Abstract. MicroRNAs (miRNAs or miRs) play important roles in cardiovascular disease. miR-21-5p is known to be involved in the regulation of cardiomyocyte injury under high glucose and high fat (HG-HF) conditions, but its mechanism of action remains unclear. In the present study, a cardiomyocyte cell line, H9c2, was treated with 33 mM glucose and 250 µM sodium palmitate for 24, 48, and 72 h to produce HG-HF injury. After treatment, miR-21-5p expression was detected by reverse transcription-quantitative PCR. A miR-21-5p mimic was then constructed and transfected into the cells and the potential molecular mechanism was investigated using Cell Counting Kit-8, TUNEL, flow cytometry and western blot assays. Expression of miR-21-5p was significantly downregulated by HG-HF treatment of H9c2 cells for 24, 48, and 72 h. In subsequent experiments, cells were treated for an intermediate period (48 h). Compared with the control group, HG-HF treatment significantly inhibited H9c2 proliferation and promoted apoptosis, while these effects were significantly reduced in the miR-21-5p mimic. Compared with the control group, HG-HF treatment significantly increased reactive oxygen species, while miR-21-5p mimic significantly reduced this effect. Compared with the control group, HG-HF treatment significantly increased the expression of the pro-apoptotic proteins Bax and phosphorylated (p)-Akt and decreased the expression of the anti-apoptotic proteins Bcl-2, p-PTEN, and p-FOXO3a, while overexpression of miR-21-5p significantly reduced these effects. The results revealed that miR-21-5p inhibited apoptosis and oxidative stress in H9c2 cells induced by HG-HF, likely through the PTEN/Akt/FOXO3a signaling pathway.

Introduction
Epidemiological studies have indicated that the incidence of diabetes mellitus (DM) is increasing annually worldwide, while diabetic cardiomyopathy (DCM) is the main factor contributing to heart failure in diabetic patients without coronary heart disease or hypertension (1,2). However, the pathogenesis of DCM is very complex and not yet fully understood. Long-term hyperglycemia can act on the respiratory chain, increase production of reactive oxygen species (ROS) and oxidative stress, and further induce myocardial apoptosis (3,4). Due to the non-renewable characteristics of myocardial cells, cardiac function gradually declines with cardiomyocyte apoptosis.

MicroRNAs (miRNAs or miRs) are non-coding small molecule RNAs regulating post-transcriptional gene expression. They can inhibit mRNA translation or target mRNA degradation by binding to specific mRNAs, thus regulating gene expression (5). MiRNAs play important roles in cardiovascular disease. For example, miR-17-5p and miR-1594 are important regulators of cellular responses to heart injury (6,7). miR-21-5p is involved in numerous diseases, including lung and endometrial fibrosis (8,9), but its exact function in heart disease remains controversial. Qiao et al reported that miR-21-5p can enhance angiogenesis and myocardial cell survival by regulating the phosphatase and tensin homolog (PTEN)-Akt signaling pathway, thus contributing to cardiac repair (10). Expression of miR-21-5p is affected by isoflurane preconditioning in a rat model of myocardial infarction (11). However, the mechanism by which miR-21-5p regulates cardiomyocyte injury under high glucose and high fat (HG-HF) conditions is unclear.

In the present study, a miR-21-5p mimic was constructed to study its effect on apoptosis in cardiomyocytes under HG-HF conditions and to provide improved understanding of its mechanism of action. The results of the present study have important implications for the pathogenesis of DCM and for research on possible treatments.
Materials and methods

Cell culture and treatments. H9c2 cells were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences). The cells were passaged when their density reached 80-90%. The supernatant was then discarded and the cells were washed twice with 1X PBS. The cells were then treated with 0.25% trypsin (containing 0.02% EDTA; 3 min at 37°C) to detach them from the culture vessel. Subsequently, the cells became round, and complete medium was added to terminate the digestion. The cells were centrifuged at 875 x g for 3 min at room temperature. The cell suspension was divided into new culture dishes at a ratio of 1:3, marked and placed in a 5% CO₂ incubator at 37°C. H9c2 cells were cultured in DMEM containing 33 mM glucose (HG) and 250 µM sodium palmitate (HF) for 24, 48 and 72 h to induce HG-HF injury as previously described (12). DMEM supplemented with 5.5 mM glucose was used as a control.

