ventricular fraction and relative wall thickness, the two-dimensional echocardiography was applied. Dual luciferase assay was applied to determine the target gene of miR-208. Compared with normal control, the level of miR-208 was significantly reduced in the hearts of CIR injury mouse models. Further studies revealed that reduction of miR-208 contributed to reactive oxygen species (ROS) production in the cardiomyocytes. We also found that inhibition of miR-208 prompted cardiomyocyte apoptosis. More importantly, the phosphorylation level of Akt and p38 was enhanced in primary cardiomyocytes transfected with miR-208 inhibitor, indicating a potential stress-response after CIR injury in primary cardiomyocytes. Dual luciferase assay and western blot analysis showed that transfection with miR-208 markedly suppressed the protein expression of p21, suggesting p21 was a target gene of miR-208. To conclude, we showed that reduced miR-208 level enhanced cardiomyocyte apoptosis mainly by targeting p21.

1. Introduction

Myocardial infarction is a serious health problem that leads to significant threat to human health (Yang et al. 2015). It is well accepted that increased cardiomyocyte apoptosis and necrosis play a key role in the process of cardiac injury (Hansson et al. 2015; Kumphune et al. 2015). After proper treatment, reperfusion is necessary for the infarction heart (Wang et al. 2015). However, the patient has to cope with a second hit, which is known as cardiac ischemia/reperfusion (I/R) injury (Yang et al. 2015; Yu et al. 2015). In previous years, researchers have been working on effective treatment methods to solve the problem (Zhang et al. 2015).

MicroRNAs (miRNAs) are small non-coding RNAs that act as important posttranscriptional regulators of gene expression (Wu et al. 2015). Previous studies have demonstrated that miRNAs widely participate in the regulation of cell proliferation, differentiation and apoptosis (Wu et al. 2015; Xia et al. 2015; Wu et al. 2016). Recently, it is reported that miR-1 contributes to cardiac ischemia reperfusion injury by targeting Hsp90aa (Zhu et al. 2016). Moreover, enhanced miR-195 expression was reported to enhance cardiomyocyte apoptosis mainly by binding the 3'untranslated region (3'UTR) of Bcl-2 after CIR injury (Gao et al. 2016). In previous studies, aberrant expression of miR-208 was identified after IR. However, its specific molecular mechanism has never been elucidated.

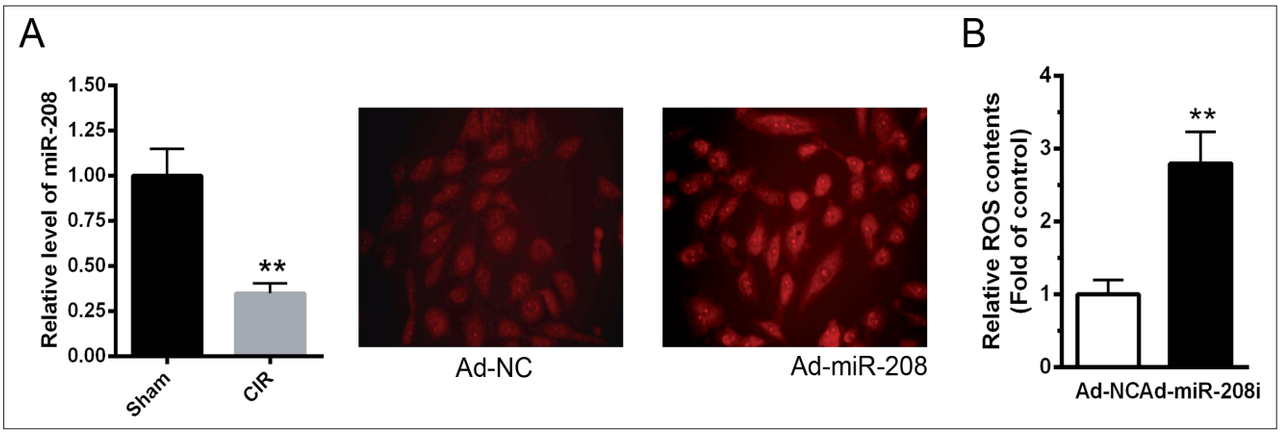

Fig. 1: Decreased miR-208 expression increased ROS production in primary cardiomyocytes. (A) The level of miR-208 was markedly reduced in the hearts of CIR mice. (B) Inhibition of miR-208 enhanced ROS production in primary cardiomyocytes. Data were presented as mean ± SE. n=5, *P<0.05, **P<0.01.

