ARTICLE
Journal of Cellular Biochemistry 115:93–101 (2014)
Journal of Cellular Biochemistry

MicroRNA-214 Protects Cardiac Myocytes Against H_2O_2-Induced Injury

Guangwei Lv,[1] Suxia Shao,[2] Hua Dong,[2] Xiaohua Bian,[1] Xingwei Yang,[1] and Shimin Dong[1]*

[1]*Department of ICU, The Third Hospital of Hebei Medical University, Shijiazhuang City, Hebei Province, P.R. China*
[2]*Department of histology and embryology, Hebei Medical University, Shijiazhuang City, Hebei Province, P.R. China*

ABSTRACT
Reactive oxygen species (ROS)-induced cardiac myocyte injury resulting from changes in the expression levels of multiple genes plays a critical role in the pathogenesis of numerous heart diseases. The purpose of this study was to determine the potential roles of microRNA-214 (miR-214) in hydrogen peroxide (H_2O_2)-mediated gene regulation in cardiac myocytes. In this study, we used quantitative real-time RT-PCR (qRT-PCR) to demonstrate that miR-214 was upregulated in cardiac myocytes after treatment with H_2O_2. We transfected cells with pre-miR-214 to upregulate miR-214 expression and transfected cells with a miR-214 inhibitor (anti-miR-214) to downregulate miR-214 expression. H_2O_2-induced cardiac cell apoptosis was detected by flow cytometry. The level of apoptosis was increased by the miR-214 inhibitor and decreased by pre-miR-214. Therefore, we believe that miR-214 plays a positive role in H_2O_2-induced cardiac cell apoptosis. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is constitutively active and is considered to be the primary downregulator of the pro-oncogenic PI3K/Akt pathway. Western blot analysis revealed that the expression of the PTEN protein in cardiac myocytes decreased after H_2O_2 induction. Anti-miR-214 increased PTEN protein expression level, in contrast, pre-miR-214 decreased the PTEN protein expression level in cultured cardiac myocytes. These results indicate that PTEN is regulated by miR-214 and serves as an important target of miR-214 in cardiac myocytes. In conclusion, miR-214 is sensitive to H_2O_2 stimulation, and miR-214 protects cardiac myocytes against H_2O_2-induced injury via one of its targets, PTEN. J. Cell. Biochem. 115: 93–101, 2014. © 2013 Wiley Periodicals, Inc.

KEY WORDS: MicroRNA-214; CARDIAC MYOCYTES; H_2O_2; APOPTOSIS; PHOSPHATASE AND TENSIN HOMOLOG (PTEN)

MicroRNAs (miRNAs\miRs) are small non-coding RNAs (ncRNAs) of approximately 20 nucleotides (nt) in length that regulate gene expression post-transcriptionally by binding to the 3′-untranslated regions (UTRs), coding sequences or 5′UTRs of target messenger RNAs (mRNAs), resulting in the inhibition of translation or in the degradation of the bound mRNA [Ambros, 2004; Zhang, 2008], over the last few years, both basic and clinical studies suggest that miRNAs are important regulators of cell differentiation, growth, proliferation and apoptosis [Ambros, 2004; Bartel, 2004; Filipowicz et al., 2008; Zhang, 2008; Almeida et al., 2011].

Diseases of the cardiovascular system represent the primary cause of human morbidity and mortality, although large progress has been made in its therapy in recent years. Several studies have demonstrated that miRNAs play important roles not only in cardiovascular development, but also in cardiovascular disease, such as cardiac hypertrophy, heart failure, and Ischemic heart disease [van Rooij et al., 2006; Care et al., 2007; Cheng et al., 2007; Ji et al., 2007; Sayed et al., 2007; Zhang, 2008]. For example, 11 miRNAs were downregulated and 16 were upregulated, with 22 persisting up to day 14 post-transverse aortic constriction (TAC). Among these miRNAs, miRNA-199a, -199a*, -199b, -21, and -214 exhibited the greatest changes (twofold upregulation) [Sayed et al., 2007]. Studies have shown that miRNA-21 has a protective effect against on H_2O_2-induced injury cardiac myocytes via its target gene PDCD4 [Cheng et al., 2009; Cheng et al., 2010]. Our studies also have shown that miRNA-21 has a protective effect against on I/R injury rat hearts and miRNAs might play critical roles in the pathophysiology of acute myocardial infarction (AMI) [Yang et al., 2007; Dong et al., 2009].

During cardiac infarction, reactive oxygen species (ROS) was increased in both infarct and non-infarct area, which may induce cells apoptosis. ROS have been reported to be generated at an accelerated level in the postischemic myocardium. Accumulating

Competing Interests: The authors have declared that no competing interests exist.
Grant sponsor: Nature Science Foundation of China; Grant number: 81070122.
*Correspondence to: Shimin Dong, The Third hospital of Hebei Medical University, Shijiazhuang City, HeBei Province, P.R.China. E-mail: lvguangweiwei@163.com
Manuscript Received: 5 March 2013; Manuscript Accepted: 24 July 2013
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 31 July 2013.
DOI 10.1002/jcb.24636 • © 2013 Wiley Periodicals, Inc.

evidence suggests that ROS function as signal transduction intermediates to induce transcription factor activation, gene expression, cell growth, and apoptosis [Thannickal and Fanburg, 2000; Martindale and Holbrook, 2002]. Multiple cell types and different enzymes contribute to the enhanced ROS production and oxidative stress associated with ischemia and reperfusion (I/R) [Lefer and Granger, 2000; Pratico and Delanty, 2000; Takano et al., 2003; Berg et al., 2005; Seddon et al., 2007]. ROS-mediated gene expression regulation has recently been extensively studied at the epigenetic and transcriptional levels [Cerda and Weitzman, 1997; Weigel et al., 2002; Vandenbroucke et al., 2008]. H_2O_2 is one of ROS that may cause cell damage. However, the effects of H_2O_2 on gene expression regulation at the translational level in heart cells are currently uncertain. Our present study has confirmed that miRNA-214 (miR-214) in cardiac myocytes is sensitive to H_2O_2 stimulation.

There are many researches involves the biological function of miR-214in different cells. miR-214 was shown to alter early cell differentiation, thereby influencing muscle cell development [Flynt et al., 2007]. Studies on human ovarian cancer showed changes in miR-214 expression. miR-214 can promote cell survival and has anti-apoptotic effects [Flynt et al., 2007; Yang et al., 2008]. It is also reported that miR-214 expression in rat-injured arteries is confirmed by qRT-PCR or Northern.

