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Original Article

MicroRNA-152-3p negatively regulates ischemia-reperfusion induced cardiomyocyte apoptosis by inhibiting PTEN

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Abstract: microRNAs (miRNAs) play a vital role in the treatment of many cardiovascular diseases. Myocardial ischemia/reperfusion (I/R) injury has been associated with adverse cardiovascular outcomes. However, whether miR-152-3p is involved in I/R induced cardiomyocyte injury remains ill-defined. In this study, it was found that miR-152-3p expression was markedly down-regulated in H9c2 cells after hypoxia/reoxygenation (H/R). H/R treatment significantly elevated the levels of LDH and MDA, and enhanced cell apoptosis in H9c2 cells. Ectopic expression of miR-152-3p markedly blocked LDH & MDA production and apoptosis in H9c2 cells after H/R, while depletion of miR-152-3p in H9c2 cells induced cell apoptosis. Mechanistic analyses indicated that phosphatase and tensin homolog (PTEN) was regulated by miR-152-3p directly. Additionally, subsequent investigations identified that the anti-apoptotic effect of miR-152-3p was partially reversed when H9c2 cells were co-transfected with PTEN mimic. In addition, up-regulation of miR-152-3p significantly elevated the levels of p-AKT and p53 under H/R. Furthermore, therapeutic promotion of miR-152-3p decreased LDH content, cardiac hypertrophy, and apoptosis after I/R in *vivo*. These findings show that miR-152-3p could suppress I/R injury induced cardiomyocyte apoptosis by targeting PTEN. Therefore, miR-152-3p and PTEN could be regarded as potential therapeutic targets for the clinical study of I/R injury.

Keywords: Cardiomyocyte, miR-152-3p, ischemia-reperfusion, PTEN, apoptosis

Introduction

Ischemic heart disease is one of the leading causes of mortality and morbidity all over the world. The current standard treatment for myocardial ischemic incidents is the rapid reperfusion [1, 2]. However, the acute restoration of blood flow can cause the cardiac injury by itself, a process termed myocardial I/R injury. Myocardial I/R induce cardiac injury in the process of myocardial ischemia, cardiac arrest, or cardiac surgery, which contributes to irreversible damage to the myocardium [3-5]. It has been elucidated that the molecular mechanisms underlying myocardial I/R injury are complex and multifaceted, including oxygen free radicals, intracellular calcium overload, inflammation, and mitochondrial permeability [6, 7]. However, the complete profile of molecular pathways underlying biologic processes involved in reperfusion injury remains mostly unknown. Recently, apoptosis was suggested to be the major mechanism leading to the myocardial I/R injury [8-10]. Therefore, further studies on the apoptotic mechanisms underlying the development of myocardial I/R injury, may lead to the identification of effective targeted therapy.

miRNAs are a family of short non-coding RNA molecules that regulate target gene expression, through specific binding to the 3'-untranslated region (3'UTR) of target messenger RNAs at the post-transcriptional level [11, 12]. Increasing

evidence has indicated that miRNAs are involved in the regulation of various biological processes that are essential for normal development and cellular homeostasis [13, 14]. Recent studies have reported that several miRNAs are involved in the regulation of pathological and physiological process of cardiac diseases [15, 16]. As an example, miRNA microarray analysis shows that miR-320 is up-regulated in cardiomyocytes after I/R treatment, and that silencing of endogenous miR-320 inhibits I/R-induced cardiomyocyte apoptosis by targeting HSP-20 [17]. Similarly, miR-320 has been shown to promote cardiomyocyte apoptosis and myocardial I/R injury, and its downregulation reduces cardiomyocyte apoptosis and death in mice [18]. Conversely, miR-17 is sensitive to I/R injury and attenuated I/R-induced apoptosome formation by inhibiting apoptotic protease activation factor 1 expression [19].

Among many miRNAs, miR-152-3p has been identified to play a key role in a number of essential biological processes, including tumorigenesis, cell proliferation, differentiation, and apoptosis [20, 21]. However, the role of miR-152-3p in cardiac I/R injury remains largely unknown. Therefore, the purpose of this study was to investigate the effects of miR-152-3p on myocardial I/R injury, and to identify mechanisms by which miR-152-3p may ameliorate the cardiomyocyte apoptosis and apoptosis related genes. In this study, miR-152-3p was found to have anti-apoptotic effects on cardiomyocyte and protective effects against myocardial I/R injury. These results provide novel mechanistic insights into the role of miR-152-3p in myocardial I/R injury.

Materials and methods

Cell culture and reagents

H9c2 cells was purchased from the Cell Bank of Chinese Academy of Science and cultured in a medium of Dulbecco's Modified Eagle Medium (Invitrogen, CA, USA) supplemented 10% FBS. Cells were cultured with 1% penicillin/streptomycin and 1% glutamine in a humidified incubator with 5% CO_2 at 37°C. Lactate dehydrogenase (LDH) and malondialdehyde (MDA) commercial kits were obtained from Beyotime Institute of Biotechnology (Shanghai, China). In situ cell death detection kit was purchased from Roche (Basel, Switzerland). The antibodies against Bax, Bcl-2 and caspase-3 were obtained from Santa Cruz Biotechnology (Santa Cruz, St Louis, Mo, USA). The antibodies against PTEN, phosopho-AKT, AKT, p53, HIF-α and β-actin were purchased from Abcam Biotechnology (Abcam, Cambridge, MA, USA).

