

MicroRNA-423-5p mediates H₂O₂-induced apoptosis in cardiomyocytes through O-GlcNAc transferase

PENG LUO¹ and WEI ZHANG²

Departments of ¹Cardiology and ²Geriatric Medicine, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu, Sichuan 610072, P.R. China

Received July 20, 2015; Accepted May 16, 2016

DOI: 10.3892/mmr.2016.5344

Abstract. Reactive oxygen species (ROS) have been clinically shown to be relevant in the progression of various apoptosis-associated heart diseases. Our previous study demonstrated that microRNA (miR)-423-5p is involved in congestive heart failure (CHF) through the direct targeting of O-GlcNAc transferase and the induction of apoptosis in cardiomyocytes. However, the role of miR-423-5p during ROS-induced apoptosis remains to be elucidated. In the present study, the expression of miR-423-5p in cardiomyocytes following exposure to H₂O₂ was determined using reverse transcription-quantitative polymerase chain reaction analysis. In addition, the effects of silencing of miR-423-5p, by using an miR-423-5p-mimic during H₂O₂ treatment, on cardiomyocyte apoptosis were detected using a terminal deoxynucleotidyl transferase-deoxyuridine triphosphate nick-end labeling assay. The expression levels of the direct target of miR-423-5p and associated downstream targets were also examined. The results demonstrated that H₂O₂ significantly induced the expression of miR-423-5p in cardiomyocytes, in a time- and concentration-dependent manner. The silencing of miR-423-5p by transfection with the miR-423-5p-mimic eliminated the H₂O₂-induced cardiomyocyte apoptosis and decrease in viability. The expression levels of the downstream targets of miR423-5p were also increased by H₂O₂, and were decreased following the silencing of miR-423-5p. Collectively, the results of the present study demonstrated that miR-423-5p mediated H₂O₂-induced apoptosis in the cardiomyocytes. Silencing of miR-423-5p significantly protected the cardiomyocytes from H₂O₂-induced apoptosis, and this may provide a novel therapeutic target for apoptosis-associated heart diseases.

Introduction

Reactive oxygen species (ROS), a product of normal cellular metabolism, are usually managed effectively by the cellular defense systems, thereby having minimal effect on cellular health (1). However, under situations of exaggerated stress or hypoxia, the cellular defenses may be insufficient to overcome ROS overload (1). Of note, in the hypertrophied and failing heart, elevated levels of ROS and oxidative stress in cardiomyocytes are associated with maladaptive ventricular remodeling and a progressive decline in cardiovascular function (2,3). Oxidative stress has been clinically shown to be relevant in the progression of heart failure, myocardial infarction, cardiac ischemia/reperfusion injury and atrial fibrillation (4,5). Excess ROS can cause various types of cellular damage, including mitochondrial dysfunction and DNA damage, and can ultimately lead to apoptosis, with apoptosis of cardiomyocytes being critical in tissue damage and eventual heart failure (6-8). Therefore, understanding the mechanism underlying the effects of ROS and apoptosis are necessary for further improvement in the diagnosis and therapy of heart diseases.

MicroRNAs (miRNAs) are small, non-coding RNAs, which inhibit the translation of target mRNAs through imperfect sequence-specific binding to the 3'-untranslated region of target mRNAs (9). Various studies have demonstrated that the expression of miRNAs is tightly regulated in a tissue-specific and a time-dependent manner (10,11). By affecting protein translation, miRNAs have been identified as potent regulators of a wide range of biological processes, including cardiac morphogenesis, heart failure and arrhythmias (12,13). It has been recognized that miRNAs can be efficiently inhibited for prolonged periods using antisense technologies, which has led to increased interest in the inhibition of specific miRNAs as a feasible therapeutic option for selected cardiovascular diseases (14). According to these characteristics, miRNAs may represent an optimal target for disease therapy.

In our previous study, it was demonstrated that miRNA (miR)-423-5p is involved in congestive heart failure (CHF) through the direct targeting of O-GlcNAc transferase (OGT) and the induction of apoptosis in cardiomyocytes (15). Clinical experiments indicated that miR-423-5p is associated with CHF and the expression levels of prohormone brain natriuretic peptide (proBNP). Furthermore, the expression of

Correspondence to: Dr Wei Zhang, Department of Geriatric Medicine, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, 32 West 2nd Section, 1st Ring Road, Chengdu, Sichuan 610072, P.R. China
E-mail: 250325743@qq.com

Key words: cardiomyocytes, microRNA-423-5p, apoptosis, reactive oxygen species, H₂O₂

miR-423-5p significantly regulates the expression of OGT and associated downstream targets, and induces apoptosis in cardiomyocytes. However, the role of miR-423-5p during ROS-induced apoptosis remains to be elucidated. The present study investigated the expression of miR-423-5p in cardiomyocytes following exposure to H₂O₂ for different durations and at different concentrations. Furthermore, the role of miR-423-5p during H₂O₂-induced apoptosis in cardiomyocytes was also determined. The present results demonstrated that miR-423-5p mediates H₂O₂-induced apoptosis in cardiomyocytes. Silencing of miR-423-5p significantly protects cardiomyocytes from H₂O₂-induced apoptosis. It may provide a novel therapeutic target for apoptosis-related heart diseases.

Materials and methods

Cell culture and treatment. The mouse cardiomyocytes used in the present study were obtained from the Shanghai Cell Bank (Shanghai, China). The cardiomyocytes were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). All cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Cell transfection was performed using FuGENE[®] HD transfection reagent (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's protocol. Briefly, cardiomyocytes were seeded into 6-well plates at a density of 2×10⁵ cells/well and cultured for 24 h to reach 70–80% confluence. Subsequently, 2 µg of plasmids encoding m-miR-423-5p and miR-Ctrl were diluted in 100 µl serum-free medium, and 5 µl FuGENE[®] HD transfection reagent was added to the tubes containing the diluted DNA. The contents were mixed, and the transfection complex was incubated for 15 min at room temperature, followed by its addition to the 6-well plates. Medium alone was used as a blank control. When the cardiomyocytes reached 70–80% confluence in the 6-well plates, H₂O₂ was added to the wells at different concentrations (0, 0.1, 0.2, 0.4 and 0.8 mM). At 12, 24 and 48 h following transfection and/or H₂O₂ treatment, the cardiomyocytes were collected for further experiments.