Blot analysis was highly upregulated [Ji et al., 2007; Cheng et al., 2009]. Our recent study showed that miR-214 is over expressed in the infarcted area and in border area of the rat hearts at 6 h after AMI [Dong et al., 2009]. One study has confirmed that MicroRNA-214 protects the mouse heart from ischemic injury by controlling Ca^{2+} overload and cell death [Aurora et al., 2012]. These studies speculated that miR-214 in myocardial apoptosis also had specific effects. However, in cardiac myocytes, the effects of miRNA-214 represent a new layer of gene expression regulation at the translational level, the effects of H_2O_2 on miRNA-214 expression are uncertain. In addition, the roles of miRNA-214 in H_2O_2-mediated gene regulation and the biological functions in cardiac myocytes remain to be elucidated. Here we show that miRNA-214 expression in cardiac myocytes is sensitive to H_2O_2 stimulation and miR-214 plays a protective role against the H_2O_2-induced cardiac cell apoptosis. These findings provide new insights into the molecular basis of heart disease and point to miR-214 as a potential therapeutic target in this setting.

In human ovarian cancer PTEN is negatively regulated by miR-214 at the protein level [26]. PTEN also is one of the target genes of miRNA-214 in gastric cancer cells [Xiong et al., 2011]. In our study we began to explore the correlation of miRNA-214 and PTEN in cardiac myocytes.

MATERIALS AND METHODS

CELL CULTURE

Primary neonatal rat cardiac ventricular myocytes were cultured as previously described [Lewis et al., 2005; Cheng et al., 2007]. In brief, the hearts of 1- to 3-day-old Sprague–Dawley rats (Hebei Medical University Laboratory Animal Center) were removed after hypothermia-induced anesthesia by immersion in ice water and were placed in ice-cold 1× HBSS (Life Technologies GIBCO C14175). After repeated rinsing, the atria were removed, and the ventricles were minced with scissors. The minced tissue was dispersed by digestion with 0.08% trypsin and 0.0067% EDTA (Safc Biosciences 59428C). The cardiac myocytes (0.5×10^6 cells/ml) were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; GIBCO C11330) supplemented with 13% bovine serum (Germany, PAA The Cell Culture Company, A15-101), 1% penicillin, 1% streptomycin (Germany, PAA The Cell Culture Company, p11-010), and 100 μmol/L 5-bromo-2-deoxyuridine (Sigma) to restrict fibroblast growth. Then, the cardiac myocytes were seeded onto the appropriate plates. The medium was replaced every 48 h.

MEASUREMENT OF CARDIAC MYOCYTE VITALITY

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) is a yellowish aqueous solution that, after reduction by dehydrogenases and reducing agents present in metabolically active cells, yields a water insoluble violet-blue formazan. The lipid-soluble formazan product may be extracted with organic solvents and estimated by spectrophotometry. The amount of MTT formazan is currently widely thought to be directly proportional to the number of living cells [van Meerloo et al., 2011]. Briefly, rat cardiac myocytes cultured in DMEM/F12 were treated with either the vehicle (0 μmol/L) or H_2O_2 (30–200 μM) for 6 h. Afterwards, cell vitality was measured by MTT. Cells cultured on 96-well plates (20,000 cells/well) were exposed to H_2O_2 (30–200 μM) for 6 h. MTT (0.2 mg/ml; Sigma) was added, and the cells were incubated for 4 h at 37°C in the dark. DMSO was added, and the optical density (OD) was measured at 490 nm. The cell viability was calculated as a percentage of the control OD [Kwon et al., 2003; Stockert et al., 2012].

ANALYSIS OF CARDIAC MYOCYTE APOPTOSIS INDUCED BY H_2O_2

The cells ($>2 \times 10^6$) that were exposed to H_2O_2 (30–100 μmol/L) for 6 h were digested with 0.25% trypsin and collected by centrifugation. After being washed twice with ice-cold phosphate-buffered saline (PBS), the cells were fixed and stained with annexin V-FITC and PI (BD) for 15 min. The apoptotic cells were identified by EPICS XL flow cytometry (Beckman Coulter, CA). Cells that were negative for both PI and annexin V-FITC staining were normal healthy cells. PI-negative, annexin V-FITC-positive cells were early apoptotic cells. PI and annexin V-FITC-positive cells were late apoptotic cells [Sheng et al., 2010; Jia et al., 2012]. After preliminary experiments we confirmed that cell death include cell apoptosis and necrosis, and the majority of cells being apoptotic, especially at doses of H_2O_2 lower than 100 μM. Others have also reported that H_2O_2 induce the production of TNF-a and the apoptosis of cardiomyocytes [Aikawa et al., 2002; Takano et al., 2003].

THE EFFECT OF H_2O_2 ON miR-214 EXPRESSION

Cultured rat cardiac myocytes were treated with either vehicle (normal cells) or H_2O_2 (30–100 μmol/L) for 6 h. RNA was then isolated from the cultured cells using an RNA isolation kit (Invitrogen Trizol reagent). miR-214 expression was determined by quantitative real-time RT-PCR (qRT-PCR) using a kit provided by GeneCopoeia. (The details will be accounted in 2.7).

TRANSFECTION OF CULTURED CARDIAC MYOCYTES

Oligo transfection was performed according to an established protocol [Cheng et al., 2007; Ji et al., 2007]. Briefly, the cells were incubated for 48 h after seeding and then transfected using a

transfection reagent (Qiagen, Chatsworth, CA). Transfection complexes were prepared according to the manufacturer's instructions. To knock down miR-214, a miR-214 inhibitor (anti-miR-214; Ambion, Inc.) was added to the culture media at a final oligonucleotide concentration of 30 nM. To upregulate miR-214, pre-miR-214 (Ambion, Inc.) was added directly to the complexes at a final oligonucleotide concentration of 30 nM. The transfection medium was replaced by regular culture medium at 3.5 h post-transfection. The vehicle control, an oligo control for anti-miR-214 (LNA-scramble), an oligo control for pre-miR-214 (pre-scramble, Ambion, Inc.), and a scramble control were also applied.

PTEN activity is inhibited by VO-OHpic (Sigma) [Mak et al., 2010]. VO-OHpic was dissolved in DMSO (100 μM) and diluted to the required concentration (100 nM) with 1% DMSO. The cells were incubated with the inhibitor at room temperature (RT) for 10 min, and then, the proteins isolated from the cultured cardiac myocytes were analyzed by Western blotting.

WESTERN BLOT ANALYSIS

Proteins isolated from cultured normal cardiac myocytes, H_2O_2-induced cardiac myocyte, the cells which transfected with pre-miR-214, anti-miR-214 and also from the sham group at the same times. Proteins isolated from cultured cardiac myocytes were analyzed by Western blotting. Equal amounts of protein were subjected to SDS–PAGE. Standard Western blot analysis was conducted using the PTEN antibody (1:1,000 dilution; Epitomics), and a GAPDH antibody (1:2,000 dilution; Epitomics) was used as a loading control.