In vitro I/R injury model

A simulated H/R cell model was performed as previously description [14, 15]. Briefly, H9c2 cells were firstly perfused in normal Hank's solution with 95% oxygen and 5% CO_2. To simulate ischemia, nitrogen gas was flushed into the incubator to decrease oxygen to 1%. To simulate reperfusion, cardiomyocytes were subjected to 6 hours of hypoxia followed by re-oxygenation by incubation in normal Hank's solution with a gas mixture of 95% oxygen and 5% CO_2 for 1 hour. Cells under normoxia throughout the experiments were set as control group.

Cell transfection

miR-152-3p mimic and its negative control (mimic control), as well as miR-152-3p inhibitor and its negative control (anti-control) were purchased from QIAGEN (Duesseldorf, Germany). The plasmid for PTEN over-expression (PTEN) was constructed by Sesh-biotech (Shanghai, China). Cells were seeded in 6-well or 96-well plates and transfected using Lipofectamine 2000 (Invitrogen, CA, USA) for 24 hours, according to the manufacturer's protocol. Transfected cells were used for further analysis.

Quantitative real-time PCR

Total RNAs from either cultured cells or tissue samples were isolated using Trizol Reagent (QIAGEN, Duesseldorf, Germany). First-strand cDNA was synthesized using a TaqMan Reverse Transcription Reagents (Life Technologies). The levels of mRNAs were detected using Taqman MicroRNA Assay Kits (Applied Biosystems, Foster City, CA) specific for miR-152-3p. The universal reverse quantitative PCR primer was provided in the miScript SYBR Green PCR Kit. The qRT-PCR results were analyzed using the comparative cycle threshold method (2-ΔΔCT). Each sample was performed in triplicate.

Detection of LDH and MDA

The levels of intracellular LDH and MDA were measured using commercial kits according to manufacturer's instructions.

Apoptotic cell determination

Apoptotic cells were determined by TUNEL method as per manufacturer's instructions. The percentage of TUNEL-positive cardiomyocytes per high powered field was observed under a fluorescence microscope (Olympus, Tokyo, Japan) by two different pathologists. For quantification, the apoptotic index was evaluated by counting the number of TUNEL-positive apoptotic cells per 100 cells in three randomly selected myocardial areas with 400 × magnification.

Western blot analysis

Cells or tissues were lysed in RIPA buffer (Beyotime, Jiangsu, China) containing protease inhibitors (Beyotime), and the protein concentrations were measured using a BCA Kit (Sigma, St. Louis, MO, USA). A 25 μg sample of protein was separated by 10% SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 5% non-fat powdered milk in PBS and then probed with primary antibodies. After primary antibody incubation, membranes were probed with peroxidase-labeled secondary antibodies. The results were normalized to β-actin as the relative density.

Dual luciferase reporter assay

The wild-type PTEN 3'UTR containing the predicted miR-152-3p binding sites was amplified by specific primers using genomic DNA as a template and then inserted into the downstream of the luciferase gene sequence in the psiCHECK-2 vector (Promega, WI, USA). The mutant-PTEN 3'UTR (deletion of AAAAUGU) was also accomplished by PCR. All vectors were identified by DNA sequencing. Cells were plated into 96-well plates in 5 replicates. For transfection, 2 μl of 20 μM of either miR-152-3p mimic or miR-152-3p inhibitor and 150 ng reporter plasmids (either wt-PTEN or mut-PTEN) were mixed with 2 μl Lipofectamine 2000 (Invitrogen, CA, USA). Firefly luciferase activity was measured using a Dual-Luciferase Assay System (Promega, WI, USA) and was normalized to Renilla activity.

In vivo gene transfection myocardial I/R model

A total of 60 female Sprague-Dawley rats (weight, 250 g-350 g; age, 10-14 weeks) were purchased from the animal experimental center of the Central South University. Animals were housed at a temperature of 25±2°C and the humidity was set at 60%. Photoperiod was maintained a 12:12 hour light-dark cycle with standard forage and drinking water ad libitum. This study's protocols were approved by the animal ethics committee of the Xiangya Hospital of Central South University. The 60 rats were randomly divided into three groups (n = 20). 1) Sham-operated group: the heart was exposed after left thoracotomy without ligating the left anterior descending artery (LAD). 2) I/R group: The LAD was ligated reversibly using silk suture. Following 30 min of ischemia, the ligature was loosened, allowing the ischemic myocardium to re-perfuse for 2 h. 3) I/R + Ad-miR-152-3p group: Four days after the rats received intramyocardial injection of Ad-miR-152-3p recombinant adenovirus, myocardial I/R treatment was induced as above. Rats were sacrificed at the time of coronary artery flow of 120 minutes to obtain the hearts and blood samples for subsequent tests. MiR-152-3p mimic lentivirus and empty viral vectors were purchased from QIAGEN (Duesseldorf, Germany).

Histology

Heart samples were cut transversely into blocks and fixed in 4% paraformaldehyde. Tese were then embedded in OCT compound (BHD, UK) and then transversely cut into 4 μm sections. Next, the slides were incubated with Masson's trichrome for assessment of infarct size. Results were analyzed as the sum of the epicardial and endocardial scar length, divided by the sum of LV epicardial and endocardial circumferences. Cardiomyocyte hypertrophy was assessed by calculating the average cardiomyocyte cross-sectional areas in five random fields.

Statistical analysis

All statistical results were analyzed using SPSS 18.0 statistical software. Experimental data from 3 independent experiments are presented as the mean ± SD. Quantitative variables between groups were analyzed using either *t*-test or ANOVA. *P* values less than 0.05 (two-sided) were considered to be statistically significant.