In the present study, we first demonstrated that through suppression the expression of p21, decreased miR-208 expression significantly led to the activation of cardiomyocyte apoptosis, thereby enhancing CIR injury.

2. Investigations, results and discussion

2.1. Decreased miR-208 expression increased ROS production in primary cardiomyocytes

Firstly, we explored the expression of miR-208 level in the hearts of CIR mice models. As shown in Fig. 1A, the level of miR-208 was markedly reduced in the hearts of CIR mice. Since enhanced ROS production significantly contributes to CIR injury, we determined ROS production in primary cardiomyocytes transfected with ad-miR-208 inhibitor. We found that inhibition of miR-208 enhanced ROS production in primary cardiomyocytes (Fig. 1B).

2.2. Inhibition of miR-208 enhanced caspase-3 activation and prompted cardiomyocytes apoptosis

Next, we explored the effects of miR-208 on cell apoptosis in primary cardiomyocytes. Hoechst staining demonstrated that transfection of ad-miR-208 inhibitor increased the nuclear apoptosis morphological changes of primary cardiomyocytes (Fig. 2A). Flow cytometry analysis also demonstrated that miR-208 inhibition enhanced cell apoptosis by nearly two fold (Figs. 2B and 2C). More importantly, we determined that transfection of ad-miR-208 inhibitor enhanced cell apoptosis in a time-dependent manner (Fig. 2D). These data indicated that inhibition of miR-208 prompted cardiomyocyte apoptosis.

2.3. Decreased cardiac function by miR-208 inhibition in primary cardiomyocytes

Then, the cardiac function was determined in mice injected with ad-miR-208 through jugular vein injection. As shown in Fig. 3A, inhibition of miR-208 decreased FS after seven days post injection. Meanwhile, echocardiography examination indicated that inhibition of miR-208 reduced RWT at 7 days and 10 days post injection (Fig. 3B). More importantly, the phosphorylation levels of p38 and Akt were both markedly enhanced in the primary cardiomyocytes, suggesting a potential stress response in the hearts of mice (Fig. 3C).

2.4. p21 was the target gene of miR-208

To explore the possible target gene of miR-208, the bioinformatics predictions were applied. As shown in Fig. 4A, there was a conserved binding site of miR-208 on the 3'UTR of p21. Dual luciferase reporter assay showed that miR-208 markedly suppressed the relative luciferase activity of pmirGLO-p21-3'UTR (Fig. 4B). More importantly, overexpression of miR-208 significantly decreased the protein level of p21 (Fig. 4C), while inhibition of miR-208 prompted the protein expression of p21 (Fig. 4D), indicating p21 was a target gene of miR-208.

More importantly, we also explored the possible effect of p21 on cardiomyocyte apoptosis. As shown in Fig. 4E, overexpression of p21 in primary caridomyocytes significantly enhanced the protein level of Bax and suppressed the protein expression of Bcl-2 (Fig. 4E). These data showed that the upregulation of p21 contributed to primary caridomyocytes apoptosis.