Vector construction. The miR-423-5p and miR-423-5p-mimic expression plasmids were constructed, as previously (15). Briefly, each miRNA and plasmid oligonucleotide were annealed at 90°C for 3 min, cooled to 37°C and incubated for 1 h. The annealed double-stranded DNA oligonucleotides were ligated between the *Hind*III and *Sac*I sites (Fermentas; Thermo Fisher Scientific, Inc.) on the pMIR-REPORT vector (Ambion Life Technologies; Thermo Fisher Scientific, Inc.). The resulting recombinant plasmids were termed pMIR-miR-423-5p, pMIR-miR-423-5p-mimic and pMIR-miR-control (Ctrl). The three constructs were verified using DNA sequencing. The plasmids, pMIR-miR-423-5p, pMIR-miR-423-5p-mimic and pMIR-miR-control, were extracted and sent to the technology center of Invitrogen Life Technologies at Shanghai for DNA sequencing. The results were comprised with Jellyfish software (version 3.2). The plasmids were extracted using EndoFree Plasmid Giga kits (Qiagen GmbH, Hilden, Germany) from DH5α *Escherichia coli* transformants (Genewiz, Suzhou, China) and stored at -20°C until further use. The concentra-

tion was determined by measuring the A260/A280 ratio using a Thermo ND 2000 spectrophotometer (Thermo Fisher Scientific, Inc.).

Cell viability detection assay. A Cell Counting Kit-8 (CCK8) assay (Beyotime Institute of Biotechnology, Shanghai, China) was used to detect cell viability. The absorbance was determined at a wavelength of 450 nm using a plate reader. The same experiments were performed three times.

RT-qPCR. Total RNA was extracted from each experimental group using TRIzol[®] reagent (Invitrogen Life Technologies, Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The RNA concentration was assessed spectrophotometrically at 260 nm (Thermo ND 2000; Thermo Fisher Scientific, Inc.). Reverse transcription was performed on the isolated total RNA using a Reverse Transcription kit (Takara Bio, Inc., Otsu, Japan), and qPCR was performed using a Real Time PCR kit (Takara Bio, Inc.). Reverse transcription was performed using 1 µg total RNA in 2 µl water at 65°C for 5 min, 30°C for 10 min, 42°C for 10–30 min and 2°C for 3 min. The qPCR conditions were as follows: Denaturation at 94°C for 2 min, amplification for 30 cycles at 94°C for 0.5 min, annealing at 60°C for 0.5 min and extension at 72°C for 1 min, followed by a terminal elongation step at 72°C for 10 min. The RT-qPCR analysis was performed on a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). U6 was amplified as an internal control and the Cq value of each PCR product was calculated, from which the fold change was analyzed (15). The m-miR-423-5p and m-U6 primers were supplied by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The sequences were not supplied due to the rules of the company.

26S proteasome activity assay. 26S proteasome function was assayed, as described previously (16). Briefly, the cells were washed with phosphate-buffered saline, followed by washing with buffer I, containing 50 mM Tris (pH 7.4), 2 mM DTT, 5 mM MgCl₂ and 2 mM ATP, and pelleted by centrifugation at 1,500 g for 3 min at 4°C. Glass beads and homogenization buffer containing 50 mM Tris (pH 7.4), 1 mM DTT, 5 mM MgCl₂, 2 mM ATP and 250 mM sucrose, were added and vortexed for 1 min. The beads and cell debris were removed by centrifugation at 1,000 × g for 5 min and 10,000 × g for 20 min. Protein concentration was determined using a bicinchoninic acid assay protocol (Pierce Biotechnology, Inc., Rockford, IL, USA). The protein from each sample (100 µg) was diluted with buffer I to a final volume of 1,000 µl, and the fluorogenic proteasome substrate, SucLLVY-7-amido-4-methylcoumarin (chymotrypsin-like; Sigma-Aldrich) was added to a final concentration of 80 µM in 1% DMSO. To access 26S function, buffer I was replaced with ATP-free buffer containing 20 mM HEPES (pH 7.8), 0.5 mM EDTA and 0.03% SDS (16). Cleavage activity was monitored continuously by the detection of free 7-amido-4-methylcoumarin using a fluorescence plate reader (Gemini, Molecular Devices LLC, Sunnyvale, CA, USA) at 380/460 nm and 37°C. As controls for the drug experiments, 7-amido-4-methylcoumarin (2 µM) was incubated with the drugs in buffer I without cell extracts, and measurements of proteasome function were corrected when necessary.