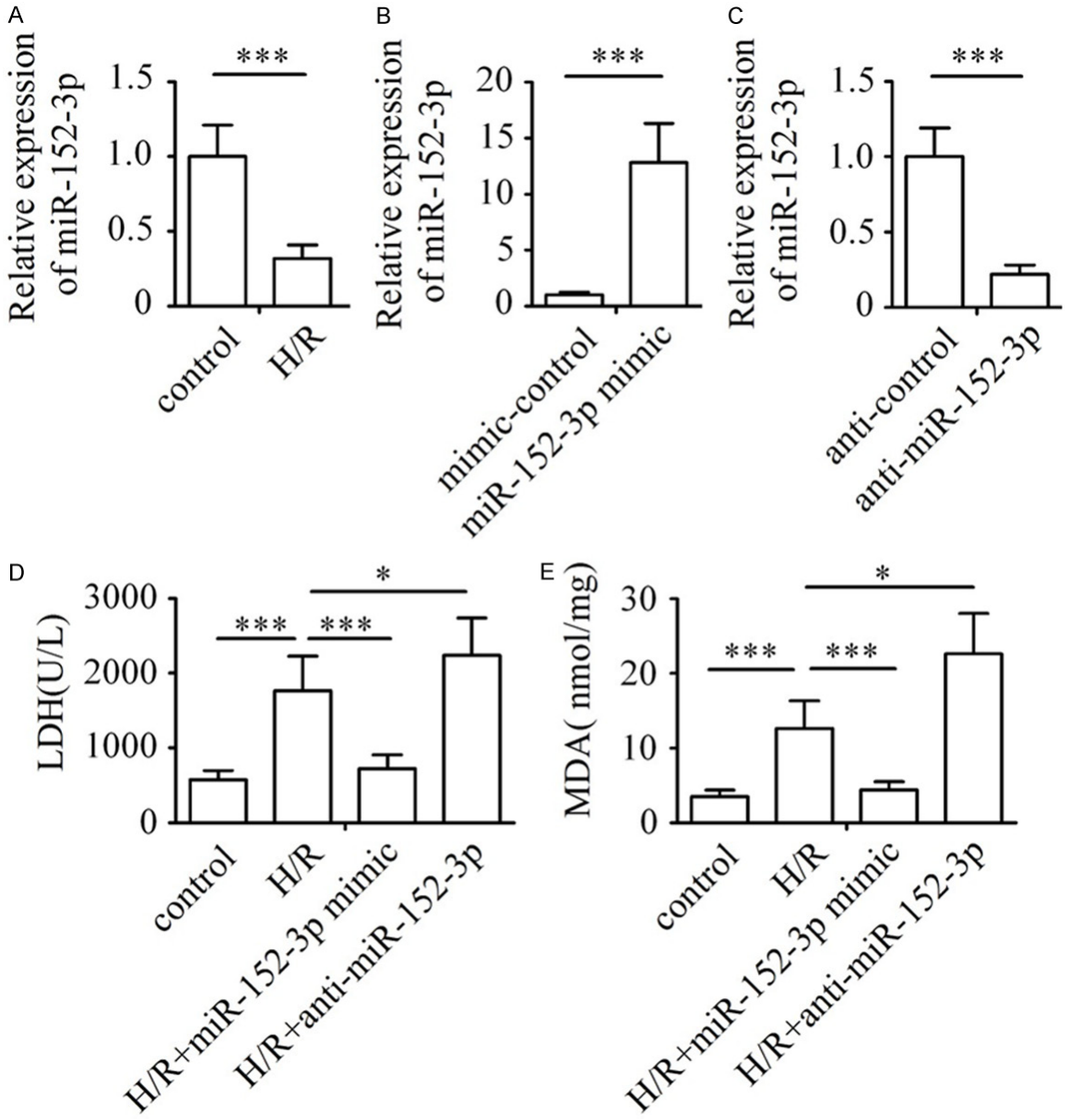

Figure 1. Effects of miR-152-3p on H9c2 cells following H/R injury. (A) The expression of miR-152-3p was decreased significantly after H/R injury. (B) H9c2 cells were transfected with miR-152-3p mimic or mimic-control, and miR-152-3p expression was detected by qRT-PCR. (C) H9c2 cells were transfected with anti-miR-152-3p or anti-control, and expression of miR-152-3p was detected by qRT-PCR. The levels of LDH (D) and MDA (E) were measured in H9c2 cells. ***$P < 0.001$, *$P < 0.05$.

Results

miR-152-3p is down-regulated in H9c2 after H/R

We first detected miR-152-3p expression in H9c2 cells after 10 hours of hypoxia and 2.5 hours of reoxygenation by qRT-PCR. The results demonstrate that the expression of miR-152-3p is reduced markedly in H9c2 cells following H/R treatment (**Figure 1A**). This finding indicates that miR-152-3p may play a vital role in the process of H/R injury.

miR-152-3p regulates H/R-induced cardiomyocyte injury in vitro

The effects of miR-152-3p modification on the H/R injury of H9c2 cells were then explored. *In vitro* gain and loss-of-function assays were per-