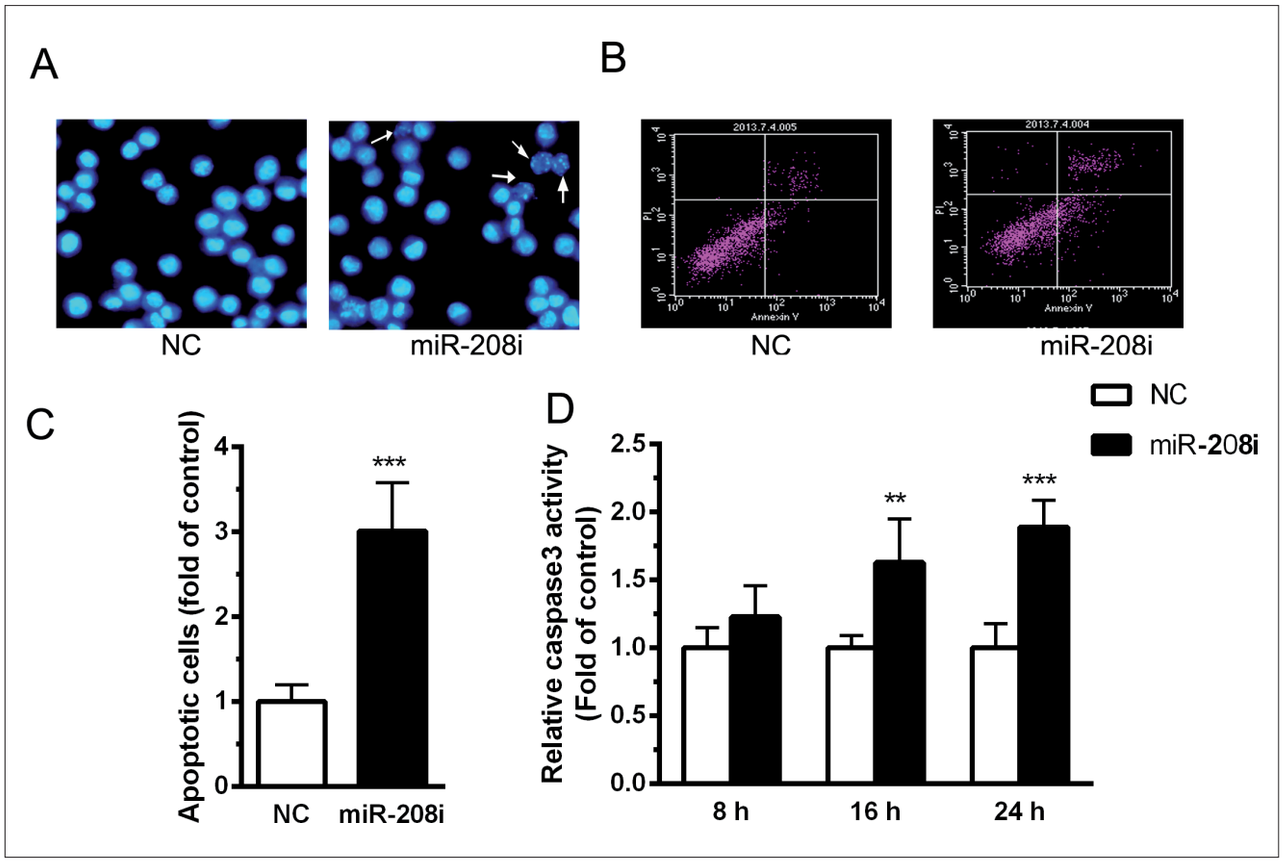

Fig. 2: Inhibition of miR-208 enhanced caspase-3 activation and prompted cardiomyocytes apoptosis. (A) Hoechst staining demonstrated that transfection of ad-miR-208 inhibitor increased the nuclear apoptosis morphological changes of primary cardiomyocytes. (B) Flow cytometry analysis also demonstrated that miR-208 inhibition enhanced cell apoptosis. (C) Statistical analysis showed that miR-208 inhibition enhanced cell apoptosis by nearly two fold. (D) Transfection of ad-miR-208 inhibitor enhanced cell apoptosis in a time-dependent manner. Data were presented as mean ± SE. n=3, *P<0.05, **P<0.01.