DETECTION OF miRNAs BY qRT-PCR

miRNAs were isolated from the cultured cells using an RNA isolation kit (Invitrogen Trizol reagent), and the miR-214 expression level was determined by quantitative real-time RT-PCR (qRT-PCR) using a kit provided by GeneCopoeia. Total RNA was obtained separately from the normal cardiac myocyte, H_2O_2-induced cardiac myocyte, the cells which transfected with pre-miR-214, anti-miR-214 and also from the sham group at the same times. The RNA isolation was performed using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The total RNA concentration was measured at 260 nm using a NanoDrop 2000c spectrophotometer (Thermo Fisher). Quantitative miRNA analysis was performed using the All-in-One miRNA First-strand cDNA synthesis kit (GeneCopoeia). Approximately 300 ng of total RNA from the cells was reverse-transcribed into cDNA using poly A polymerase primers. qPCR was performed using the All-in-One miRNA qRT-PCR kit and probes (GeneCopoeia). U6 was used as an internal normalization control for the cells. The miRNA-214 forward primer (Cat# RmiRQP1208), the U6 forward primer (Cat# RmiRQP9003), and the reverse primer (#Po1011A) were provided by GeneCopoeia Inc. The Ct values for the target were normalized by subtracting the U6 Ct value, which provided the ΔCt value. Our instrument for PCR is ABI7500. The difference in the expression level between the treatments was then calculated using the following equation: relative gene expression $= 2^{-(\Delta Ct,\, sample - \Delta Ct,\, control)}$.

STATISTICAL ANALYSIS

All data are presented as the mean and standard deviation (SD). One-way ANOVA followed by Student's *t*-test was used for statistical analysis in SPSS 13.0 software. A *P*-value <0.05 was considered statistically significant.

RESULTS

THE EFFECT OF H_2O_2 ON CULTURED CARDIAC MYOCYTE VITALITY

Although low concentrations (<10 μM) of H_2O_2 had no effect on cell vitality (data not shown), the cardiac myocyte vitality was decreased by high concentrations (30–200 μM) of H_2O_2 in a dose-dependent manner after 6 h of treatment under our experimental conditions (Fig. 1). We confirmed that the myocardial cell vitality decreased as the concentration of H_2O_2 increased and that the cell vitality reflected the process of cell apoptosis or necrosis changes to a certain extent. We have chosen the relative lower concentration of H_2O_2 (<100 μM), for the further experiment.

THE EFFECT OF H_2O_2 ON THE APOPTOSIS OF CULTURED CARDIAC MYOCYTES

The cells ($>10^6$) were exposed to H_2O_2 (30–100 μM) for 6 h. Then, the apoptosis rate was measured by flow cytometry method (FCM). In the present study, the apoptosis rate was significantly higher in the H_2O_2-treated cells than in the normal cells, and this effect was dose dependent (Fig. 2A). Flow cytometry analysis results of 0, 30, 50, or 100 μM of H_2O_2 were displayed in Fig. 2B. The fourth quadrant (% Gated) shows the early apoptotic cells, and the second quadrant (% Gated) represents the late apoptotic cells.

THE EFFECT OF H_2O_2 ON miR-214 RELATIVE EXPRESSION IN CULTURED CARDIAC MYOCYTES

MiR-214 is sensitive to H_2O_2. As shown in Figure 3, the short-term exposure (6 h) of cardiac myocytes to H_2O_2 resulted in the increased expression of miR-214, and the effect was dose dependent.

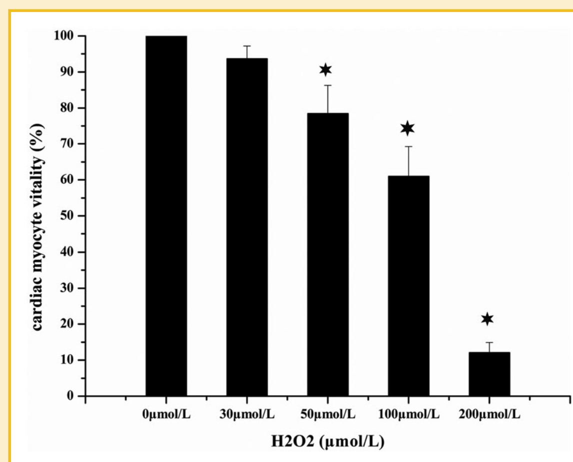

Fig. 1. The effect of H_2O_2 on rat cardiac myocyte vitality. Cultured neonatal rat cardiac myocytes were treated with normal cells or H_2O_2 (30–200 μM) for 6 h. The cell vitality was measured by MTT. Note: n = 5; $^*P < 0.05$ compared with the vehicle control (0 μM).

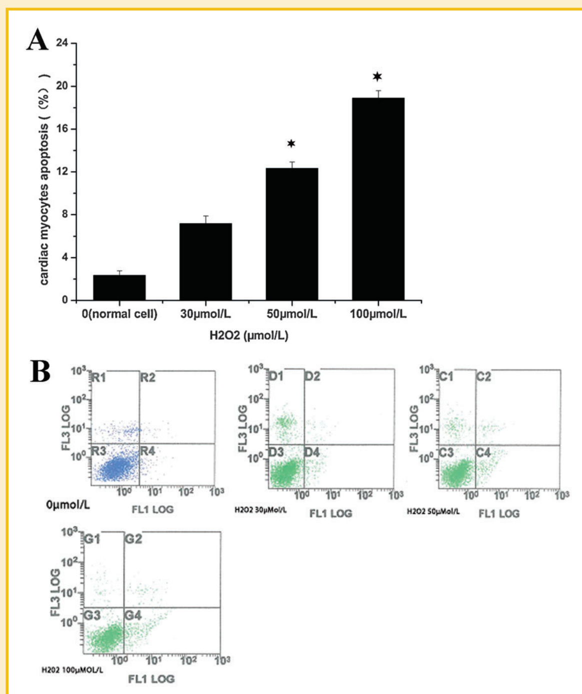

Fig. 2. A: The effect of H_2O_2 on rat cardiac myocyte apoptosis. Cultured neonatal rat cardiac myocytes were treated with vehicle (0 μM) or H_2O_2 (30–100 μM) for 6 h. Cell apoptosis was measured by flow cytometry. Note: n = 5; $^*P < 0.05$ compared with the vehicle control (0 μM). B: Flow cytometry analysis results. The fourth quadrant (% Gated) shows the early apoptotic cells, and the second quadrant (% Gated) represents the late apoptotic cells.