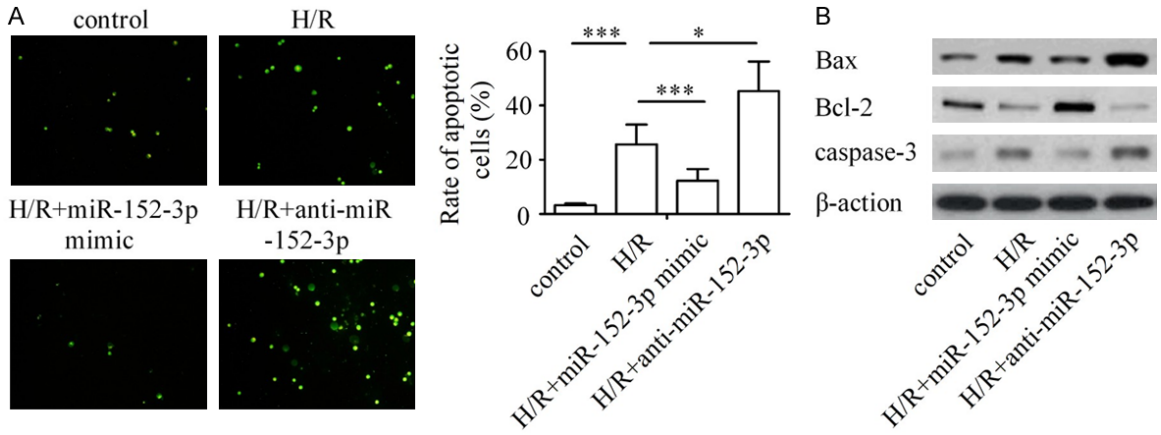

Figure 2. Effects of miR-152-3p on H/R-induced cardiomyocyte apoptosis. A: Ectopic expression of miR-152-3p attenuates cardiomyocyte apoptosis during H/R injury and its down-regulation promotes myocyte apoptosis. B: Western blot analysis of Bax, Bcl-2 and caspase-3 staining in H9c2 cells. β-action was used as endogenous control. ***$P < 0.001$, *$P < 0.05$.

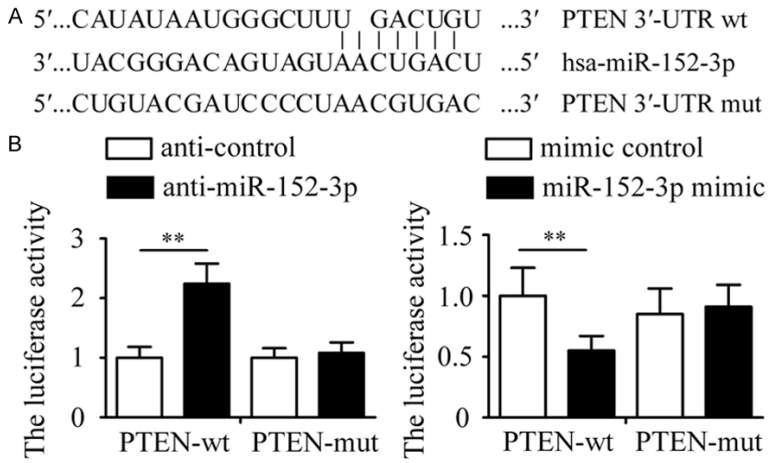

Figure 3. miR-152-3p down-regulated PTEN by interacting with its 3'UTR. A: miR-152-3p and its putative binding sequences in the PTEN 3'UTR. B: H9c2 cells were co-transfection of PTEN-3'UTR-wt or PTEN-3'UTR-mut with miR-152-3p mimic, anti-miR-152-3p or appropriate controls and the relative luciferase activities were measured. **$P < 0.01$.

formed by the transfection of miR-152-3p mimic or inhibitor in H9c2 cells. As a result, transfection of miR-152-3p mimic increased expression of miR-152-3p dramatically (**Figure 1B**). Moreover, the relative level of miR-152-3p was reduced significantly after transfection with miR-152-3p inhibitor (**Figure 1C**). LDH release is an indicator of cellular injury. Our results demonstrate that the level of LDH significantly increases in response to H/R treatment in the culture media. Expression of LDH was repressed significantly by miR-152-3p over-expression. In contrast, the miR-93 inhibitor promoted LDH release after H/R (**Figure 1D**). MDA is regarded as a biomarker for cardiomyocyte oxidative damage. As shown in **Figure 1E**, the H/R-induced MDA release was reduced in H9c2 cells with over-expression of miR-152-3p, whereas the depletion of miR-152-3p in H9c2 cells increased MDA expression (**Figure 1E**).

miR-152-3p down-regulation is required for H/R-induced cardiomyocyte apoptosis

H/R is a potent inducer of apoptosis in cardiomyocyte [14]. To investigate whether the down-regulation of miR-152-3p is required for H/R-induced cardiomyocyte apoptosis, the miR-152-3p inhibitor was transfected into H9c2 cells to inhibit the expression of miR-152-3p and the responses to H/R were investigated. The results show that the ratio of apoptotic cardiomyocyte was promoted notably after H/R treatment compared with that in the control group. However, transfection with miR-152-3p mimic significantly inhibited H/Rinduced cell apoptosis, and silencing of miR-152-3p promoted H/R-induced apoptosis in H9c2 cells (**Figure 2A**).