Western blot analysis. Following transfection of the cardiomyocytes with m-miR-423-5p and miR-Ctrl (48 h), total protein was collected. The cells were lysed on ice for 30 min with radioimmunoprecipitation assay lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40; 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μ g/ml each of aprotinin, leupeptin and pepstatin, 1 mM Na_3VO_4 and 1 mM NaF. The proteins (20 μ g) were separated by 10% SDS-polyacrylamide gel electrophoresis and electronically transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Bedford, MA, USA). Following blocking with 5% non-fat milk in Tris-buffered saline containing 0.5% Tween-20 (TBST), the membranes were incubated with the recommended dilutions of primary antibodies against OGT (rabbit polyclonal; 1:800; cat. no. sc-32921; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at 37°C, phosphorylated (p) adenosine monophosphate (AMP)-activated protein kinase (p-AMPK; cat. no. 4188; 1:1,000), AMPK (cat. no. 2532; 1:1,000), p53 (cat. no. 2527; 1:1,200) and caspase 3 (cat. no. 9662; 1:600) for 1 h at 37°C (all purchased from Cell Signaling Technology, Inc., Danvers, MA, USA) and GAPDH (cat. no. sc-25778; 1:5,000; Santa Cruz Biotechnology, Inc.). Following washing with TBST, the membranes were incubated with peroxidase-conjugated secondary antibodies against goat anti-mouse immunoglobulin G (cat. no. ab97040; 1:5,000) or goat anti-rabbit (cat. no. ab191866; 1:5,000; each from Abcam, Cambridge, MA, USA) at 37°C for 1 h. The peroxidase-labeled bands were visualized using an enhanced chemiluminescence kit (Pierce Biotechnology, Inc.). The ratios of OGT and p-AMPK to GAPDH were calculated using densitometry, and values were normalized by dividing the ratios by the ratio in the blank control.

Terminal deoxynucleotidyl transferase-deoxyuridine triphosphate nick-end labeling (TUNEL) assay. In order to detect apoptotic cells in the cardiomyocytes, a TUNEL assay was performed using a DeadEnd™ Fluorometric TUNEL system (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol. Cell nuclei with dark green fluorescent staining were defined as TUNEL-positive nuclei, which were visualized using a fluorescence microscope (DTX500; Nikon Corporation, Tokyo, Japan). In order to quantify the TUNEL-positive cells, the number of green fluorescent-positive cells were counted in randomly selected fields (n=5) at magnification, x200. The cell nuclei were then counterstained with 4',6-diamidino-2-phenylindole (Beyotime Institute of Biotechnology).

Statistical analysis. Statistical comparisons of all results were analyzed using one-way analysis of variance. Statistical analyses were performed using SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). Values are expressed as the mean \pm standard error of the mean. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

H₂O₂ decreases cell viability in cardiomyocytes. The effect of H_2O_2 treatment on cardiomyocyte viability was determined using a CCK8 assay. As shown in Fig. 1A, H_2O_2 treat-

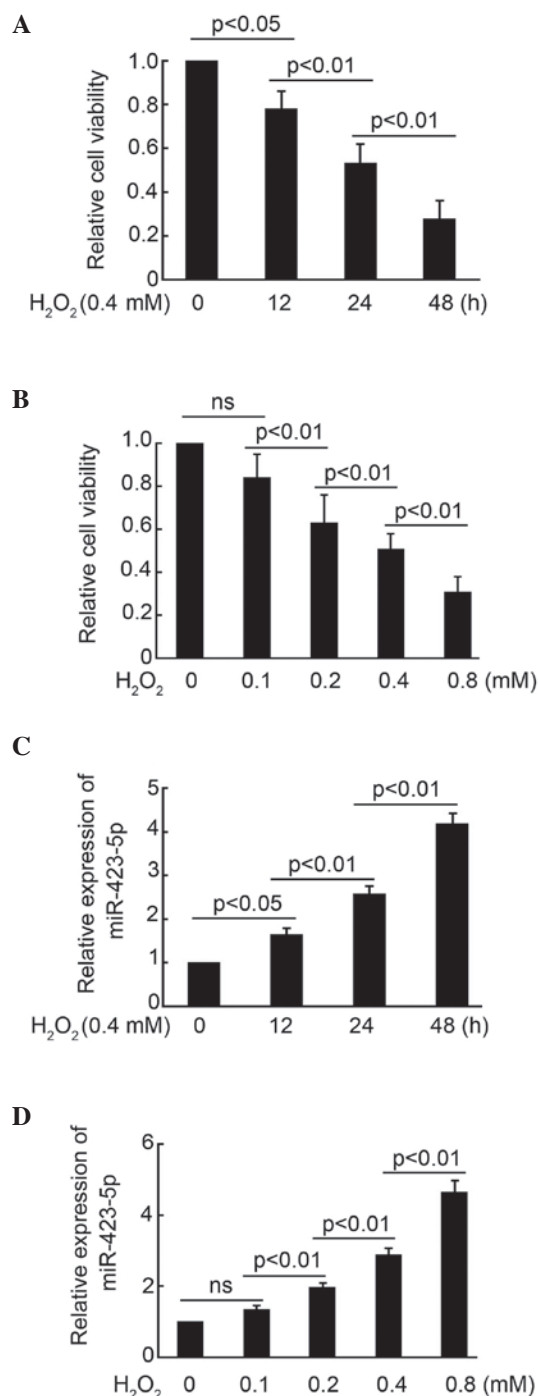


Figure 1. H_2O_2 decreases cell viability and induces the expression of miR-423-5p in cardiomyocytes. (A) Cardiomyocyte viability detection using a CCK8 assay following exposure to H_2O_2 (0.4 mM) for 0, 12, 24 and 48 h. (B) Cardiomyocyte viability detection using a CCK8 assay following exposure to H_2O_2 (0, 0.1, 0.2, 0.4 and 0.8 mM) for 24 h. (C) RT-qPCR analysis of m-miR-423-5p in cardiomyocytes following exposure to H_2O_2 (0.4 mM) for 0, 12, 24 and 48 h. (D) Real-time PCR of m-miR-423-5p in cardiomyocytes following exposure to H_2O_2 (0, 0.1, 0.2, 0.4 and 0.8 mM) for 24 h. Data are presented as the mean \pm standard error of the mean. The same experiments were performed three times. miR, microRNA; CCK8, Cell Counting Kit-8; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ns, no significant difference.

ment (0.4 mM) for 12 h significantly reduced cell viability. Increasing the duration of treatment with H_2O_2 to 24 and 48 h, markedly inhibited the viability of the cardiomyocytes (Fig. 1A). Furthermore, the effect of H_2O_2 concentration on