Transfection. A total of two sterilized Eppendorf tubes were prepared for each group of cells. Each tube was filled with 62.5 µl Opti-MEM™ (Thermo Fisher Scientific, Inc.). In addition, one tube was filled with 2.5 µl Lipofectamine™ 3000 (Thermo Fisher Scientific, Inc.) while the other with 6.25 µl of either a negative control (NC) mimic (5'-UCACAA CCUCUCAAAGAGUAUCUACUCUUUCAGGA GUUUGUGA-3') or a miR-21-5p mimic (5'-UAGCUUAUC AGACUGAUGUUAUACACUGACUGUAAGC UA-3') from General Biosystems (Anhui) Co., Ltd., and both were then incubated at room temperature for 5 min. The two tubes were evenly mixed and incubated at room temperature for 15 min, after which the mixed solution was dropped into wells in a 6-well plate before the cells were returned to the incubator. Following 48 h of transfection of the H9c2 cells, transfection efficiency was detected by reverse transcription-quantitative PCR (RT-qPCR) and the treatment groups were cultured in HG-HF medium for an additional 48 h at 37°C.

TUNEL assay. Cells (3x10⁵ cells/ml) were fixed with 4% paraformaldehyde for 15 min at room temperature, washed 3 times with PBS, and then permeated for 20 min at room temperature in PBS containing 0.5% Triton X-100. PBS was used to wash the culture dishes 3 times (3 min each). TUNEL solution was added to each well and incubated for 1.5 h at 45°C. DAPI (5 µg/ml) was added to stain the nuclei for 5 min at room temperature. The culture dish was sealed with 50% glycerol, and images from at least five fields in each section were taken under a fluorescence microscope (magnification, x200).

Measurement of ROS. To assess the levels of ROS, the cells (3x10⁵ cells/ml) were incubated with DCFH-DA (10 µM) (Beyotime Institute of Biotechnology) at 37°C for 20 min. Subsequently, they were washed three times with serum-free medium to remove excess DCFH-DA. ROS levels were then analyzed by flow cytometry (FACSCalibur; BD Biosciences). The data were analyzed by FlowJo 7.6 (FlowJo LLC).

Measurement of nitric oxide (NO) level. The levels of NO were measured in the cells (3x10⁵ cells/ml) using the double antibody sandwich method according to the manufacturer’s instructions (cat. no. MM-20607R1; Jiangsu Enzyme Industry Co., Ltd.).

RT-qPCR. Total RNA was extracted from cells using an Ultrapure RNA extraction kit according to the manufacturer’s instructions (CoWin Biosciences). The concentration and purity of RNA (OD₂₆₀/OD₂₃₀) were determined by a UV-Vis spectrophotometer. RNA was reversely transcribed into cDNA using a miRNA cDNA Synthesis Kit according to the manufacturer’s instructions (cat. no. CW2141S; CoWin Biosciences). The reaction system for qPCR was as follows: RNase free dH₂O, 9.5 µl; cDNA, 1 µl; upstream primer, 1 µl; downstream primer, 1 µl; and 2X SYBR Green PCR Master Mix (miRNA qPCR Assay Kit; cat. no. CW2142S; CoWin Biosciences), 12.5 µl. The reaction steps were as follows: Pre-denaturation at 95°C for 10 min; denaturation at 95°C for 10 sec; annealing at 58°C for 30 sec; and extension at 72°C for 30 sec, carried out over 40 cycles. The primers were designed based on poly(A) tailing reaction method (13) and synthesized by General Biosystems (Anhui) Co., Ltd., using the following sequences: miR-21-5p forward, 5'-TAGCCTTATCGACTGATGTGA-poly(A)-3' and the reverse primer (5'-AGTGCAGGTCGGAGTTT-3') was a general primer in the kit (2X SYBR Green PCR Master Mix; CoWin Biosciences); U6 forward, 5'-GCTTTCGAGCAGCAATT ACTACTATAAAT-3' and reverse, 5'-CGCTTCCAGGAATT TGCGTGTCAT-3'. The target gene expression was calculated using the 2⁻ΔΔCt method (14).