Consistent with morphologic changes, the representative Western blot analysis showed that

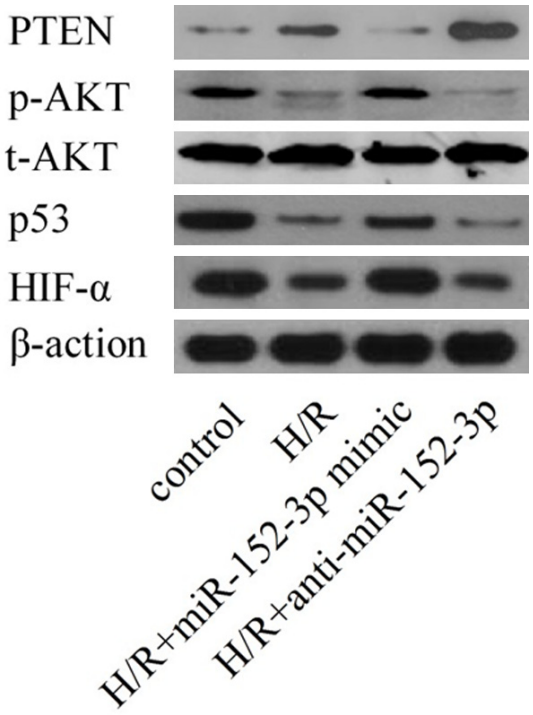

Figure 4. Western blot analysis of PTEN, phosphorylated PI3K/Akt and HIF-α in H9c2 cells after transfected with either miR-152-3p inhibitor or miR-152-3p mimic. β-actin was used as endogenous control.

significant down-regulation in Bcl-2 expression dramatically increased Bax & caspase-3 expression in H9c2 cells after H/R treatment. However, expression of Bcl-2 was reduced significantly, Bax was decreased & caspase-3 increased in the H/R + miR-152-3p inhibitor group compared with the H/R group, whereas ectopic expression of miR-152-3p markedly rescued the effect of H/R on expression of Bax, Bcl-2, and caspase-3 (**Figure 2B**). These results demonstrate that up-regulation of miR-152-3p reverses H/R-induced apoptosis ability.

PTEN is a direct target of miR-152-3p

To determine which gene was affected by miR-152-3p in cardiomyocytes, a bioinformatic analysis was performed on TargetScan to obtain putative target gene of miR-152-3p. The results indicate that human PTEN mRNA contains a binding site for miR-152-3p (**Figure 3A**). In addition, dual-luciferase reporter assay was employed to reveal whether miR-152-3p regulates PTEN directly or indirectly. When the wt-PTEN plasmid was co-transfected with the miR-152-3p inhibitor, the luciferase activity of H9c2 cells was decreased significantly, whereas this effect was almost abolished when the PTEN-3'UTR binding site was mutated. In contrast, the promotional effect of miR-152-3p on luciferase activity was obviously abrogated by the co-transfection of PTEN-3'UTR-mut in H9c2 cells (**Figure 3B**). These data support the assumption that miR-152-3p directly targets PTEN.

To further validate whether miR-152-3p regulates endogenous PTEN, the effects of miR-152-3p on PTEN were then explored in H9c2 cells by Western blot. As shown in **Figure 4**, the level of endogenous PTEN protein was significantly repressed in miR-152-3p-ectopic H9c2 cells and markedly enhanced in miR-152-3p-depleted H9c2 cells.

The PI3K/Akt signaling pathway has been reported as a main regulator of H/R induced myocardial cell apoptosis [7]. As depicted in **Figure 4**, compared with the control group, the levels of p53 and phosphorylation of Akt were inhibited by H/R treatment, and this inhibition was ameliorated by over-expression of miR-152-3p. Furthermore, miR-152-3p knockdown reduced the levels of p53 and phosphorylated-Akt in H9c2 cells. These observations indicate that miR-152-3p may protect against H/R-induced myocardial apoptosis via up-regulation of PI3K/Akt signaling.

PTEN restoration partially reverses the effect of miR-152-3p on H/R-induced cardiomyocyte injury

To assess the role of PTEN in H/R-induced cardiomyocyte injury, expression of PTEN was up-regulated in H9c2 cells after transfection with PTEN mimic. The effects of PTEN restoration on cardiomyocyte injury following the H/R treatment were determined. As shown in **Figure 5A** and **5B**, PTEN up-regulation reversed the inhibition of LDH and MBA induced by miR-152-3p mimic.

Our results demonstrate that up-regulation of miR-152-3p markedly inhibited cardiomyocyte apoptosis induced by H/R. However, the effects of miR-152-3p mimic were reversed by co-transfection with PTEN mimic (**Figure 5C**). These data present that the effects of miR-152-3p on H/R-induced cardiomyocyte injury were achieved by direct targeting of PTEN.