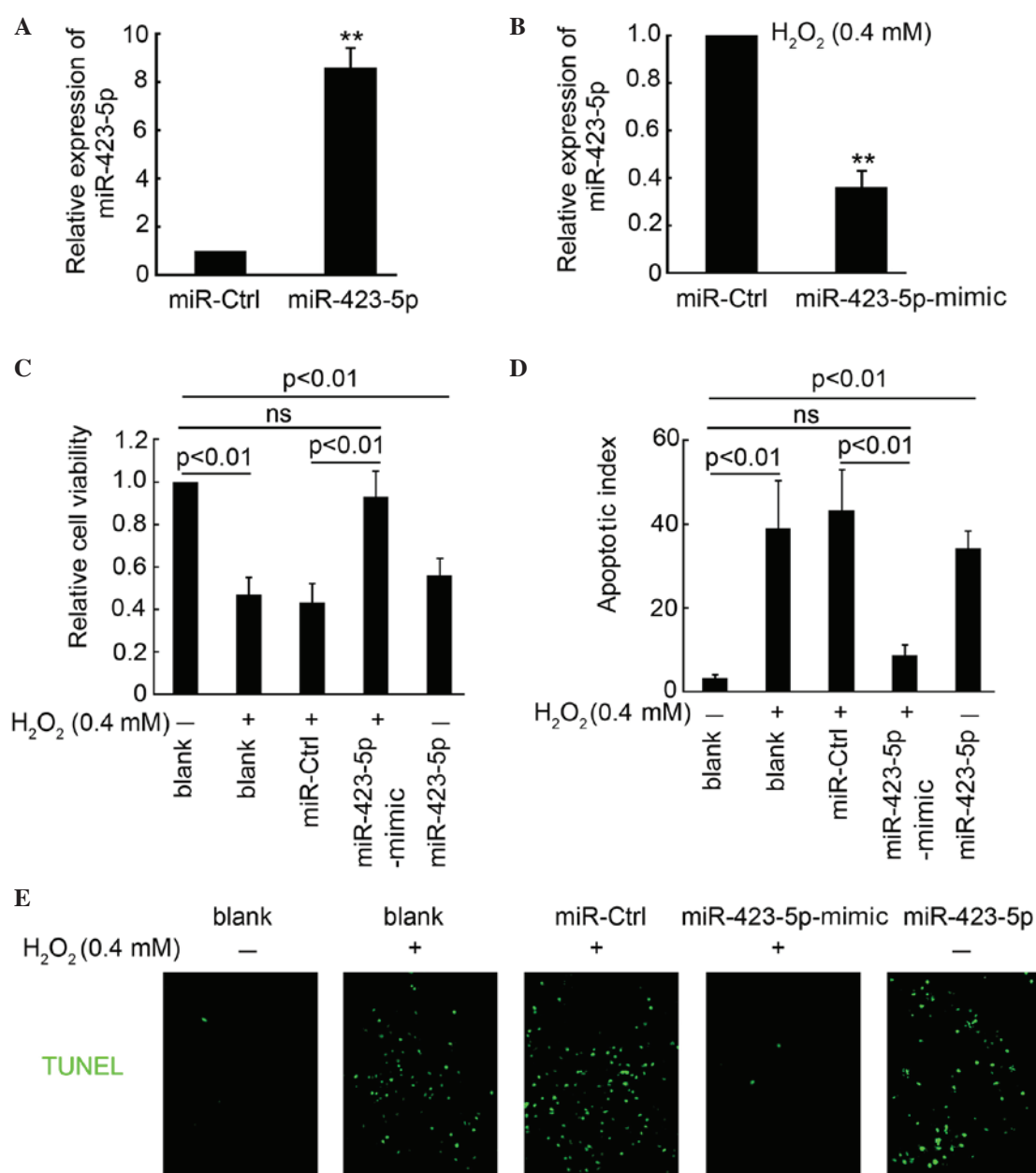


Figure 2. miR-423-5p mediates H₂O₂-induced apoptosis in cardiomyocytes. (A) RT-qPCR analysis of m-miR-423-5p in cardiomyocytes following transfection with the m-miR-423-5p expression plasmid. (B) RT-qPCR of m-miR-423-5p in cardiomyocytes following exposure to H₂O₂ (0.4 mM) and transfection with miR-Ctrl and m-miR-423-5p-mimic plasmids for 48 h. **P < 0.01 compared with the miR-ctrl group. (C) Cardiomyocyte viability detection using a CCK8 assay in the following treatment groups: Blank, H₂O₂ (0.4 mM) treatment, H₂O₂ combined with miR-Ctrl transfection, H₂O₂ combined with miR-423-5p-mimic transfection and miR-423-5p transfection without H₂O₂ treatment. (D) Statistical analysis of the apoptotic index of cardiomyocytes in the different treatment groups (number of apoptotic cells / number of total cells), which was determined by performing a TUNEL assay, according to the kit instructions. (E) Positive cells are indicated in green; DAPI was used to indicate all cells present. Data are presented as the mean \pm standard error of the mean. The same experiments were performed three times. Original magnification, x200. miR, microRNA; Ctrl, control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CCK8, Cell Counting Kit-8; TUNEL, terminal deoxynucleotidyl transferase-deoxyuridine triphosphate nick-end labeling; ns, no significant difference.

cardiomyocyte viability was determined. As the results in Fig. 1B show, treatment with 0.1 mM H₂O₂ for 24 h had no significant effect on cardiomyocytes viability. However, treatment with 0.2, 0.4 and 0.8 mM H₂O₂ for 24 h significantly inhibited cardiomyocyte growth (Fig. 1B). These results indicated that the effect of H₂O₂ treatment on cell viability was time- and concentration-dependent.