THE REGULATORY EFFECT ON miR-214 IN CULTURED CARDIAC MYOCYTES

To investigate the regulatory effects on miR-214 in cultured cardiac myocytes, both gain-of-function and loss-of-function approaches

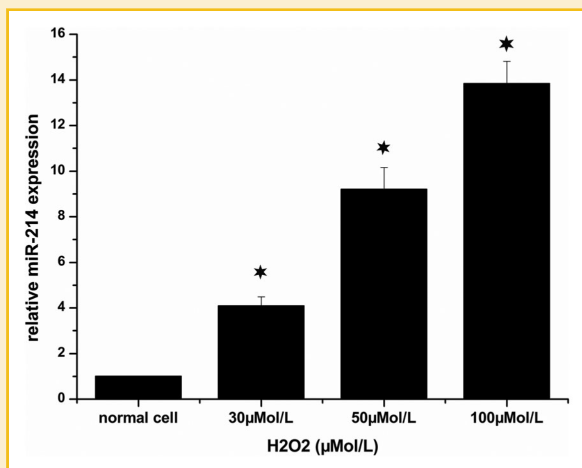

Fig. 3. The effect of H_2O_2 on miR-214 expression in cultured rat cardiac myocytes. Cultured neonatal rat cardiac myocytes were treated with normal cell or H_2O_2 (30–100 μM) for 6 h. The miR-214 levels were determined by qRT-PCR. Note: n = 5; $^*P < 0.05$ compared with the vehicle control (0 μM).

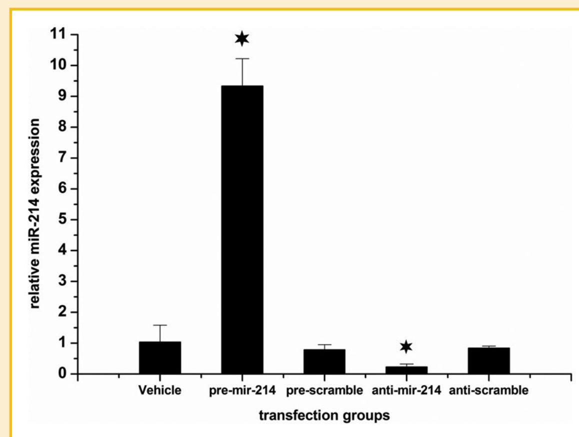

Fig. 4. Modulating miR-214 expression in cultured cardiac myocytes. Cultured cardiac myocytes were treated with vehicle (normal cells), pre-miR-214 (30 nM), pre-miR-214 scramble (30 nM), miR-214 inhibitor (antimiR-214, 30 nM), anti-miR-214 scramble (anti-scramble, 30 nM), for 3.5 h. The miR-214 levels were determined 24 h later by qRT-PCR. Note: n = 5; $^*P < 0.05$ compared with the vehicle control.

were applied. As shown in Figure 4, anti-miR-214 decreased the miR-214 level, and pre-miR-214 increased the miR-214 level in cardiac myocytes. In contrast, their respective control oligos (scrambled oligos) had not effect on miR-214 expression. In addition, the effects of both anti-miR-214 and pre-miR-214 on miR-214 expression were miR-214 specific, as no effects were found on other miRNAs (data not shown).

THE EFFECT OF miR-214 ON THE H_2O_2-INDUCED APOPTOSIS OF CARDIAC MYOCYTES

Pre-miR-214 decreased the rate of H_2O_2-induced cardiac myocyte apoptosis, in contrast, the apoptosis rate in cardiac myocytes increased after treatment with anti-miR-214 as determined by FCM Figure 6A. Representative results for the cardiac myocytes treated with the vehicle control (normal cells, H_2O_2 induced), the control scramble oligo, pre-miR-214 and anti-miR-214 are shown in Figure 6B. The results indicate that miR-214 had a protective effect against the H_2O_2-induced apoptosis of cardiac myocytes.

THE CORRELATION OF miRNA-214 AND PTEN IN CARDIAC MYOCYTES

If PTEN is a miR-214 target, H_2O_2 should decrease the expression of this protein in cardiac myocytes because miR-214 expression was upregulated after H_2O_2 stimulation (Fig. 3). To confirm this hypothesis, we incubated cardiac myocytes with either vehicle (normal cells) or H_2O_2 (100 μM) for 2 h and then determined the protein level of PTEN by Western blotting. As shown in Figure 6A, H_2O_2 decreased the PTEN expression level. These results suggest that PTEN is a potential miR-214 target in cardiac myocytes stimulated by H_2O_2.

To verify that PTEN is a target of miR-214 in cardiac myocytes, both gain-of-function and loss-of-function approaches were applied. As shown in Figure 6, anti-miR-214 increased and pre-miR-214