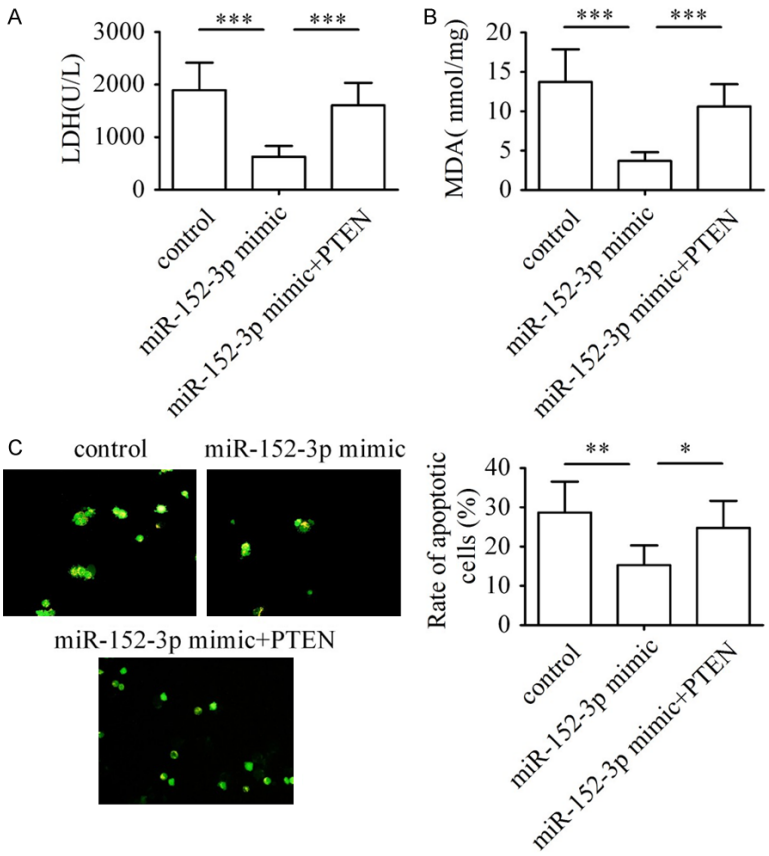

Figure 5. PTEN restoration partially reversed the protective effect of miR-152-3p on cardiomyocyte. The inhibition of LDH (A) and MBA (B) induced by miR-152-3p over-expression was almost completely abrogated by the raised expression of PTEN. (C) The anti-apoptotic effect of miR-152-3p was markedly rescued by the up-regulation of PTEN in H9c2 cells. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

miR-152-3p protects against myocardial I/R injury in vivo

As the miR-152-3p plays a vital role in suppressing cardiomyocyte injury in *vitro*, the next step was to explore the effects of miR-152-3p on cardiomyocyte injury in *vivo*. As shown in **Figure 6A**, the expression of miR-152-3p was significantly decreased in rat hearts after I/R treatment. Elevated levels of miR-152-3p in myocardium were observed after transfection of Ad-miR-152-3p by qRT-PCR. Furthermore, consistent with the results obtained from the in *vitro* assays, the activity of serum LDH was notably up-regulated in the I/R group in comparison with the sham group, whereas elevation in LDH level was significantly down-regulated in the I/R + Ad-miR-152-3p group than the I/R group (**Figure 6B**). Then, the effect of miR-152-3p on I/R-induced myocardial infarct was examined. Our results show that the myocardial infarct area size is reduced to ~63% by the Ad-miR-152-3p transfection (**Figure 6C**).

In addition, the TUNEL detection of cell apoptosis in the myocardium tissues was also assessed. As indicated in **Figure 6D**, the percentage of TUNEL-positive stained cells was higher in the tissues derived from I/R group than its expression in the sham group. Forced overexpression of miR-152-3p suppressed cardiomyocyte apoptosis compared with that in the I/R group. Subsequently, levels of apoptosis related proteins in the myocardium were examined by Western blot. The results demonstrate the expression of Bcl-2 and p-Akt were decreased in the I/R group compared with that in the sham group, which were both reverted in the I/R + Ad-miR-152-3p group. Moreover, the caspase-3 level was promoted in the I/R group, which was attenuated in the I/R + Ad-miR-152-3p group (**Figure 6E**). Taken together, such outcomes indicate that miR-152-3p can inhibit myocardial I/R injury in *vivo* effectively.

Cardiomyocyte hypertrophy is a pathological response associated with heart failure and sudden death [19, 20]. This study attempted to investigate the effect of miR-152-3p upon cardiomyocyte hypertrophy in rats. The results disclose that the cell size of cardiomyocytes was significantly decreased in the myocardium by I/R treatment, whereas transfection with Ad-miR-152-3p markedly increased the cell size of cardiomyocytes following I/R treatment (**Figure 7**).

Discussion

Myocardial I/R injury after coronary reperfusion is a complex biologic process which ultimately

