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Original Article

MicroRNA-182-5p protects H9c2 cardiomyocytes from hypoxia-induced apoptosis by down-regulation of PTEN

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Abstract: Recent studies suggest that miRNA may play an important role in the process of cell oxidative damage. In this study we made an attempt to explore the functional role of miR-182-5p in acute myocardial infarction. H9c2 cells were cultured and hypoxic H9c2 cells were incubated in hypoxic incubator to simulate hypoxia. Total RNA was extracted and quantified for miR-182-5p. MiR-182-5p mimic, si-miR-182-5p and the NC controls were synthesized and transfected into H9c2 cells. Transfected cells were then then assessed for cell-viability by CCk8 assay, cell-apoptosis and cell migration. Result showed that hypoxia led to proportionate decrease in cell-viability, an increased cell-apoptosis, and significant reduction in cell-migration at 24 hrs (*P*-value < 0.001). A significant increase in relative expression of miR-182-5p was observed with time at 24 hrs (*P*-value < 0.001). Over-expression of miR-182-5p alleviated myocardial injury induced by hypoxia (*P*-value < 0.0001) while knockdown of miR-182-5p showed contrary results. Cells transfected with miR-182-5p mimic down-regulated the expression of phosphatase and tensin homolog (PTEN) (*P*-value < 0.05) and PTEN expression was significantly up-regulated in silenced miR-182-5p cells. The results also showed that over-expression of miR-182-5p inhibited PTEN expression and promoted Akt/GSK-3 beta signaling pathway, resulting in promotion of myocardial cell survival and reduction of cell injury. It was safely concluded that over-expression of miR-182-5p inhibited PTEN expression and enhanced Akt/GSK-3 beta signaling pathway which led to the promotion of myocardial cell survival and reduction of cell injury.

Keywords: Myocardial infarction, hypoxia, mir-182-5p, H9c2 cells, phosphatase and tensin homologue (PTEN), Akt/GSK-3 beta signaling pathway

Introduction

MicroRNAs (miRNAs) are produced endogenously and are short noncoding RNAs which regulate target messenger RNA translation by binding to the 3' untranslated region of mRNA transcript, thereby leading to the degradation of RNA and/or inhibition of protein synthesis [1-3]. miRNAs are not only crucial for the development and maintenance of physiological homeostasis, but are also equally important in diverse pathological condition [4, 5]. A number of studies have proved that miRNAs play an indispensable role in gastrointestinal development and physiology [6-8]. Moreover, characteristic changes in miRNA expression are associated with cell apoptosis, oxidative stress and inflammation [7, 9, 10]. miR-182-5p is a family member of miR-182, which is highly expressed in prostate cancer cell. miR-182-5p can promote cell invasion and proliferation by down-regulating the expression of FOXF2, RECK and MTSS1 [11].

Cell apoptosis play an important role in both normal development and pathology of a variety of tissues. Hypoxia affects the extent of cell injury and death by triggering apoptosis in cardiomyocytes [12]. Myocardial hypoxia-induced apoptosis was associated with cardiac dysfunctions, especially in myocardial infarction (MI), myocardial ischemia, and heart failure [13]. Hence, therapeutic strategies are being investigated and developed to prevent or delay the hypoxia-induced cardiac apoptosis in the treatment of related heart diseases. It is well established fact that activation of the PI3K/Akt pathway protects the heart from ischemia-reperfusion injury (IRI), where phosphatase and tensin homolog (PTEN) is the main negative regulator.

Recent studies suggest that miRNA may play an important role in the process of cell oxidative damage. In this study we made an attempt to explore the functional role of miR-182-5p in acute myocardial infarction (AMI).

Materials and methods

Cell culture and treatment

H9c2 cells derived from rat embryonic ventricular cardiomyocytes were cultured in DMEM contained with 10% (v/v) FBS, 100 U/ml penicillin and 100 lg/ml streptomycin at 37°C in an atmosphere of 95% air and 5% CO_2. Hypoxic H9c2 cells were incubated in hypoxic incubator containing 94% N_2, 5% CO_2, and 1% O_2 to simulate hypoxia [11].