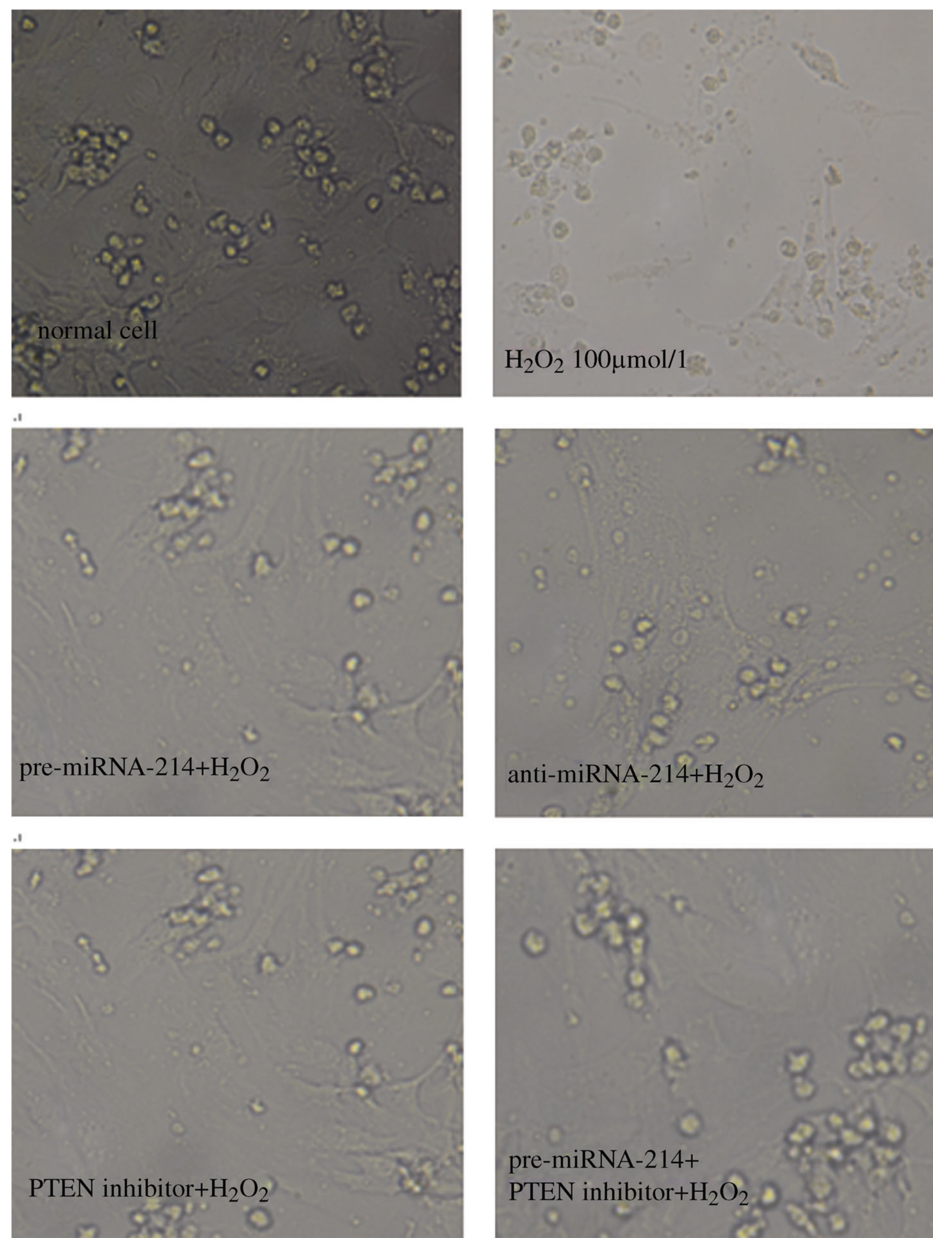

Fig. 5. Representative cell pictures from different treatment groups. The state of different cells group observed in the inverted microscope. The group of normal cells, pre-miR-214 + H_2O_2, anti-miR-214 + H_2O_2, PTEN inhibitor + H_2O_2, and pre-miR-214 + PTEN inhibitor + H_2O_2.

decreased PTEN protein level in cultured cardiac myocytes. These results also suggest that PTEN is a target of miR-214, at least, there is a correlation between them.

To verify the functional involvement of PTEN in the cellular effects of miR-214, we assessed the role of PTEN in H_2O_2-induced cardiac myocyte apoptosis. As shown in Figure 7C, pre-miR-214 had a protective effect against H_2O_2-induced cardiac myocyte apoptosis. However, we observed the protective effect of pre-miR-214 against the H_2O_2-induced apoptosis of cardiac myocytes become noticeable when the activity of PTEN was decreased, as shown in Figure 7C. When the activity of PTEN was decreased the H_2O_2-induced cardiac myocyte apoptosis also decreased. Representative FCM results for the cardiac myocytes treated with the vehicle control (normal cells, H_2O_2 induced), the pre-miR-214 (cells with pre-mir-214 and H_2O_2), anti-miR-214 (cells with anti-mir-214 and H_2O_2) and PTEN inhibitor (VO-OHpic) (cells with PTEN inhibitor and H_2O_2) are shown in Figure 7D.

These results suggest that PTEN is a functional target gene involved in the miR-214-mediated protective effect against H_2O_2-induced injury in cardiac myocytes.

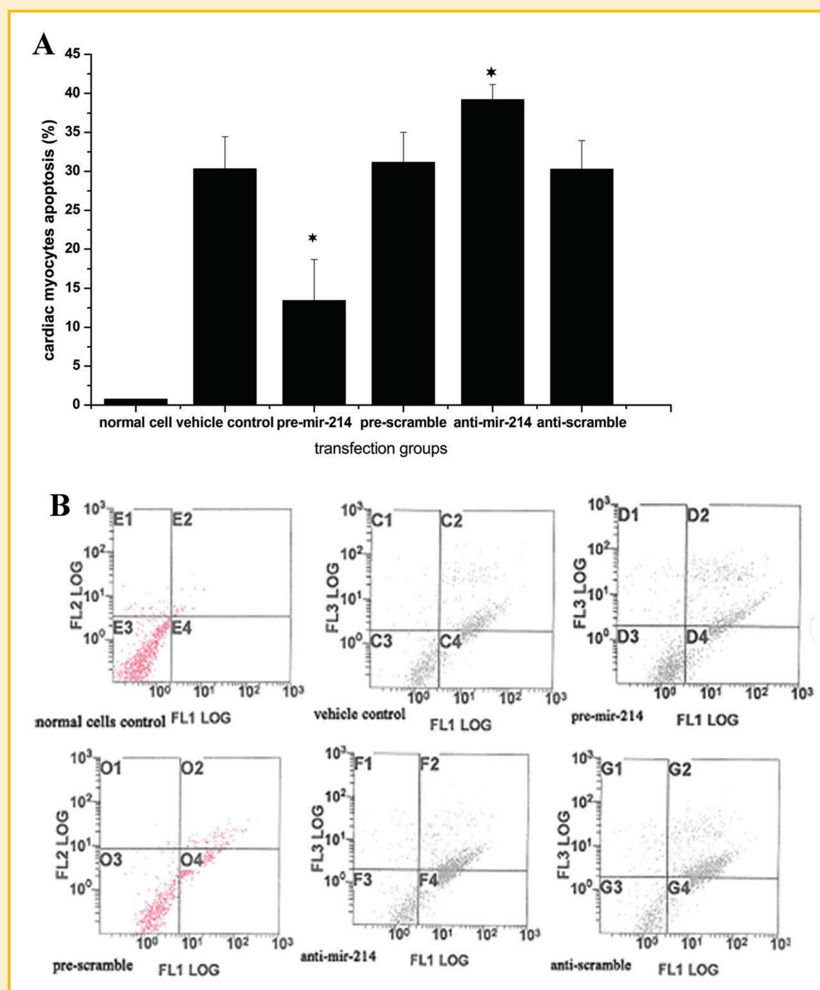

Fig. 6. A: The effect of miR-214 on H_2O_2-induced cardiac myocyte apoptosis. Cultured normal rat cardiac myocytes (normal cells) and pre-treated with vehicle (no treated cells), miR-214 inhibitor scramble (antiscramble, 30 nM), miR-214 inhibitor (anti-miR-214, 30 nM), pre-miR-214 scramble (30 nM), or pre-miR-214 (30 nM) were all treated with H_2O_2 (100 μM) for 6 h. Note: n = 6; $^*P < 0.05$ compared with the vehicle control (normal cell, H_2O_2-induced). B: Representative flow cytometry analysis results from normal cells and cells treated with vehicle (no treated cells), anti-scramble, anti-miR-214, pre-miR-214 scramble, or pre-miR-214. Note: n = 6; $^*P < 0.05$. The fourth quadrant (% Gated) represents the early apoptotic cells, and the second quadrant (% Gated) represents the late apoptotic cells.

DISCUSSION

In our study, to assess the potential role of miR-214 in H_2O_2-mediated cardiac myocyte injury, miR-214 expression was modulated using a miR-214 inhibitor and pre-miR-214. After cells were treated with these regulators, the level of H_2O_2-induced cell apoptosis was determined using FCM. Interestingly, the upregulation of miR-214 expression inhibited the H_2O_2-mediated apoptosis of neonatal rat cardiac myocytes. In contrast, H_2O_2-mediated cardiac myocyte apoptosis was exacerbated after the downregulation of miR-214 expression. The expression of miR-214 conferred resistance to H_2O_2-induced cell apoptosis, suggesting that miR-214 is an anti-apoptotic factor in cardiac myocyte.