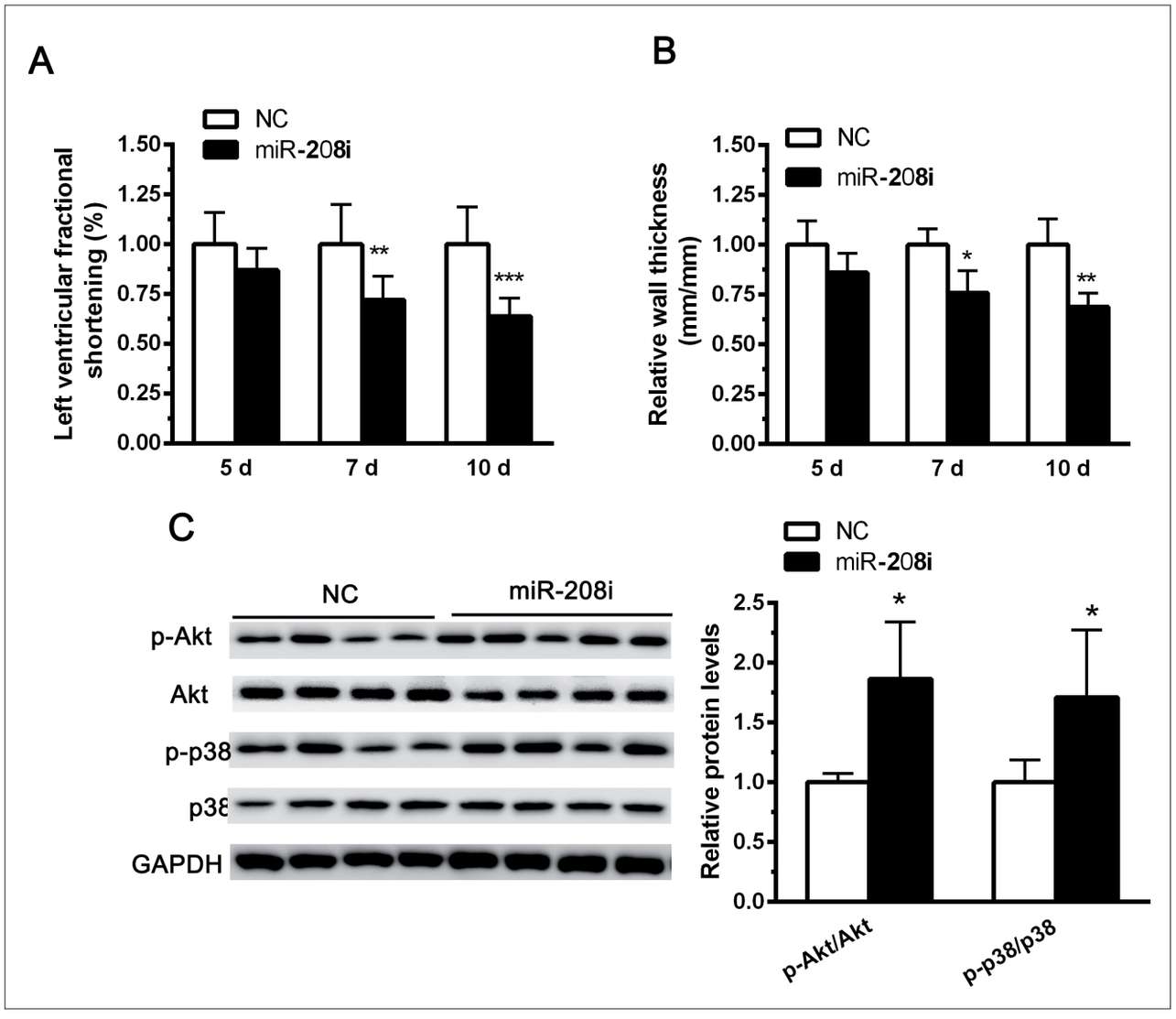

Fig. 3: Decreased cardiac function by miR-208 inhibition in primary cardiomyocytes. (A) Inhibition of miR-208 decreased FS after seven days post injection. (B) The echocardiography examination indicated that inhibition of miR-208 reduced RWT at 7 days and 10 days post injection. (C) The phosphorylation levels of p38 and Akt were both markedly enhanced in the primary cardiomyocytes. Data were presented as mean ± SE. n=5, *P<0.05, **P<0.01.

Accumulating evidence has suggested the important role of miRNAs in the progression of CIR injury (Wang et al. 2015; Zhu et al. 2016). Several miRNAs have been reported to be differentially expressed in the hearts of CIR injury, including miR-29a, let-7, miR-34, miR-130a (Lu et al. 2015; Wang et al. 2015; Joshi et al. 2016). In the present study, we first showed that the level of miR-208 was significantly decreased in both the hearts of CIR mice. We have shown that inhibition of miR-208 enhanced the primary cardiomyocyte apoptosis. However, the specific underlying mechanism of miR-208 on HCC progression has never been explored.