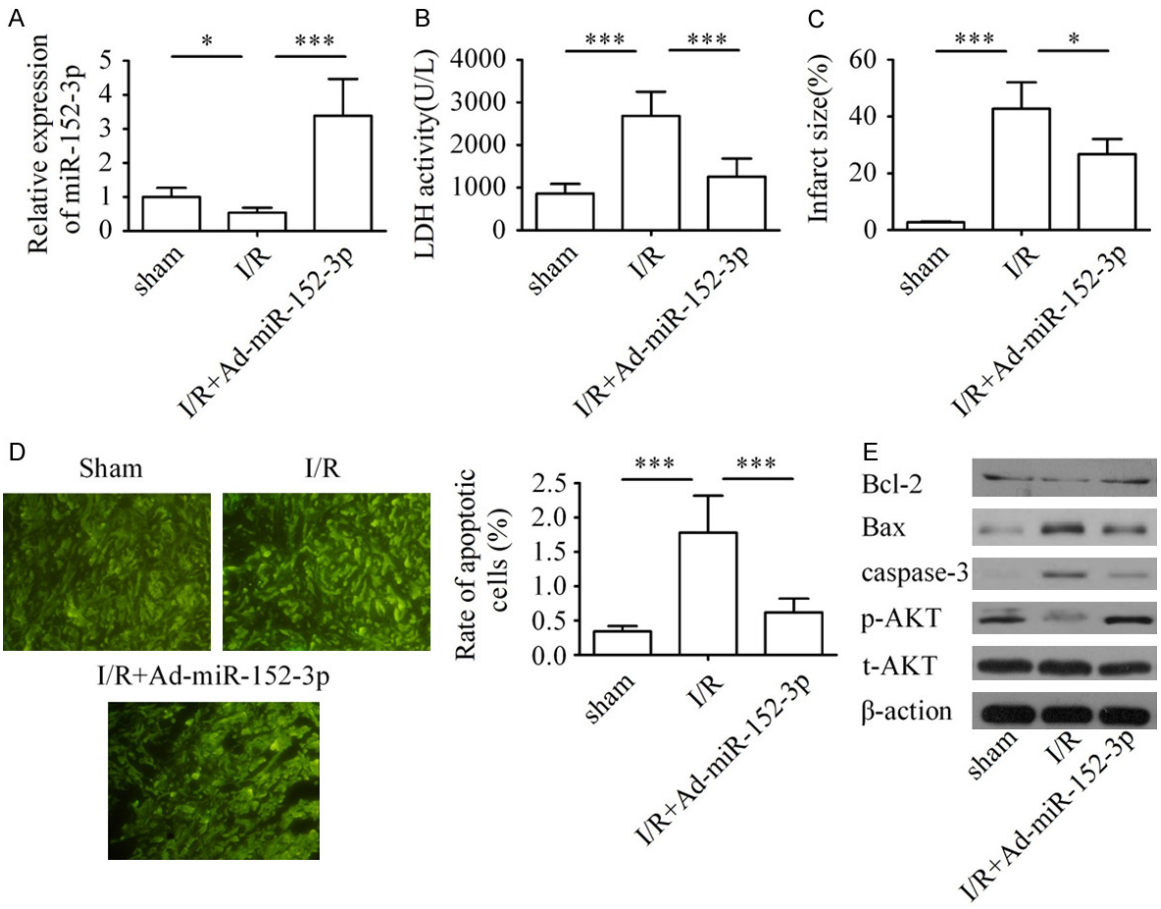

Figure 6. miR-152-3p exerts protective effect against myocardial I/R injury in *vivo*. (A) Expression of miR-152-3p was determined after myocardial I/R injury. Effects of miR-152-3p on the release of LDH (B) and myocardial infarct size (C) following I/R injury. (D) Representative TUNEL stained images of apoptotic cardiomyocytes. (E) Effects of miR-152-3p on the levels of apoptosis-related factors after myocardial I/R injury. ***$P < 0.001$, *$P < 0.05$.

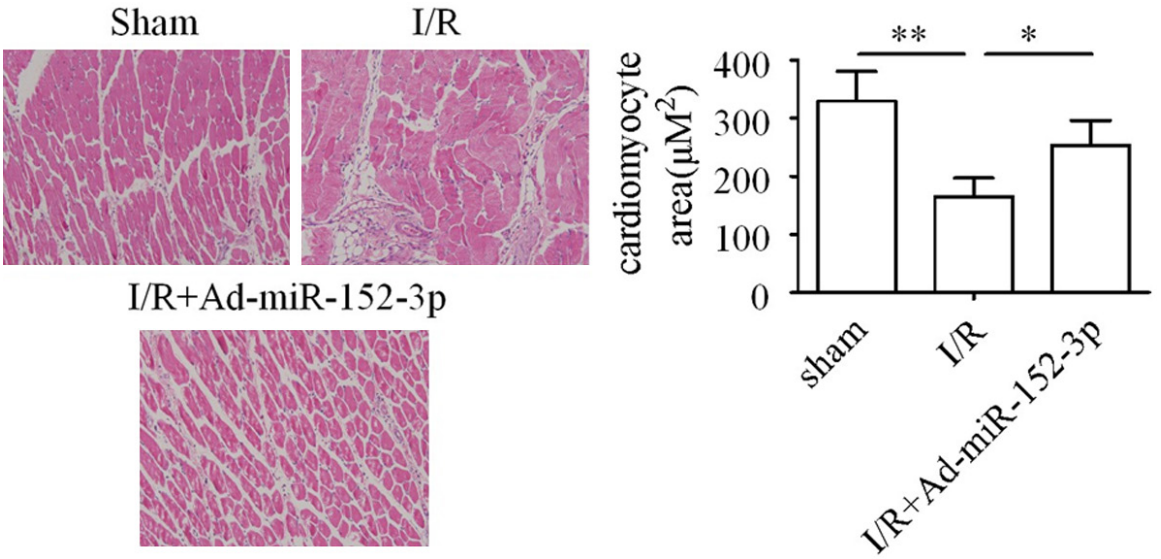

Figure 7. Overexpression of miR-152-3p attenuates cardiomyocyte hypertrophy following I/R injury in *vivo*. **$P < 0.01$, *$P < 0.05$.

causes further damage to ischemic myocardium [1-4]. It has been well established that apoptosis plays a vital role in cardiac I/R injury [8-10]. Hence, investigations of underlying apoptotic mechanisms will help develop new strategies for the treatment of I/R injury. Recently, several miRNAs have been shown to correlate with the regulation of pathological and physiological processes of heart disease owing to their effects on targeted genes associated with apoptosis [15, 16]. Here, the role of miR-152-3p was explored during the cardiac I/R injury. In *vitro* experiments, RT-PCR showed that the level of miR-152-3p was reduced after H/R treatment as compared with controls. Therefore, miR-152-3p may act as a valuable I/R-related miRNA in cardiomyocyte. However, the potential role of miR-152-3p in the cardiomyocyte apoptosis of I/R injury remains unclear.