H_2O_2 is one of ROS and has been widely used in the experiment to mimic situation with oxidative stress. However, the concentration of H_2O_2 used in experiments differs widely in different cell types. Different type of cells showed different response to oxidative stress induced by H_2O_2 [Djordjevic et al., 2008]. In this study, we are not first to evaluate the effect of H_2O_2 on cardiac myocyte. But we have found that cardiac myocytes damage induced by H_2O_2 was almost time- and dose-dependent. Also, we have chosen the relative lower concentration of H_2O_2 (<100 μM), for the further experiment. Similar idea has also been described by Tanaka and Aikawa [Tanaka et al., 2000; Takano et al., 2003].

It is also reported that miR-214 expression in rat injured arteries is confirmed by qRT-PCR or Northern blot analysis was highly upregulated [Ji et al., 2007; Cheng et al., 2009]. Recent study also showed that miRNA-214 is over expressed in the infarcted area and in border area of the rat hearts at 6 h after AMI [Dong et al., 2009]. One study has confirmed that MicroRNA-214 protects the mouse heart from ischemic injury by controlling Ca^{2+} overload and cell death [Aurora et al., 2012]. These studies speculated that miR-214 in myocardial apoptosis also had specific effects. It has been confirmed that miR-21 expression is sensitive to H_2O_2 stimulation in cardiac

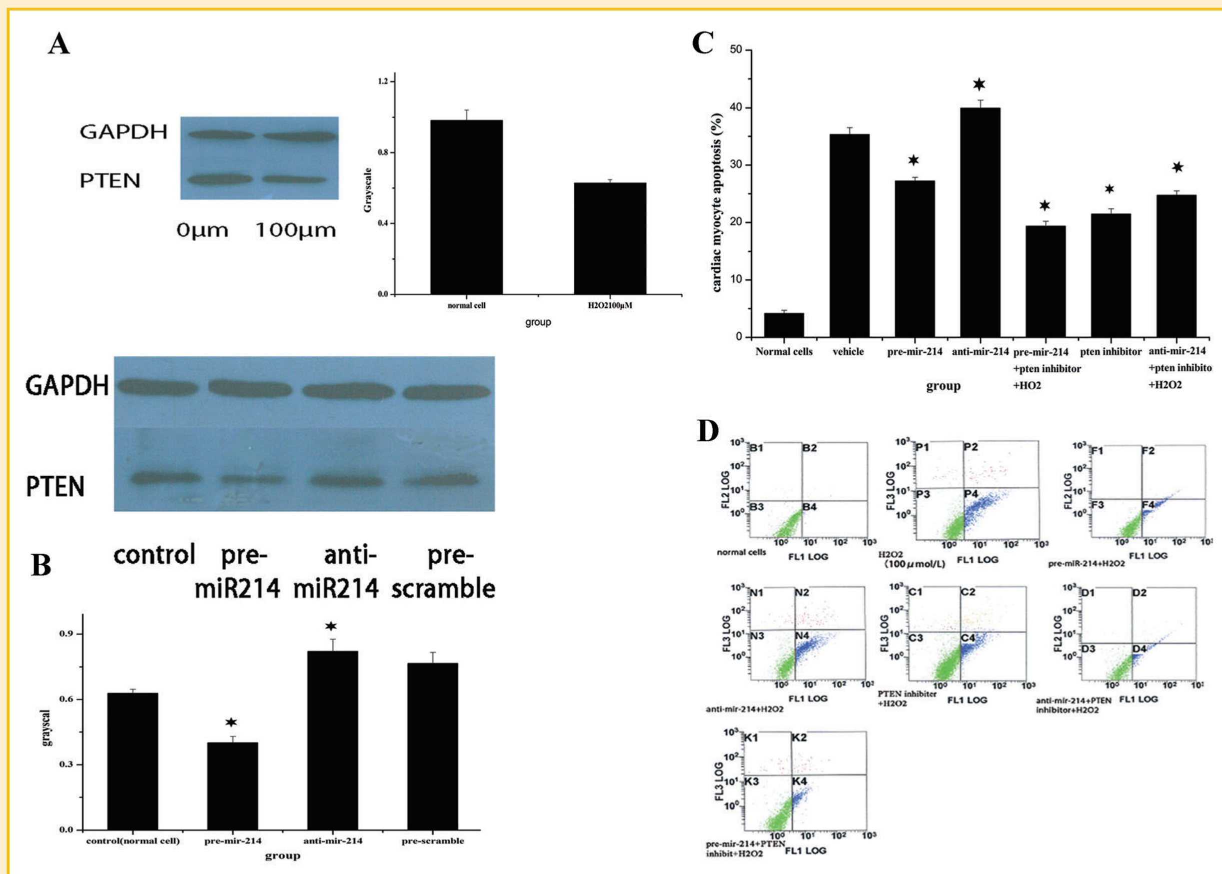

Fig. 7. PTEN is a gene target of miR-214. A: H_2O_2 (100 μM) decreased PTEN expression in cultured cardiac myocytes. B: The miR-214 inhibitor (30 nM) increased PTEN expression in cardiac myocytes compared with the vehicle control. pre-miR-214 (30 nM) decreased PTEN expression in cardiac myocytes compared with the vehicle or pre-miR-214 scramble (30 nM) controls. Image J software for Western Blotting quantitative analysis, A standard Western blot analysis was conducted using GAPDH antibody, gray value of the target protein PTEN in proportion to the gray value of GAPDH represents the relative expression level of target protein. The bar in (A) represent that the relative expression level of target protein in normal cardiac myocytes and cardiac myocytes after H_2O_2 exposure. The bar in (B) represent that the relative expression level of target protein in normal cardiac myocytes, cardiac myocytes after pre-mir-214 exposure, cardiac myocytes after anti-mir-214 exposure and scramble exposure. C: The pre-miR-214-mediated protective effect on cardiac myocyte apoptosis was intensified in H_2O_2-treated cells via VO-OHpic, which decreases PTEN activity. Note: n = 6; $^*P < 0.05$ compared with the vehicle (normal cell, H_2O_2-induced). D: Representative flow cytometry analysis results.

myocytes and protects against the H_2O_2-induced injury on cardiac myocytes [Cheng et al., 2009]. But the effect of miR-214 in cardiac myocytes was not known. In our current study, we found that miR-214 expression is sensitive to H_2O_2 stimulation in cardiac myocytes. Six hours after H_2O_2 treatment, the level of miR-214 was upregulated in a dose-dependent manner. The expression changes of miRNAs after ROS stimulation could be very important in ROS-mediated modulations of multiple gene expression and signaling transduction pathways, in this respect, the expression changes other miRNAs induced by ROS stimulation in cardiac myocytes should be investigated in future.