H₂O₂ induces the expression of miR-423-5p in cardiomyocytes. In the subsequent experiment, the expression of

miR-423-5p in cardiomyocytes following H₂O₂ treatment was examined using RT-qPCR analysis. As shown in Fig. 1C, H₂O₂ treatment (0.4 mM) for 12 h significantly induced the expression of miR-423-5p in the cardiomyocytes. The expression of miR-423-5p in the cardiomyocytes was higher following H₂O₂ treatment for 24 and 48 h, compared with that at 12 h. Treatment with 0.1 mM H₂O₂ for 24 h had minimal effect on the expression of miR-423-5p in the cardiomyocytes, compared with 0.2, 0.4 and 0.8 mM H₂O₂ treatment, which significantly induced the expression of miR-423-5p in

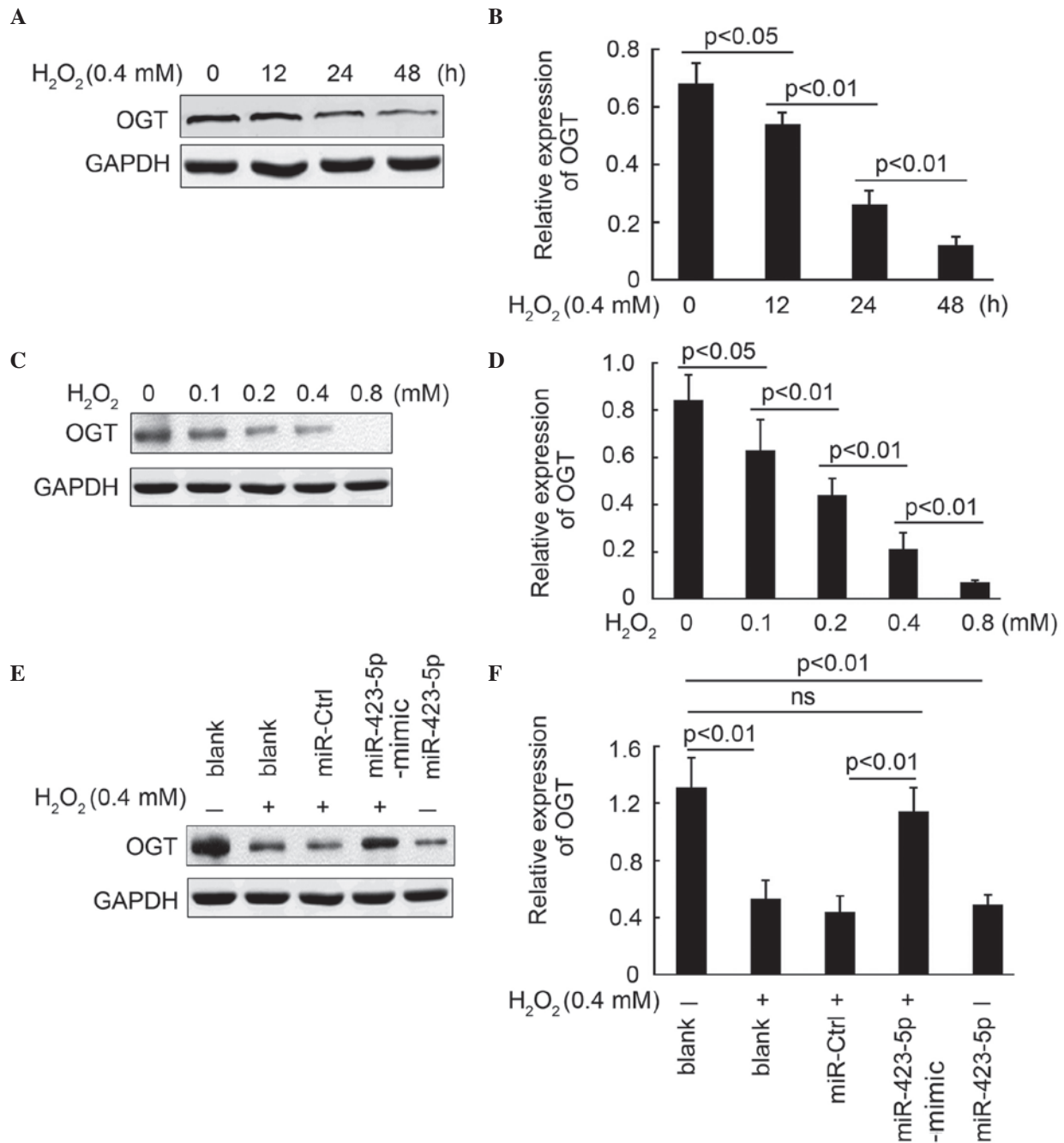


Figure 3. miR-423-5p mediates H₂O₂-regulated expression of OGT. (A) Expression of OGT in cardiomyocytes following exposure to H₂O₂ (0.4 mM) for 0, 12, 24 and 48 h, determined using western blot analysis and (B) quantified using statistical analysis. GAPDH was used as the loading control. (C) Expression of OGT in cardiomyocytes following exposure to H₂O₂ (0, 0.1, 0.2, 0.4 and 0.8 mM) for 24 h, determined using western blot analysis and (D) quantified using statistical analysis. GAPDH was used as the loading control. (E) Expression of OGT in cardiomyocytes in the following treatment groups: Blank, H₂O₂ (0.4 mM) treatment alone, H₂O₂ combined with miR-Ctrl transfection, H₂O₂ combined with miR-423-5p-mimic transfection and miR-423-5p transfection without H₂O₂ treatment. Expression was determined using western blot analysis and (F) quantified using statistical analysis. GAPDH was used as the loading control. Data are presented as the mean \pm standard error of the mean. The same experiments were performed three times. miR, microRNA; Ctrl, control; OGT, O-GlcNAc transferase; ns, no significant difference.