Quantitative-real-time PCR (QRT-PCR)

Total RNA was extracted from cells and tissues using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay of miR-182-5p and U6 (Applied Biosystems, Foster City, CA, USA) were used for testing the expression levels of miR-182-5p in cells.

MiRNAs transfection

MiR-182-5p mimic, si-miR-182-5p and the NC controls were synthesized by GenePharma Co. (Shanghai, China). Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol.

CCK-8 assay

Cells were seeded in 96-well plates with 5000 cells/well, cell proliferation was assessed by a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Briefly, after stimulation, the CCK-8 solution was added to the culture medium, and the cultures were incubated for 1 hour at 37°C in humidified 95% air and 5% CO_2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

Apoptosis assay

Flow cytometry analysis was performed to identify and quantify the apoptotic cells by using Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). The H9c2 cells (100,000 cells/well) were seeded in 6 well-plates. Treated cells were washed twice with cold PBS and resuspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with flow cytometer (Beckman Coulter, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

Migration assay

Cell migration was determined by using a modified two-chamber migration assay with a pore size of 8 mm. For migration assay, cells suspended in 200 ml of serum-free medium were seeded on the upper compartment of 24-well Transwell culture chamber, and 600 ml of complete medium was added to the lower compartment. After incubation at 37°C, cells were fixed with methanol. Non-traversed cells were removed from the upper surface of the filter carefully with a cotton swab. Traversed cells on the lower side of the filter were stained with crystal violet and counted.

Western blot

The protein used for Western blotting was extracted using RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. GAPDH antibody was purchased from Sigma. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane at 4°C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 hour at room temperature. After rinsing, the polyvinylidene difluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 µl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

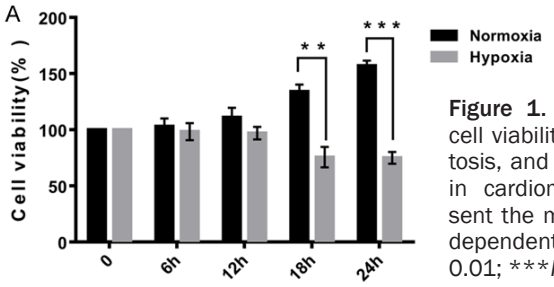
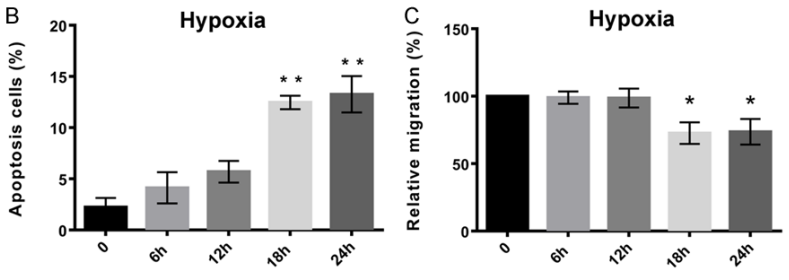


Figure 1. Hypoxia inhibited the cell viability, promoted cell apoptosis, and reduced the migration in cardiomyocytes. Data represent the mean ± SD of three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.



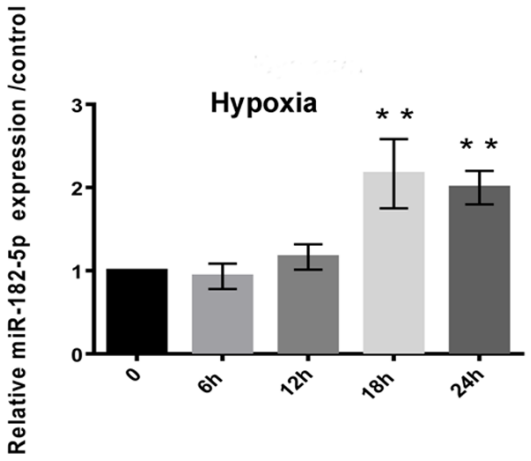


Figure 2. MiR-182-5p was highly expressed by hypoxia induction. Data represent the mean ± SD of three independent experiments. $^{**}P < 0.01$.