In the present study, it is demonstrated that up-regulation of miR-152-3p inhibits H/R-induced apoptotic cell death and further study shows that the silencing of miR-152-3p increases the cardiomyocyte apoptosis in response to H/R in *vitro*. It is known that the Bax/Bcl-2 ratio is relevant to the regulation of myocardial apoptosis during reperfusion [23]. This study reveals that miR-152-3p down-regulated the ratio of Bax/Bcl-2 by decreasing Bax as well as increasing Bcl-2 expression, indicating that miR-152-3p participates in H/R injury process by regulating the Bax/Bcl-2 ratio. Our prior studies demonstrated that free radicals and myocardial enzymes are generated excessively during ischemic conditions [3-5]. These results confirm that the release of LDH and MDA increased significantly owing to H/R injury, while overexpression of miR-152-3p could reduce LDH and MDA contents. These findings elucidate that miR-152-3p serves as a protector of myocardium against I/R injury in *vitro*.

Further experiments were conducted to verify the hypothesis that the protective effect of miR-152-3p on cardiomyocyte are sustained in *vivo*. *In vivo* studies have shown that the inhibition of apoptosis by the ectopic expression of miR-152-3p in rats is associated with decreased LDH release, infarct area and the ratio of Bax/Bcl-2.

A growing body of evidence points that I/R injury induces cardiac hypertrophy, the remarkable reduce in myocyte hypertrophy may be a contributor to the noted improvement in cardiac function [23, 24]. Several studies have demonstrated that miRNAs protect against cardiac hypertrophy [25]. In the present study, upon histological examination, a cell size decrease was observed in the non-infarcted area of the LV following I/R injury, whereas miR-152-3p suppressed myocyte hypertrophy that were induced by the I/R injury. On this ground, miR-152-3p may be useful in alleviating I/R injury. These in *vivo* findings are coincide with the changes observed in the cultured cardiomyocyte. These data also could strongly support the idea that miR-152-3p plays a vital role in the protection of I/R injury both in *vitro* and in *vivo*.

Previous studies have already outlined that miR-152-3p is deceased and functions as a tumor suppressor in malignant melanoma by targeting HOTAIR, which was further regulated by the activation of the downstream PI3k/Akt/mTOR signaling pathway [20]. Although miR-152-3p has been proven to be proapoptotic under certain circumstance, our findings disclose that it is anti-apoptotic in the ischemic setting [20, 21]. It is common for miRNAs have the opposite property under different conditions [26, 27]. PTEN has been identified as a key regulator of splicing events related to a series of physiological and pathologic conditions. PTEN up-regulation is able to promote apoptosis and inhibit proliferation and migration in several solid cancers [28-30]. A previous study indicated that PTEN acts as an essential regulator in myocardial remodeling, myocardial fibrosis, and cardiac hypertrophy, as well as in augmentation of calcium signaling [31, 32]. However, the interaction between miR-152-3p and biological functions of PTEN has not been investigated to date. In our study, up-regulation of miR-152-3p could decrease the expression of PTEN in H9c2 cells, whereas the level of PTEN was significantly promoted in miR-152-3p-depleted H9c2 cells. Moreover, PTEN expression was markedly up-regulated in myocardial cells following H/R injury, and the anti-apoptotic effect of miR-152-3p was almost completely abrogated by the up-regulation of PTEN. More importantly, dual-luciferase reporter assays further confirmed that miR-152-3p directly targeted PTEN 3'UTR. These results indicate that PTEN is a functional target gene of miR-152-3p and they contribute to improving the cardiac function after I/R injury.

The PI3K/Akt signaling pathway is a classical protective pathway, and it has been involved in the maintenance of cell proliferation, apoptosis and inflammatory responses in most tissues [33, 34]. A study conducted by Chen et al. indicated that the PI3K/Akt signaling pathway can act as an endogenous negative feedback regulator to exert anti-apoptotic and proliferative effects via the phosphorylation of anti-apoptotic and pro-apoptotic substrates, such as Bax, Bcl-2, p70s6k, and caspase9 [35]. The PI3K/Akt signaling pathway can be critical for the development of myocardial infarction and cardiac dysfunction under the condition of ischemia [36]. Increased PI3K/Akt activity has been reported to correlate with attenuation of myocardial I/R injury [37]. Moreover, PTEN was demonstrated to be a major negative regulator of PI3K/Akt pathway [38]. Hence, we hypothesized that activation of PI3K/Akt signaling pathway could play a role in cardioprotection against I/R injury by miR-152-3p. In this study, the levels of phosphorylated PI3K/Akt were down-regulated in cardiomyocyte following I/R injury, while up-regulation of miR-152-3p in cardiomyocyte significantly increased the PI3K/Akt activity both in *vivo* and in *vitro*. Thus, these data indicate that cardiomyocyte protection properties of miR-152-3p are likely to be mediated by activation of the PI3K/Akt pathway. Our results only open a window and more investigations are needed to further decode the mechanism for the anti-apoptotic property of miR-152-3p.

Acknowledgements

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Disclosure of conflict of interest

None.

Abbreviations

miRNAs, microRNAs; I/R, ischemia/reperfusion; H/R, hypoxia/reoxygenation; PTEN, Kirsten rat sarcoma viral oncogene homolog; UTRs, untranslated regions; LDH, lactate dehydrogenase; LDA, malonic dialdehyde; TUNEL, TdT-mediated dUTP Nick-End Labeling; LAD, left anterior descending; qRT-PCR, quantitative real-time polymerase chain reaction; PI3K, phosphoinositide 3-kinase; wt, wild-type; mut, mutant.

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