the cardiomyocytes (Fig. 1D). Taken together, these results indicated that H₂O₂ treatment significantly induced the expression of miR-423-5p in cardiomyocytes, in a time- and concentration-dependent manner.

miR-423-5p mediates H₂O₂-induced apoptosis in cardiomyocytes. To examine the effect of miR-423-5p on mediation of H₂O₂-induced apoptosis in cardiomyocytes, plasmids encoding miR-423-5p and miR-423-5p-mimic, which neutralized

the activity of miR-423-5p, were constructed. RT-qPCR analysis demonstrated that transfection with the miR-423-5p plasmid significantly induced the expression of miR-423-5p in the cardiomyocytes (Fig. 2A). To examine the effect of the miR-423-5p-mimic on the expression of miR-423-5p, the cardiomyocytes were exposed to H₂O₂ (0.4 mM) following miR-423-5p-mimic transfection. After 48 h, the cells were collected for RT-qPCR analysis. The results demonstrated the ability of the miR-423-5p-mimic to inhibit the expres-

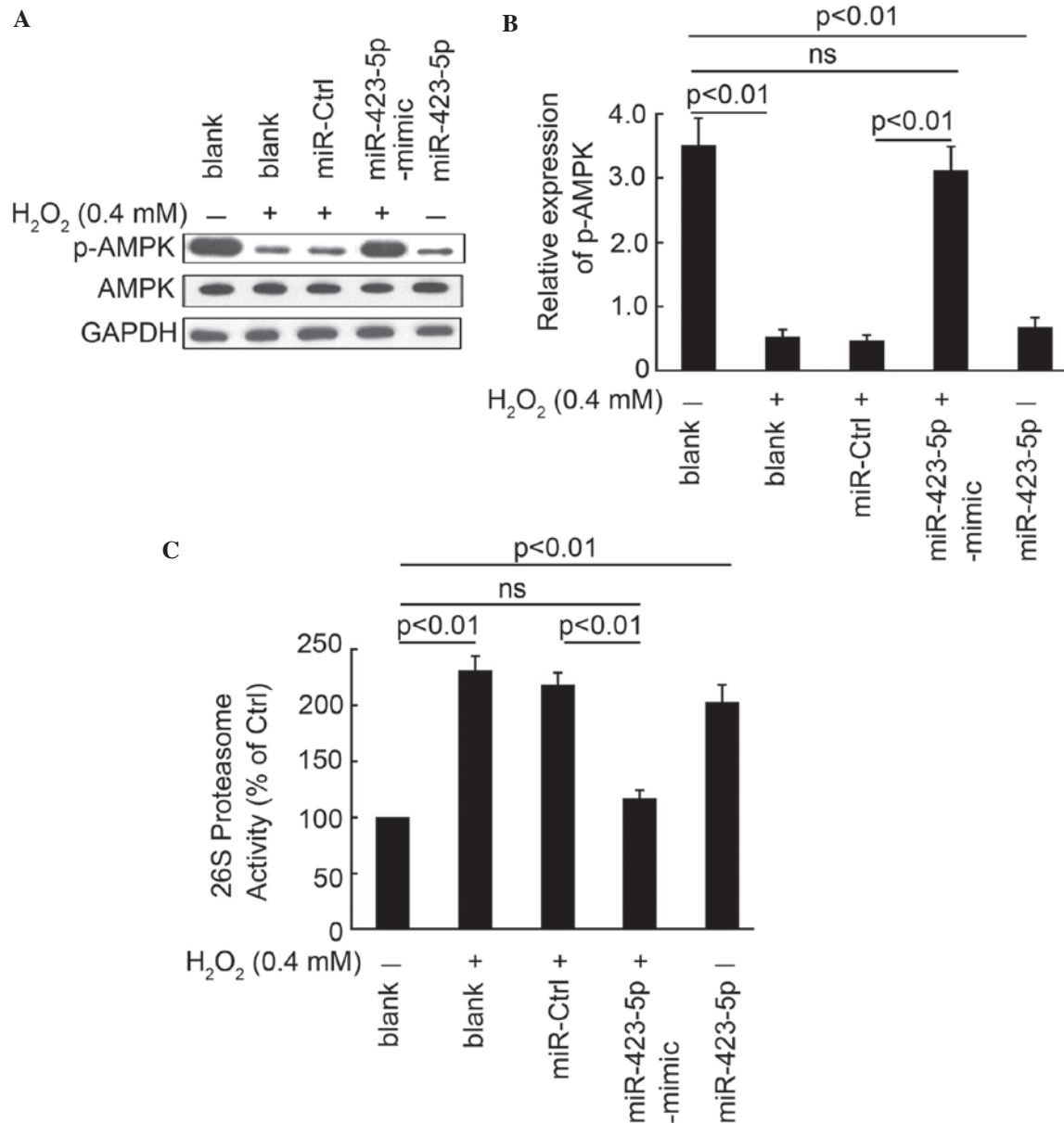


Figure 4. miR-423-5p mediates H₂O₂-regulated expression of OGT downstream targets. (A) Expression of AMPK and p-AMPK in cardiomyocytes in the following treatment groups: Blank, H₂O₂ (0.4 mM) treatment alone, H₂O₂ combined with miR-Ctrl transfection, H₂O₂ combined with miR-423-5p-mimic transfection and miR-423-5p transfection without H₂O₂ treatment. Expression was determined using western blot analysis and was (B) quantified using statistical analysis. GAPDH was used as the loading control. (C) 26S proteasome activity in cardiomyocytes in the following treatment groups: Blank, H₂O₂ (0.4 mM) treatment alone, H₂O₂ combined with miR-Ctrl transfection, H₂O₂ combined with miR-423-5p-mimic transfection and miR-423-5p transfection without H₂O₂ treatment, determined according to the kit instructions. Data are presented as the mean \pm standard error of the mean. The same experiments were performed in three times. miR, microRNA; Ctrl, control; OGT, O-GlcNAc transferase; AMPK, adenosine monophosphate-activated protein kinase; p-AMPK, phosphorylated AMPK; ns, no significant difference.