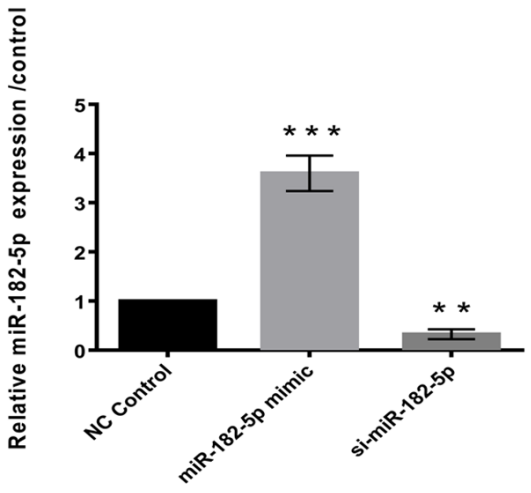


Figure 3. Over-expression or silenced the expression of miR-182-5p in cardiomyocytes. Data represent the mean ± SD of three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as mean ± SD. Statistical analyses were performed using SPSS 19.0 statistical software. *P*-values were calculated using one-way analysis of variance (ANOVA). A *P*-value of < 0.05 was considered to indicate a statistically significant result.

Results

Hypoxia induced cardiomyocytes injury

Hypoxia led to a proportionate decrease in cell-viability and increase in cell-apoptosis with time compared to the percentage of normoxia. A statistically significant decrease in cell viability ($P < 0.0001$) and an increase in cell apoptosis were observed at 24 hrs. A significant reduction in cell-migration was observed at 24 hrs ($P < 0.001$, **Figure 1**). These results suggest that hypoxia induced injury in H9c2 cells.

Hypoxia induced high expression of miR-182-5p

A significant increase in relative expression of miR-182-5p was observed with time at 18 and 24 hrs (*P*-value < 0.001, **Figure 2**). These results suggest that hypoxia induced high expression of miR-182-5p in H9c2 cells.

Over-expression or silencing of miR-182-5p expression in cardiomyocytes

MiR-182-5p mimic, si-miR-182-5p and the NC controls were synthesized and transfected into H9c2 cells. Over-expression or silencing of miR-182-5p expression was observed in cardiomyocytes when compared to the control cells ($P < 0.0001$ and $P < 0.001$, respectively, **Figure 3**). These findings suggested that miR-182-5p can

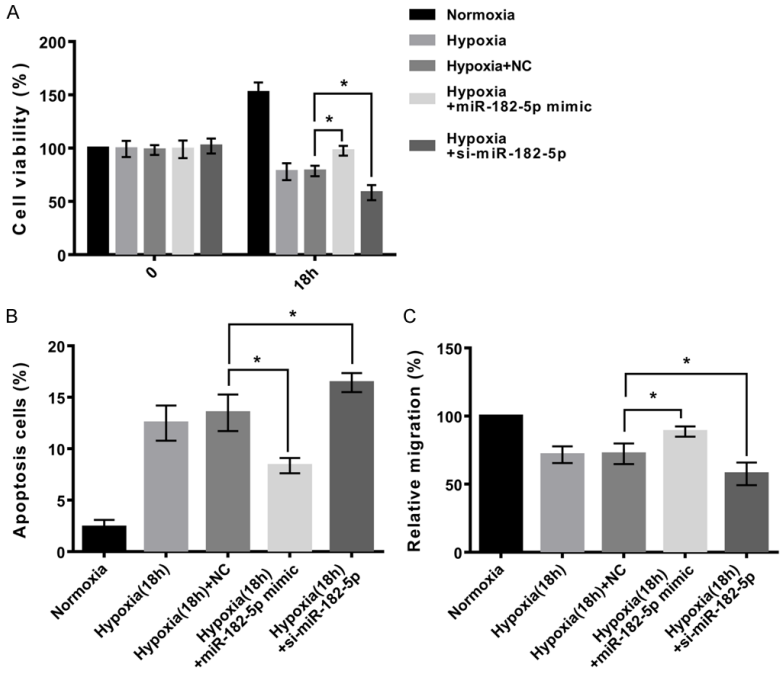


Figure 4. Over-expression of miR-182-5p alleviated hypoxia-induced proliferation inhibition, reduced cell apoptosis, and promoted cell migration. Data represent the mean ± SD of three independent experiments. *$P < 0.05$.