Western blotting. Cells were collected and total protein was extracted using TriplePrep kit according to the manufacturer’s instructions (cat. no. 28-9425-44; ReadyPrep; Cytiva). After 30 min on ice, the lysate was centrifuged at 8798 x g for 10 min at 4°C. Protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology). A total of (20 µg) per protein sample was denatured, and the samples were separated by 12% SDS-PAGE for 2 h, followed by transfer to a PVDF membrane. Following blocking in 5% skim milk for 2 h at room temperature, the membranes were incubated with primary antibodies at 4°C overnight, and then the membranes were incubated with a secondary antibody at room temperature for 2 h. The primary antibodies included mouse monoclonal anti-GAPDH (1:2,000; cat. no. TA-08; ZSGB-BIO), rabbit anti-phosphorylated (p)-PTEN (1:1,000; cat. no. AF3351; Affinity Biosciences), rabbit anti-p-AKT (1:1,000; cat. no. AF0016; Affinity Biosciences) rabbit anti-p-FoxO3a (1:1,000; cat. no. AF5302; Affinity Biosciences), rabbit anti-Bax (1:1,000; cat. no. A0207; ABClonal Biotech Co., Ltd.), and mouse anti-Bcl-2 (1:500; product code ab692; Abcam). The secondary antibodies (1:10,000) were HRP-labeled goat anti-rabbit IgG (cat. no. 65-6120; Thermo Fisher Scientific, Inc.) and HRP-labeled goat anti-mouse IgG (cat. no. 31430; Thermo Fisher Scientific, Inc.). Enhanced chemiluminescence exposure solution (cat. no. CW0049; CoWin Biosciences) was added to the membrane and exposed in a gel imaging system. The gray value was analyzed by Quantity One software (version 4.62; Bio-Rad Laboratories, Inc.).
Statistical analysis. All data are expressed as the mean ± standard deviation (SD; n=6 in each group) and analyzed by Graphpad Prism version 7 (GraphPad Software, Inc.). Results from the two groups were compared using unpaired Student's t-test and one-way ANOVA followed by Bonferroni's correction were applied to compare three or more groups. P<0.05 was considered to indicate a statistically significant difference.

Results

HG-HF downregulates the expression of miR-21-5p in H9c2 cells. To explore the effect of HG-HF on the expression of miR-21-5p in H9c2 cells, RT-qPCR was used to detect the expression of miR-21-5p mRNA. The results are revealed in Fig. 1. Compared with the control group, the expression of miR-21-5p in H9c2 cells was significantly reduced by treatment with HG-HF for 24, 48, and 72 h.

Figure 1. Effects of high levels of glucose and fat on the expression of microRNA-21-5p in H9c2 cells. **P<0.01 compared with the control group (n=6 in each group). HG-HF, high glucose-high fat; miR, microRNA.

Figure 2. Effects of high levels of glucose and fat on expression of apoptosis-related and PTEN/Akt/FOXO3a proteins in H9c2 cells. **P<0.01 compared with the control group (n=6 in each group). HG-HF, high glucose-high fat; p-, phosphorylated.