It is well accepted that oxidative stress plays a key role in the CIR injury and enhanced ROS production was reported to trigger cell apoptosis (Xu et al. 2012; Muzi-Filho et al. 2013). Thus, we explored the effects of miR-208 on primary cardiomyocyte apoptosis. Interestingly, we found that inhibition of miR-208 in primary cardiomyocytes resulted in ROS production, suggesting a potent detrimental effect of miR-208 on cardiomyocytes after CIR injury in mice. Next, several different cellular signaling pathways that may be triggered by ROS production were further explored. Previous studies have suggested that PI3K/Akt signaling pathway are of great importance for cardiomyocyte survival (Rong and Xijun 2015; Qin-Wei and Yong-Guang 2016). In contrast, abnormal activation of PI3K/Akt signaling was reported to be a negative feedback in response to abnormal stimuli (Ravingerova et al. 2007; Fang et al. 2010). Based on our study, we found the phosphorylation level of Akt was enhanced in mice cardiomyocytes transfected with miR-208 inhibitor, indicating a potential stress-response after CIR injury in primary cardiomyocytes. Moreover, the activation of MAPK signaling, including p38, ERK, and JNK, was also studied (Kim and Choi 2010). Here, we determined that the activation of p38 was significantly increased, which also showed a stress response. Moreover, after transfection with miR-208 inhibitor, cardiomyocytes apoptosis was significantly enhanced. Altogether, these data showed that reduced miR-208 expression in the hearts of CIR injury mice contributed to cardiomyocyte injury mainly by enhancing ROS production and cardiomyocytes apoptosis.

Then, we tried to explore the specific mechanism in which miR-208 contributed to CIR injury. Dual luciferase reporter assay showed that p21 was a target gene of miR-208. In a previous study, it was reported that upregulation of the p53-p21 pathway controls cardiomyocyte hypertrophy and apoptosis in diabetic cardiomyopathy (Raut et al. 2016). Here, we identified that overexpression of p21