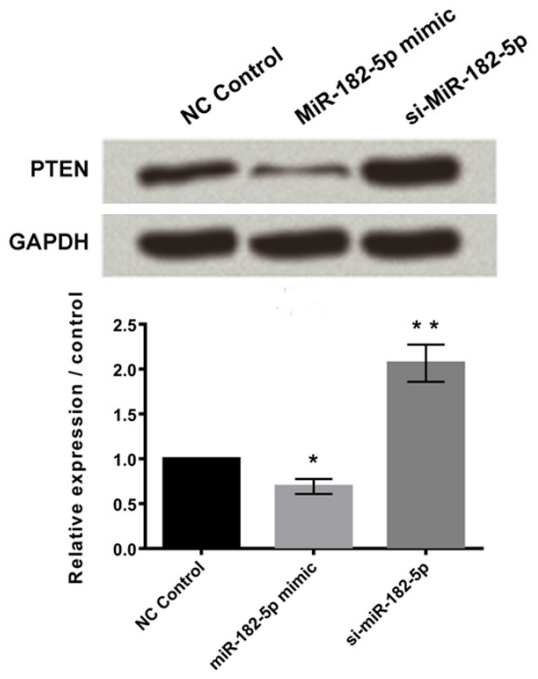


Figure 5. MiR-182-5p negatively regulated the expression of PTEN. Data represent the mean ± SD of three independent experiments. *$P < 0.05$; **$P < 0.01$.

be either overexpressed or silenced in cardiomyocytes.

Effects of over-expression and silencing of miR-182-5p of hypoxia induced injury

At 18 hrs, cell-viability increased significantly due to over-expression of miR-182-5p ($P < 0.05$) and decreased significantly in silenced miR-182-5p ($P < 0.05$) as compared to the control cells. Additionally, cell apoptosis decreased significantly due to over-expression of miR-182-5p ($P < 0.05$) and increased when miR-182-5p was silenced ($P < 0.05$) compared to control cells. A significant increase in relative cell-migration was observed in cells with over-expression of miR-182-5p ($P < 0.05$) and decrease was observed in cells with silenced miR-182-5p with $P < 0.05$ (**Figure 4**). These results indicated the effects of miR0182-5p in hypoxia induced injury.

MiR-182-5p negatively regulated the expression of PTEN

Western blotting results showed that cells transfected with miR-182-5p mimic down-regulated the expression of phosphatase and tensin homolog (PTEN) with $P < 0.05$. Similarly, significant up-regulation was observed in cells transfected with silenced miR-182-5p, both with $P < 0.001$ (**Figure 5**).

MiR-182-5p alleviated hypoxia-induced cardiomyocytes injury by regulation of PTEN expression via activating Akt/GSK-3β signaling pathway

PTEN is an inhibitory factor of AKT pathway. Hypoxia induced slight increase of AKT/GSK3β pathway. This was confirmed by the over-expression of miR-182-5p which inhibited the expression of PTEN, leading to the activation of AKT/GSK3β pathway, and then promoted cell survival and reduced apoptosis. The results were reversed by silencing of miR-182-5p ($P < 0.05$, **Figure 6**).