Figure 3. miR-21-5p mimic promotes miR-21-5p expression in H9c2 cells. **P<0.01 compared with the control group (n=6 in each group). miR, microRNA.
HG-HF triggers apoptosis-related protein expression and modulates PTEN/Akt/FOXO3a signaling. The expression levels of the apoptosis-related proteins Bax/Bcl-2 and PTEN/Akt/FOXO3a were detected by western blotting. Compared with the control group, HG-HF treatment for 24, 48, and 72 h significantly increased the expression of the pro-apoptotic proteins Bax and p-Akt, while it significantly decreased the expression of the anti-apoptotic proteins Bcl-2, p-PTEN and p-FOXO3a (Fig. 2).

miR-21-5p mimics reduce apoptosis of H9c2 cells caused by HG-HF. TUNEL staining was used to detect apoptosis, and the results were revealed in Fig. 4. Compared with the control H9c2 cells, HG-HF treatment significantly promoted apoptosis while the miR-21-5p mimic inhibited apoptosis induced by HG-HF (Fig. 4).

Effects of the miR-21-5p mimic on NO levels and oxidative stress in H9c2 cells. In order to explore the effect of miR-21-5p mimic on oxidative stress in H9c2 cells, flow cytometry was used to detect ROS. Compared with the control group, HG-HF treatment significantly increased the level of ROS, while the miR-21-5p mimic significantly decreased ROS levels in the HG-HF group (Fig. 5A and B). Conversely, the miR-21-5p mimic had no significant effect on NO levels in H9c2 cells (Fig. 5C).

miR-21-5p mimic reduces apoptosis of H9c2 cells caused by HG-HF, likely via the PTEN/Akt/FOXO3a signaling pathway. To further explore the effect of miR-21-5p on apoptosis of H9c2 cells induced by HG-HF and to identify its molecular mechanism, the expression levels of the apoptosis-related proteins Bax/Bcl-2 and the signaling pathway proteins PTEN/Akt/FOXO3a were detected by western blotting. Compared with the control group, HG-HF treatment significantly increased the expression of the pro-apoptotic proteins Bax and p-Akt and decreased the expression of the anti-apoptotic proteins Bcl-2, p-PTEN and p-FOXO3a, and these effects were greatly reduced in the miR-21-5p mimic group (Fig. 6). These data indicated that the miR-21-5p mimic inhibited H9c2 cell apoptosis induced by HG-HF, likely via the PTEN/Akt/FOXO3a signaling pathway.

Discussion

The steady increase in the number and mortality of diabetic patients is partly due to DM-related heart disease (15), including abnormal cardiac structure and function such as left ventricular dysfunction, myocardial apoptosis, and myocardial fibrosis (16). Cardiomyocyte apoptosis has been considered a
potential mechanism for the development of cardiomyopathy and heart failure (17,18).

It has been observed that the PTEN/Akt signaling pathway is important for regulation of cell apoptosis, inflammation, and synaptic plasticity (19,20). PTEN was initially recognized as a tumor suppressor that can antagonize the effect of PI3K and downregulate PIP3 (21‑23). PIP3 can increase the level of p‑Akt and participate in the growth and survival of cells.
PTEN is a negative regulator of the PI3K/Akt pathway and plays an important role in regulation of cell growth, differentiation, apoptosis, migration, and neuronal plasticity (19,20). The FOXO protein is a member of the forkhead transcription factor family. Its common characteristic is the forkhead protein (Fox) domain, which plays an important role in apoptosis via regulation of the PI3K/Akt pathway. Li et al (24) found that the PTEN/Akt/FOXO3a pathway plays an important role in hypoxia and ischemia-induced neuronal apoptosis in rats. In the present study, it was revealed that p-Akt was significantly upregulated and p-PTEN and p FOXO3a were significantly downregulated. Additionally, upregulation of the pro-apoptotic protein Bax and downregulation of the anti-apoptotic protein Bcl-2 were observed after 24, 48, and 72 h of HG-HF treatment, which indicated that HG-HF promoted H9c2 cell apoptosis through the PTEN/Akt/FOXO3a signaling pathway.