sion of miR-423-5p (Fig. 2B). Cardiomyocyte viability was determined in the following treatment groups: Blank, H₂O₂ (0.4 mM) alone, H₂O₂ combined with miR-Ctrl transfection, H₂O₂ combined with miR-423-5p-mimic transfection, and miR-423-5p transfection without H₂O₂ treatment. As shown in Fig. 2C, H₂O₂ treatment or transfection with miR-423-5p significantly decreased cardiomyocyte viability (Fig. 2C). No significant difference in cardiomyocyte viability was found between the blank control group and the H₂O₂ combined with miR-423-5p-mimic transfection group (Fig. 2C). These results indicated that cardiomyocyte viability was restored following inhibition of miR-423-5p activity. A TUNEL assay

was used to detect apoptotic cardiomyocytes. It was found that H₂O₂ treatment or the overexpression of miR-423-5p significantly induced cardiomyocyte apoptosis, compared with the miR-423-5p-mimic, which markedly inhibited H₂O₂-induced apoptosis (Fig. 2D and E). These results demonstrated that miR-423-5p mediated H₂O₂-induced apoptosis in the cardiomyocytes.

miR-423-5p mediates H₂O₂-regulated expression of OGT. In our previous study, it was demonstrated that OGT is the direct target of miR-423-5p. Thus, in the present study, the expression of OGT was examined. As shown in Fig. 3A and B, the expres-

sion of OGT was significantly inhibited following exposure to H_2O_2 for 12, 24 and 48 h. Further investigation demonstrated that H_2O_2 markedly decreased the expression of OGT in a concentration-dependent manner (Fig. 3C and D). To examine the role of miR-423-5p on mediation of the H_2O_2 -regulated expression of OGT, the cardiomyocytes were treated in the following groups: Blank control, H_2O_2 (0.4 mM) treatment, H_2O_2 combined with miR-Ctrl transfection, H_2O_2 combined with miR-423-5p-mimic transfection, and miR-423-5p transfection without H_2O_2 treatment. The results indicated that treatment with H_2O_2 or transfection with miR-423-5p significantly inhibited the expression of OGT. Of note, the expression of OGT in cardiomyocytes was restored following inhibition of miR-423-5p activity by the miR-423-5p-mimic. No significant difference were observed between blank control group and the H_2O_2 combined with miR-423-5p-mimic transfection group. Collectively, miR-423-5p mediated the H_2O_2 -induced downregulation of OGT.

miR-423-5p mediates H_2O_2 -regulated expression of OGT downstream targets. The activities of AMPK and 26S proteasome activity, as the downstream targets of OGT, were also determined in the present study. The phosphorylation of AMPK was inhibited following H_2O_2 treatment or the overexpression miR-423-5p through a reduction in OGT (Fig. 4A). Whereas, no significant change in the expression of p-AMPK was observed between the blank control group and the H_2O_2 combined with miR-423-5p-mimic transfection group. According to the change in the expression of p-AMPK, 26S proteasome activity was also increased following H_2O_2 treatment or the overexpression miR-423-5p (Fig. 4C). Transfection with the miR-423-5p-mimic also restored 26S proteasome activity to a normal level (Fig. 4C). These results demonstrated that miR-423-5p mediated the H_2O_2 -regulated expression of p-AMPK and activity of 26S proteasome, which are downstream targets of OGT.

Discussion

In the present study, it was found that the expression of miR-423-5p in cardiomyocytes was induced by H_2O_2 in a time- and concentration-dependent manner. The results indicated that the silencing of miR-423-5p by transfection with miR-423-5p-mimic significantly inhibited H_2O_2 -induced apoptosis in cardiomyocytes. Furthermore, the direct target of miR-423-5p, OGT, and downstream targets, p-AMPK and 26S proteasome, were also demonstrated to be involved in H_2O_2 -induced apoptosis in cardiomyocytes. Collectively, the results of the present study demonstrated that miR-423-5p mediated H_2O_2 -induced apoptosis in the cardiomyocytes. Silencing of miR-423-5p significantly protected the cardiomyocytes from H_2O_2 -induced apoptosis, and miR-423-5p may provide a novel therapeutic target for apoptosis-associated heart diseases.

As is already known, apoptosis is involved in the pathological process of several cardiovascular diseases, including heart failure, myocardial infarction, cardiac ischemia and reperfusion injury, and atrial fibrillation (17-20). Excess ROS can cause a variety of cellular damage, including mitochondrial dysfunction and DNA damage, and ultimately leads to apoptosis, with apoptosis of cardiomyocytes being critical in

tissue damage and eventually heart failure (6-8). The administering of antioxidants, including vitamin C, have been effective in preventing oxidative stress-mediated cardiovascular dysfunction (21). Thus, inhibiting the apoptosis of cardiomyocytes appears the most effective clue to protecting the heart from injury. To examine the correction between miR-423-5p and H_2O_2 -induced apoptosis in cardiomyocytes, the expression of miR-423-5p was examined following exposure of the cells to H_2O_2 . It was found that miR-423-5p was a sensor of H_2O_2 -induced apoptosis.