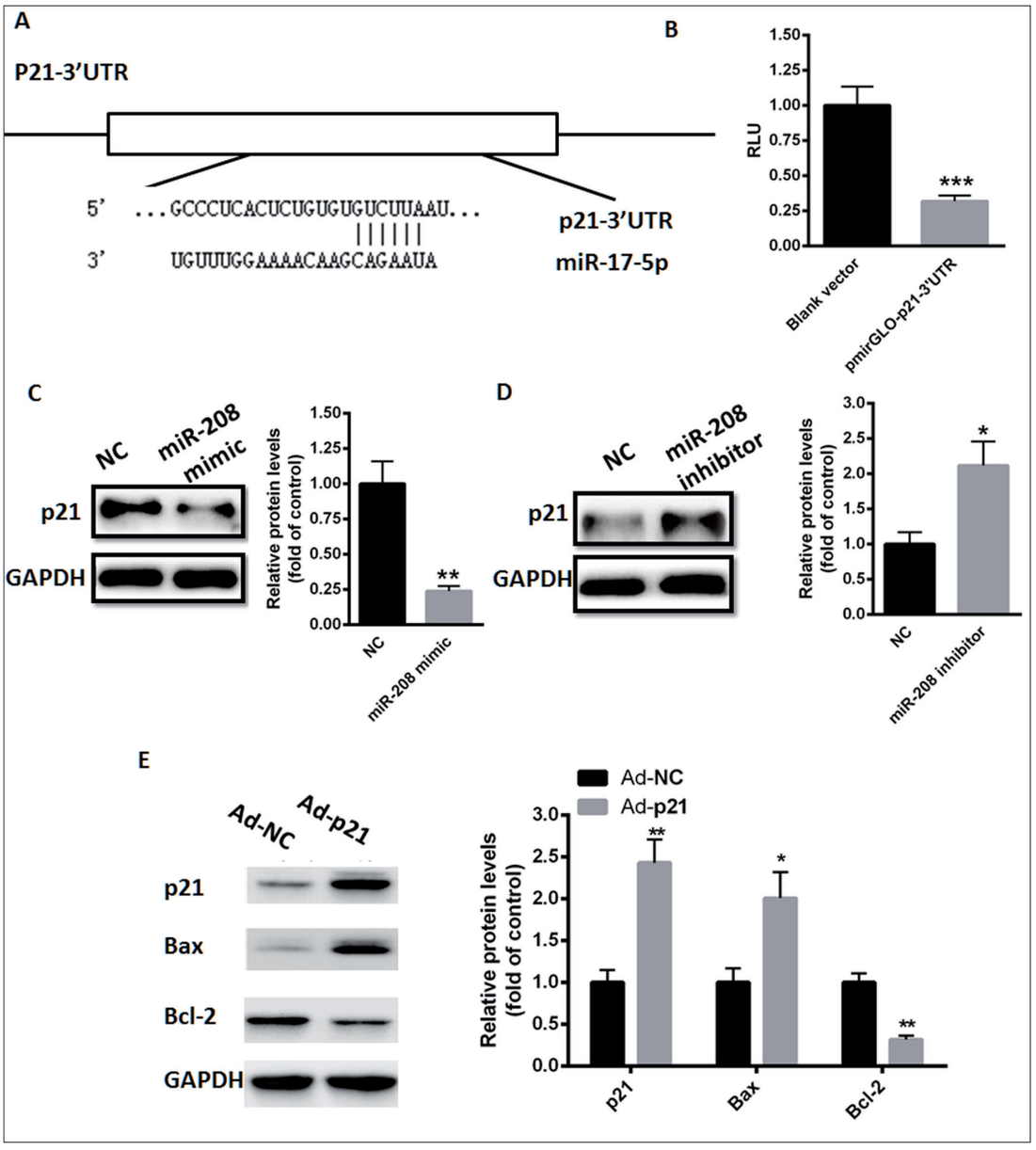

Fig. 4: Decreased cardiac function by miR-208 inhibition in primary cardiomyocytes. (A) Inhibition of miR-208 decreased FS after seven days post injection. (B) The echocardiography examination indicated that inhibition of miR-208 reduced RWT at 7 days and 10 days post injection. (C) The phosphorylation levels of p38 and Akt were both markedly enhanced in the primary cardiomyocytes. Data were presented as mean ± SE. n=5, *P<0.05, **P<0.01.

in cardiomyocytes significantly prompted the protein expression of Bax and suppressed the protein level of Bcl-2, suggesting the pro-apoptotic role of p21 in cardiomyocytes.

To conclude, in the current study, we first determined that the expression level of miR-208 was significantly decreased in the hearts of CIR mice. Moreover, reduction of miR-208 expression contributed to ROS production and cardiomyocytes apoptosis mainly by targeting p21.

3. Experimental

3.1. Primary cardiomyocyte culture

Primary cardiomyocytes from rat neonatal hearts were isolated and maintained as described (Ieda et al. 2009). Animal protocol was approved by Zhongshan Hospital Affiliated to Dalian University. All procedures conform to NIH guidelines. Briefly, hearts were minced and digested with collagenase type II (Worthington) solution. Digested cells were preplated for 2 h to enrich cardiomyocytes. The attached cells after 2 h plating were considered to be non-myocytes and discarded, while the unattached cells were primarily cardiomyocytes.

3.2. Adenovirus vectors

Adenovirus vector overexpressing miR-208 or inhibiting miR-208 was constructed by Genechem (Shanghai, China).

3.3. RNA extraction and real-time PCR

The total RNA from cultured cells was isolated with TriZol (Invitrogen) according to the manufacturer's instructions. The total RNA was reverse transcribed into complementarty DNA (cDNA) with TaqMan RNA Reverse Transcription Kit (Applied Biosystems). A quantitative real-time PCR assay was performed using SYBR Green Supermix (Bio-Rad) in a BIO-RAD iCycleriQ real-time PCR detection system as previously described (Guo et al. 2014).

3.4. Protein extraction and Western blot analysis

Proteins samples were extracted in RIPA buffer (1% TritonX-100, 15 mmol/L NaCl, 5 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.0) (Solarbio, China) supplemented with a protease and phosphatase inhibitor cocktail (Sigma) and then separated by 10% SDS-PAGE, followed by electrophoretical transfer to a PVDF membrane. After soaking with 8% milk in PBST (pH 7.5) for 2 h at room temperature, the membranes were incubated with the following primary antibodies: anti-p21, anti-p38, anti-p-Akt

and anti-GAPDH (Cell signaling). Immunodetection was performed by enhanced chemiluminscence detection system (Millipore) according to the manufacturer's instructions. The house-keeping gene GAPDH was used as the internal control.