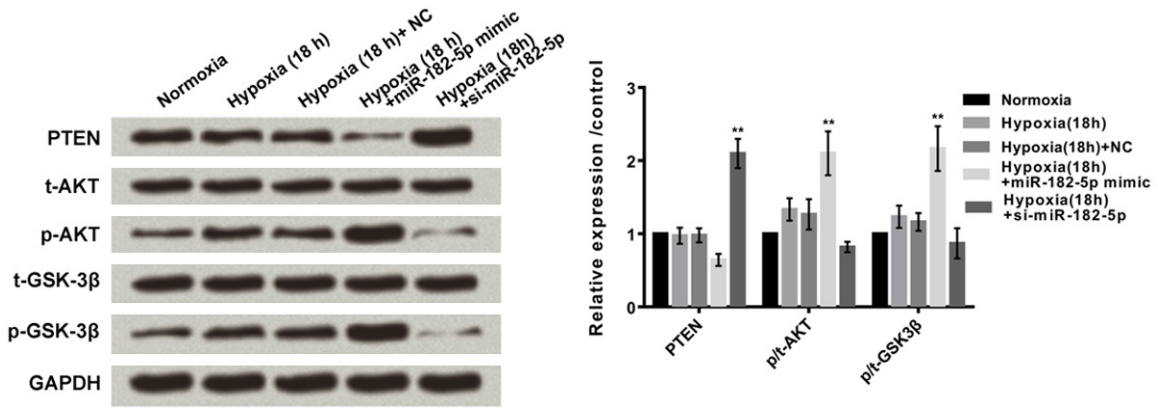


Figure 6. PTEN is a inhibitory factor of AKT pathway: Hypoxia induced weak increase of AKT/GSK3β pathway: Over-expression of miR-182-5p inhibited the expression of PTEN, leading to the activation of AKT/GSK3β pathway, and then promoted cell survival and reduced apoptosis. The results were reversed by silencing of miR-182-5p. Data represent the mean ± SD of three independent experiments. $^{**}P < 0.01$.

Discussion

Myocardial hypoxia is known to trigger cell injury and apoptosis, which play a key role in the pathogenesis of many cardiovascular diseases including myocardial infarction (MI), heart failure, myocardial ischemia, and reperfusion injury [14]. A number of scientific reports indicated that miRNAs are of vital importance in cardiovascular diseases, thereby increasing the importance of demonstration of miRNAs in controlling apoptosis and identifying their direct and indirect targets [15-17].

A study by Zhang et al. showed that miR-182 is a tumor suppressor in lung cancer cell line [18], while a number of reports suggested miR-182-5p to be an oncogene in several types of cancers [19-24].

In this paper, the effects and mechanisms of miR-182-5p on hypoxia-induced injury of cardiomyocytes were explored. Hypoxia induced upregulation of miR-182-5p. Over-expression of miR-182-5p led to alleviation of myocardial injury induced by hypoxia while knockdown of miR-182-5p showed contrary results. Further, the results showed that over-expression of miR-182-5p inhibited PTEN expression and promoted Akt/GSK-3 beta signaling pathway, resulting in promotion of myocardial cell survival and reduction of cell injury. PTEN when deleted on chromosome ten was found to antagonize the actions of phosphatidylinositol (PI)-3-kinase (PI3K) by de-phosphorylation of PI-3,4,5-trisphosphate (PIP3) to PI-4,5-bisphophate (PIP2) and Pi [25], which lead to enhanced cell death and increased myocardial contractility in the heart [26-29]. Thus, pharmacological inhibition of PTEN was found to enhance PI3K activity and attenuate cardiac injury after ischemia-reperfusion [30]. Moreover, inactivation of cardiac-specific PTEN protects the heart from functional failure and fibrosis as observed in a mouse model of pressure overload [31]. Further, constitutive PI3K (p110 alpha) and chronic Akt activation attenuates post-MI remodeling [32, 33]. Hence, the findings of our study revealed that the expression of PTEN inhibition led to the activation of Akt/GSK-3 beta signaling pathway which was subsequently followed by promotion of myocardial cell survival and reduction of cell injury was in line with observations in aforementioned reports. This paper thus lays a theoretical foundation for further study on the function of miR-182-5p, and also provides a new strategy for the clinical treatment of myocardial infarction.

Disclosure of conflict of interest

None.

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