In recent years many researches has confirmed the tumor-suppressor effect of miR-214 [Narducci et al., 2011; Xia et al., 2012]. A study of human ovarian cancer showed that the miR-214 expression pattern differed between normal tissue and cancer tissue, with an 8.63-fold higher level in the cancer tissue, miR-214 can promote cell survival and has an anti-apoptotic effect [Flynt et al., 2007; Yang et al., 2008]. In this study, we began to elucidate the effects of miR-214 related to H_2O_2-induced cardiac myocyte injury. Interestingly, the upregulation of miR-214 expression inhibited the H_2O_2-mediated apoptosis of neonatal rat cardiac myocytes. In contrast, H_2O_2-mediated cardiac myocyte apoptosis was exacerbated after the downregulation of miR-214 expression. The expression of miR-214 confers resistance to H_2O_2-induced cell apoptosis, suggesting that miR-214 is an anti-apoptotic factor. Further confirmed the effect of miRNA-214 on cell survival and cell apoptosis will become new research direction about miRNA-214.

PTEN is a dual protein–lipid phosphatase that dephosphorylates the second messenger produced by PI3K and interrupts the downstream activation of Akt [Rosivatz, 2007]. PI3K/Akt is an intracellular signaling pathway, which plays significant roles in a variety of biological processes involving cell survival, growth, and migration [Wetzker and Rommel, 2004]. The activation of the PI3K (phosphoinositide-3 kinase)/Akt (protein kinase B, PKB) signaling pathway has been demonstrated to be essential for protection against ischemia/reperfusion injury [Siddall et al., 2008]. Importantly, PTEN

(phosphatase and tensin homolog) is the primary phosphatase that negatively regulates the PI3K/Akt pathway [Mocanu and Yellon, 2007]. MiR-214 induces cell survival and cisplatin resistance through targeting the UTR of PTEN, which leads to down-regulation of PTEN and the activation of the Akt pathway [Yang et al., 2008]. PTEN is also targeted by 13 other miRNAs [Schmeier et al., 2011]. Similarly, in cardiac myocytes, the effect of PTEN has been proved. MicroRNA-1 transfected embryonic stem cells enhance cardiac myocyte differentiation and inhibit apoptosis by modulating the PTEN/Akt pathway in the infarcted heart [Glass and Singla, 2011]. A report has been identified that three proapoptotic proteins (PTEN, ROCK1, and CaMKIIδ) and two antiapoptotic proteins (FGFR2 and LIF) were authentic targets for miR-494. Importantly, the Akt-mitochondrial signaling pathway was activated in miR-494-over-expressing myocytes [Wang et al., 2010]. In our study we also confirmed that H_2O_2 decreases PTEN expression in cultured cardiac myocytes. In addition, PTEN expression in cardiac myocytes is regulated by miR-214, anti-miR-214 increased and pre-miR-214 decreased PTEN expression in cultured cardiac myocytes. These results suggest that PTEN may be a potential miR-214 target gene in cardiac myocytes stimulated with H_2O_2. To validate the role of PTEN in the anti-apoptotic function of miR-214, we decreased the activity of PTEN using a PTEN inhibitor (VO-OHpic) and found that the protective effect of pre-miR-214 against H_2O_2-induced cardiac myocyte apoptosis was stronger.

In summary, the current study reveals that the miR-214 in cardiac myocytes is sensitive to H_2O_2 stimulation. MiR-214 protects against the H_2O_2-induced injury on cardiac myocytes. There is a correlation between miR-214 and PTEN in cardiac myocyte. These novel findings may have extensive implications for the diagnosis and therapy of a variety of heart diseases related to ROS such as cardiac hypertrophy, heart failure, myocardial infarction, and myocardial ischemia/reperfusion injury. The potential physiological roles of miRNA-214 in other cardiac myocytes disease models should be evaluated for future study.

ACKNOWLEDGMENTS

This study was supported by the Nature Science Foundation of China (no. 81070122). For all studies involving the use of animals, we have received permission from the Institutional Animal Care and Use Committee.

REFERENCES

Aikawa R, Nitta-Komatsubara Y, Kudoh S, Takano H, Nagai T, Yazaki Y, Nagai R, Komuro I. 2002. Reactive oxygen species induce cardiomyocyte apoptosis partly through TNF-alpha. Cytokine 18:179–183.

Almeida MI, Reis RM, Calin GA. 2011. MicroRNA history: Discovery, recent applications, and next frontiers. Mutat Res 717:1–8.

Ambros V. 2004. The functions of animal microRNAs. Nature 431:350–355.

Aurora AB, Mahmoud AI, Luo X, Johnson BA, van Rooij E, Matsuzaki S, Humphries KM, Hill JA, Bassel-Duby R, Sadek HA, Olson EN. 2012. MicroRNA-214 protects the mouse heart from ischemic injury by controlling Ca(2)(+) overload and cell death. J Clin Invest 122:1222–1232.

Bartel DP. 2004. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116:281–297.

Berg K, Jynge P, Bjerve K, Skarra S, Basu S, Wiseth R. 2005. Oxidative stress and inflammatory response during and following coronary interventions for acute myocardial infarction. Free Radic Res 39:629–636.

Care A, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, Bang ML, Segnalini P, Gu Y, Dalton ND, Elia L, Latronico MV, Hoydal M, Autore C, Russo MA, Dorn GW II, Ellingsen O, Ruiz-Lozano P, Peterson KL, Croce CM, Peschle C, Condorelli G. 2007. MicroRNA-133 controls cardiac hypertrophy. Nat Med 13:613–618.

Cerda S, Weitzman SA. 1997. Influence of oxygen radical injury on DNA methylation. Mutat Res 386:141–152.

Cheng Y, Ji R, Yue J, Yang J, Liu X, Chen H, Dean DB, Zhang C. 2007. MicroRNAs are aberrantly expressed in hypertrophic heart: Do they play a role in cardiac hypertrophy? Am J Pathol 170:1831–1840.

Cheng Y, Liu X, Zhang S, Lin Y, Yang J, Zhang C. 2009. MicroRNA-21 protects against the H(2)O(2)-induced injury on cardiac myocytes via its target gene PDCD4. J Mol Cell Cardiol 47:5–14.

Cheng Y, Zhu P, Yang J, Liu X, Dong S, Wang X, Chun B, Zhuang J, Zhang C. 2010. Ischaemic preconditioning-regulated miR-21 protects heart against ischaemia/reperfusion injury via anti-apoptosis through its target PDCD4. Cardiovasc Res 87:431–439.

Djordjevic VB, Zvezdanovic L, Cosic V. 2008. Oxidative stress in human diseases. Srp Arh Celok Lek 136(Suppl2):158–165.

Dong S, Cheng Y, Yang J, Li J, Liu X, Wang X, Wang D, Krall TJ, Delphin ES, Zhang C. 2009. MicroRNA expression signature and the role of microRNA-21 in the early phase of acute myocardial infarction. J Biol Chem 284:29514–29525.