In previous years, miR-423-5p has been widely investigated as a potential biomarker and therapeutic target for various heart diseases. miR-423-5p has been reported in array studies to be upregulated in the failing myocardium in humans (22). Tijssen *et al* (23) showed that circulating levels of miR-423-5p are increased in subjects with clinical heart failure, defined by the Framingham criteria and elevated levels of proBNP, and that the levels of miR-423-5p are associated with proBNP and ejection fraction in this patient group. Our previous study (15) demonstrated that miR-423-5p corrects with CHF through the direct targeting of OGT and inducing apoptosis in cardiomyocytes. Clinical experiments indicated that miR-423-5p is associated with CHF and the expression levels of proBNP. Furthermore, the expression of miR-423-5p significantly regulated the expression levels of OGT and its associated downstream targets, and induced apoptosis of the cardiomyocytes. However, the role of miR-423-5p during ROS-induced apoptosis remains to be fully elucidated. In the present study, it was demonstrated that the expression of miR-423-5p in cardiomyocytes was induced by H_2O_2 in a time- and concentration-dependent manner. The silencing of miR-423-5p by transfection with the miR-423-5p-mimic significantly inhibited H_2O_2 -induced apoptosis in cardiomyocytes. These results demonstrated that miR-423-5p mediated H_2O_2 -induced apoptosis in cardiomyocytes, and that silencing of miR-423-5p significantly protected the cardiomyocytes from H_2O_2 -induced apoptosis.

In conclusion, the results of the present study demonstrated that the expression of miR-423-5p was induced by H_2O_2 in a time- and concentration-dependent manner, and silencing of miR-423-5p significantly protected the cardiomyocytes from H_2O_2 -induced apoptosis. These results indicated that miR-423-5p may be an effective therapeutic target for the treatment of apoptosis-associated heart diseases.

References

1. Khurana S, Hollingsworth A, Piche M, Venkataraman K, Kumar A, Ross GM and Tai TC: Antiapoptotic actions of methyl gallate on neonatal rat cardiac myocytes exposed to H_2O_2 . *Oxid Med Cell Longev* 2014: 657512, 2014.
2. Dhalla AK, Hill MF and Singal PK: Role of oxidative stress in transition of hypertrophy to heart failure. *J Am Coll Cardiol* 28: 506-514, 1996.
3. Hill MF and Singal PK: Right and left myocardial antioxidant responses during heart failure subsequent to myocardial infarction. *Circulation* 96: 2414-2420, 1997.
4. Dhalla NS, Temsah RM and Netticadan T: Role of oxidative stress in cardiovascular diseases. *J Hypertens* 18: 655-673, 2000.
5. Maulik SK and Kumar S: Oxidative stress and cardiac hypertrophy: A review. *Toxicol Mech Methods* 22: 359-366, 2012.
6. Peng YW, Buller CL and Charpie JR: Impact of N-acetylcysteine on neonatal cardiomyocyte ischemia-reperfusion injury. *Pediatr Res* 70: 61-66, 2011.

7. Rodrigo R: Prevention of postoperative atrial fibrillation: Novel and safe strategy based on the modulation of the antioxidant system. *Front Physiol* 3: 93, 2012.
8. Tsutsui H, Kinugawa S and Matsushima S: Oxidative stress and heart failure. *Am J Physiol Heart Circ Physiol* 301: H2181-H2190, 2011.
9. Ambros V: The functions of animal microRNAs. *Nature* 431: 350-355, 2004.
10. Cordes KR and Srivastava D: MicroRNA regulation of cardiovascular development. *Circ Res* 104: 724-732, 2009.
11. Liu N and Olson EN: MicroRNA regulatory networks in cardiovascular development. *Dev Cell* 18: 510-525, 2010.
12. Ivey KN, Muth A, Arnold J, King FW, Yeh RF, Fish JE, Hsiao EC, Schwartz RJ, Conklin BR, Bernstein HS and Srivastava D: MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. *Cell Stem Cell* 2: 219-229, 2008.
13. Zhao Y, Ransom JF, Li A, Vedantham V, von Drehle M, Muth AN, Tsuchihashi T, McManus MT, Schwartz RJ and Srivastava D: Dysregulation of cardiogenesis, cardiac conduction and cell cycle in mice lacking miRNA-1-2. *Cell* 129: 303-317, 2007.
14. van Rooij E, Marshall WS and Olson EN: Toward microRNA-based therapeutics for heart disease: The sense in antisense. *Circ Res* 103: 919-928, 2008.
15. Luo P, He T, Jiang R and Li G: MicroRNA-423-5p targets O-GlcNAc transferase to induce apoptosis in cardiomyocytes. *Mol Med Rep* 12: 1163-1168, 2015.
16. Fekete MR, McBride WH and Pajonk F: Anthracyclines, proteasome activity and multi-drug-resistance. *BMC Cancer* 5: 114, 2005.
17. Al-Gubory KH, Fowler PA and Garrel C: The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. *Int J Biochem Cell Biol* 42: 1634-1650, 2010.
18. Hauton D: Hypoxia in early pregnancy induces cardiac dysfunction in adult offspring of *Rattus norvegicus*, a non-hypoxia-adapted species. *Comp Biochem Physiol A Mol Integr Physiol* 163: 278-285, 2012.
19. Nanduri J, Makarenko V, Reddy VD, Yuan G, Pawar A, Wang N, Khan SA, Zhang X, Kinsman B, Peng YJ, *et al*: Epigenetic regulation of hypoxic sensing disrupts cardiorespiratory homeostasis. *Proc Natl Acad Sci USA* 109: 2515-2520, 2012.
20. Patterson AJ and Zhang L: Hypoxia and fetal heart development. *Curr Mol Med* 10: 653-666, 2010.
21. Kane AD, Herrera EA, Camm EJ and Giussani DA: Vitamin C prevents intrauterine programming of in vivo cardiovascular dysfunction in the rat. *Circ J* 77: 2604-2611, 2013.
22. Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, Doevendans PA, Mummery CL, Borlak J, Haverich A, *et al*: MicroRNAs in the human heart: A clue to fetal gene reprogramming in heart failure. *Circulation* 116: 258-267, 2007.
23. Tijssen AJ, Creemers EE, Moerland PD, de Windt LJ, van der Wal AC, Kok WE and Pinto YM: MiR423-5p as a circulating biomarker for heart failure. *Circ Res* 106: 1035-1039, 2010.