miR-21 (miRBase Accession number: MI0000077) is a stem-loop precursor sequence and is processed into two mature miRNA sequences, miR-21-5p (miRBase Accession number: MIMAT0000076) and miR-21-3p (miRBase Accession number: MIMAT0004494). miR-21-5p and miR-21-3p are derived from 5' and 3' ends of miR-21, respectively (25). MiR-21 is differentially expressed in numerous cardiovascular diseases, including neointimal injury, myocardial infarction, heart failure, as well as other pathological states, and in cardiomyocyte apoptosis associated with a variety of conditions (10). Sayed et al (26) revealed that miR-21 was sensitive to sustained hypoxia, which could downregulate the expression of miR-21 in cardiomyocytes. Cheng et al (27) identified that miR-21 was sensitive to hydrogen peroxide, which could upregulate the expression of miR-21 in cardiomyocytes. Regulation of PTEN by miR-21 has been reported in cancer and cardiovascular injury (28,29). Interestingly, miR-21-5p has also been revealed to perform key roles in heart diseases. Knockdown of miR-21-5p decreases myocardial infarction injury, indicating that miR-21-5p may play an active role in post-myocardial infarction repair (30,31). In the present study, it was revealed that the expression of miR-21-5p in HG-HF-induced cardiomyocytes was significantly lower than in a normal control group. miR-21-5p mimic inhibited Bax expression and increased Bcl-2 expression to inhibit cell apoptosis, and also reduced the effect of HG-HF on the PTEN/Akt/FOXO3a signaling pathway, indicating that miR-21-5p regulates the PTEN/Akt/FOXO3a signaling pathway to inhibit HG-HF-induced apoptosis in cardiomyocytes. Although the specific function of miR-21 and miR-21-5p in heart diseases was not distinguished, the mimic of miR-21 may also exert a similar function, since miR-21-5p is a mature product of miR-21. In addition, the function of miR-21-3p should also be investigated in future.

Oxidative stress is widely reported in numerous pathophysiological processes, such as aging, inflammation, and psychiatric disorders (32). Studies have revealed that autophagy and apoptosis are ROS-dependent (33), and ROS are involved in regulation of apoptosis (34). In the present study, ROS levels in the experimental groups were also detected using flow cytometry. HG-HF treatment increased ROS in H9c2 cells, while the miR-21-5p mimic decreased ROS in the HG-HF group. These data may suggest that the miR-21-5p mimic inhibits HG-HF-induced apoptosis of cardiomyocytes by regulating ROS. NO generated within the heart has long been known to influence vascular homoeostasis (35), but it was revealed that the miR-21-5p mimic had no significant effect on NO levels in the H9c2 cells. These data indicated that regulation of apoptosis by miR-21-5p is independent of NO.

There are a few limitations in the present study. Firstly, whether miR-21-5p has other anti-apoptotic mechanisms and whether the PTEN/Akt/FOXO3a pathway is the downstream target of miR-21-5p in cardiomyocyte apoptosis induced by HG-HF remains unclear. Therefore, the relationship between miR-21-5p and PTEN/Akt/FOXO3a signaling in inhibiting apoptosis in cardiomyocytes needs further study. Secondly, our research was limited to in vitro cell experiments; animal experiments and human clinical trials have not yet been carried out. The present study provided a preliminary conceptual and experimental basis for the use of miR-21-5p in the treatment of diabetic cardiomyopathy.

In conclusion, our results indicated that miR-21-5p inhibits apoptosis in cardiomyocytes induced by HG-HF, and may act via the PTEN/Akt/FOXO3a signaling pathway.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
YH, XC, MP, JG, WC, DL and CX performed the experiments and analyzed the data. YH designed the study and wrote the manuscript. YH, XC, MP, JG, WC, DL and CX confirmed the authenticity of the raw data.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.
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