3.5. Luciferase target assay

The 3'untranslated region (UTR) of p21 containing the predicted binding site was cloned into the pmirGLO (Promega) luciferase reporter vector. The PCR procedures are as follows: a hot start step at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 55 °C for 45 s, 72 °C for 30 s. To construct the mutant vector, the Fast Mutagenesis System was applied (TransGen Biotech, Beijing, China).

For luciferase reporter assay, cells were seeded at 5×10^4 cells/ well in 24-well plates in a 500 µl for 18 h. Then, the modified firefly luciferase vector (500 ng/µl) was mixed with Vigofect transfection reagent according to the manufacturer's instruction. After transfection for 48 h, the Dual-luciferase reporter assay system (Promega) was applied to determine the changes of relative luciferase units (RLU). Renilla activity was used as the internal control.

3.6. Two-dimensional echocardiography

Two-dimensional echocardiography of rats was performed as previously reported with certain modifications (Cittadini et al. 1996). For evaluation, the rat was anesthetized and the chest was shaved. Then, the the echocardiographic systems (model SSD-900; Aloka, Tokyo) was applied to perform two-dimensional echocardiography. To determine the papillary muscle level of the left ventricle (LV), two-dimensional short-axis images was taken. Then, a single observer was responsible for the examination of end-diastolic posterior wall thickness, end-diastolic and end-systolic internal diameters of the LV.

The calculation of relative wall thickness (RWT) was calculated as follows:

RWT = 2 × LVPWTd / LVDd,

Here, LVPWTd means an end-diastolic posterior wall thickness of LV, while LVDd refers to an end-diastolic internal diameter of LV.

The calculation of fractional shortening (FS) was as follows:

FS = 100 × (LVDd − LVDs) / LVDd, where LVDs refers to an end-systolic internal diameter of the LV.

3.7. Hoechst 33258 staining

To determine the effects of ad-miR-208 inhibitor on cell apoptosis, primary cardiomyocytes were transfected with ad-208 inhibitor or ad-NC for 48 h. The, the cells were washed with PBS for three times (5 min/time) and examined under a fluorescence microscope.

3.8. Apoptosis assay

Firstly, the cells were washed with PBS for three times. To determine cell apoptosis, an Annexin-V FITC–PI Apoptosis Kit (Invitrogen, Carlsbad, CA) was used. Breifly, the cells were washed with 1 × Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of $2\text{-}3\times10^6$ cells/mL. Then, the annexin-v fitc and propidium iodide buffer was added followed by incubation at room temperature for 15 min. After treatment, the cells were filtered by a 300 mesh filter and analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) within 1 h of staining.

3.9. Determination of ROS

After appropriate treatment, the cells were washed with PBS for three times (5 min/time) and incubated with ROS Fluorescent Probe-DHE (Vigorous Biotechnology Beijing Co., Ltd) in serum-free DMEM F-12 medium for 30 min at 37 °C in the dark. The, the cells were fixed in 4% paraformaldehyde for 30 min at room temperature and the slides were mounted. The fluorescence was examined by fluorescence microscopy. To quantify the relative fluorescence, the cells stained with Probe-DHE was collected at the concentration of 2.5×10^6 cells/mL and analyzed using flow cytometry (Becton Dickinson, Franklin Lakes, NJ) within 1 h of staining.

3.10. Determination of caspase-3activity

An assay kit (Medical & Biological Laboratory, Nagoya) was applied to determine the activity of caspase3 in accordance with the instructions. In brief, the cell lysates were collected and incubated with the substrates for 1h at 37 °C. To determine the enzyme activity, the intensity of fluorescence was measured by a spectrophotometer (Molecular Dynamics, Sunnyvale, CA, USA). The activity was calculated by comparison with the level of the untreated control.

3.11. Statistical analysis

Data were presented as mean±SE from three independent experiments. Statistical analysis was carried out with Student's t test. $P < 0.05$ was considered as statistically significant difference.

Conflicts of interest: None declared.

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