Filipowicz W, Bhattacharyya SN, Sonenberg N. 2008. Mechanisms of post-transcriptional regulation by microRNAs: Are the answers in sight? Nat Rev Genet 9:102–114.

Flynt AS, Li N, Thatcher EJ, Solnica-Krezel L, Patton JG. 2007. Zebrafish miR-214 modulates Hedgehog signaling to specify muscle cell fate. Nat Genet 39:259–263.

Glass C, Singla DK. 2011. MicroRNA-1 transfected embryonic stem cells enhance cardiac myocyte differentiation and inhibit apoptosis by modulating the PTEN/Akt pathway in the infarcted heart. Am J Physiol Heart Circ Physiol 301:H2038–H2049.

Ji R, Cheng Y, Yue J, Yang J, Liu X, Chen H, Dean DB, Zhang C. 2007. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. Circ Res 100:1579–1588.

Jia LQ, Yang GL, Ren L, Chen WN, Feng JY, Cao Y, Zhang L, Li XT, Lei P. 2012. Tanshinone IIA reduces apoptosis induced by hydrogen peroxide in the human endothelium-derived EA.hy926 cells. J Ethnopharmacol 143:100–108.

Kwon SH, Pimentel DR, Remondino A, Sawyer DB, Colucci WS. 2003. H(2)O(2) regulates cardiac myocyte phenotype via concentration-dependent activation of distinct kinase pathways. J Mol Cell Cardiol 35:615–621.

Lefer DJ, Granger DN. 2000. Oxidative stress and cardiac disease. Am J Med 109:315–323.

Lewis BP, Burge CB, Bartel DP. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120:15–20.

Mak LH, Vilar R, Woscholski R. 2010. Characterisation of the PTEN inhibitor VO-OHpic. J Chem Biol 3:157–163.

Martindale JL, Holbrook NJ. 2002. Cellular response to oxidative stress: Signaling for suicide and survival. J Cell Physiol 192:1–15.

Mocanu MM, Yellon DM. 2007. PTEN, the Achilles′ heel of myocardial ischaemia/reperfusion injury? Br J Pharmacol 150:833–838.

Narducci MG, Arcelli D, Picchio MC, Lazzeri C, Pagani E, Sampogna F, Scala E, Fadda P, Cristofoletti C, Facchiano A, Frontani M, Monopoli A, Ferracin M, Negrini M, Lombardo GA, Caprini E, Russo G. 2011. MicroRNA profiling reveals that miR-21, miR486 and miR-214 are upregulated and involved in cell survival in Sezary syndrome. Cell Death Dis 2:e151.

Pratico D, Delanty N. 2000. Oxidative injury in diseases of the central nervous system: Focus on Alzheimer's disease. Am J Med 109:577–585.

Rosivatz E. 2007. Inhibiting PTEN. Biochem Soc Trans 35:257–259.

Sayed D, Hong C, Chen IY, Lypowy J, Abdellatif M. 2007. MicroRNAs play an essential role in the development of cardiac hypertrophy. Circ Res 100: 416–424.

Schmeier S, Schaefer U, Essack M, Bajic VB. 2011. Network analysis of microRNAs and their regulation in human ovarian cancer. BMC Syst Biol 5:183.

Seddon M, Looi YH, Shah AM. 2007. Oxidative stress and redox signalling in cardiac hypertrophy and heart failure. Heart 93:903–907.

Sheng R, Gu ZL, Xie ML, Zhou WX, Guo CY. 2010. Epigallocatechin gallate protects H9c2 cardiomyoblasts against hydrogen dioxides-induced apoptosis and telomere attrition. Eur J Pharmacol 641:199–206.

Siddall HK, Warrell CE, Yellon DM, Mocanu MM. 2008. Ischemia-reperfusion injury and cardioprotection: Investigating PTEN, the phosphatase that negatively regulates PI3K, using a congenital model of PTEN haploinsufficiency. Basic Res Cardiol 103:560–568.

Stockert JC, Blazquez-Castro A, Canete M, Horobin RW, Villanueva A. 2012. MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. Acta Histochem 114:785–796.

Takano H, Zou Y, Hasegawa H, Akazawa H, Nagai T, Komuro I. 2003. Oxidative stress-induced signal transduction pathways in cardiac myocytes: Involvement of ROS in heart diseases. Antioxid Redox Signal 5:789–794.

Tanaka M, Miyazaki T, Tanigaki S, Kasai K, Minegishi K, Miyakoshi K, Ishimoto H, Yoshimura Y. 2000. Participation of reactive oxygen species in PGF2alpha-induced apoptosis in rat luteal cells. J Reprod Fertil 120:239–245.

Thannickal VJ, Fanburg BL. 2000. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol 279:L1005–L1028.

van Meerloo J, Kaspers GJ, Cloos J. 2011. Cell sensitivity assays: The MTT assay. Methods Mol Biol 731:237–245.

van Rooij E, Sutherland LB, Liu N, Williams AH, McAnally J, Gerard RD, Richardson JA, Olson EN. 2006. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci USA 103:18255–18260.

Vandenbroucke K, Robbens S, Vandepoele K, Inze D, Van de Peer Y, Van Breusegem F. 2008. Hydrogen peroxide-induced gene expression across kingdoms: A comparative analysis. Mol Biol Evol 25:507–516.

Wang X, Zhang X, Ren XP, Chen J, Liu H, Yang J, Medvedovic M, Hu Z, Fan GC. 2010. MicroRNA-494 targeting both proapoptotic and antiapoptotic proteins protects against ischemia/reperfusion-induced cardiac injury. Circulation 122:1308–1318.

Weigel AL, Handa JT, Hjelmeland LM. 2002. Microarray analysis of H_2O_2-, HNE-, or tBH-treated ARPE-19 cells. Free Radic Biol Med 33:1419–1432.

Wetzker R, Rommel C. 2004. Phosphoinositide 3-kinases as targets for therapeutic intervention. Curr Pharm Des 10:1915–1922.

Xia H, Ooi LL, Hui KM. 2012. MiR-214 targets beta-catenin pathway to suppress invasion, stem-like traits and recurrence of human hepatocellular carcinoma. PLoS ONE 7:e44206.

Xiong X, Ren HZ, Li MH, Mei JH, Wen JF, Zheng CL. 2011. Down-regulated miRNA-214 induces a cell cycle G1 arrest in gastric cancer cells by up-regulating the PTEN protein. Pathol Oncol Res 17:931–937.

Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, Zhang Y, Xu C, Bai Y, Wang H, Chen G, Wang Z. 2007. The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat Med 13:486–491.

Yang H, Kong W, He L, Zhao JJ, O'Donnell JD, Wang J, Wenham RM, Coppola D, Kruk PA, Nicosia SV, Cheng JQ. 2008. MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN. Cancer Res 68:425–433.

Zhang C. 2008. MicroRNAs: Role in cardiovascular biology and disease. Clin Sci (Lond) 114:699–706.